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Exosomes associated with human ovarian tumors harbor a reversible checkpoint of T cell responses

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Abstract

Nano-sized membrane-encapsulated extracellular vesicles isolated from the ascites fluids of ovarian cancer patients are identified as exosomes based on their biophysical and compositional characteristics. We report here that T cells pulsed with these tumor-associated exosomes during TCR-dependent activation inhibit various activation endpoints including translocation of NF κ B and NFAT into the nucleus, upregulation of CD69 and CD107a, production of cytokines and cell proliferation. Additionally, the activation of virus-specific CD8⁺ T cells that are stimulated with the cognate viral peptides presented in the context of class I MHC is also suppressed by the exosomes. The inhibition occurs without loss of cell viability, and coincidentally with the binding and internalization of these exosomes. This exosome-mediated inhibition of T cells was transient and reversible: T cells exposed to exosomes can be reactivated once exosomes are removed. We conclude that tumor-associated exosomes are immunosuppressive, and represent a therapeutic target blockade of which would enhance the antitumor response of quiescent tumor-associated T

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cells and prevent the functional arrest of adoptively transferred tumor-specific T cells or chimeric antigen receptor (CAR) T cells.

Keywords

Ovarian cancer; Immunomodulation; Ascites Fluid; Exosomes; T cell function

Introduction

Effector memory T cells present in the microenvironments of human tumors are hypo-responsive to activation via the T cell receptor (TCR) (1–5). Multiple cells and factors have been reported to contribute to the arrest of the antitumor response of T cells present in the microenvironment of human tumors (6). The unresponsiveness of T cells in tumors is due in part to an arrest in the T cell signaling cascade that occurs following activation (7). The T cells are quiescent but not functionally inert, as the tumor-associated T cells can be activated in tumor xenografts by treatment with IL12-loaded liposomes, a mechanism that bypasses TCR-induced activation (1). These activated T cells can kill tumor cells.

Previously, it was established that a non-cellular component of ovarian tumor microenvironments induced the TCR signaling blockade in both tumor-associated T cells and in T cells isolated from normal donor PBL (7). Many biologically active immunosuppressive soluble factors have been reported to be present in ovarian tumor ascites fluids (7). Ovarian tumor ascites fluids also contain extracellular vesicles that may impact tumor progression (8–10). The tumor-associated vesicles, often referred to as exosomes, are spherical membrane bound particles with an average diameter of 50 nm with characteristic marker proteins (11–14). These exosomes have been reported to be both immunosuppressive and immunostimulatory, depending upon their surface phenotype and the intravesicular cargo (15, 16). Exosomes increase in number with tumor progression (15). A considerable controversy currently exists as to whether exosomes mediate a loss or gain of an antitumor immune response and how these vesicles function. Given their presence in immunosuppressive tumor microenvironments, most emphasis has been placed upon determining mechanisms by which exosomes (present in tumor ascites, solid tumors and serum) inhibit immunocompetent cells in cancer patients.

Exosomes isolated from tumor microenvironments have been suggested to suppress antitumor responses indirectly by augmenting the function or preventing the apoptosis of T regulatory cells, generating myeloid-derived suppressor cells (MDSCs), and by blocking the maturation of dendritic cells and macrophages (15, 17–20). Several direct mechanisms have also been proposed to explain how exosomes may arrest T cell function. These include the induction of T cell apoptosis that is mediated by exosomes expressing apoptosis-inducing ligands such as FasL, PDL1 and TRAIL (21) and a time dependent inhibition of CD3 ζ chain in T cells (22). Cell culture-derived exosomes that bound to but were not internalized by T cells, were found to regulate expression of several genes that collectively result in a loss of T cell function (23).

Although some or all of these mechanisms may be contributing to the exosome-mediated immune suppression of T cells, they all would result in a relatively slow and nonreversible arrest in T cell functions. The actual mechanism by which the exosomes inhibit the activation of T cells remains poorly understood.

We report here that exosomes incubated with T cells rapidly (within 2h) bind to and internalize into the cells. T cells so treated lose the ability to respond to activation via their T cell receptor. This immunosuppressive effect on T cells occurs in response to exosomes and without loss of T cell viability. In view of differences with previous reports we began by characterizing the exosomes with regard to their morphology, size, and composition and evaluating the immunosuppressive ability of exosomes derived from tumor ascites fluids of 12 patients with ovarian cancer and T cells derived from 8 different normal donor PBL. To validate our findings we have used multiple different and independent activation endpoints, and have established the ability of the exosomes to inhibit the activation of virus-specific CD8⁺ T cells that are stimulated with viral peptides in the context of class I MHC. Finally, we demonstrate here that the exosome-mediated inhibition of T cell activation is reversible, which makes this system function as a checkpoint that could be a useful immunotherapeutic target in ovarian cancer patients.

