Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production

Aled Clayton, Saly Al-Taei, Jason Webber, Malcolm D. Mason and Zsuzsanna Tabi

J Immunol published online 15 June 2011
http://www.jimmunol.org/content/early/2011/06/15/jimmunol.1003884

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/06/15/jimmunol.1003884.4.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production

Aled Clayton, Saly Al-Taei, Jason Webber, Malcolm D. Mason, and Zsuzsanna Tabi

Extracellular adenosine is elevated in cancer tissue, and it negatively regulates local immune responses. Adenosine production from extracellular ATP has attracted attention as a mechanism of regulatory T cell-mediated immune regulation. In this study, we examined whether small vesicles secreted by cancer cells, called exosomes, contribute to extracellular adenosine production and hence modulate immune effector cells indirectly. We found exosomes from diverse cancer cell types exhibit potent ATP- and 5′-AMP-phosphohydrololytic activity, partly attributed to exosomally expressed CD39 and CD73, respectively. Comparable levels of activity were seen with exosomes from pleural effusions of mesothelioma patients. In such fluids, exosomes accounted for 20% of the total ATP-hydrolytic activity. Exosomes can perform both hydrolytic steps sequentially to form adenosine from ATP. This exosome-generated adenosine can trigger a cAMP response in adenosine A2A receptor-positive but not A2A receptor-negative cells. Similarly, significantly elevated cAMP was also triggered in Jurkat cells by adding exosomes with ATP but not by adding exosomes or ATP alone. A proportion of healthy donor T cells constitutively express CD39 and/or CD73. Activation of T cells by CD3/CD28 cross-linking could be inhibited by exogenously added 5′-AMP in a CD73-dependent manner. However, 5′-AMP converted to adenosine by exosomes inhibits T cell activation independently of T cell CD73 expression. This T cell inhibition was mediated through the adenosine A2A receptor. In summary, the data highlight exosome enzymatic activity in the production of extracellular adenosine, and this may play a contributory role in negative modulation of T cells in the tumor environment.

The sequential activities of a pair of molecules catalyze the two-step process to produce adenosine extracellularly. CD39 (ectonucleoside triphosphate diphosphohydrolase-1) converts extracellular ATP (or ADP) to 5′-AMP. Thereafter, CD73 (5′-nucleotidase) converts 5′-AMP to adenosine. Natural regulatory T cells of mice express both CD39 and CD73, whose coordinated enzymatic actions produce adenosine as a mechanism for immune suppression that is independent of IL-10 and TGF-β (12, 13). Several cancer cell types also express CD39 and CD73, whose coordinated enzymatic actions produce adenosine from extracellular substrate directly, without the requirement of nucleotidases expressed by infiltrating cells or by endothelial cells. In murine cancer models, targeting cancer cell CD73 inhibits tumor growth not only by direct inhibition of tumor cell migration and metastasis (16) but also by freeing antitumor T cells from the suppressive effects of extracellular adenosine (15, 17).

Adenosine receptors (of four known subtypes A1, A2A, A2B, and A3) are expressed broadly by leukocytes such as neutrophils and macrophages (expressing all four receptors), B lymphocytes (A2A), and T cells (A2A, A2B, and A3). These primarily drive anti-inflammatory effects such as reduced TNF-α, IL-1β, IL-2, IFN-γ, and elevated IL-10 secretion (5, 18–21), although the precise cellular response can be influenced by other factors (22). The predominant adenosine receptor expressed by T cells is the high-affinity A2A receptor, which becomes elevated after cell activation (23). This induction may render activated T cells more sensitive to adenosine-mediated suppression when they migrate into the tumor microenvironment. Signals emanating from A2A trigger intracellular cAMP production (23) and increase Src-homology region 2 domain-containing phosphatase-2, dephosphorylating STAT-5 (24). This pathway can inhibit responses to IL-2 and TCR ligation providing a potent mode for attenuating multiple T cell effector functions (19, 25). The same receptor can attenuate NK cell cytotoxic production and cytokotoxic activity (26, 27), and promote the generation of regulatory T cells (Treg) (28). Adenosine also
impairs maturation and function of dendritic cells, but these effects are mediated principally through the A<sub>3</sub> receptor (29).

