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## Dominant immunosuppression of dendritic cell function by prostate-cancer-derived exosomes

Josephine Salimu\*, Jason Webber, Mark Gurney , Saly Al-Taei, Aled Clayton  and Zsuzsanna Tabi 

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### ABSTRACT

Exosomes are a distinct population of extracellular vesicles of endocytic origin with a protein repertoire similar to the parent cell. Although tumour-derived exosomes harbour immunosuppressive characteristics, they also carry tumour antigens and thus potentially contribute to immune activation. The aim of this study was to examine the impact of prostate cancer exosomes on tumour antigen cross-presentation. DU145 cells, transduced with shRNA to knockdown Rab27a (DU145<sup>KD</sup>) that inhibits exosome secretion, triggered significantly stronger tumour-antigen-specific T cell responses when loaded onto dendritic cells (DC) than control DU145 cells. Enhanced T cell response was prevented by adding purified exogenous DU145 exosomes to DU145<sup>KD</sup> cells, demonstrating that the dominant effect of tumour exosomes is immunosuppression and not antigen delivery. CD8<sup>+</sup> T cell responses were impaired via exosomal regulation of DC function; exosomes triggered the expression of CD73, an ecto-5-nucleotidase responsible for AMP to adenosine hydrolysis, on DC. CD73 induction on DC that constitutively express CD39 resulted in an ATP-dependent inhibition of TNF $\alpha$ - and IL-12-production. We identified exosomal prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) as a potential driver of CD73 induction, as inhibition of PGE<sub>2</sub> receptors significantly reduced exosome-dependent CD73 induction. The results reveal a hitherto unknown suppression of DC function via exosomal PGE<sub>2</sub>, adding a new element to tumour exosome-immune cell cross-talk.

**Abbreviations:** AMP: adenosine monophosphate; ATP: adenosine triphosphate; BLCL: B lymphoblastoid cell line; CME: exosomes enriched from cell line conditioned media; DC: dendritic cell; DMSO: dimethyl-sulfoxide; DU145<sup>c</sup>: DU145 cells with irrelevant knockdown control; DU145<sup>KD</sup>: DU145 cells with Rab27a knockdown; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; GM-CSF: granulocyte-macrophage colony stimulating factor; HLA: human lymphocyte antigen; IL: interleukin; LPS: lipopolysaccharide; mfi: mean fluorescence intensity; PBMC: peripheral blood mononuclear cells; PBS: phosphate buffer solution; PGE<sub>2</sub>: prostaglandin E<sub>2</sub>; TRF: time-resolved fluorescence.

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## Introduction

Cancer cells influence tumour-infiltrating immune cells mainly via cell-to-cell contact and soluble factors. However, accumulating evidence indicates an immunoregulatory role for tumour-derived microvesicles, known as “exosomes” [1]. Exosomes are a distinct population of membranous vesicles of endocytic origin. They range from 30 to 150 nm in diameter and are released by cells upon fusion of intracellular multivesicular bodies with the plasma membrane [2].

Exosomes express a protein repertoire similar to that of the cell of origin. This results in the formation of functionally diverse exosomes, capable of immune activation or immune suppression, respectively. Tumour-derived exosomes harbour the immunosuppressive characteristics of tumour cells [3]. These exosomes can directly impair the capacity of CD14<sup>+</sup> monocytes

to differentiate into functional dendritic cells (DC), thus having a potential effect on both antigen presentation and subsequent T cell responses [4]. Tumour-derived exosomes have also been shown to generate myeloid-derived suppressor cells in mice [5]. Furthermore, tumour-derived exosomes may induce apoptosis of activated tumour-specific T cells [6], impair the cytotoxicity, activation and proliferation of lymphocytes [7–9] and generate regulatory T cells [10].

On the other hand, as tumour-derived exosomes carry tumour antigens, they may contribute to antigen cross-presentation by DC to initiate antitumour immune responses [11,12]. Tumour antigen cross-presentation occurs when antigen-presenting cells take up, process and present extracellular tumour antigens on MHC class I molecules to CD8<sup>+</sup> T cells. We have previously shown that irradiated prostate cancer cells (DU145), expressing

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the 5T4 tumour-associated antigen, are taken up by DC, which then efficiently cross-present the antigen to 5T4-specific CD8<sup>+</sup> T cells that kill 5T4<sup>+</sup> tumour cells [13].

The aim of this study was to examine whether antigen transfer or immunosuppression is the dominant effect of exosomes during tumour antigen cross-presentation. In order to answer this question, we developed a DU145 prostate cancer cell line where Rab27a, a key mediator of exosome release from the cell [14], had been knocked down (DU145<sup>KD</sup>). We found an immunosuppressive effect of exosomes during tumour antigen cross-presentation, mediated by CD73 induction on DC. The resulting net effect of exosomes was adenosine-mediated immunosuppression. The findings of this study will greatly contribute to our understanding of the role exosomes play in tumour-immune cell cross-talk in cancer.

## Materials and methods

### Tumour cells

DU145 prostate cancer cells were obtained from the American Type Culture Collection and maintained in culture with regular passaging for less than 6 months. Authentication was carried out by the supplier using cytogenetic, isoenzymatic and DNA profile analysis. The HLA type of DU145 cells is HLA-A03/A33/B50/B57 (Welsh Blood Transfusion Service, Cardiff, UK). The cells were mycoplasma free and tested regularly using a MycoAlert Mycoplasma Detection Kit (LT07-418; Lonza). Irradiation was carried out using a <sup>137</sup>Cs-source (with regular dosimetry quality assurance) at a rate of 0.627 Gy/min.

### Media, reagents and inhibitors

RPMI-1640 (BE12-167F; Lonza) was supplemented with fetal bovine serum (10270106; Thermo Fisher Scientific), AB-serum (H4522; Sigma-Aldrich) where indicated, 100 U/ml penicillin, 100 mg/ml streptomycin (DE17-603E; Lonza), 2 mmol/l L-glutamine (BE17-605E; Lonza), 25 mmol/l HEPES and 1 mmol/l sodium pyruvate (Sigma-Aldrich). FBS was depleted of bovine exosomes by pelleting at 100,000 g for 2 h. Lipopolysaccharide (LPS, L4391), ATP (A6419) and AMP-CP (CD73 inhibitor, M8386) were obtained from Sigma. PGE<sub>2</sub>, (2292), NECA (1691) and SCH58261 (adenosine A<sub>2A</sub> receptor inhibitor, 2270) were obtained from Tocris. PGE<sub>2</sub>-receptor inhibitors AH-6809 (EP2 inhibitor; CAY14050) and AH-23848 (EP4 inhibitor; CAY-19023) were obtained from Cayman Chemicals.