Materials and Methods

Study Design

The study was designed to assess the effect of exosomes on T cell function in response to antigen-specific as well as polyclonal stimuli. The source of exosomes were ascites fluids of ovarian cancer patients and lymphocytes isolated from several different healthy donors, who were randomly selected based on availability. Forty-one different ascites fluids and 13 different lymphocyte specimens were used in the study. 300–500 µg exosomes (by protein weight) were used in the assays. All experiments reported were reproducibly repeated at least three times. Seven different independent endpoints of T cell activation were monitored. For the analysis of transcription factor translocation by confocal microscopy, a minimum of 400 cells were counted. For flow cytometry and imaging cytometry experiments, data acquisition was stopped after acquiring 5×10^4 lymphocytes or nucleated cells respectively.

Specimens

Ascites fluids from Stage III or Stage IV ovarian cancer patients were received from the Roswell Park Cancer Institute (RPCI) Tissue Procurement Facility. Experiments were done using cell-free ascites fluids that had been stored at -80°C . Normal donor peripheral blood was provided by the Flow and Image Cytometry Facility at RPCI. Normal donor peripheral blood lymphocytes (NDPBL) were obtained by monocyte depletion and Ficoll-Hypaque density separation. Cells were frozen and stored in liquid nitrogen until use, as previously reported (1, 7). In order to perform MHC multimer studies (which are haplotype-specific), we required peripheral blood from individuals that had been previously haplotyped and tested positive for a specific AST population. All specimens were obtained under sterile conditions and using IRB approved protocols.

Reagents

See Supplementary Table S1.

Isolation of exosomes

Ascites fluids were first centrifuged at $300 \times g$ to separate cells and large debris, followed by another round of centrifugation at $1150 \times g$ to remove smaller debris and membrane fragments. They were then diluted to 50% (with RPMI-1640 or PBS), passed through a $0.22 \mu\text{m}$ PVDF filter (Millipore) and ultracentrifuged at $200,000 \times g$ for 90 min. The pellet was resuspended in RPMI-1640 + 1% HSA (for functional experiments) or PBS (for biophysical characterization).

Transmission Electron microscopy (TEM)

For TEM studies, exosomes were isolated and fixed using 2% paraformaldehyde. $10 \mu\text{L}$ of exosome suspension was coated on formvar-carbon coated grids and negatively stained with 2% uranyl oxalate. The grids were air dried for 5 min. The specimens were analyzed with a 100CX Transmission Electron Microscope (JEOL USA Inc.).

Size measurement of exosomes

The size of exosomes was measured using nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern). The exosomes were diluted appropriately to give counts in the linear range of the instrument (i.e., 3×10^8 to 10^9 per mL). Videos of the particles undergoing Brownian motion in the laser beam were recorded and analyzed using the NTA software which determines the exosome concentration and size distribution. Three videos of 10s duration each were recorded for each sample.

Anisotropy measurements

The exosome pellet was re-suspended in 1mL of PBS and labeled with $0.6 \mu\text{M}$ of the membrane probe, diphenyl hexatriene (DPH) (Invitrogen). Fluorescence anisotropy experiments were conducted on a PTI Quantamaster fluorescence spectrophotometer (Photon Technology International), fitted with a Peltier unit. The sample was excited at 355 nm and the emission monitored at 430 nm. Fluorescence polarization and anisotropy were calculated as described previously (24). The phase behavior and transition was monitored using fluorescence anisotropy as a function of temperature over a temperature range of 4°C to 50°C .

Exosome antibody array

The identification of protein markers on the isolated exosomes was done using the commercially available Exo-Check exosome antibody array (System Biosciences Inc.) kit as described by the manufacturer. The membrane was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and analyzed using ChemiDoc MP System (BioRad).

Detection of NFAT translocation following T cell activation with MHC dextramers

The method for detection of NFAT translocation following T cell activation with MHC dextramers was as previously described (25) with the following modifications specific to studying the effects of ascites fluid-derived exosomes. Whole blood from EBV- or CMV-positive donors was incubated with peptide-loaded dextramers with or without exosomes for 2h at room temperature or for 10 minutes on ice, after which cells were immunophenotyped.

T cell activation with antibodies to CD3 and CD28

Antibodies were immobilized on maxisorb 12 × 75 mm tubes (Nunc) by incubating 0.1 µg of purified anti-CD3 (Bio X Cell, catalog number BE001-2; clone OKT3) and 5 µg of purified anti-CD28 (Life Technologies, catalog number CD2800-4; clone 10F3) in 500 µl of PBS, at 4°C overnight. PBL from normal donors were thawed, resuspended in RPMI-1640 + 1% human serum albumin, and 5×10^5 total cells were incubated in anti-CD3/anti-CD28 in coated tubes at 37°C/5% CO₂ for the duration of activation.