We recently showed that CD73 is expressed by nanometer-sized vesicles called exosomes, secreted by human bladder cancer cells (30). In this report, we address the hypothesis that cancer cell-derived exosomes may use CD73 or other expressed phosphatases such as CD39 to participate in extracellular adenosine production. Our data reveal ATP- and 5'-AMP-hydrolitic activities are associated with exosomes isolated from several cancer cell lines or from malignant effusions of mesothelioma patients. This exosomally associated enzyme activity accounted for ∼20% of the total ATP-hydrolitic activity within freshly obtained pleural fluid. Such enzyme activity leads to formation of adenosine, which in turn is associated with exosomes isolated from several cancer cell lines or from malignant effusions of mesothelioma patients. This exosomally associated enzyme activity accounted for ∼20% of the total ATP-hydrolitic activity within freshly obtained pleural fluid. Such enzyme activity leads to formation of adenosine, which in turn may contribute toward negative regulation of T cell function. We propose this mechanism may have broad implications for attenuation of effector immune responses in cancer and in other settings.

Materials and Methods

Cell culture

Human cancer cell lines, as a source of exosomes, included bladder-HT1376 and colorectal-CACO2 (from Cancer Research-UK, London, U.K.), prostate-DU145 and PC3 (from American Type Culture Collection, Teddington, U.K.), and the breast cancer line MCF7 (from ECACC, Salisbury, U.K.). All purchased cells were obtained during 2008–2009 and maintained for <6 mo in culture. Lines were authenticated by the supplier cell bank by combinations of cytogenetic, isoenzyme, and DNA-profile analyses. A mesothelioma cell line (Meso) was developed in the department from pleural effusions (31, 32). The HT1376 cells were maintained in DMEM supplemented with nonessential amino acids, penicillin, streptomycin, and 5% FBS (which had been depleted of exosomes by overnight ultracentrifugation at 100,000 × g). All other cells were maintained in RPMI 1640 with the same supplements excluding nonessential amino acids. The cells were seeded into bioceiver flasks (Integra Biosciences, SLS, Nottingham, U.K.) and maintained at high-density culture for exosome production as described (33). Cells were confirmed negative for mycoplasma contamination by monthly screening (Mycoalert; Lonza, Slough, U.K.).

Collection of pleural fluid

Pleural fluid was collected from patients with malignant pleural mesothelioma into sterile containers at the patients’ own homes. The collections took 2–24 h depending on the volume present in the patient. Samples were kept at room temperature during collection and during transport to the laboratory, which took 20–30 min. Fluid was subjected to centrifugation (300 × g, 10 min, twice) to remove cells and the supernatants processed for exosome purification as described later. In some experiments, cells and thereafter exosomes were removed from the fluid by centrifugation, and the impact of this on ATP-hydrolitic capacity of the pleural fluid was measured. Ethical approval for obtaining patient-derived materials or blood from healthy donors was obtained from the South East Wales Research Ethics Committee.

Exosome purification

Cell culture medium was subjected to serial centrifugation, removing cells (300 × g, 10 min) and removing noncellular debris (2000 × g for 15 min). The supernatant was then centrifuged at 10,000 × g for 30 min, and the supernatant was retained. This was underlayered with a 30% sucrose/D<sub>2</sub>O cushion and subjected to ultracentrifugation at 100,000 × g for 2 h. Sucrose and D<sub>2</sub>O was purchased from Sigma (Dorset, U.K.) and maintained at high-density culture for exosome production as described (33). Cells were confirmed negative for mycoplasma contamination by monthly screening (Mycoalert; Lonza, Slough, U.K.).

Determining exosome density

To quantify the density of exosomes, we used a protocol similar to that previously described, based on ultracentrifugation on a linear sucrose gradient (36). Briefly, cell culture medium was preclarified of debris by 30 min centrifugation at 10,000 × g. The supernatant was subjected to ultracentrifugation at 100,000 × g to pellet exosomes, and the pellet was resuspended in 300 µl PBS. The preparation was overlaid on a continuous sucrose gradient (0.2 M sucrose to 2.5 M sucrose). Specimens were centrifuged at 4°C, overnight at 210,000 × g, using an MLS-50 rotor in an Optima-Max ultracentrifuge (Beckman Coulter). Fractions of 300 µl were collected (from the top of the tube), and the refractive index (at 20°C) was measured using an automatic refractometer (J57WR-SV; Rudolph Research Analytical, Hackettstown, NJ). This instrument is subject to weekly 2-point calibration (with H<sub>2</sub>O and calibration standard certified as 1.36099 ± 0.00001 at 20°C by Cargille Laboratories, Cedar Grove, NJ). The density (g/ml) of each fraction was calculated from the published table in the Beckman Ultracentrifugation catalogue as described (37). Each fraction was washed in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.025 M MES, 125 mM NaCl), and subjected to 150,000 × g ultracentrifugation. After removing all supernatant, pelleted material was resuspended in 30 µl MES buffer. This was split 1/2 for hydrolysis activity assays and 2/3 for Western blotting as described (38).

