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IMMUNO-ONCOLOGY AND TECHNOLOGY

ORIGINAL ARTICLE

Assessment of CD73 activity in breast cancer-derived small extracellular vesicles: application to monitoring of patients' responses to immunotherapy

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Background: We previously discovered that small extracellular vesicles (sEV) isolated from melanoma cells produce immunosuppressive adenosine (ADO) via the ATP→ADP→AMP→ADO pathway and that CD73 is the 'gateway' ecto-nucleotidase used by melanoma sEV to generate ADO. Here we extend these findings to CD39(+)CD73(+) and CD39(+)CD73(−) sEV from breast cancer cells.

Materials and methods: sEV were isolated from supernatants of a triple-negative breast cancer cell line ± the genetic knockout of CD73. A newly developed high pressure liquid chromatography assay with fluorescence detection was used for assessment of N^6-etheno-AMP conversion to N^6-etheno-ADO by sEV. PSB12379 (selective CD73 inhibitor) and anti-CD73 antibodies were used to inhibit/neutralize CD73 activity in sEV.

Results: Untreated sEV isolated from CD39(+)CD73(+) breast cancer cells readily metabolized N^6-etheno-AMP to N^6-etheno-ADO, and this activity was abolished by PSB12379. sEV from CD39(+)CD73(−) breast cancer cells were unable to metabolize N^6-etheno-AMP to N^6-etheno-ADO. Effects of three different anti-CD73 antibodies on CD73 activity in sEV were examined. Only one antibody, the direct binding pocket inhibitor of CD73, but not antibodies that allosterically inhibit recombinant CD73, attenuated conversion of N^6-etheno-AMP to N^6-etheno-ADO by cancer-derived sEV.

Conclusions: In breast cancer-derived sEV, as in melanoma-derived sEV, CD73 is the gateway enzyme regulating ADO formation from upstream AMP. The quantitation in sEV of N^6-etheno-AMP conversion to N^6-etheno-ADO ± neutralizing anti-CD73 antibodies provides a measure of the ability of these antibodies to suppress ADO production and could potentially serve as a personalized predictor of CD73 activity in patients with cancer.

Key words: tumor-derived small extracellular vesicles, N^6-etheno-adenosine, adenosine production, neutralization by anti-CD73 antibodies

INTRODUCTION

Cancer progression is associated with suppression of anti-tumor immune responses.[1] Numerous mechanisms of tumor-induced immune defects in patients with solid or hematological malignancies have been described.[2,3] In advanced disease, the tumor microenvironment (TME) is characterized by the presence and activation of multiple immunoinhibitory pathways, which might be responsible for a lack of response of cancer patients to immunotherapy. The inability of immune checkpoint inhibitors (ICIs) to restore adequate antitumor responses has been reported to occur in 20%-50% of cancer patients treated with immune therapies.[4,5] This lack of response to ICIs has stimulated an intense search for additional factors, mechanisms and cellular components that contribute to tumor resistance to immune therapies with an overall goal to identify and silence immunosuppression.

Among various cellular and molecular pathways contributing to tumor-induced immune suppression, the adenosinergic pathways play a major role.[6] One such adenosinergic pathway is the conversion of ATP to adenosine (ADO), a pathway that is mediated by cell membrane associated ecto-nucleotidases and is accelerated in the TME, resulting in ADO-driven immune suppression.[7,8] This ATP to ADO adenosinergic pathway involves CD39 (converts ATP to ADP and ADP to AMP) and CD73 (converts AMP to ADO) and is an actionable therapeutic target in cancer. In this regard, various antibody-based therapies specific for CD39 or CD73 are currently in clinical trials to evaluate their efficacy for reducing/eliminating ecto-nucleotidase activity and restoring antitumor immunity.[9,10] We have recently

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reported that CD39 and CD73 are surface components of small extracellular vesicles (sEV), also called exosomes, produced by tumor cells and released into the extracellular space.[11] These small tumor-derived vesicles, also called TEX, are derived from the endocytic cell compartment, carry a molecular/genetic cargo that resembles parent tumor cells, resemble tumor cells functionally and mediate immune suppression *in vitro*[3,12] and *in vivo* in experimental animals.[13] TEX are ubiquitous and present in high numbers (e.g. 10^{12}/ml) in cancer patients' plasma, interact with all cell types in the TME, can produce ADO *in vitro* and *in vivo*, and can deliver CD39 and CD73 to recipient cells, endowing them with the ability to *de novo* produce ADO.[14-16] Thus, TEX have emerged as a major source of immunosuppressive exogenous ADO which mediates pro-tumor activities in cancer patients.