Detection of NFAT and NFκB translocation following T cell activation

After activation, cells were attached to alcian blue coverslips in a humid chamber (10 min) and fixed in 2% Formaldehyde in 1x PBS (40 min), the cells were permeabilized and blocked with 30µg NMIgG in 5% normal mouse serum in 1x PBS + 0.4% Triton X-100. The cells were then stained for intracellular CD3 for 20 minutes. After washing once with NGS block (5% normal goat serum in 1X PBS), the cells were incubated with 2 µg/mL goat anti-mouse IgG-Alexa Fluor 568 for 15 minutes. This was followed by staining with purified rabbit anti-human NFκB p65 or NFAT in NGS block/perm for 1 hour. After washing twice with NGS block, the cells were incubated with 2 µg/mL goat anti-rabbit IgG-Alexa Fluor 488 in 100µL NGS block/perm for 30 minutes. The cells were washed twice with NGS block and twice with 1X PBS before mounting the coverslips on glass slides with Vectashield Mounting Medium (Vector Laboratories, Burlingame). Cells were then observed on a Zeiss LSM 510 Confocal Microscope with at least 400 CD3+ cells counted per condition.

Detection of NFAT and NFκB translocation following T cell activation

Human NDPBL were activated for 2h at 37°C with immobilized anti-human CD3/CD28 with or without ovarian ascites fluid-derived exosomes. The percentage of activated T cells was determined by monitoring the translocation of NFAT or NFκB from the cytosol into the nucleus using fluorescence microscopy as previously reported (7).

Detection of CD69 expression following T cell activation

Human NDPBL were activated for 2h at 37°C with immobilized anti-human CD3/CD28 with or without ovarian ascites fluid-derived exosomes. The cells were then incubated for 18h in RPMI-1640 + 1% HSA at 37°C/5% CO₂ in the absence of stimulation or exosomes. For flow cytometry, the cells were labeled with the recommended amounts of fluorochrome-conjugated antibodies to CD3, CD4, CD8 and CD69 for 30 mins at 4°C. The cells were then washed with 2 mL of PBS, acquired on an LSR Fortessa (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star Inc. OR).

Detection of CD107a expression following T cell activation

Human NDPBL were activated for 6h at 37°C/5% CO₂ with immobilized anti-human CD3/CD28 in the presence of 1 µL/mL GolgiStop (BD Biosciences) and 20 µL/mL fluorochrome labeled antibody to CD107a with or without exosomes derived from ovarian ascites fluid. For flow cytometry, the cells were labeled with fluorochrome-conjugated antibodies to CD3, CD4 and CD8 for 30 mins at 4°C, washed, fluorescence emission acquired, and results analyzed as above.

Proliferation assay

Human NDPBL were labeled with CellTrace Violet Proliferation kit (Thermo Fisher Scientific) as recommended by the manufacturer. The labeled cells were incubated in the presence or absence of ascites fluid derived exosomes in tubes that were coated with immobilized antibodies to human CD3 and CD28 for 7 days. Fresh medium was added after 3 days. On day 7, the cells were labeled with fluorochrome-conjugated anti-human CD3. Sytox Red was added 15 min before flow cytometry at a final concentration of 5 nM to label the dead cells. The fluorescence was acquired on an LSR Fortessa (BD Biosciences) flow cytometer. The data were analyzed using FlowJo software (Tree Star Inc.) and ModFit software (Verity Software House) to calculate the proliferation index.

Detection of intracellular IL-2 and IFN-γ expression following T cell activation

Human NDPBL were activated for 6h at 37°C/5% CO₂ with immobilized anti-human CD3/CD28 in the presence of 1 µL/mL GolgiStop (BD Biosciences) with or without ovarian ascites fluid derived exosomes. For flow cytometry, the cells were labeled with fluorochrome-conjugated antibodies to CD3, CD4 and CD8 for 30 mins at 4°C. The cells were then fixed and permeablized with the fixation/permeablization solution from the Cytofix/Cytoperm kit (BD Biosciences) as described by the manufacturer and labeled with fluorochrome-conjugated antibodies to IL-2 and IFN-γ at 4°C for 30 min, washed, fluorescence emission acquired, and results analyzed as above.