Measuring ATP-hydrolitic activity in exosome preparations

The ApoGlow kit (Lonzra), was used to measure ATP-hydrolis activity associated with exosomes. In brief, exosomes were incubated at room temperature with typically 1000–4000 nM ATP (Sigma), and after incubations (5–60 min), Nucleotide Monitoring Reagent (containing luciferin) was added. The plate was gently shaken for 1 min, and light was measured (1 s, integrated) using a Wallac Victor-2 Multilabel Counter (PerkinElmer, Cambridge, U.K.). Values were compared with a standard curve to quantify ATP levels. The sensitivity of the assay was 10–20 nM ATP. The CD39 inhibitor sodium metatungstate (POM-1) (38) (Tocris Bioscience, Bristol, U.K.) was found to interfere with this luciferase assay, hence production of inorganic phosphate from ATP was measured by the malachite green assay (described later).

Immuno-affinity depletion of exosomes

To examine the effect CD39- or CD73-positive exosomes on ATP hydrolysis, we incubated purified exosomes (10 µg/sample) with primary Ab (10 µg/sample) (BioLegend, Cambridge, U.K.) in a final volume of 0.5 µl in PBS. After overnight incubation, rolling at 4°C, 1 ml PBS was added and exosomes were pelleted at 100,000 × g for 10 min. Pelleted exosomes were resuspended in 10 µl PBS, prior to adding 30 µl protein G-coated Dynal beads (Invitrogen, Paisley, U.K.). Samples were shaken at room temperature for 3 h, and beads were pelleted using a magnet. The ATP-hydrolitic activity associated with the bead-free supernatants or in association with the beads was measured as above after 60-min incubation with ATP substrate (2000 nM). As a positive control, an Ab against CD9 was used (R&D Systems, Abingdon, U.K.). As a control for nonspecific binding, we used the same dose of IgG1 isotype-matched Ab (BioLegend), or no Ab.

Measuring 5’AMP-hydrolitic activity in exosome preparations

The malachite green colorimetric assay was used to quantify the production of inorganic phosphate from ATP or 5’AMP substrate (Sigma) using the SensoLyte kit (AnaSpec, Fremont, CA). Exosomes that had been previously prepared and frozen in PBS required washing to remove this phosphate that would significantly interfere with the assay. This was done by adding excess volume of MES buffer (0.025 M MES, 125 mM NaCl) and re-pelleting exosomes by ultracentrifugation. 5’AMP diluted in MES buffer at doses up to 500 µM was added to specified exosome doses, and after incubation at room temperature for 30 min, the copper-based colorimetric reagent was added and absorbance measured at 660 nm after 5-min gentle shaking. Values were compared with a standard curve to quantify phosphate levels. The sensitivity of the assay was 0.39–0.78 µM phosphate. The CD73 inhibitor α,β-methylene-ADP (APCP, Sigma) was used in some assays.

Measuring cAMP production in response to adenosine

An adenin e A<sub>3</sub> receptor-overexpressing HEK293 cell line (CB-80200-26) was obtained from Codex Biosolutions (Montgomery Village, MD) and confirmed by real-time quantitative PCR to express ≈70-fold the A<sub>3</sub> receptor mRNA levels of the parent cell (data not shown). The cells were cultured to 70–80% confluence in DMEM/10% FBS in 6-well plates and incubated with 50 µM of a cAMP phosphodiesterase inhibitor [4-(3-butoxy-4-methoxy-benzyl)imidazolidin-2-one; Sigma] 30 min prior to addition of adenosine or other stimuli. After 20 min incubation at 37°C, the cells were lysed on ice and cAMP levels determined using the reagents of the manufacturer’s protocol for the Parameter cAMP immunoassay kit (R&D Systems). Similarly, the Jurkat T cell line (ECACC, Salisbury, U.K.) was also used in some cAMP assays.
T cell purification and analyses