Numerous reports in the literature emphasize the role of CD73 as a gateway to ADO production. Specifically, it was shown that CD73 expression in triple-negative breast cancer (TNBC) is associated with worse clinical outcomes and increased resistance to chemotherapy.[17] Also, high levels of CD73 expression in epithelial tumor cells were associated with reduced disease-free survival as well as overall survival and significantly correlated with immune cell infiltration into TNBC. Patients with high levels of CD73 and low levels of tumor infiltrating leucocytes had poor clinical outcomes.[18] Additionally, antibodies that target the cell surface CD73 were shown to reduce growth of primary and metastatic tumors in mice by inhibiting catalytic activity of CD73 and thus increasing antitumor activity of cytotoxic T cells in various tumors, including TNBC.[19] In aggregate, these studies suggest that measuring levels of CD73 activity in cancer plasma might serve as an indicator of cancer-induced immunosuppression and predictor of response to therapy. ADO levels in plasma or tumor tissues, however, cannot be measured with any precision because the half-life of free ADO in human blood is only ~1 s. TEX are equipped to generate extracellular ADO, and measuring adenosinergic activity of TEX offers an alternative approach to estimate the role of ADO in tumor-induced immunosuppression. Our preliminary data suggest that levels of TEX-mediated adenosinergic activity differ among cancer patients.[11] This suggests that measurements of adenosinergic activity in TEX could potentially be useful as guidance for immunotherapy and might have prognostic value.

We recently developed a rapid, highly sensitive and specific novel assay[20] for measuring the activity of the ATP to ADO pathway in TEX and for assessing the efficacy of blocking strategies to reduce or eliminate ecto-nucleotidase activities on the TEX surface that mediate the ATP to ADO pathway. We showed that TEX produced by melanoma cells produce more ADO from ATP than sEV produced by non-malignant cells, and that CD73 in melanoma TEX is the key 'gateway' ecto-nucleotidase responsible for ADO production from upstream extracellular AMP in TEX.[20] Here, using TEX isolated from supernatants of a TNBC cell line, with and without genetic knockout (KO) of CD73, we show that these results extend to TEX derived from cancer cells other than melanoma cells. We also illustrate the effects of CD73 blockade by pharmacologic and biologic inhibitors on ADO production by breast cancer-derived TEX. This assay approach could serve as a personalized predictor of the pathological significance of CD73 activity in TEX and could gauge the susceptibility of TEX-based CD73 activity to inhibition by specific anti-CD73 antibodies or pharmacological inhibitors.

MATERIALS AND METHODS

Cell lines

A human breast cancer cell line MDA-MB-2310 (wt) and the same cell line with CD73 KO were obtained from Dr Sarita Sehra at Incyte Corp, Wilmington, DE. The cell lines were grown at 37°C in an atmosphere of 5% CO_2 in air. Cultures were routinely tested and found to be mycoplasma free. Cells were cultured in RPMI-1640 medium, 1% (v/v) penicillin/streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (Thermo-Fisher Scientific, Waltham, MA) previously depleted of extracellular vesicles by ultracentrifugation at 100 000 *g* for 3 h. Cells were cultured in 150 cm^2 cell culture flasks containing 25 ml of the culture medium. Each flask was seeded with 4×10^6 cells, and following 72 h of incubation, supernatants were collected, while the cells were harvested using 2 ml of Tryp-LE Express (Gibco, Grand Island, NY) and washed in serum-containing medium. For subsequent passages, cells were re-seeded in new flasks using the cell numbers described above. Supernatants were collected for isolation of TEX by size exclusion chromatography (SEC).

Generation of CRISPR-Cas9-mediated CD73KO MDA-MB-231 cells

Pre-designed CRISPR sgRNA (crRNA + tracrRNA) targeting three independent sequences of CD73 (NT5E, IDT, Ref 281738000, 281738001 and 281738002) were used. Additionally, CRISPR sgRNAs targeting HPRT and non-target control were used as positive and negative controls, respectively. CRISPR sgRNA were transduced to 600 k cells/gRNA by nucleofection. Single-cell clones were isolated and expanded in monolayer culture conditions. CD73 KO in cells was confirmed for the transcript and protein loss by sequencing and immunoblotting. The CD73KO MDA-MB-231 cells were generated by Dr Sarita Sehra, Incyte, Corp.