Detection of secreted IFN-γ expression following T cell activation

Human NDPBL were activated for 2h at 37°C with immobilized anti-human CD3/CD28 with or without ovarian ascites fluid derived exosomes. The cells were then incubated for 18h in RPMI-1640 + 1% HSA at 37°C/5% CO₂ in the absence of stimulation, but with exosomes present in a 24-well plate. The amount of IFN-γ secreted in the supernatant was determined using ELISA as previously reported (26).

Exosome Labeling

Exosomes were labeled with CellTrace Violet using the CTV Proliferation kit (Thermo Fisher Scientific), or with PKH67 using the PKH67 Cell Linker kit (Sigma-Aldrich) as recommended by the respective manufacturer.

ImageStreamX acquisition

Imaging flow cytometry acquisition and analysis was performed as previously described (27). Data acquisition was performed on an imaging flow cytometer (ImageStreamX Mk-II;

Amnis, part of EMD Millipore, WA). The selected laser outputs prevented saturation of pixels in the relevant detection channels as monitored by the corresponding Raw Max Pixel features during acquisition. Cell classifiers were set for the lower limit of size of the bright field image to eliminate debris, the upper limit of size of the brightfield image to eliminate aggregates, and a minimum intensity classifier on the DAPI channel to exclude non-cellular (DAPI negative) images.

ImageStreamX data analysis

Following compensation for spectral overlap based on single color controls, image analysis was performed with IDEAS® software (Amnis, part of EMD Millipore). The internalization score is a standard feature available in the IDEAS image analysis software. The Internalization feature is defined as the ratio of the intensity inside the cell to the intensity of the entire cell. The so-called masked area (region of interest) to define the inside of the cell was created by eroding the object mask of the brightfield by 3 pixels (Erode (Object (M01, Ch01, Tight), 3) and the internalization features were calculated using this mask for the CD3 and exosome-specific channels (Ch3 and Ch2 respectively). Note that the internalization feature is invariant to cell size and accommodates concentrated bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range. The spatial relationship between the transcription factors and nuclear images was measured using the 'Similarity' feature in the IDEAS® software, as described previously (28, 29). Briefly, a 'Morphology' mask is created to conform to the shape of the nuclear DAPI image, and a 'Similarity Score' (SS) feature is defined. The SS is a log-transformed Pearson's correlation coefficient between the pixel values of two image pairs, and provides a measure of the degree of nuclear localization of a factor by measuring the pixel intensity correlation between the NFAT images and the DAPI images within the masked region. Cells with a low SS exhibit poor correlation between the images (corresponding with a predominant cytoplasmic distribution of NFAT or NFκB), whereas cells with a high SS exhibit positive correlation between the images (corresponding with a predominant nuclear distribution of the transcription factor).

Statistics

All statistics were calculated using Excel 2013 (Microsoft). Paired or unpaired Student's t test was applied to determine whether the differences between groups could be considered significant. A *P* value higher than 0.05 was not significant (NS), whereas **P* < 0.05; ** *P* < 0.01 and *** *P* < 0.001 were considered significant.

Results

Characterization of immunosuppressive vesicles from ovarian tumor ascites fluids

Vesicles isolated from ovarian cancer patients' tumor ascites fluid by ultracentrifugation were examined for ultrastructural morphology and size by transmission electron microscopy (TEM). Uranyl oxalate stained vesicles were homogeneously spherical, membrane bound particles consistent with the morphology of exosomes (Fig. 1A).

Orthogonal biophysical techniques such as nanoparticle tracking analysis (NTA) and fluorescence anisotropy were employed to determine size and lamellarity of the vesicles. NTA analysis of the vesicles revealed a size distribution of 50–200 nm with a modal diameter of 60–80 nm (Fig. 1B). The lamellarity of these vesicles was analyzed by labeling these vesicles with diphenyl hexatriene (DPH); lipid order and dynamics were measured at various temperatures using fluorescence anisotropy (Fig. 1C). At lower temperatures, anisotropy values were higher, consistent with a rigid acyl chain packing, but anisotropy values decreased with higher temperatures due to increased acyl chain mobility. The anisotropy values as a function of temperature showed a broad transition centered around 37°C suggesting lamellarity in lipid organization. We conclude that the vesicles present within ovarian tumors are surrounded by a lipid bilayer.

Vesicles isolated by ultracentrifugation from ovarian tumor ascites fluids were assayed for the presence of marker proteins that are typically found on exosomes (30) using a commercially available antibody platform called Exosome Antibody Array. Five of the exosome marker proteins (CD81, Tsg-101, Flotillin-1, EpCAM, and Annexin V) were found to be abundant in the vesicles; two other markers, CD63 and Alix, were detected but less abundant (Fig. 1D). The absence of a positive spot for GM130 indicated that our exosome preparations were not contaminated with cellular material. As we and others have previously reported, tumor-associated exosomes also express a negatively charged glycerophospholipid, phosphatidylserine (PS) (31), representing a lipid marker expressed on the surface of exosomes.