### Rab27a lentiviral knockdown

Briefly, DU145 cells were plated in 48-well flat-bottomed plates at 18,000 cells/well. On day one, cells were infected with lentiviral particles, at a multiplicity of infection = 10, delivering Rab27a or non-mammalian control shRNA (SHCLNV; Sigma-Aldrich) in the presence of hexadimethrine bromide (8 mg/ml, H9268; Sigma-Aldrich). Puromycin (1.25 mg/ml, P9620, Sigma-Aldrich) was added on day 2. Media were changed on day 5, and cells were cultured in the presence of puromycin for a further six passages prior to experimental use. Rab27a expression was quantified by qRT-PCR (Applied Biosystems; Thermo Fisher Scientific), and expressed relative to the control cells. Exosome secretion was measured by nanoparticle tracking analysis (Nanosight, Malvern Instruments, Amesbury, UK) as described [15] and confirmed by immunofluorescence-based quantification of exosomes.

### Exosome enrichment from conditioned media of cell lines

Culture media was collected from confluent cultures of DU145<sup>C</sup> or DU145<sup>KD</sup> cells. The cells were trypsinized and counted. The media were subjected to serial centrifugation, at 400 g for 7 min to remove cells and at 2000 g for 15 min to remove any non-cellular debris. A filtration step using a 0.22 µm membrane filter was also carried out to remove any fragments and vesicles larger than 200 nm. The supernatant was then centrifuged at 200,000 g for 2 h, to pellet the microvesicles. These exosome-containing pellets were resuspended in fresh culture media, normalized to original cell numbers (in 7 ml/10<sup>7</sup> cells). We refer to them as “exosomes enriched from conditioned media” (CME) in this paper. CME preparations, derived from the two cell lines, were added at 50% to T cells or DC.

### Exosome purification

Exosome purification was performed as we have previously described [16]. Briefly, prostate cancer cell conditioned media was subjected to serial centrifugation to remove cells (400 g, 10 min) and cellular debris (2000 g, 15 min). The supernatant was then filtered (0.22 µm), to remove remaining debris and larger vesicles. The clarified media was underlaid with a 4 ml cushion of 30% sucrose/D<sub>2</sub>O (S9378; 151882; Sigma-Aldrich), and following 2 h ultracentrifugation at 100,000 g (SW32 rotor; Beckman Coulter), the cushion was collected, and washed in excess PBS (BE17-512F; Lonza) by ultracentrifugation. The pellet was resuspended in 50–100 µl PBS and frozen in aliquots at –80°C. Protein concentrations were evaluated using a micro-BCA protein assay (23235; Thermo); the

nanoparticles in each preparation were quantified by nanoparticle tracking analysis (see above).

### **Use of CellGS-Exo-Spin Midi Columns**

Exo-Spin Midi Columns (EX04; Cell Guidance Systems) were pre-packed in a preservative-containing buffer. This buffer was removed and the columns were washed twice with 20 ml of PBS. Cell conditioned medium (1 ml), freshly defrosted at room temperature, was added to the column prior to elution with PBS, and up to 30 separate 500  $\mu$ l fractions were collected. A proportion of each fraction was assayed using a micro-BCA protein assay, CD81 protein binding assay and PGE<sub>2</sub> competitive enzyme immunoassay (KGE004B; R&D Systems).

### **Analysis of column fractions by immunostaining**

Immunostaining was performed as previously described [17]. Briefly, 50  $\mu$ l of each fraction was diluted 1:1 with PBS and then added to high-binding enzyme-linked immunosorbent assay (ELISA) strips (756071; Greiner Bio-One Ltd). After overnight incubation at 4°C, the plates were washed three times in wash buffer (42-01; Kaivogen Oy) using an automated plate washer (Thermo Fisher Scientific), and blocked for 2 h at room temperature using 1% bovine serum albumin (DY995; R&D Systems) in PBS. Plates were then washed as above, and primary antibodies added at 1  $\mu$ g/ml. These included anti-CD81 (MCA1847EL; AbD Serotec) and isotype controls (14-4714-85; e-Bioscience). After 2 h at room temperature, the strips were washed, and goat anti-mouse IgG-biotin conjugated antibody was added (1:2500; NEF823001EA; Perkin Elmer) and incubated for an hour. After another wash, a streptavidin-europium conjugate was added (1:1000; 1244-360; PerkinElmer) in red assay buffer (42-01; Kaivogen Oy) and incubated for 40–60 min. The strips were washed six times prior to addition of europium fluorescent intensifier (42-04; Kaivogen Oy), and time-resolved fluorescence (TRF) was performed on a PHERAstar FS multi-mode plate reader (BMG Labtech).

### **Donors and DC**

Ethical approval to collect venous blood was granted and informed consent was obtained from healthy HLA-A2<sup>+</sup> volunteers. Peripheral blood mononuclear cells (PBMC) from venous blood, collected in EDTA vacutainers, were isolated by density gradient centrifugation. CD14<sup>+</sup> monocytes were enriched by negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (19058; StemCell Technologies).

Average purity of CD14<sup>+</sup> cells was 70–80%, determined by flow cytometry (CD14 antibody from Affymetrix, 25-0149). Cells were incubated at  $5 \times 10^6$  cells per well in 6-well trays in 5 ml/well of 10% FBS-RPMI plus 500 ng/ml rhGM-CSF (CYT-221; ProSpec) and 500 U/ml IL-4 (Gentaur) for 5–6 days. DC were exposed either to purified exosomes at 200  $\mu$ g/ml for 48 h or to CME at 50% of culture media, unless otherwise indicated.

### **Generation of a 5T4 specific CD8<sup>+</sup> T-cell line**

A CD8<sup>+</sup> T-cell line was developed from a HLA-A2<sup>+</sup> healthy donor by repeated stimulation of nonadherent PBMCs with autologous DC loaded with 2 mg/ml 5T4 [17–25] peptide (RLARLALVL; ProImmune), as described previously [18].

### **Flow cytometry**

Cells were labelled in flow cytometry buffer (PBS, 1 mmol/l EDTA and 2% FBS) with fluorochrome-conjugated antibodies and incubated on ice for 40 min. For intracellular labelling, the cells were fixed and permeabilized using e-Bioscience Fixation (00-82249) and Permeabilization (00-8333) reagents before antibodies were added for 40 min at room temperature. Antibodies used in this study: CD73 (550257, BD Bioscience), CD14 as above, CD39 (17-0399, e-Bioscience), 5T4 (FAB49751F, R&D). For cytokine flow cytometry, T cells were fixed and permeabilized as above and CD3 (47-0036), CD8 (45-0088) and IFN $\gamma$  (12-7319) antibodies (e-Bioscience) were added together for 40 min. Flow cytometry was carried out using a FACSVerser flow cytometer with FACSuite software (BD Bioscience).

### **T cell and DC stimulation**

**Primary T cell stimulation.** Untouched T cells were separated from PBMC using the EasySep T cell enrichment kit (19051; StemCell Technologies). Tumour cells ( $10^4$ /well) or enriched exosomes (CME) were added for 45 min before adding CD3/CD28 antibody coated beads to T cells ( $10^5$ /well) (11131D; Thermo Fisher Scientific) at 1:1 bead : T cell ratio. Overnight cytokine flow cytometry for IL-2 production was carried out, or IL-2 was detected in the supernatant after 24 h stimulation by ELISA.