PBMCs of healthy donors were isolated by centrifugation on a Histopaque gradient (Sigma). Flow cytometric analysis of PBMCs was performed with a FACSCanto cytomter running FACS Diva version 6.1.2 software (Becton Dickinson, Oxford, U.K.). Compensation was performed for each Ab panel using FACScomp beads (Becton Dickinson). Conjugated Abs used included CD39–allophycocyanin (BioLegend), Foxp3–FITC, CD24–PE–Cy5, CD3–FITC (eBioscience, Hatfield, U.K.), CD73–PE, CD8–PE–Cy7, and CD4–allophycocyanin–Cy7 (Becton Dickinson). CD3+ T cells were purified using immuno-magnetic beads (Miltenyi Biotec, Surrey, U.K.), according to the manufacturer’s protocols. For functional analyses, purified T cells (10^6 cells/well) were activated using CD3/CD28 Ab-coated beads (Invitrogen), at a bead to cell ratio of 2:1. Doses of 5′AMP (0 to 50 μM) were added at the same time. For some treatments, the 5′AMP substrate was pretreated with exosomes (at concentrations of 100–150 μg/ml) for 1 h and the exosomes removed by ultracentrifugation (150,000 g, 1 h) in an Optima-Max ultracentrifuge in a TLA110 rotor. The supernatant above the exosome pellet was carefully removed and used as “exosome-modified 5′AMP.” At day 3, proliferation was assessed by pulsing cells with [3H]thymidine as described (32), and cytokines (IL-2 and TNF-α) were quantified using DuoSet ELISA, following the manufacturer’s protocol (R&D Systems). The adenosine A_2A receptor antagonist SCH 58261 (Sigma) was used in some assays.

Statistics

Comparisons between treatments was made using one-way ANOVA with Tukey’s multiple comparison test or using two-way ANOVA with Bonferroni posttests. The p values <0.05 were considered significant. These were calculated using Prism-4 (version 4.03) graphing and statistical software (GraphPad, San Diego, CA).

Results

Cancer exosomes exhibit ATP-hydrolytic activity, which is partly CD39 dependent

For our first approach, we analyzed exosomes from HT1376 bladder cancer cells and took advantage of the natural property of exosomal vesicles to float at characteristic densities when subjected to ultracentrifugation on continuous sucrose gradients (0.2–2.5 M sucrose range). Classically, exosomes float at densities of ~1.1–1.2 g/ml, and this property is a useful analytical tool for discriminating exosomal from non-exosomal factors secreted by cancer cells. Fractions collected from the gradients were subjected to refractometry to quantify the density, and the capacity of each fraction to hydrolyze ATP was measured using a luciferase-based ATP assay. Fractions at densities of <1.1 g/ml showed no loss in the starting level of ATP after 30 min incubation. However, there was almost a total loss in ATP, reaching the detection limits of the assay, with fractions of 1.1–1.2 g/ml density. Fractions of densities >1.2 g/ml contained some ATP-hydrolytic activity, but this diminished with increasing density (Fig. 1A). The presence of TSG101 protein, commonly used as a marker to indicate a multivesicular body origin of vesicles (i.e., exosomes), coincided in fractions harboring ATP-hydrolytic function. Similarly, CD39 was also detected within the fractions bearing ATP-hydrolytic capacity (Fig. 1A, Western blot).