Based upon the morphology, size, and presence of relevant protein and lipid markers, we conclude that the extracellular vesicles we are isolating from ovarian cancer patients' tumor ascites fluids are exosomes.

Exosomes inhibit nuclear translocation of NFAT and NF κ B following activation

Extracellular vesicles derived from cancer patients' sera/plasma (32) or from patients' ovarian tumor ascites fluids (31) have been reported to inhibit the activation of T cells. However, those studies used a method to activate the T cells that depended on antibodies to CD3 and CD28 immobilized on antibody-coated beads (32). Such a protocol represents an artificial stimulus for T cells of unknown specificity. Because exosomes may simply block CD3 and/or CD28 antibody binding to T cells, we asked whether tumor ascites-derived exosomes would similarly inhibit an antigen-induced activation of T cells.

To address this question, we utilized Class I MHC multimers (dextramers) loaded with peptides known to bind to antigen receptors on either EBV- or CMV-specific T cells and activate them (25). T cell activation is determined by a translocation of NFAT from the cytosol into the nucleus and has been confirmed by cells' production of cytokines (25). Peripheral blood from HLA-A2 donors known to have EBV- or CMV-specific T cells were incubated either on ice (non-permissive for activation) or at room temperature (permissive for activation) with EBV peptide (Fig. 2A, B) or CMV peptide (Fig. 2C, D) loaded HLA-A2 dextramers with or without exosomes. The location of the transcription factor NFAT in CD3⁺ CD8⁺ T cells (either in the cytosol or nucleus) was determined using imaging flow cytometry as previously reported (25). Prior to activation, NFAT is present in the cytosol of

the T cells. At the permissive temperature only, the virus specific CD8⁺ T cells incubated without exosomes, but with the appropriate peptide loaded dextramer, translocated NFAT from the cytosol into the nucleus. The presence of exosomes resulted in a significant inhibition of the activation of both EBV-specific (Fig. 2A, B) and CMV-specific (Fig. 2C, D) T cells incubated with the appropriate dextramer (82% and 42% inhibition respectively). No significant activation was observed with cells incubated on ice (Fig. 2A–D). Although prolonged exposure to tumor derived vesicles eliminates tumor-specific T cells by driving them to apoptosis (33), we demonstrate here that these exosomes can inhibit the activation of antigen-specific T cells with a brief (2h) exposure. We conclude that the tumor-derived exosomes induce an arrest in an early activation endpoint (a blockade in the activation of NFAT) of a proportion of the virus-specific T cells that are stimulated by their cognate antigen in the context of MHC.

To better understand the kinetics and durability of the exosome-mediated inhibition of T cells, we studied the effects of exosomes on additional early and later endpoints of T cell activation. In these studies, PBL derived from normal donors were incubated for two hours in the presence or absence of exosomes, and with a T cell stimulus of immobilized antibodies specific for CD3 and CD28. Early activation was monitored by detecting the translocation of the transcription factors NFAT and NFκB into the nucleus of CD3⁺ T cells by confocal microscopy. We found that, similar to the inhibition of viral peptide-induced NFAT translocation to the nucleus, the translocation of NFAT in response to polyclonal stimulation was also inhibited by 42% in the presence of exosomes (Fig. 2E). The translocation of another key transcription factor downstream of TCR signaling, NFκB, was also inhibited by 59% (Fig. 2F), consistent with our previous report (31). The percentage of exosome-mediated inhibition in T cells varies with the ascites fluids from which the exosomes are derived (Supplementary Fig. S1). The mean inhibition for 41 different ascites fluid-derived exosomes was found to be $41 \pm 6.4\%$.

Exosomes inhibit the upregulation of activation marker CD69 in CD4⁺ and CD8⁺ T cells

We next tested the effect of the presence of exosomes during activation on a later activation endpoint – the upregulation of CD69. Following a brief pulse with the polyclonal activation stimulus and exosomes, cells were washed and incubated overnight in culture. Using flow cytometry with gates set on viable CD3⁺CD4⁺ or CD3⁺ CD8⁺ T cells, expression of the activation marker CD69 was assessed. After a 2 h activation without exosomes, followed by overnight culture without further stimulation, expression of CD69 on both CD4⁺ and CD8⁺ T cells was upregulated. However, after a 2 h activation with exosomes, followed by overnight culture, expression of CD69 was significantly inhibited in both CD4⁺ T cells (Fig. 3A and B) and CD8⁺ T cells (Fig. 3C–D).