**Antigen cross-presentation assays** were carried out by plating out DU145 cells in two 96-well U-bottomed plates ( $5 \times 10^3$  cells/well). After irradiating one plate with 12 Gy, plates were incubated for 72 h. DC were then added at  $5 \times 10^3$  to the wells and, after 48 h, 5T4-specific CD8<sup>+</sup> T cells were added at  $2.5 \times 10^4$  cells/well. Golgi Plug (0.2  $\mu$ l/200  $\mu$ l; 55509; BD) and Golgi Stop (0.14  $\mu$ l/200  $\mu$ l; 554724;

BD) were added to the wells 1 h later and the cultures were incubated overnight. Cytokine flow cytometry was carried out to determine the percentage of IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells [13].

*Peptide stimulation assay* of 5T4-specific T cells was carried out by loading autologous DC with the 5T4 peptide (20  $\mu$ g/ml) for 1 h, adding 10<sup>5</sup> T cells to 10<sup>4</sup> DC in an overnight cytokine flow cytometry assay as described. The following treatments were also carried out before co-culturing T cells and DC: (a) T cells were pre-treated with NECA (0.5–2  $\mu$ M) for 1 h; (b) CD73 inhibitor (10  $\mu$ M) and/or A<sub>2A</sub>R inhibitor (10  $\mu$ M) were added for 1 h and the excess removed; DC were pre-treated with PGE<sub>2</sub> receptor inhibitors EP2 and EP4 (100  $\mu$ M each) for 30 min. AMP (200  $\mu$ M) was added to DC 30 min before T cells were added.

*LPS stimulation of DC*, co-cultured with 100  $\mu$ g/ml exosomes for 24 h, was carried out with or without 40  $\mu$ M ATP added for 30 min. This was followed by adding 200 ng/ml LPS in the presence of 100 ng/ml IFN $\gamma$  for 18 h. Cytokine flow cytometry to detect IL-12 (554575, BD) and TNF $\alpha$  (17-7349, e-Bioscience) produced by DC was carried out as above.

## IL-2 ELISA

The IL-2 Duo-Set ELISA kit was purchased from R&D Systems (DY202). T cell supernatants were harvested after 24 h culture and kept at –20°C before assaying them according to the manufacturer's instructions.

## Statistical analysis

Statistical analysis was carried out by applying Student's *t*-test, paired *t*-test and ANOVA with Tukey's post-hoc test (GraphPad InStat 3.06). Statistically significant differences are marked as \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

## Results

### Knockdown of Rab27a decreases exosome secretion by DU145 cells

In order to assess the influence of exosomes on tumour antigen cross-presentation, we generated a DU145 prostate cancer cell line with deficient exosome secretion, by knocking down Rab27a [14] using lentiviral particles. (DU145<sup>KD</sup>) Quantification by qPCR and western blotting revealed 80% reduction in Rab27a expression at both mRNA and protein level, compared to that of the DU145<sup>C</sup> control cell line. Knockdown efficiency was validated at different passage numbers to verify long-term stable gene silencing (Figure 1(a)). To establish if knocking

down Rab27a expression successfully inhibited the secretion of particles ranging from 30 to 150 nm in diameter, which we will call here “exosomes”, nanoparticle tracking analysis was carried out (Figure 1(c), i and ii). Particle secretion by the DU145<sup>KD</sup> cell line was less than 30% of that secreted by the DU145<sup>C</sup> cell line (Figure 1(c), ii). Immunofluorescence-based quantification of exosomes confirmed a similar level of reduction in exosome release by DU145<sup>KD</sup> cells (Figure 1(c), ii).

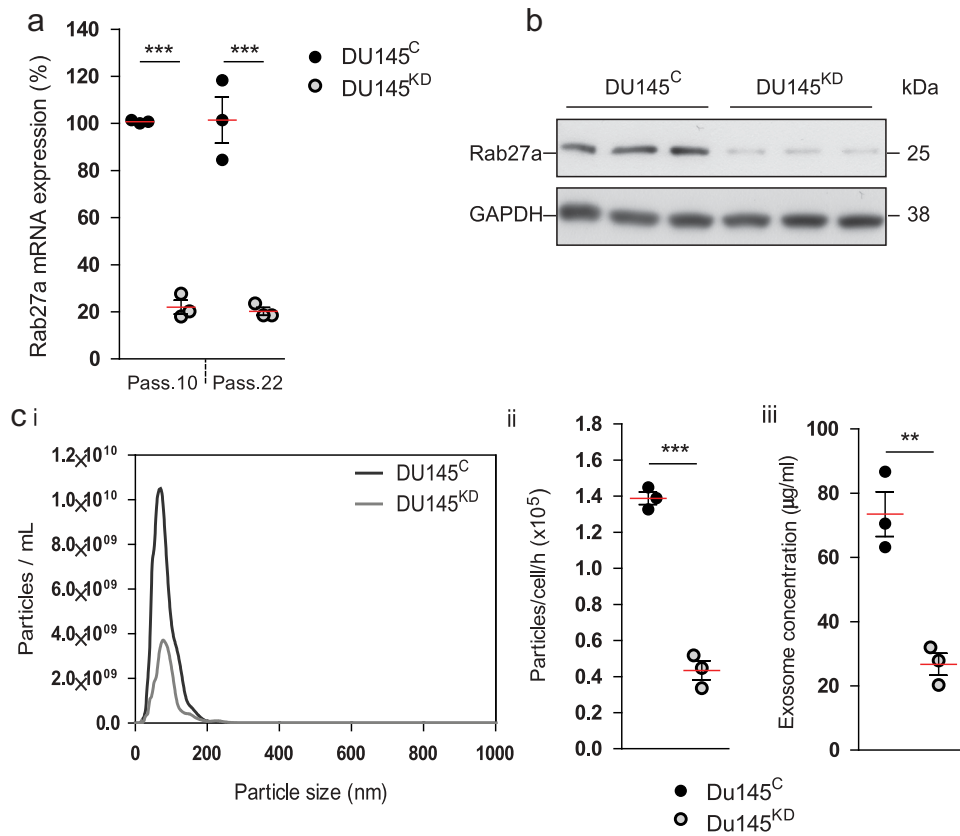
### DU145<sup>KD</sup> cells induce superior T cell responses

To study the effect of tumour-derived exosomes on antigen cross-presentation, we used a previously established antigen cross-presentation model [13]. Irradiated (12Gy) or untreated DU145<sup>KD</sup> and DU145<sup>C</sup> cells were co-cultured with HLA-A2<sup>+</sup> DC followed by co-culture with tumour-antigen-specific T cells. A 5T4-specific, HLA-A2-restricted CD8<sup>+</sup> T cell line was used and the percentage of T cells producing IFN $\gamma$  upon stimulation with these DC was determined. DC co-cultured with DU145<sup>KD</sup> cells generated significantly stronger T cell responses than those co-cultured with DU145<sup>C</sup> cells when the tumour cells received no radiation. The results (Figure 2(a)) show that inhibition of exosome secretion is beneficial for this immune response. In contrast, cross-presentation from 12Gy irradiated DU145<sup>C</sup> and DU145<sup>KD</sup> cell lines generated similar levels of T cell responses (Figure 2(a)). We established that 5T4 antigen expression levels were comparable in the two untreated cell lines and upregulated at a similar extent by irradiation in both lines (Figure 2(b)). To confirm whether it was the lack of exosomes that influenced T cell responses in the cross-presentation of antigen from non-irradiated cells, exosomes were added back to DC together with DU145<sup>KD</sup> cells at 200  $\mu$ g/ml. The addition of exosomes together with DU145<sup>KD</sup> cells to DC reduced T cell responses to the same level as induced by DU145<sup>C</sup> cells (Figure 2(c)). This confirms that the presence of tumour exosomes dampens T cell responses during tumour antigen cross-presentation from prostate cancer cells.