Next examined if this activity was present in exosomes isolated from other cell types. Exosomes were purified using the 30% sucrose/D_2O cushion method, and after quantification by protein assay, the ATP-hydrolytic activity was measured as described above as a function of exosome dose. Whereas hydrolysis of ATP was clearly demonstrable in exosomes from various cell types, there were considerable differences in the level of activity among the cellular sources (Fig. 1B). The breast cancer cell line MCF7 demonstrated very strong hydrolysis of ATP, with almost complete elimination (99% decrease) of ATP levels after 30-min incubation using only 5 μg of exosomes/well. In contrast, exosomes isolated from a mesothelioma cell line showed weak ATP-hydrolytic activity, with only a 2% decrease in ATP levels under the same conditions. CD39 was present in the fractions containing peak hydrolytic activity (Fig. 1A), suggesting it may play a role in this reaction. We briefly examined the relative levels of CD39 in cell lysates and exosomes by Western blot (Supplemental Fig. 1). In the three cell lines examined, a band for CD39 was detectable within cell lysates. In comparison, exosomes revealed detectable but relatively weak staining for CD39. Given this weak staining, it was not readily possible to correlate the level of ATP hydrolysis with CD39 expression levels by exosomes, and because cell surface CD39 measured by flow cytometry (Supplemental Fig. 1, bars) was broadly comparable, this could not account for the differences seen in exosomal ATP-hydrolytic activity (Fig. 1B). To address possible involvement of CD39 in ATP hydrolysis, sucrose cushion-purified exosomes were assessed for ATP hydrolysis in...
the presence of increasing doses of POM-1, an inhibitor of CD39 (38). For this assay, we measured phosphate production, using the malachite green assay, as POM-1 had some luciferase-inhibitory effect. The assay revealed POM-1 inhibition of exosomal ATP hydrolysis (up to 50% at 50 μM POM-1) indicating CD39 is at least partly responsible for the hydrolytic activity (Fig. 1C). We also attempted to examine the effect of eliminating CD39-positive exosomes on ATP-hydrolytic activity, using immunoaffinity depletion with protein G beads. Using an Ab against the tetraspanin CD9, which is very strongly expressed by carcinoma exosomes (A. Clayton and J. Webber, unpublished observations), we achieved ~90% reduction of ATP hydrolysis in the supernatant, whereas the corresponding bead-associated material revealed strong ATP hydrolysis (of >98%), consistent with an efficient immunoprecipitation of exosomes. In contrast, this level of efficiency was not achieved using several different Abs against CD73 or CD39, an issue related to either Ab affinity or more likely due to relatively low levels of expression of these molecules on the exosome surface that make this approach comparatively inefficient. Nevertheless, immuno-depletion with either of these Abs resulted in reduced ATP hydrolysis (of ~30% and 20%, respectively), whereas isotype-matched control Ab or control samples lacking Ab showed no loss in ATP-hydrolytic function (Fig. 1D, left). There was some general nonspecific binding of exosomes to the protein G beads, but the ATP-hydrolytic activity related to the beads was at least twice this background level when using CD39 or CD73 Ab (Fig. 1D, right). The data show ATP hydrolysis is associated with CD9-, CD73-, and CD39-positive exosomes. Furthermore, targeting CD9 by chemical inhibition or immuno-depletion methods is not sufficient to eliminate fully this ATP-hydrolytic activity. This highlights the possibility that additional exosomal constituents may also exhibit ATP-hydrolytic activity in a CD39-independent manner.

Cancer exosomes exhibit 5′AMP-hydrolytic activity, which is CD73 dependent

We also performed similar experiments to investigate whether exosomes exhibit 5′AMP-hydrolytic activity. Fractions from continuous sucrose gradients were assessed for their capacity to produce inorganic phosphate from exogenously added 5′AMP substrate. This was measured colorimetrically using the malachite green assay after 30-min incubation of each fraction with 5′AMP. This yielded similar results as for ATP hydrolysis, with the 5′AMP-hydrolytic activity focused to fractions of classical exosomal density, with little detectable at densities <1.1 g/ml or >1.22 g/ml (Fig. 2A).

We also observed differences in hydrolysis of 5′AMP between different exosome sources (Fig. 2B). Those exosomes that exhibited strong ATP hydrolysis (e.g., CACO2) did not necessarily harbor strong 5′AMP activity, and similarly DU145 exosomes, which were potent at 5′AMP hydrolysis, were among the poorest at ATP hydrolysis (Figs. 1B, 2B). Nevertheless, all cancer exosomes tested to date exhibit some level of ATP and 5′AMP hydrolytic activity.

We investigated if CD73 was the principal molecule mediating 5′AMP hydrolysis. HT1376 exosomes were incubated with increasing doses of 5′AMP in the absence or presence of the CD73-specific inhibitor APCP. The lowest dose of APCP (10 μM) was sufficient to attenuate phosphate generation by exosomes by >75% (at 500 μM 5′AMP dose), and increasing the dose of APCP 20-fold only slightly inhibited this further (83% inhibition at 500 μM 5′AMP) (Fig. 2C). The data show CD73 to be responsible principally for the exosomal 5′AMP-hydrolytic activity.

In summary, we show that cancer exosomes exhibit striking ATP and 5′AMP hydrolytic activities involving exosomal CD39 and CD73, respectively.

Exosomes in malignant effusions contribute to ATP-hydrolytic function

To examine the possible physiological relevance of ATP hydrolysis by exosomes, we performed some analyses of pleural fluid taken from patients with pleural malignant mesothelioma. This fluid is a tumor effusion previously described by us and others as containing exosomes (31, 39), and although it does not represent the tumor microenvironment per se, it does reflect an in vivo tumor-conditioned environment.