Isolation and characterization of sEV

Supernatants were collected for isolation of sEV by SEC as previously described.[21] Briefly, a 50 ml aliquot of cell culture supernatant was centrifuged at room temperature (RT) for 10 min at 2000 *g* to sediment cells and cell fragments. Further centrifugation at 10 000 g at 4°C for 30 min removed microvesicles (MVs). Next, the supernatant was filtrated using a 0.22 μm bacterial filter. Afterwards, the supernatant was concentrated to 1 ml by using Vivacell 100 concentrators (Sartorius Corporation, Bohemia, NY) at 2000 *g*.[21] For sEV isolation by SEC, an aliquot (1 ml) of

concentrated supernatant was loaded on to a 10 cm-long Sepharose 2B column and was eluted with phosphate-buffered saline (PBS). Individual 1 ml fractions were collected. Fraction 4, containing the bulk of non-aggregated morphologically intact sEV, was harvested, concentrated using 100 000 MWCO Amicon Ultracel centrifugal concentrators (UFC510096) and evaluated for protein, vesicle size, molecular content and sEV functions as illustrated in Supplementary Figure S1, available at https://doi.org/10.1016/j.iotech.2025.101052 and previously described.[22]

Transmission electron microscopy

Transmission electron microscopy (TEM) of sEV was carried out at the University of Pittsburgh Center for Biologic Imaging as previously described.[22] Freshly isolated sEV were placed on copper grids coated with 0.125% Formvar in chloroform and stained with 1% (v/v) uranyl acetate in ddH_2O. A JEM 1011 microscope was used for sEV visualization.

Western blot analysis

Following concentration of isolated sEV, vesicle aliquots were lysed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and separated using 4%-15% SDS/PAGE gels. Each lane was loaded with 10 μg of fraction 4 protein. After transfer from gels to the polyvinylidene fluoride membranes, proteins were detected using antibodies specific for CD81 (Thermo-Fisher, MA5-13548), ALIX (Thermo-Fisher, MA5-32773), calnexin (Cell Signaling Technology, #2433, Danvers, MA), CD39 (Santa Cruz #33558) and CD73 (Abcam #81720). Immunodetection by blotting showed the protein profile consistent with sEV.

NanoSight measurements

The sEV concentration and size distribution were measured by nanoparticle tracking analysis (NTA) using NanoSight 300 (Malvern, UK). The vesicles were diluted in ddH_2O and then the video image was captured at a camera level of 14. The captured videos were analyzed using NTA software, maintaining the screen gain and the detection threshold at 1 and 5, respectively. To determine mean particle size/concentration in each sample, five consecutive measurements were obtained and averaged. NanoSight measurements yielded particle sizes consistent with sEV.

Protein concentration

Protein concentrations of sEV were determined by using a BCA protein assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The ratio of EV numbers by NTA to total protein level in sEV was used to estimate relative purity of sEV.

sEV functional activity

The ability of isolated sEV (10 μg protein) to induce apoptosis of CD8-positive Jurkat T cells during a 6-h co-incubation was measured by flow cytometry using the FITC Annexin-V total (ANXV) Apoptosis Detection Kit (BD Biosciences, #55647, San Jose, CA) in a Cytoflex flow cytometer (Beckman, Indianapolis, IN) as previously described.[23] Annexin V/PI assays with isolated sEV were used to assess their intact apoptotic activity (see Supplementary Figure S1, available at https://doi.org/10.1016/j.iotech.2025.101052).

On-bead flow cytometry analysis of sEV

On-bead flow cytometry of sEV or TEX was carried out as previously described by us.[24] Briefly, sEV (10 μg/100 μl PBS) were incubated with a cocktail of biotin-labeled anti-CD63 monoclonal antibodies (0.5 μg, Biolegend, #353018) and biotin-labeled anti-CD9 monoclonal antibody (0.5 μg, Biolegend, #349514) for 2 h at RT. Next, streptavidin-coated magnetic beads (50 μl aliquot; ExoCap™, MBL International, Woburn, MA) were added to the vesicle-antibody mixtures and incubated for 2 h at RT. The bead/antibody/vesicle complexes were washed with PBS and dispersed in 50 μl PBS. For detection of target antigens, 4 μl aliquots of the complex were dispersed in 100 μl PBS and blocked with 2% mouse serum. The pre-titered labeled detection antibodies were added; beads were washed with PBS and diluted in PBS for antigen detection using flow cytometry. Antigen expression concentrations were measured as relative fluorescence intensity (RFI) calculated as the ratio of Ag RFI/isotype control RFI.

Preparation of sEV for purine analysis

Total sEV were concentrated using an Amicon ultra-filter (100 000 MWCO) and diluted in PBS for high pressure liquid chromatography with fluorescence detection (HPLC—FL) analysis at a protein concentration of 100 μg/ml. Mean estimated particle concentrations were similar in preparations of MDA-MD-231 cells (4.9×10^{10}/ml) and MDA-MD-231-KO cells (4.5×10^{10}/ml).