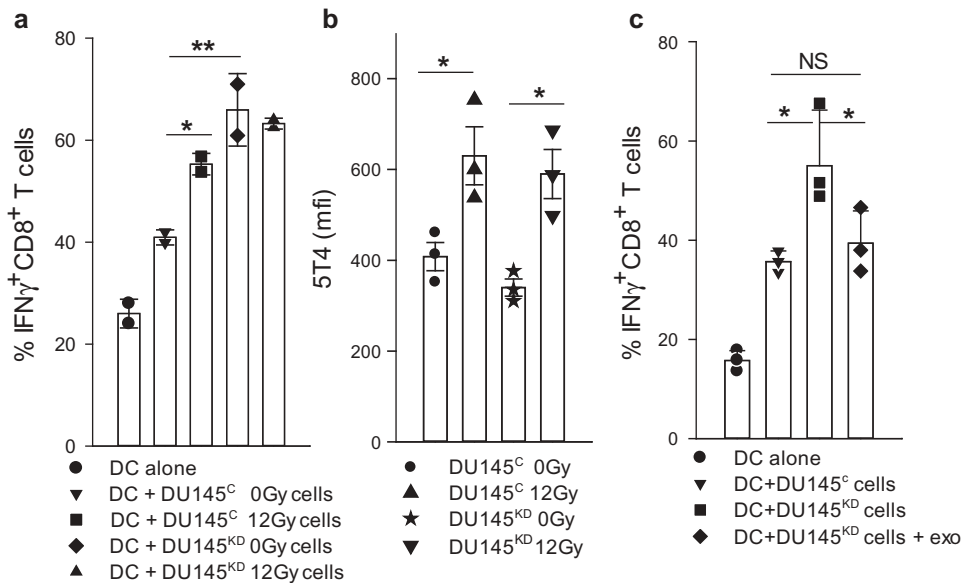
### Exosomes inhibit IL-2 production by CD4<sup>+</sup> but not CD8<sup>+</sup> T cells

We have previously reported that exosomes directly inhibit IL-2 production by T cells, preferentially by CD4<sup>+</sup> T cells [7,9]. Here we wanted to examine the extent to which exosomes directly affect T cell function in the cross-presentation model. First, freshly isolated primary T cells were co-cultured with DU145<sup>C</sup> and DU145<sup>KD</sup> cells before stimulation with CD3/CD28 antibody-coated beads for 24 h (Figure 3). IL-2 ELISA of the supernatant indicated that IL-

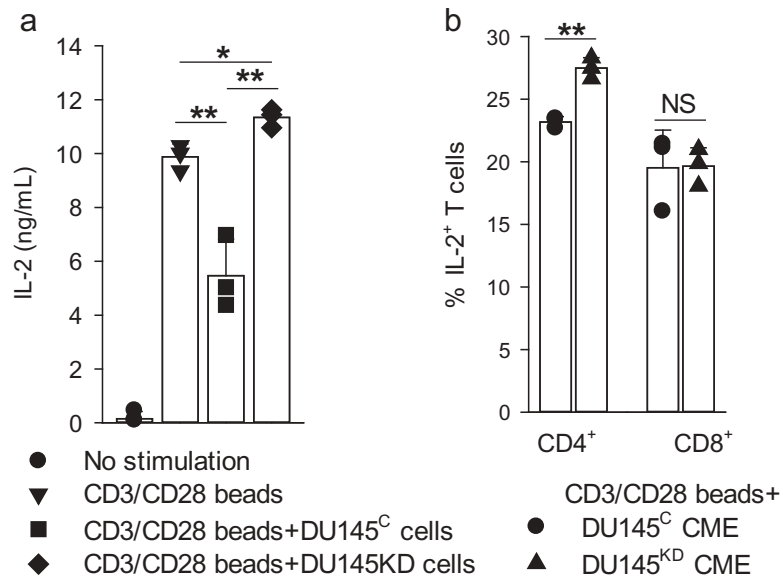




**Figure 1.** Knockdown of Rab27a decreases exosome secretion by DU145 cells. (a) Rab27a expression at mRNA level at 12 and 22 passages in DU145<sup>KD</sup> cells. Relative expression compared with that in DU145<sup>C</sup> cells shown. (b) Rab27a protein levels detected by western blotting in DU145 cells. (c) Exosome secretion, measured by nanoparticle tracking analysis (i, ii) or by an in-house exosome ELISA-like assay (iii). (a, c) ii and iii: Raw data are shown as symbols; means + SE are also shown. \*\*\**p* < 0.001, \*\**p* < 0.01. Representatives of at least three repeat experiments are shown.



**Figure 2.** Exosomes inhibit antigen cross-presentation. (a) 5T4-specific T cells were stimulated overnight in the cross-presentation model, as shown. (b) 5T4 expression (mfi) is shown on DU145 cells with or without irradiation after 48 h culture. Representatives of two to three repeated experiments are shown. (c) T cells as in (a), DC were loaded with non-irradiated DU145 cells with or without exosomes, as indicated. Raw data are shown as symbols; means + SD of results are also shown. \**p* < 0.05, \*\**p* < 0.01.