We first isolated exosomes from pleural fluid of three donors and examined the ATP-hydrolytic activity of such exosomes, comparing directly with exosomes from the HT1376 cell line (Fig. 3A). Data reveal exosomes isolated from this ex vivo source do exhibit ATP-hydrolytic activity that is generally comparable with if not more potent than that of HT1376-derived exosomes. The physiological levels of exosomes within this fluid are ~3 to 4 μg/ml (Supplemental Fig. 2A). Although this is lower than the exosome doses used in these assays, we can confirm that ATP substrate is significantly hydrolyzed given sufficient time of incubation with low-dose exosomes (Supplemental Fig. 2B).

We next examined what proportion of the total ATP-hydrolytic activity within the pleural fluid is attributable to the exosomes therein. A fresh pleural fluid specimen was subject to differential centrifugation. Native (untouched) fluid exhibited strong ATP hydrolysis, eliminating ~90% of available substrate (Fig. 3B). However, by removing the mixed cellular component of the fluid

![FIGURE 2. Cancer exosomes can hydrolyze exogenous 5′AMP. A. The sucrose gradient method was used to examine hydrolysis of exogenously added 5′AMP (100 μM). Levels of inorganic phosphate were quantified after 30 min at room temperature and plotted against fraction density. B, Exosomes (5–20 μg/well) purified by sucrose/D2O cushion from assorted cancer cell types were incubated with 5′AMP (50 μM) for 30 min at room temperature. Graph represents percent hydrolysis of substrate against exosome dose. C, The capacity of HT1376 exosomes to hydrolyze 5′AMP in the presence of the CD73 inhibitor APCP was almost entirely abrogated, demonstrating CD73 as a principal mediator of 5′AMP hydrolysis by exosomes. Symbols represent mean ± SEM of triplicates. ***p < 0.001 at each inhibitor dose (two-way ANOVA with Bonferroni post tests).](http://www.jimmunol.org/guestonJune122017)
The supernatant was drastically reduced to ∼demonstrate this, we used an HEK293 cell line that overexpresses Cancer exosomes convert ATP to adenosine and trigger human pleural effusions. Nevertheless, a factor contributing toward the total activity within the principal source of enzymes driving ATP hydrolysis, they are together, the data demonstrate that although exosomes are not centrifugation at 100,000 g, the majority of hydrolytic activity. The residual activity present indicating that it is cells present within this environment that drive the human pleural effusions.

Cancer exosomes convert ATP to adenosine and trigger intracellular cAMP production

We next examined whether exosomes were capable of converting exogenous ATP to form adenosine in a sequential manner. To demonstrate this, we used an HEK293 cell line that overexpresses the high-affinity adenosine A2A receptor, and we measured an elevation in the intracellular production of cAMP in cell lysates after exposure to exosomes and ATP.

The cells responded efficiently to the addition of exogenous adenosine, used as a positive control, producing readily measurable intracellular cAMP levels within 20 min of stimulation. In contrast, adding exogenous ATP at the same doses had no effect on cAMP levels at all but the highest dose, in which there was a small increase (from 14.5 pM at 250 nM ATP to 39 pM at 500 nM ATP). At the same doses, however, ATP became a potent stimulator of a cAMP elevation in the intracellular production of cAMP in cell lysates after exposure to exosomes and ATP.

We next examined if exosomally produced adenosine had a functional impact on T cells. We used multicolor flow cytometry to examine the constitutive expression of CD39 and CD73 by human PBMCs (Fig. 5A). The data show that unlike mice, the expression of CD39 and CD73 is not particularly limited to human Treg. Approximately 20% of T cells are positive for these enzymes. The CD4+ subset had an equal proportion of CD39 and CD73 single-positive cells, there was a clear bias in the CD8+ subset for CD73 single-positive cells. Similarly, there was a bias within the Treg population for CD39 single-positive cells. The proportion of double-positive cells in any population was <2% (average of five healthy donors) (Fig. 5B, bar graph). Circulating T cells in healthy donors therefore do express significant levels of nucleotidase that could participate in extracellular adenosine production.