Assessment of N^6-etheno-AMP metabolism by sEV in breast cancer cells

Cell line-derived sEV (5 μg protein) were incubated at 37°C in 50 μl of PBS with N^6-etheno-AMP (eAMP) ± enzyme inhibitors. As recently shown, both serum[25] and tumor[26] levels of phosphate are markedly elevated in cancer patients. Therefore, we selected to use a phosphate buffer for evaluation of ecto-nucleotidase activity which would better approximate the TME. Also as recently shown, in MDA-MB-231 cells phosphate concentrations from 1 to 8 mmol/l mildly (~20%) reduce ecto-nucleotidase activity,[27] an effect mediated by generation of H_2O_2.[27] Indeed, we also see a modest reduction in eAMP metabolism with recombinant tissue-nonspecific alkaline phosphatase (5 ng) in PBS versus HEPES buffer (17.5 versus 19.3 μmol/l/20 min, $n = 2$, with 1000 μmol/l of eAMP). As we previously described,[20] to provide strong signals, high concentrations of eAMP were employed (100 μmol/l), and the incubation period of 20

min was selected in preliminary experiments to completely convert eAMP to N^6-etheno-ADO (eADO). After incubation, samples were rapidly heat inactivated at 95°C for 90 s to denature ectoenzymes, centrifuged at 4500 *g* at 4°C and diluted 10-fold before analysis of eAMP and N^6-etheno-ADO (eADO). eAMP and eADO (BioLog Life Science Institute, Hayward, CA) were quantified using HPLC—FL as recently described by us in detail.[28] We previously determined and reported the sensitivity (detection limit, 1 pmol injected on column), precision (coefficient of variation, <2%) and accuracy (excellent match between assay values versus known concentrations of standards) of this assay system.[28] Specificity was confirmed by demonstrating baseline separation of all chromatographic peaks generated from samples of a mixture of N^6-etheno-purines (ePurines) and from samples of medium conditioned by four different cell lines incubated with eATP.[28] Moreover, we confirmed that: (i) ePurines are metabolized by ecto-nucleotides with an efficiency similar to their corresponding natural substrates; (ii) there is no 'off-target' (non-nucleotidase-mediated) metabolism of ePurines; and (iii) the metabolism of ePurines is restricted to the membrane surface, i.e. is not due to intracellular nucleotidases.[28]

Selection of ecto-nucleotidase inhibitors

Many different ecto-nucleotidase inhibitors are available for pharmacological testing of the role of specific ecto-nucleotidases in the metabolism of the extracellular ATP pathway (ATP/ADP/AMP/ADO). Here, the objective was to block CD73. We selected PSB12379 at 20 µmol/l as a pharmacologic inhibitor of CD73 activity and neutralizing antibodies Cx00182, Cx00379 and Cx00167 provided by Incyte for blocking experiments. Cx00182 and Cx00379 block recombinant CD73 via allosteric inhibition, whereas Cx00167 is a direct binding pocket inhibitor. Several assays were carried out to determine where/how the antibodies bind and function, including competitor-based assays with binding in the presence of AMPCP (CD73 competitive inhibitor); these assays demonstrated that molecules binding to the active site were blocked in the presence of this competitor. Further, antibody epitope on CD73 was identified by hydrogen—deuterium exchange mass spectrometry (HDX—MS). In the CD73 blocking experiments we carried out, Cx00376 was used as an isotype control. Each antibody was incubated at a range of concentrations with substrate (eAMP, 100 µmol/l) at 30°C for 30 min and the product was measured by HPLC—FL.

Statistical analysis

Statistical analysis was conducted using NCSS 2019 Statistical Software (NCSS, LLC. Kaysville, Utah). Data were analyzed with a 1- factor analysis of variance (1F-ANOVA) and *post hoc* comparisons were conducted using the Bonferroni method. $P < 0.05$ was the criteria for significance. Values are means and standard error of means.

RESULTS

Characterization of sEV isolated from supernatants of breast cancer cells

Characteristics of sEV isolated from supernatants of MDA-MB-231(wt) are shown in Supplementary Figure S1, available at https://doi.org/10.1016/j.iotech.2025.101052 and were previously described.[23] Based on the presence of endocytic markers (ALIX, TSG101), the absence of cytosolic proteins (calnexin and grp94), vesicular morphology by TEM and their size, these vesicles were characterized as tumor-derived sEV or TEX. Further, the isolated EVs induced apoptosis of activated human T cells in Annexin V binding assays, an indication that the EVs are tumor derived.[23] The vesicular morphology by TEM, vesicle size and concentration by NTA and functions of sEV isolated from supernatants of MDA-MB-231CD73KO were comparable to those reported by us for vesicles produced by MDA-MB-231 (wt). The western blots, however, showed the absence of CD73 protein in sEV obtained from supernatants of MDA-MB-231CD73KO cells (Figure 1A). The presence of CD81 and ALIX in vesicle lysates and absence of calnexin confirms the sEV endocytic origin of these vesicles (Figure 1A). Expression levels of vesicular CD39 are somewhat higher in sEV produced by the CD73 KO cells than by sEV produced by the wt cells (Figure 1A). On-bead flow cytometry confirmed the absence of CD73 on the surface of sEV produced by MDA-MB-231CD73KO cells (Figure 1B).