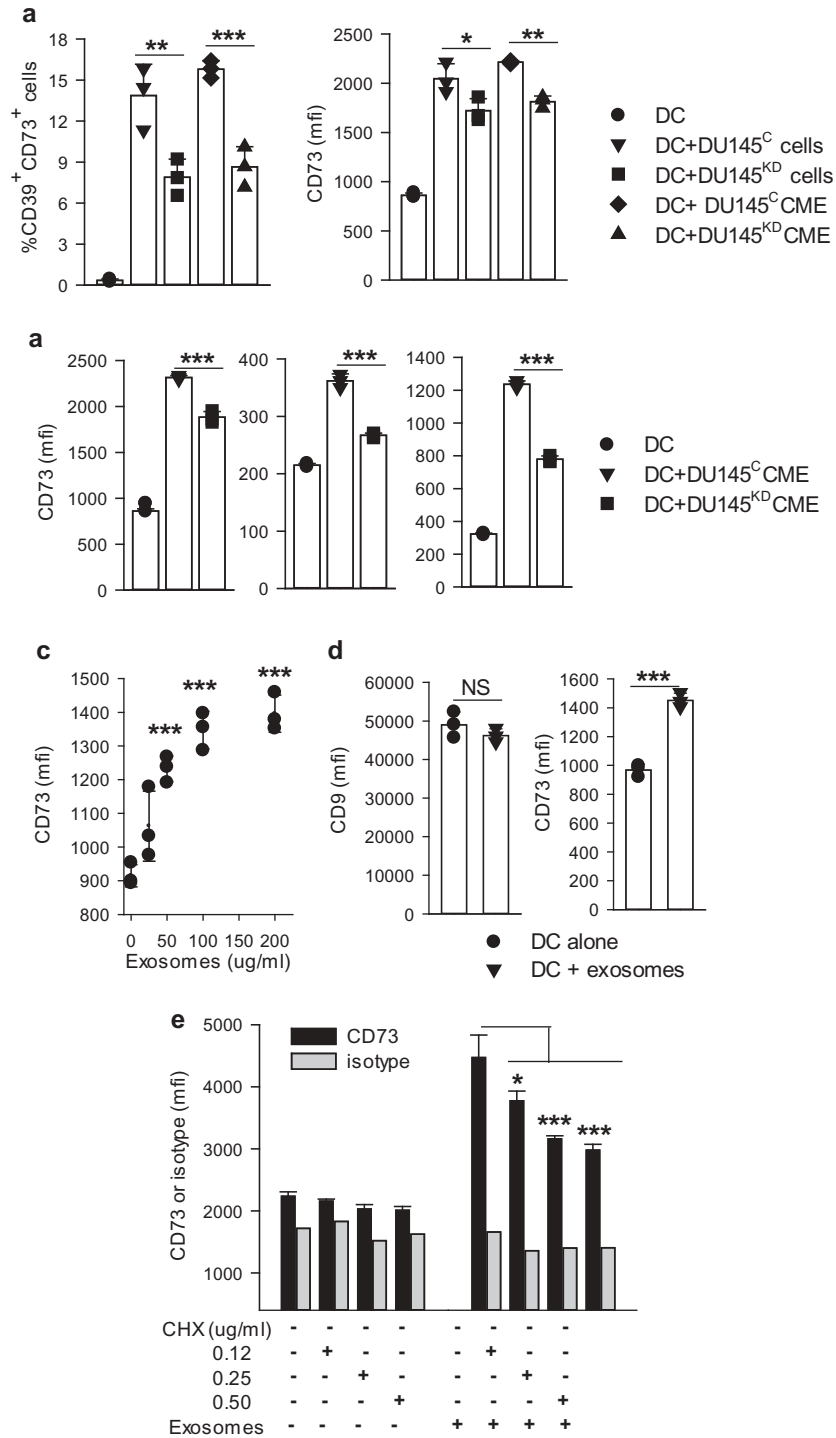
**Figure 3.** Direct effect of exosomes on T cell function. Primary T cells were stimulated with CD3/CD28 antibody-coated beads in the presence of (a) DU145<sup>C</sup> or DU145<sup>KD</sup> cells or (b) DU145<sup>C</sup> or DU145<sup>KD</sup> CME. (a) IL-2 secretion 24 h after T cell stimulation was measured by ELISA. (b) Intracellular cytokine staining of percentage of IL-2<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells after an overnight incubation with CD3/CD28 beads in the presence CME preparations, as shown. Raw data are shown as symbols; means + SD of results are also shown. \* $p < 0.05$ , \*\* $p < 0.01$ . Representatives of two repeated experiments are shown.

2 production by T cells was comparable when stimulated with beads alone or with beads in the presence of DU145<sup>KD</sup> cells. Conversely, 50% less IL-2 was detected in response to beads in the presence of DU145<sup>C</sup> cells, confirming the direct immunosuppressive effects of exosomes on T cells (Figure 3(a)). Next, we wanted to confirm whether exosomes were able to elicit the same immunosuppressive effects in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. This objective was achieved by using exosomes enriched by serial centrifugation of the supernatants from DU145<sup>C</sup> and DU145<sup>KD</sup> cell cultures. These exosome preparations reflected the exosome concentrations of the conditioned media from the two cell lines (CME). Significantly more CD4<sup>+</sup> T cells produced IL-2 in the presence of DU145<sup>KD</sup> CME, compared with DU145<sup>C</sup>, while CD8<sup>+</sup> T cells were not sensitive to the presence of exosomes (Figure 3(b)). This suggests that the impact of exosomes on the function of 5T4-specific CD8<sup>+</sup> T cells in the cross-presentation assay is distinct from the observed and published direct effect of exosomes on CD4<sup>+</sup> T cells.

### Exosomes induce CD73 expression on DCs

Next, we studied whether exosomes have exerted any immune-regulatory effects on antigen-presenting cells, as this may indirectly lead to impaired CD8<sup>+</sup> T cell responses. DC were co-cultured with tumour cells or exosome fractions from the control and knockdown cell

lines for 48 h before assessing the expression of immunosuppressive markers. DC treatment with both cell lines or with exosomes from both cell lines resulted in the induction of CD73 on DC. The percentage of CD39<sup>+</sup>CD73<sup>+</sup> cells and the mean fluorescence intensity (mfi) of CD73 expression on DC (Figure 4(a)) were significantly higher in the presence of DU145<sup>C</sup> cells and CME than in the presence of DU145<sup>KD</sup> cells and DU145<sup>KD</sup> CME. This was confirmed with DC from three different donors (Figure 4(b)). CD73 induction on DC proved to be exosome dose dependent (Figure 4(c)), which explains the less efficient CD73 induction by DU145<sup>KD</sup> cells. As it has been previously shown that exosomes express membrane-bound CD73 [7], we wanted to confirm that CD73 expression on DC was not due to exosome binding. To do this, expression of CD73 and the exosome marker CD9 were assessed on DC, treated for 24 h with or without exosomes, by flow cytometry (Figure 4(d)). While the presence of exosomes induced expression of surface CD73 on DC, the level of CD9 expression remained unchanged, indicating that CD73 expression was independent of exosome attachment to the cell surface. Furthermore, blocking protein synthesis by pre-treating DC for 30 min before adding exosomes with cyclohexamide decreased CD73 induction (Figure 4(e)), proving that CD73 expression on DC is not, or at least not exclusively, the result of these cells being “decorated” by CD73-expressing exosomes binding or adhering to DC.



**Figure 4.** De novo induction of surface expression of CD73 on DC by exosomes. (a) DC were co-cultured for 48 h with DU145 cells or CME. Raw data as symbols and means + SD of % CD39<sup>+</sup>CD73<sup>+</sup> cells or mfi of CD73 expression from triplicate samples are shown. (b) Mfi of CD73 expression on DC, derived from three independent donors, cultured with or without CME, are shown. Representatives of two to three repeated experiments are shown. (c) DC treated with increasing concentrations or with (d) 200 µg/ml purified DU145 exosomes. Raw data are shown as symbols. Means + SD of CD73 or CD9 expression are also shown. (e) DC pre-treated with increasing concentrations of cyclohexamide (CHX) before exosome treatment. CD73 expression is shown from triplicate samples (mean + SD). \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.