To test this, purified CD3+ T cells were incubated with increasing doses of 5′AMP in the absence or presence of the CD73 inhibitor APCR. T cells were activated by adding anti-CD3/CD28 Ab-coated beads, and proliferation and cytokine (IL-2 and TNF-α) production was measured at day 3. The T cell response was attenuated in a dose-dependent manner when adding 5′AMP as a substrate. As expected, incubations in the presence of 10 μM APCR abrogated this response, restoring full activation (Fig. 5C).
The data show that human T cells have a constitutive ability to form adenosine from 5′AMP, which impairs their function, and that CD73 is essential for this to occur. In the same experiments, we treated 5′AMP substrate solutions with cancer exosomes and incubated for 1 h to allow substrate hydrolysis to occur and for adenosine to be formed. These samples were then subjected to robust ultracentrifugation (150,000 × g for 1 h) to pellet and remove the exosomes. This step ensured that the assays were measuring the effect of exosomes on the substrate, rather than measuring the direct effect of exosomes on T cell functions. The resultant supernatants were added to T cells in the same manner, in the absence or presence of APCP, and proliferative/cytokine responses were measured. We observed similar levels of attenuated function with exosome-treated substrates in the absence of APCP. By treating T cells with this CD73 inhibitor, however, the inhibitory effects of the exosome-pretreated substrate remained (Fig. 5C), which is consistent with a conversion of 5′AMP by exosomes to form adenosine, which attenuates T cell function independently of T cell CD73. In addition, when exosome-modified 5′AMP was added in the presence of an A2A receptor antagonist, T cell IL-2 production was restored, and proliferation was also restored at all but the highest substrate dose (Fig. 5D).

The data show exosome-generated adenosine can negatively regulate T cell functions.

Discussion

We present in this report a demonstration that exosomes secreted by cancer cells are capable of dephosphorylating exogenous ATP and 5′AMP to form adenosine. These hydrolytic activities are due in part to expression of functional CD39 and CD73 by exosomes. This mechanism may contribute to rising adenosine levels within the microenvironment and hence participate in attenuating T cell function. The study highlights these enzymes as relevant factors for exosome-mediated immune evasion in cancer.

Although some reports describe cancer exosomes having immune-activating functions, there are also accumulating data pointing to varied suppressive effects (40). Documented mechanisms include induction of T cell apoptosis (41), TGF-β-mediated impaired cytolytic functions and Treg induction (32), downregulation of activating receptors like NKG2D (31), and negative effects also on APCs (42). To date, these mechanisms appear to require delivery of receptor ligands, growth factors, or microRNA in a contact- and/or uptake-dependent manner to immune cells. This report, therefore, presents a novel exosome mechanism that does not require direct contact with immune cells. We argue that the expression of catalytically functional membrane-associated enzymes, such as CD73, may be a particularly important and fundamental feature of exosomes in general, in which their dissemination into the microenvironment provides a vast surface area for membrane-tethered enzymes to mediate their functions. Hence, our report describes such a role for exosomal CD39/CD73 in a contact- and/or uptake-dependent manner to immune cells. This report, therefore, presents a novel exosome mechanism that does not require contact with immune cells. We argue that the expression of catalytically functional membrane-associated enzymes, such as CD73, may be a particularly important and fundamental feature of exosomes in general, in which their dissemination into the microenvironment provides a vast surface area for membrane-tethered enzymes to mediate their functions. Hence, our report describes such a role for exosomal CD39/CD73 in a contact- and/or uptake-dependent manner to immune cells. This report, therefore, presents a novel exosome mechanism that does not require contact with immune cells.

Blockade of CD73 activity (with APCP) showed strong inhibition, suggesting CD73 accounts for ~75% of the ability of exosomes to hydrolyze AMP. It was not possible to impair this hydrolysis further by increasing the dose of inhibitor. Similarly, chemical inhibition of CD39 (with POM-1) or CD39 immunodepletion did not fully abrogate ATP hydrolysis. This suggests that there may be other exosomally associated factors that contribute to the hydrolysis of these substrates. It is important to note that several proteomics studies have highlighted that a host of proteins with NTP-binding and NTP-hydrolyzing domains are expressed...
by exosomes of diverse origin (43). It is possible, therefore, that some of these or other yet unidentified exosomal constituents account for the remaining hydrolytic activities.

Signaling downstream of the A_{2A} and A_{2B} receptors involves activation of adenylyl cyclase leading to production of cAMP. This signaling response was used to demonstrate that exosomes are capable of sequentially hydrolyzing ATP and thereafter 5’-AMP to form adenosine. Adding ATP or exosomes alone to A_{2A}-overexpressing HEK293 cells did not stimulate cAMP production, but when combined they were almost as effective as matched doses of adenosine in driving cAMP signaling. Similarly, elevated cAMP could also be triggered by cotreatment with ATP and exosomes in a T cell line (Jurkat). Extracellular adenosine generated by cancer exosomes can therefore drive cAMP signaling in A_{2A} receptor-positive cells.