Metabolism of eAMP to eADO in sEV from MDA-MB-231 cells

Cancer sEV were prepared from MDA-MB-231 cells as previously described for melanoma cell-derived sEV.[28] Here, breast cancer sEV (5 µg) were incubated with eAMP (100 µmol/l) and analyzed for eADO as described in Methods. In 20 min, MDA-MB-231 sEV converted 100% of eAMP to eADO (Figure 2). These results confirm an important role for CD73 in breast cancer sEV.

CD73 as a 'gateway' ecto-nucleotidase

In our previously reported experiments with sEV derived from melanoma cells, CD73 was shown to be the only ecto-nucleotidase on these sEV that mediates the conversion of eAMP to eADO, i.e. CD73 is the ecto-enzyme which metabolizes all AMP to ADO in melanoma sEV. We therefore refer to CD73 as the 'gateway' ecto-nucleotidase on these melanoma sEV that is responsible for biosynthesis of ADO from upstream AMP. Note that we do not refer to CD73 as the 'rate-limiting enzyme' since in the absence of inhibitors, the rate of ADO production from AMP would depend on the rate of AMP production from upstream ecto-nucleotidases (that is until AMP reaches concentrations that drive CD73 activity to Vmax). To determine whether CD73 plays a similar 'gateway' role in sEV derived from breast cancer cells, we further examined the metabolism of eAMP to eADO in sEV obtained from supernatants of breast cancer

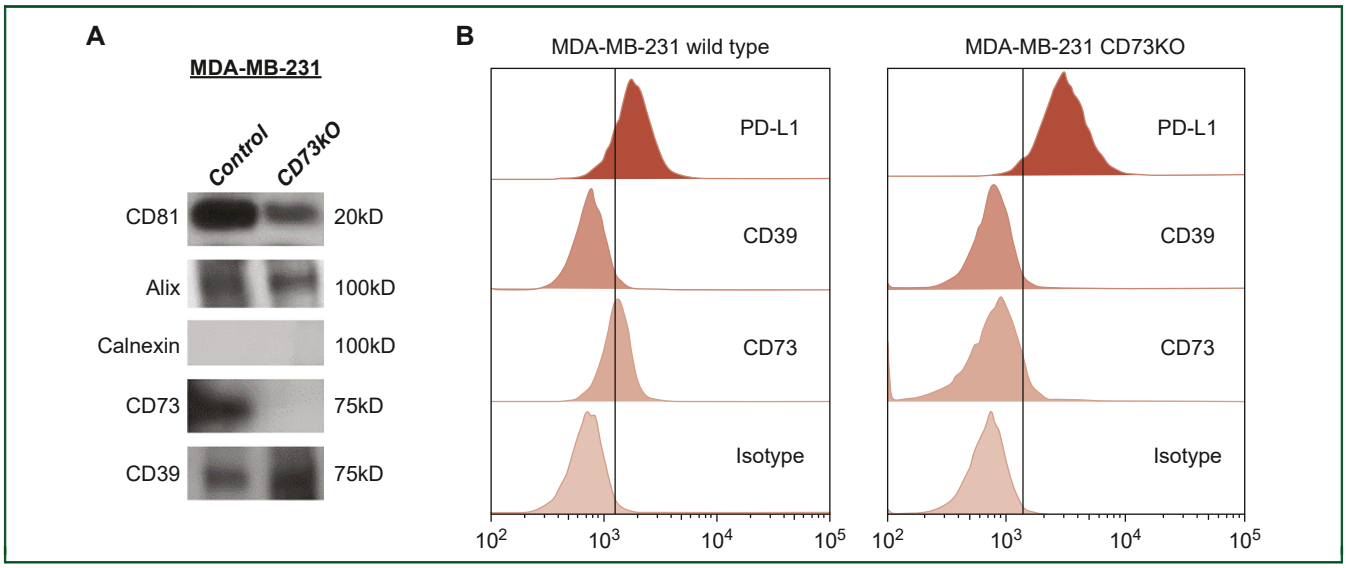

Figure 1. Validation of MDA-MB-231 sEV. (A) Western blot of protein lysates from wt MDA-MB-231 EV (control) versus CD73KOMDA-MB-231 EV. (B) On-bead flow cytometry results for expression of CD73 or CD39 on the surface of EV from control and CD73KOMDA-MB-231 EV. Note that CD39 was present in the vesicle lumen but was not detected on the EV surface by on-bead flow cytometry in either wt or CD73KO EV.
EV, extracellular vesicles; sEV, small extracellular vesicles; wt, wild type.

cells. In contrast to MDA-MB-231 sEV, MDA-MB-231-CD73KO sEV lacking CD73 were unable to metabolize any eAMP to eADO (Figure 2), as were MDA-MB-231 sEV treated with the potent and highly selective CD73 inhibitor PSB12379 (Figure 2).