### Exosomal PGE<sub>2</sub> contributes to CD73 induction on DC

We were interested in establishing the mechanism of exosome-induced CD73 expression on DC. As it has been shown in our laboratory recently that PGE<sub>2</sub> induces CD73 on primary CD14<sup>+</sup> cells [19], we tested whether PGE<sub>2</sub> was contributing to the induction of CD73 on DC. DC treatment for 24 h with PGE<sub>2</sub> (50 nM) or CME from DU145<sup>C</sup> and DU145<sup>KD</sup> cultures induced CD73 expression (Figure 5(a)). To determine if it was exosomal PGE<sub>2</sub> that was inducing the effect, DC were treated with PGE<sub>2</sub> receptor antagonists before being cultured with exosomes (Figure 5(b)) at a concentration established previously [19]. Two out of four PGE<sub>2</sub> prostanoid receptors, EP2 and EP4, that had been shown to be present on DC [20] were inhibited before the addition of exosomes to DC. PGE<sub>2</sub> receptor inhibition resulted in a significantly reduced CD73 induction compared with the control (DMSO), suggesting that exosomal PGE<sub>2</sub> is a major contributor to CD73 induction on DC (Figure 5(b)).

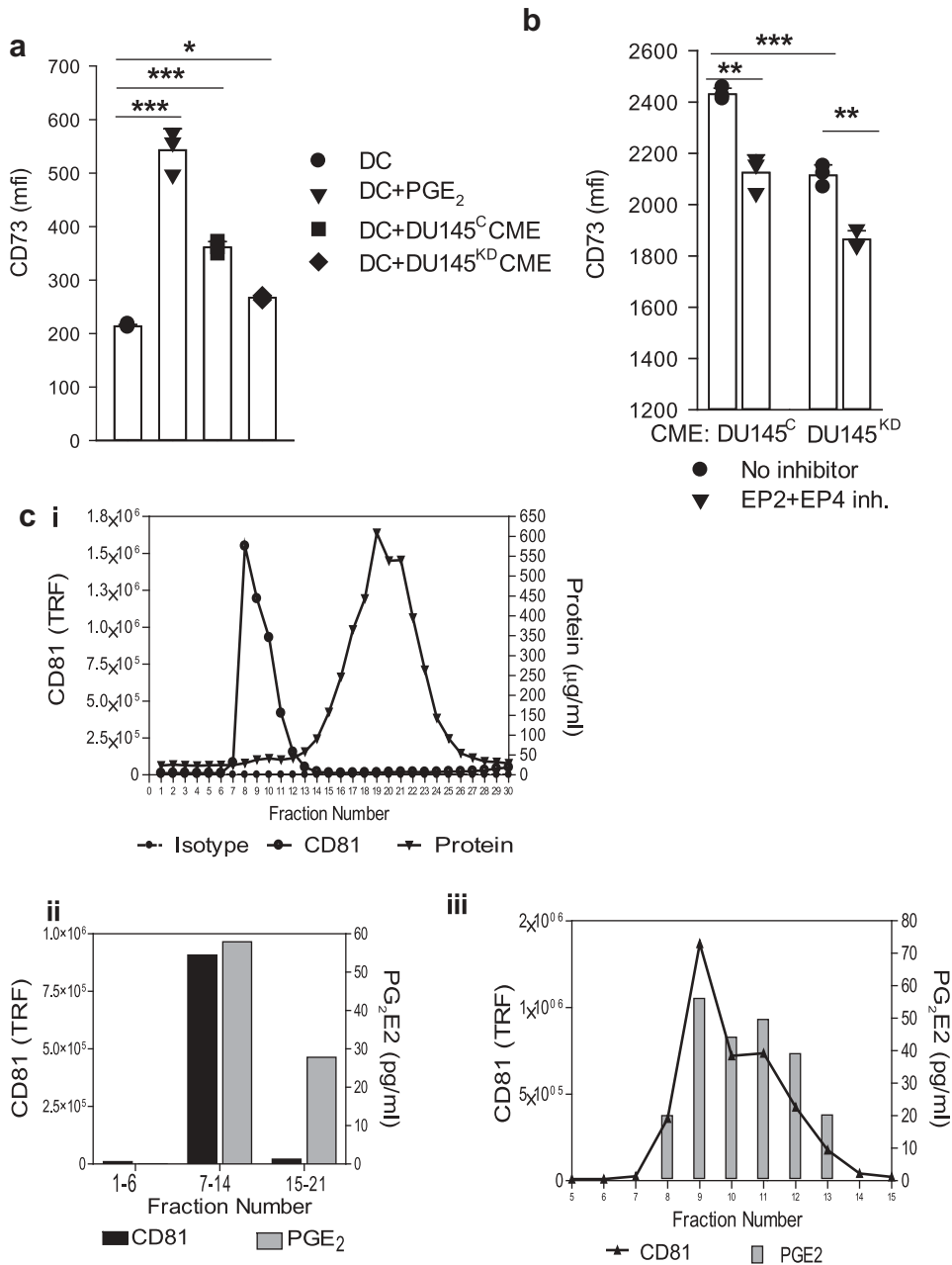
To confirm that PGE<sub>2</sub> is associated with tumour exosomes, a size exclusion chromatographic approach was used to separate vesicles, present in cell culture medium, from non-vesicular proteins (Figure 5(c)). This was carried out using commercially available, ready-made Exo-Spin Midi Columns. A proportion of each fraction was assayed for protein using the micro-BCA assay (Figure 5(c), i). This revealed a small protein peak at around fractions 8–12, and a larger peak thereafter. Another proportion of each fraction was directly coupled to protein-binding ELISA plates and stained for the tetraspanin CD81, which is also an exosome marker. A strong signal at fractions 7–13 for CD81 was observed while the signal when using an isotype control was negligible (Figure 5(c), i). The results were in agreement with previously published data from our group showing that vesicle-associated markers, such as CD81, CD9 and CD63, precede fractions containing the bulk of the proteins [21]. Based on the protein and CD81 results, aliquots were combined from fractions 1–6 (pre-exosomal fractions), fractions 7–14 (exosomal fractions) and fractions 15–21 (post-exosomal fractions) (Figure 5(c), ii). A proportion of the pre-exosomal, exosomal and post-exosomal fractions were then assayed in a PGE<sub>2</sub> competitive enzyme immunoassay. The majority of PGE<sub>2</sub> detected in the DU145 cell supernatant was detected within the exosomal fractions (Figure 5(c), ii), although a significant portion was also present in the post-exosomal fractions. Assessment of individual fractions from the exosomal fractions (7–14) revealed that the concentration of PGE<sub>2</sub> co-localized with the CD81 signal, highlighting a strong association of PGE<sub>2</sub> to exosomal vesicles (Figure 5(c), iii).