These results establish that both CD4⁺ and CD8⁺ T cells require only a 2h exposure to exosomes to achieve and observe an inhibition of an activation end point (CD69 upregulation) that occurs much later without a persistent presence of the exosomes. As the T cells were gated on viable cells, the exosomal inhibition of activation occurred without a loss of T cell viability over the period of analysis. This was confirmed by experiments which

demonstrated that the viability of T cells activated for 2h with or without exosomes were comparable following overnight culture (Supplementary Fig. S2).

Exosomes inhibit degranulation of activated cytotoxic CD8⁺ T cells

A well-defined function of CD8⁺ cytotoxic T cells is the killing of target cells that is dependent upon the release of preformed cytotoxic granules (34). Upon activation of cytotoxic T cells, these cytoplasmic granules move to and fuse with the plasma membrane of the cells and release their lytic enzymes. Surface labeling of T cells with antibodies to CD107a following activation identifies human and mouse degranulating CD8⁺ T cells (34). Six hours after activation, 35% of the CD8⁺ T cells derived from the PBL of normal donors were positive for the surface expression of CD107a (Fig. 3E–F). CD107a expression was inhibited when the T cells were incubated with tumor-associated exosomes (Fig. 3E–F).

Exosomes inhibit IL2 and IFN γ production by CD4⁺ and CD8⁺ T cells

Another function of T cells for both CD4⁺ and CD8⁺ cells is the production and secretion of cytokines following activation. An increase in the percentage of both CD4⁺ and CD8⁺ T cells that express IL2 in the cytoplasm following activation was significantly inhibited when the cells were incubated with exosomes (Supplementary Fig. S3A–D). Similarly, exosomes were also found to inhibit the production of IFN γ at the single cell level in CD4⁺ and CD8⁺ T cells (Supplementary Fig. S4A–D), and in bulk cultures of PBL following activation (Supplementary Fig. S4E).

Exosomes inhibit proliferation of T cells in response to persistent activation

The results presented above establish that multiple early and late endpoints of activation are inhibited in T cells briefly pulsed with exosomes. We next attempted to determine if the inhibition of activation could be overcome or reversed by persistent activation. To address this, we monitored another endpoint of activation - the proliferation of T cells cultured with or without exosomes for 7 days in the presence of immobilized antibodies to CD3 and CD28. Cell proliferation was quantified by the generational reduction of fluorescence intensity of T cells labelled with CellTrace Violet (CTV) (Fig. 4A). Proliferation modeling was done using the ModFit software, and the proliferation index, which represents the fold expansion during culture, was calculated. As expected, T cells cultured with persistent stimulation but without exosomes proliferated with nearly a 6-fold population expansion (Fig. 4A–B). In contrast, T cells that were persistently stimulated in the presence of exosomes proliferated, but with less than a 3-fold population expansion (Fig. 4B). We conclude that exosomes suppress but do not eliminate the proliferation of T cells in response to persistent stimulation.

Exosome-mediated inhibition of T cell activation is concurrent with internalization of exosomes

The binding of CTV-labeled exosomes to CD3⁺ T cells was quantified by flow cytometry. We found that approximately 25% of the T cells showed an intermediate increase in the MFI (Exo intermediate/Exo^{int}) of CTV. About 5% of the T cells had a higher MFI (Exo^{hi}) suggesting that exosome binding to T cells was occurring, but at two different levels of

intensity (Fig. 5A). We determined that there was no inhibition of activation in the 70% of the T cells showing no evidence of binding of CTV-labeled exosomes (Exo⁻). T cells showing moderate binding and those with high levels of exosome binding revealed 39% and 60% inhibition of activation respectively (Fig. 5B–C).

The association between exosome binding and inhibition of activation was further addressed using imaging flow cytometry. T cells were pulsed for 2h with exosomes labeled with PKH67, which labels the exosome lipid bilayer. Following the activation of the cells with immobilized antibodies to CD3 and CD28, the CD3⁺ T cells were individually interrogated by imaging flow cytometry simultaneously for their (a) binding and internalization of the PKH67 stained exosomes into the T cells, and (b) activation status as indicated by the localization of AlexaFluor 647-labeled NFκB either in the cytoplasm (for unactivated cells) or in the nucleus (for activated cells). Fig. 6A shows examples of T cells with exosome clusters labeled with PKH67 (cyan) present within the cell cytoplasm, and with NFκB (red) staining also in the cytoplasm of unactivated T cells. In contrast, T cells activated in the absence of exosomes translocate NFκB (red) to the nucleus, marked by DAPI (green) (Fig. 6B). Internalization of exosomes, defined as the ratio of the intensity inside the cell to the intensity of the entire cell, and was calculated for the CD3 signal and the exosome signal using the IDEAS® software and is demonstrated in Fig. 6C. Higher scores indicate a greater concentration of intensity inside the cell. The data in Fig. 6B establish that for the CD3⁺/exosome⁺ cells, the CD3 internalization score is predominantly negative whereas that of the exosome signal is positive consistent with the expected membrane localization of CD3 and an internalized exosome localization. The IDEAS software was also used to calculate the similarity score (SS), which is a measure of nuclear NFκB (Fig. 6D). Unactivated T cells had a similarity score of -0.66, with most cells having NFκB in the cytosol, which increased to +0.21 on activation in the absence of exosomes. However, the SS of cells that had internalized exosomes was only +0.08, consistent with the notion that exosome binding and internalization was coincident with a blockade of activation. Together, these results confirm that a proportion of T cells bind and internalize exosomes, rendering them unresponsive to activation.