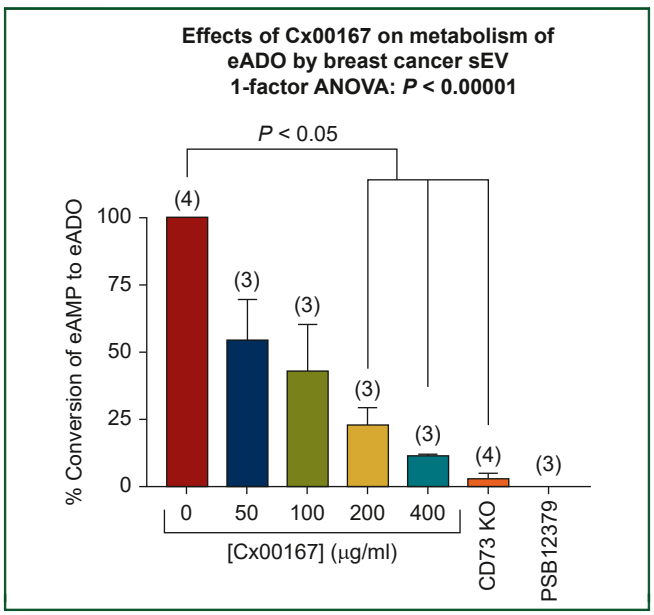

Figure 2. Metabolism of eAMP to eADO in sEV from MDA-MB-231 cells: effects of CD73 knockout and pharmacological and immunological inhibition of CD73. Cancer sEV were prepared from MDA-MD-231 and MDA-MB-231-CD73 KO cells as described in Methods. Cancer sEV (5 μg/50 μl) were incubated with eAMP (100 μmol/l) and analyzed for eADO. In 20 min, MDA-MB-231 sEV converted 100% of eAMP to eADO. In contrast to MDA-MB-231 sEV, the metabolism of eAMP to eADO was nearly abolished in MDA-MB-231-CD73KO sEV (CD73 KO) and was abolished in MDA-MB-231 sEV treated with the CD73 inhibitor PSB12379. Anti-CD73 antibody (Cx00167; Incyte) also inhibited, in a concentration-dependent manner, metabolism of eAMP to eADO in MDA-MB-231 sEV. There were no significant differences between eAMP metabolism in MDA-MB-231 sEV treated with Cx00167 at 200 and 400 μg/ml and MDA-MB-231-CD73 KO sEV (CD73KO). Values represent means and standard errors of mean with a sample size of $n = 3$-4, as indicated in parentheses.
ADO, adenosine; ANOVA, analysis of variance; KO, knockout; sEV, small extracellular vesicles.

The anti-CD73 antibody Cx00167 blocks eAMP metabolism to eADO in sEV from MDA-MB-231 cells

We also examined the effects of an anti-CD73 antibody (Cx00167; Incyte) on metabolism of eAMP to eADO in MDA-MB-231 sEV. Cx00167 is known to target the substrate binding pocket of CD73. Here we observed that Cx00167 caused a concentration-dependent inhibition of eAMP metabolism to eADO with efficacy similar to knocking out CD73 (Figure 2). Unlike Cx00167, antibody Cx00376, an inactive isotype control antibody, did not affect eAMP conversion to eADO in sEV from MDA-MB-231 cells (Figure 3A).

The data presented in Figure 2 convey three important conclusions: (i) that antibody Cx00167 inhibits AMP metabolism; (ii) that AMP metabolism here is mediated by CD73 (hence blocked by CD73KO and by PBS12379); and (iii) that Cx00167 for all intents and purposes blocks CD73 with an efficacy which is as good as CD73KO or the pharmacological inhibition of CD73.

Effects of anti-CD73 antibodies Cx00182 and Cx00379 on eAMP metabolism to eADO in sEV from MDA-MB-231 cells

We also examined whether antibodies Cx00182 and Cx00379 (Incyte) inhibit metabolism of eAMP to eADO by MDA-MB-231 sEV. Whereas antibody Cx00167 significantly blocked CD73 activity at 200 μg/ml and showed a strong tendency to do so at 50 and 100 μg/ml (Figure 2), neither Cx00182 (Figure 3B) nor Cx00379 (Figure 3C) at 50, 100 or 200 μg/ml inhibited metabolism of eAMP to eADO in MDA-MB-231 sEV. Importantly, unlike Cx00167, both Cx00182 and Cx00379 target an allosteric site on CD73 rather than the binding pocket.