### Adenosine inhibits T cell function

DC constitutively express CD39, an ectonucleotidase responsible for ATP-to-AMP hydrolysis, and CD73, responsible for AMP-to-adenosine hydrolysis. We hypothesized that CD39<sup>+</sup>CD73<sup>+</sup> DC sequester pro-inflammatory ATP from the tumour microenvironment and replace it with immunosuppressive adenosine. First we tested if adenosine inhibits the function of the 5T4-specific CD8<sup>+</sup> T cells. Titration of the adenosine analogue NECA was carried out on T cells before stimulation with peptide-pulsed DC for 6 h. IFN $\gamma$  production by the T cells was reduced in the presence of NECA in a dose-dependent manner, reaching significance at 2  $\mu$ M (Figure 6(a)), indicating the adenosine sensitivity of effector T cells. Next, to investigate the DC-mediated adenosine effect in our model, ATP was added to exosome-treated DC for 30 min before being stimulated with LPS and IFN $\gamma$  overnight. The results of TNF $\alpha$  and IL-12 intracellular cytokine staining showed that exosome-treated DC in the presence of ATP produced significantly less TNF $\alpha$  and IL-12 than DC without exosome exposure (Figure 6(b), i and ii). However, a small but significant decrease in IL-12 production by exosome-treated DC not cultured with ATP was also observed (Figure 6(a), ii), which may owe to a greater sensitivity of IL-12 production to adenosine due to the presence of residual ATP. To prove that the ultimate effect on T cells was due to adenosine, 5T4 specific CD8<sup>+</sup> T cells were pre-treated with an adenosine A<sub>2A</sub> receptor inhibitor before stimulation with 5T4 peptide-pulsed DC, treated with or without exosomes, in the presence of AMP (Figure 6(c)). To ensure we only investigated CD73 activity due to its expression on DC and not T cells, a CD73 inhibitor was added to the T cells. We also included a group of DC where CD73 induction by exosomal PGE<sub>2</sub> was inhibited with the EP4 receptor inhibitor. T cell responses were significantly reduced when AMP was added to exosome-treated DC but were partially rescued by adding the EP4 inhibitor. However, in the presence of the A<sub>2A</sub> adenosine receptor inhibitor, AMP had no inhibitory effect on T cell responses, providing evidence for adenosine-mediated inhibition. Taken together, the results show that exosome-treated DC suppress T cell function in an adenosine-dependent manner.

### Discussion

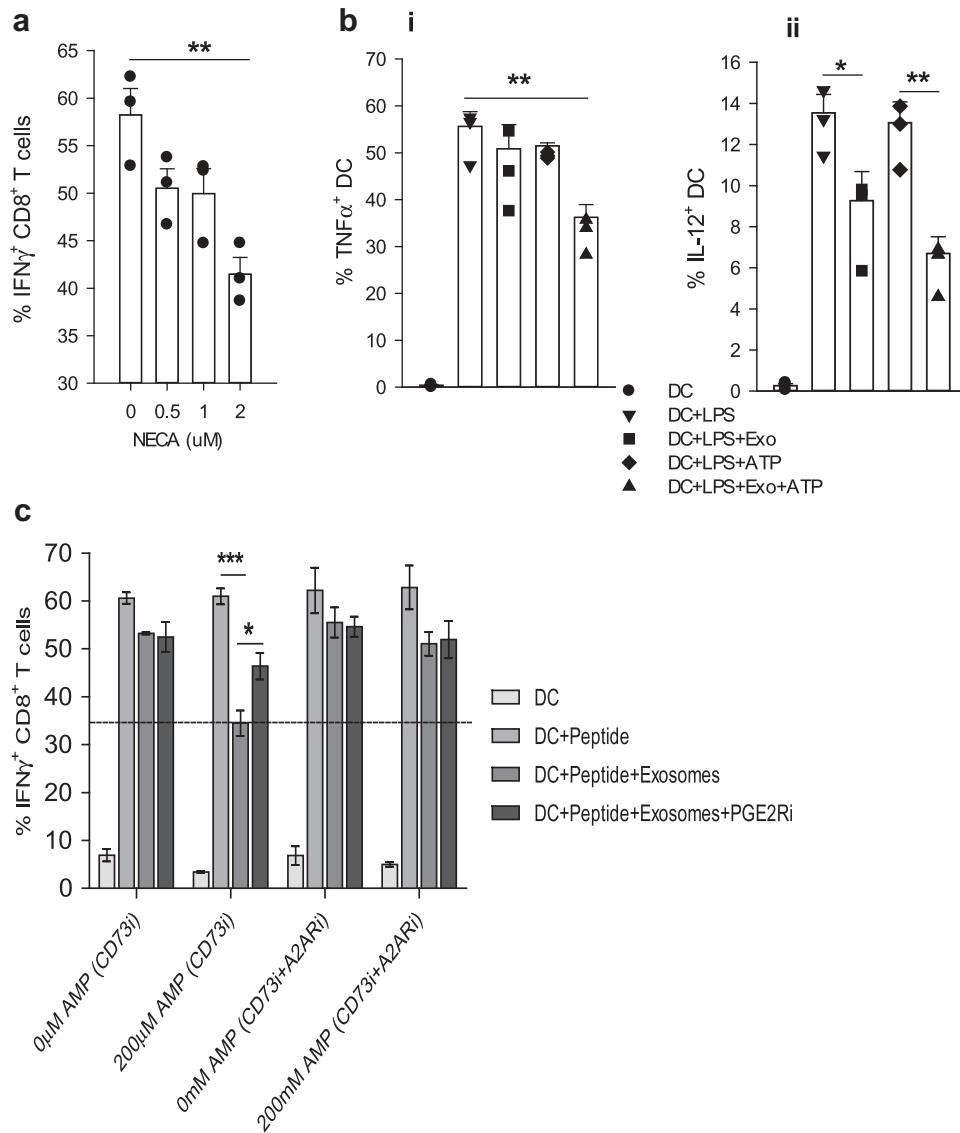
The present study reveals that tumour exosomes inhibit CD8<sup>+</sup> T cell responses when tumour-associated antigen is cross-presented by DC. We inhibited exosome secretion by knocking down Rab27a in DU145 prostate cancer cells. When these cells were added to a tumour antigen cross-presentation model, improved IFN $\gamma$  production was observed by 5T4 tumour-antigen-specific CD8<sup>+</sup> T cells.