Adenosine levels within cancerous interstitial fluid measured in murine tumor models reach micromolar concentrations, particularly in hypoxic regions, and are some 10- to 20-fold the levels found in noncancerous tissue (44). Such doses are broadly consistent with those used in this and other studies (44, 45) and have been shown to impart suppressive effects on effectors T cells. We also examined the effects of adenosine generation on T cell function and showed that 5’-AMP as substrate was effective at attenuating T cell proliferative responses and the production of inflammatory cytokines. This, however, was true even in the absence of any cancer exosome contribution and was dependent on constitutive CD73 expression by T cells. Chemically blocking CD73 hydrolytic function abrogated this inhibition. Most recent studies of T cell adenosine production have focused on the CD4^{+} CD25^{hi}Foxp3^{+} natural Treg population. These are double-positive T cells from arthritic patients and showed ATP-hydrolytic capacity of this non-Treg population. But these cells did not inhibit proliferation, probably because of the high levels of IFN-γ and IL-17 produced by these cells (47). Of interest, the patients showed decreased levels of CD73 expression by CD4^{+} T cells taken from inflamed joints compared with circulating cells. This suggests that low T cell CD73 expression levels support a proinflammatory environment. We also show in this article that CD39 is broadly expressed by CD4^{+} and to some degree CD8^{+} T cells and reveal a bias of CD8^{+} T cells for CD73 expression, in agreement with past studies (48). Few double-positive Treg were present in healthy donors, and numerically, Treg represent only a small proportion of enzyme-positive T cells. Exosomes produced by cancer cells are capable of adenosine production from ATP independently of contributions to substrate hydrolysis from T cells.

It was difficult to measure the impact of exosomally generated adenosine on T cell function because of the endogenous capacity of CD73^{+} T cells to hydrolyze 5’-AMP. Blocking T cell CD73 was effective at attenuating adenosine production from 5’-AMP, and this in turn blocked adenosine-driven hyporesponsiveness. Under such blocking conditions, adding exosome-modified 5’-AMP substrate inhibited T cell function consistent with this being an exosome-generated, rather than T cell-generated, adenosine effect. Furthermore, by removing exosomes from this substrate, the data underline this immune-suppressive mechanism to be independent of exosome–T cell contact. Although the exosome dose used in these in vitro experiments was higher than that found in the tumor-associated pleural fluid of mesothelioma patients, this approach allowed us to achieve rapid and almost complete (>95%, data not shown) substrate conversion to adenosine in ~1 h of incubation. This allowed us to add exosome-converted substrate as rapidly as possible to T cells. We do not know whether the microenvironmental doses of exosomes will ever reach such levels in vivo, nevertheless physiological exosome doses are capable of ~60% substrate conversion in 24 h. Hence in vivo, lower exosome doses, but present perhaps continuously within this microenvironment, would likely provide a sufficient source of prolonged ectoenzyme activity, contributing to rising adenosine levels.

At present, it is difficult to estimate the relative importance of exosomes compared with other cells or factors contributing to adenosine production in the tumor microenvironment in vivo. Using pleural fluid taken freshly from patients with pleural malignant mesothelioma offered an opportunity to begin addressing this question. Centrifugation to remove the mixed population of tumor cells and leukocytes present within such tumor effusions revealed that the majority (80%) of ATP-hydrolytic activity was associated with these cellular components. Nevertheless, the remainder could be accounted for by eliminating exosomes. At least in this ex vivo system, therefore, exosomes contribute ~20% of the ATP-hydrolytic activity within this tumor-associated environment. We do not yet know if this remains true within the immediate pericellular space of the tumor, as evaluating exosome dose and function within tumor interstitial fluid remains a significant challenge.

In summary, these findings implicate the secretion of exosomes by cancer cells as a mechanism contributing to rising adenosine levels in the tumor microenvironment. Exosomes may therefore contribute toward adenosine-mediated attenuation of T cell functions, even in the absence of direct T cell–exosome interactions. Abrogation of exosome secretion by cancer cells or targeting their ectonucleotidase activities may prove therapeutically attractive.

Acknowledgments
We thank Dr. J. Welton and Mrs. L. Court for assistance in cell culture maintenance, exosome purifications, and analyses.

Disclosures
The authors have no financial conflicts of interest.

References