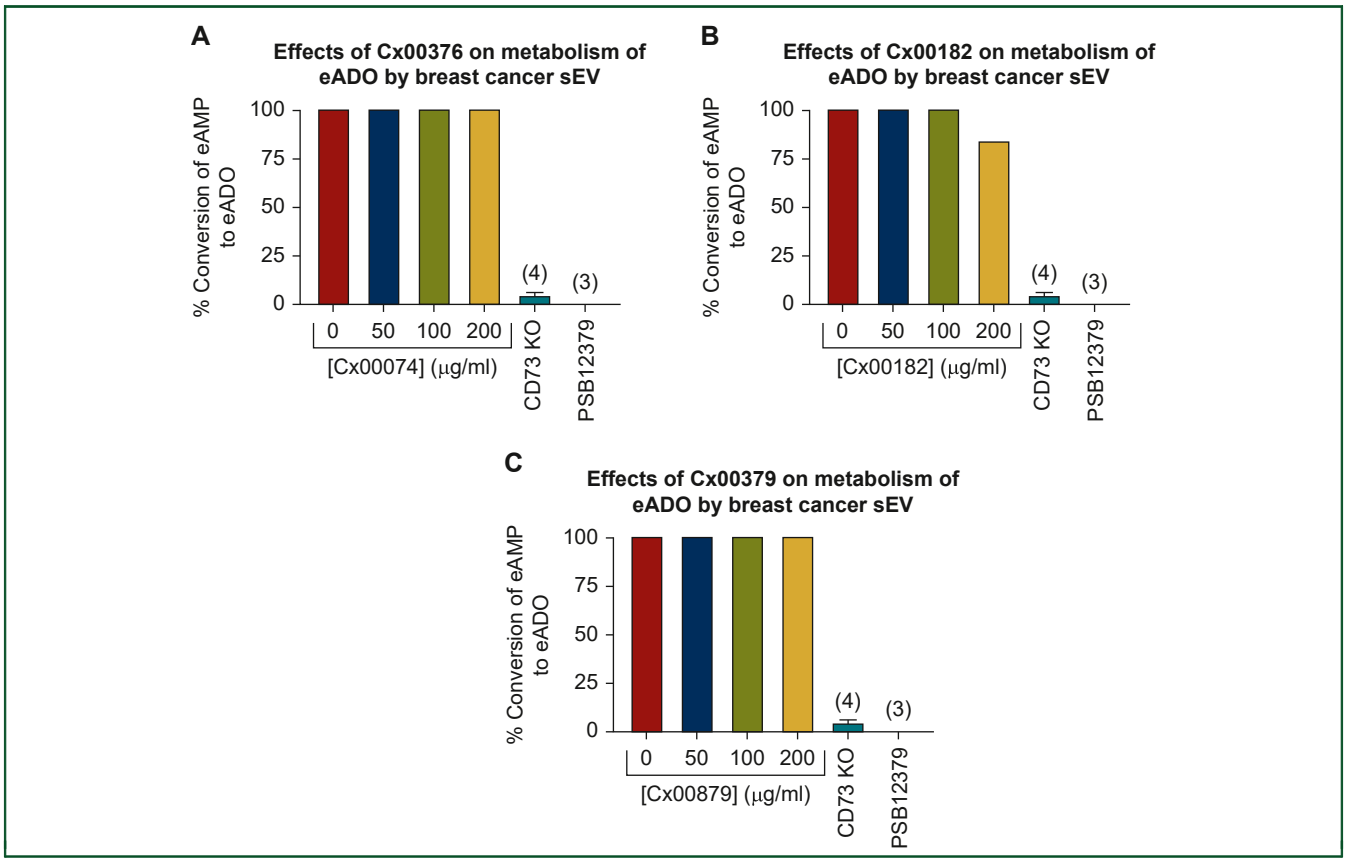

Figure 3. Metabolism of eAMP to eADO in sEV from MDA-MB-231 cells: no effect of antibodies Cx00182 or Cx00379. Cancer sEV were prepared from MDA-MB-231 cells as described in Methods. Cancer sEV (5 μg/50 μl) were incubated with eAMP (100 μmol/l) and analyzed for eADO. In 20 min, MDA-MB-231 sEV converted 100% of eAMP to eADO. Neither Cx00376 (inactive isotype control), Cx00182 nor Cx00379 affected the conversion of eAMP to eADO at concentrations of 50, 100 or 200 μg/ml (one experiment per concentration, three experiments total for each antibody). For reference, included in these bar graphs are the effects of the CD73 inhibitor, PSB12379, and knockout of CD73 (CD73KO) from Figure 2.
ADO, adenosine; sEV, small extracellular vesicles.