**Figure 5.** Exosomal PGE<sub>2</sub> is a major trigger to induce CD73 expression on DC. (a) DC were treated for 24 h with PGE<sub>2</sub> (50 nM) or DU145<sup>C</sup> and DU145<sup>KD</sup> CME. (b) DC were pre-treated for 30 h with inhibitors of the EP2 and EP4 PGE<sub>2</sub> receptors before being cultured with CME from the cell lines. Raw data are shown as symbols. Means + SE of CD73 expression are also shown. (c) Size exclusion chromatography to separate vesicles present in cell culture medium from non-vesicular proteins. (i) 30 fractions of DU145 cell supernatants were assessed for protein concentration using a BCA assay or for CD81 in an ELISA assay. TRF values from single measurements are shown. Representatives of two experiments are shown. (ii) Co-localization of the exosome marker CD81 and PGE<sub>2</sub> in pooled fractions as indicated. (iii) Detailed co-localization assay in the individual fractions of the exosome-zone. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

This effect was reversible when purified exogenous exosomes were added together with DU145<sup>KD</sup> cells to DC. We identified exosomal PGE<sub>2</sub> as a significant inducer of CD73 expression on DC, triggering adenosine-mediated immunosuppressive effects. Whilst Rab27a knockdown results in a major attenuation (66%) of vesicle secretion from the DU145 cells, it does not fully abrogate it, leaving a

population of vesicles remaining. We do not know whether or not this remainder sub-population also carries PGE<sub>2</sub> and will also impair cross-presentation like the wild type and complete vesicle population. If indeed this is the case, they remain insufficient in number following Rab27a-knockdown to achieve an effective immune-suppressing dose. We clearly present evidence that



**Figure 6.** Adenosinergic suppression of T cell and DC function by exosomes. (a) T cell line: 5T4-specific responses to DC+peptide stimulation in the presence or absence of NECA. % IFN $\gamma$ -producing T cells, determined by flow cytometry. (b) Percentage of DC that produce TNF $\alpha$  (i) or IL-12 (ii) following LPS and exosome treatment in the presence or absence of ATP. Raw data are shown as symbols. Means and SE are also shown. Representatives of two repeated experiments are shown. (c) 5T4 peptide-pulsed DC, treated with/without exosomes and PGE $_2$  receptor inhibitor (PGE2Ri) for 24 h, were incubated with AMP (0 or 200  $\mu$ M) for 20 min before being co-cultured with 5T4-specific T cells. A $_2$ A receptor inhibitor (A2ARi) was added and cytokine flow cytometry carried out after overnight incubation. Means + SE % of IFN $\gamma^+$  CD8 $^+$  T cells are shown from triplicate cultures. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

perturbation of secretion through knockdown of Rab27a is sufficient to remove enough suppressive vesicles from the system, and to restore cross-presentation function. The results demonstrate an important and dominantly negative role for vesicles in this context.

As we have reported previously, irradiated DU145 cells are more effective in cross-priming T cells than non-irradiated tumour cells, primarily due to the translocation and cell surface expression of Hsp70 and enhanced expression of 5T4 [13]. The presence or absence of exosomes did not alter the efficiency of cross-presentation from irradiated tumour cells. A

potential explanation of this observation is that stress conditions experienced by tumour cells are reflected in the exosomes they release [22–24]. Radiation of DU145 cells may generate exosomes carrying immune-activating Hsp70, as observed in heat-shocked or chemotherapy-treated tumour cells [24,25]. This would suggest that radiation impacts on the quality of tumour exosomes, potentially altering their dominant immunosuppressive nature. This is a question currently being investigated in our laboratory.

However, T cell cross-priming by non-irradiated tumour cells was influenced by the presence of tumour

exosomes in a dose-dependent manner. T cell responses improved when exosome release was inhibited, highlighting the predominant immunosuppressive effect of tumour exosomes. Although tumour-derived exosomes have been reported to generate antitumour immune responses in several murine tumour models [11,12,26,27], our results strongly suggest that, even if antigen is delivered by exosomes to DC, exosomal immunosuppressive effects override the potential antigen-delivery function. Indeed a clinical trial, with tumour-derived exosomes as a cancer vaccine in 40 patients with colorectal cancer, found no clinical benefit [28], which may reflect the immunosuppressive properties of tumour exosomes.

In this study, we have demonstrated that exosomes suppress the ability of CD4<sup>+</sup> T cells to produce IL-2 upon TCR activation, confirming previous results [9]. However, we found no direct exosomal inhibition of IL-2 production by CD8<sup>+</sup> T cells. This may be due to different T cell subset sensitivity to exosomes, as described before. The lack of direct exosomal inhibition of CD8<sup>+</sup> T cells suggested that it may be the antigen-presenting cells, i.e. the DC, that are affected by exosomes in this model. It has been previously shown that exosomes inhibit the differentiation of monocytes to DC [4,29] and also have an inhibitory effect on DC maturation and cytokine production [30]. DU145 exosomes induced DC to express CD73 on their surface. To our knowledge, this is the first study to assess the expression of this immunosuppressive marker on DC exposed to tumour exosomes. CD73 is expressed on lymphocytes, endothelial cells and epithelial cells but not on normal human monocytes, macrophages or DC. CD73 is also expressed in several types of human cancers; high expression of CD73 is associated with a poor prognosis in colorectal cancer [31] and triple negative breast cancer [32]. The observed change in DC phenotype creates a subpopulation of DC expressing both CD39 and CD73, which makes these cells uniquely able to hydrolyse ATP to adenosine in the pericellular milieu. It has been shown that under normal conditions adenosine has direct tumour-promoting, angiogenic and metastasis-inducing effects, and it is also a powerful inhibitor of antitumour immune effector cells in the tumour microenvironment [33,34]. It also impairs maturation and function of dendritic cells [35]. Addition of ATP to exosome-treated DC significantly reduced the ability of DC to produce proinflammatory cytokines TNF $\alpha$  and IL-12. Both cytokines are beneficial for enhancing numerous antitumour effects and support the maturation of DC and activation of T cells.

PGE<sub>2</sub> is a lipid mediator that elicits a wide range of biological effects associated with inflammation and cancer [36]. Although exosomal PGE<sub>2</sub> expression has not been studied in detail, the PGE<sub>2</sub> content of exosomes

was found to correlate with the frequency of myeloid-derived suppressor cells in mice [5]. We show here that PGE<sub>2</sub> is found predominantly in the exosomal fraction of the supernatant of DU145 cells. Inhibition of the PGE<sub>2</sub> receptors EP2/EP4 on DC significantly inhibited exosome-induced CD73 expression, suggesting ligand-receptor binding between exosomal PGE<sub>2</sub> and DC. PGE<sub>2</sub> is necessary for the upregulation of CCR7 and metalloproteinase 9 on DC and their consequent migration into lymph nodes [37,38]. However, PGE<sub>2</sub> is also one of the prominent inducers of IDO1 expression on DC resulting in the attraction and induction of Tregs and consequent inhibition of CD8<sup>+</sup> T cell responses [39]. Our data highlight how exosomal PGE<sub>2</sub> induces CD73 expression on DC with a significant impact on cytokine production and T cell activation.

Lastly, assessment of T cell responses in this study demonstrated that exosome-treated DC suppress T cell function in an adenosine-dependent manner in the presence of extracellular ATP or AMP. Inhibition of the A<sub>2A</sub> adenosine receptor on T cells alleviated the immunosuppression of 5T4-specific T cell responses in our experimental setting. This is in agreement with other studies that have shown that signalling via the A<sub>2A</sub> receptor inhibits pro-inflammatory cytokine production by T cells and suppresses CD8<sup>+</sup> cytolytic activity [40,41].

Taking all this together, we have demonstrated here that prostate-cancer-derived exosomes suppress tumour antigen cross-presentation via the induction of CD73 expression on DC. We also reveal a hitherto unknown effect of tumour exosomes on the suppression of DC function via exosomal PGE<sub>2</sub>, adding a new element to tumour exosome-immune cell cross-talk.

## Disclosure statement

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