Exosome-mediated inhibition of T cell activation is reversible

To determine whether the inhibition of T cell activation by tumor-associated exosomes was reversible, we incubated T cells with immobilized antibodies to CD3 and CD28 in the presence or absence of exosomes. Activation was measured by determining the nuclear translocation of NFκB (Fig. 7A) or the production of intracellular IFNγ (Fig. 7B). As expected, we saw a significant inhibition in both activation endpoints in these T cells in the presence of exosomes. These cells, and control cells that were activated without exosomes, were then rested for either 24 or 48h in the absence of stimulation and exosomes and then reactivated. When using NFκB translocation as an endpoint, we observed a complete recovery of activation potential in the T cells previously inhibited by the exosomes (Fig. 7A). We observed a similar recovery in the activation potential when using IFNγ as the activation endpoint, as the T cells that were initially inhibited by exosomes were now found to be reactivated to the same level as control T cells (Fig. 7B). The decrease in the percentage of IFNγ seen in the control T cells upon reactivation is typical when cells are reactivated

after a brief recovery period. Since these T cells recovered their activation potential within 24h, we conclude that the inhibition of T cell activation that occurs during a 2h pulse with the exosomes is reversible.

Collectively, these results show that tumor-associated exosomes bind to and are internalized rapidly by T cells, and that the binding/internalization coincides with the arrest of the activation of the T cells and does not affect viability. This T cell arrest is transient and can be reversed by removing the immunosuppressive exosomes. This suggests that tumor-associated exosomes represent a potential cancer therapeutic target.

Discussion

We have previously reported that T cells present in the ascites fluids of patients with ovarian cancer are hypo-responsive to activation via the T cell receptor (7) and that the suppression of these tumor-associated T cells appears to be mediated by small but uncharacterized extracellular vesicles (31). In this report, we have characterized these membrane encapsulated vesicles by size, morphology, composition and biophysical properties as exosomes. We have determined that the activation of T cells derived from normal donor PBL is arrested during a 2h incubation of the cells with the tumor-associated exosomes. This inhibition, which is shown here to include multiple different activation endpoints, occurs coincidentally with the binding and internalization of the exosomes, and without loss of T cell viability. The exosome induced T cell arrest is reversible as the exosome inhibited T cells lost their inhibition after incubation for 24–48h without exosomes. This recovery included two different activation endpoints, (a) the early translocation of NF κ B, and (b) the later functional activation indicated by production of IFN γ . Our results establish that tumor-associated exosomes have the ability to arrest T cells during an activation stimulus. Once exosomes are removed, this arrest is reversed over 24 to 48h.

However, the ability to reverse the exosome-mediated downregulation of the T cells may well depend upon the duration of the exposure of the cells to the exosomes. Others have reported that the T cell inhibition induced by tumor-associated extracellular vesicles, including vesicles characterized as exosomes, occurs gradually and appears to be irreversible (15). For example, extracellular vesicles isolated from tumors act over several days, and can act indirectly by augmenting the function of T regulatory cells and MDSCs or blocking the maturation of dendritic cells and macrophages (15, 17–20). It has also been proposed that the tumor-associated vesicles act directly over a period of days to permanently suppress T cells by driving them into apoptosis that occurs as a result of the suppression of the CD3 ζ chain, or through the expression of apoptosis-inducing ligands on exosomes including FasL, PDL, and TRAIL (21). These results suggest that a prolonged exposure of the T cells to the exosomes (1–4 days) may drive these cells into irreversible suppression. A similar gradual and progressive loss of T cell function is observed with antigen-driven exhaustion of T cells with an accumulation of multiple checkpoint molecules such as PD-1, CTLA4, LAG-3, TIM3 etc. leading to a deterioration of T cell functions that ultimately become irreversible (35). However, our results establish that a brief (2h) exposure of the T cells to the exosomes during the activation of the T cells results in a rapid but reversible arrest in their response to activation. Recognition of differences in the dynamic and kinetic effects of

the exosomes on T cells may help determine the mechanisms by which exosomes suppress T cell function, and for the eventual design of therapeutic strategies to enhance the antitumor effects of T cell by reversing the immunosuppressive effects of the exosomes in tumor microenvironments.