DISCUSSION

The finding that tumor-derived sEV (TEX) are a major source of extracellular ADO in plasma of cancer patients is paradigm shifting. Unlike soluble ADO in plasma, which has a very short half-life, ADO in TEX lumen is protected from exogenous degradation. Moreover, CD39-positive CD73-positive sEV interacting with and entering recipient cells can produce ADO at the tumor site and even within the tumor. It is important to remember that TEX are ubiquitous in cancer plasma and their numbers are significantly increased in the circulation of cancer patients relative to those in healthy donors.[29-31] We reported earlier that TEX readily metabolize ATP to ADO and that of 20 different purines, ADO is the most abundant purine in TEX.[11] Thus, it appears that the sEV-associated ecto-nucleotidases are the major producers of biologically active ADO in cancer. Further, TEX carrying enzymatically active CD39/CD73 can deliver the ectoenzymes and intraluminal ADO to various recipient cells, including immune cells responsible for anti-tumor immunity, and thereby disseminate and enhance immune suppression in the TME.[15,32]

Of the ecto-nucleotidases present on the surface of sEV, CD73 on melanoma sEV was recently shown by us to be the 'gateway' ecto-enzyme (the primary ecto-enzyme) that converts AMP to ADO.[20] Although the production rate of ADO may be limited by upstream AMP production, any extracellular AMP in the biophase of tumor-derived sEV must be processed by CD73 to generate ADO, regardless of the source of AMP. Here, we demonstrate that the pharmacologic or immunologic blockade of CD73 activity or its genetic silencing in TEX-producing tumor cells completely inhibits ADO production by TEX. Thus, this report extends our results from melanoma TEX to breast cancer TEX, suggesting that activation of the adenosinergic pathway in TEX and its regulation at the level of AMP conversion to ADO represent a generalized phenomenon in cancer. Consequently, targeting CD73 for inhibition would prevent ADO production from AMP regardless of the source of AMP and source of sEV; thus, CD73 represents an ideal therapeutic target for immune checkpoint inhibition in cancer.

Here, we have also demonstrated effective *in vitro* inhibition of ADO production by TEX using a neutralizing anti-CD73 antibody from Incyte. Based on our data, it appears that ADO-producing CD73-positive TEX, rather than tumor cells *per se*, are major therapeutic targets for antibody-induced suppression of CD73 activity. Of note, not all anti-CD73 antibodies effectively suppress CD73 activity in sEV. Cx00167 targets the binding pocket of CD73 and effectively blocks CD73 activity. In contrast, Cx00182 and Cx00379 that target an allosteric site on CD73 do not

potently suppress CD73 activity. CD73 forms oligomers in membranes, and these higher-level structures of CD73, which likely vary depending on the nature of the host cell membrane, may restrict the binding sites of some anti-CD73 antibodies to their targets.

The ability to reliably measure neutralizing activity of anti-CD73 antibodies, using an assay in which TEX isolated from the cancer patient's plasma serve as test materials, may be useful for estimation of ADO-induced immune suppression. The quantitation of AMP to ADO metabolic activity might offer a personalized measure of response to antibody-based therapy. As the isolation of TEX from body fluids of cancer patients is readily achievable, the *in vitro* assessment in TEX of ADO production from AMP as a substrate could potentially become a measure of existing immune suppression. At the same time, the ability to quantitate the ability of antibodies to neutralize TEX-associated ADO could serve as a predictor of each patient's capability to respond to a given anti-CD73 antibody therapy.

In conclusion, our findings suggest that testing the effectiveness of anti-CD73 antibodies against TEX CD73 may be more predictive of therapeutic efficacy than testing these antibodies with recombinant soluble CD73 or CD73 expressed on tumor cells. Testing of TEX for both basal CD73 activity and effectiveness of anti-CD73 antibodies to suppress this activity may help guide patient and antibody selection for optimal outcomes. Recently, we have successfully applied the assay we developed to testing of EVs from patients with cancer and healthy donors, confirming the assay utility in the translational setting.[33] Further studies are necessary to confirm the assay value for measuring metabolism of AMP to ADO as a potential biomarker of response to immunotherapies.

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DISCLOSURE

The authors have declared no conflicts of interest.

DATA SHARING

Data are available from the corresponding author upon reasonable request.

CONSENT FOR PUBLICATION

All co-authors agreed to publication of the acquired data.

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