We propose that exosome induced T cell arrest begins with the binding of the exosomes to a receptor on T cells that induces an immunosuppressive signal. A causal link of PS to the exosomal suppression of T cells was established by blocking this suppression with anti-PS antibodies and Annexin V as well as by a selective depletion of PS⁺ vesicles (31). A testable hypothesis relevant to the T cell suppression mechanism is that PS⁺ exosomes bind to a PS receptor such as TIM3. Further, since empty liposomes expressing PS on their surface are able to mimic the same T cell arrest induced by exosomes, it is possible that PS by itself has the capacity to modulate T cell function directly, and that PS on exosomes is capable of inducing a direct signaling arrest independent of an immunosuppressive exosome cargo (31).

PS enhances the metabolic activity of diacylglycerol kinase (DGK) (36), a negative regulator of diacylglycerol (DAG), which is part of the TCR signaling cascade. Because PMA, a DAG analog, reverses the T cell inhibitory effects of tumor-associated vesicles (31), it is plausible that PS acts to inhibit the TCR signaling cascade by a DGK phosphorylation of DAG converting it into inactive phosphatidic acid. This mechanism is supported by the finding that inhibitors of DGK block the inhibitory activity of exosomes derived from the ascites (31) and the regulation of DAG by DGK is critical in the induction of T cell anergy (37–40).

The presence of immunosuppressive exosomes in the tumor microenvironment likely contributes to local blockade of an antitumor response in patients. Treatment strategies that block or reverse the effects of exosomes may be useful alone or in combination with other immunotherapeutic approaches. We have established here that the immunosuppression of T cells following a brief (2h) exposure to exosomes during their activation is reversible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of extracellular vesicles isolated from human ovarian ascites fluid
Electron microscope images of vesicles isolated from ovarian tumor ascites fluids using ultracentrifugation (**A**). Size distribution of the vesicles was determined using nanoparticle tracking analysis (**B**) and phase transition study of vesicles isolated from ovarian tumor ascites fluid by ultracentrifugation was done using anisotropy measurements (**C**). The composition of vesicles isolated from ovarian tumor ascites fluid by ultracentrifugation was determined using an Exosome Antibody Array (**D**). Dark spots indicate presence of the marked protein. Absence of a spot for GM130 indicates absence of cellular contaminants in the preparation. Data shown are representative of 3 independent experiments.

Fig. 2. Exosomes inhibit the nuclear translocation of NFAT and NFκB following activation

A–D: NDPBL from HLA-A2 positive donors were incubated with dextramers loaded with EBV (A–B) or CMV (C–D) peptides in the presence or absence of ascites fluid-derived exosomes. The activation was monitored by determining NFAT translocation to the nucleus using an Amnis ImageStream Cytometer. The intra-patient heterogeneity in exosome-induced inhibition of the dextramer-induced NFAT activation in EBV and CMV specific T cells is represented in (A) and (C). Each data point represents an individual cell. The horizontal bars represent the mean $\pm$ the standard deviation of all the CD8⁺/dextramer⁺ cells

detected. Mean $\pm$ standard deviations of a triplicate assessment in three different EBV positive individuals are shown in (B) and three different CMV positive individuals are shown in (D). Note that NFAT translocation does not occur with cells on ice. E–F: NDPBL were either left unactivated (UN), or activated for 2 hours with immobilized antibodies to CD3 and CD28 in the absence (Act) or presence (Act + Exo) of exosomes derived from ovarian tumor ascites fluid. The translocation of the transcription factors NFAT (E) or NF κ B (F) into the nucleus of CD3⁺ T cells was monitored using fluorescence microscopy. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Fig. 3. Exosomes inhibit the activation and degranulation of T cells

NDPBL were either left unactivated (UN), or activated for 2 hours with immobilized antibodies to CD3 and CD28 in the absence (Act) or presence (Act + Exo) of exosomes derived from ovarian tumor ascites fluid. The expression of the activation marker CD69 on CD3⁺ CD4⁺ cells (A–B) or CD3⁺ CD8⁺ cells (C–D) was measured by flow cytometry following overnight culture of the activated cells. The compiled mean $\pm$ SEM from three independent experiments for CD4⁺ T cells is shown in (B) and that for CD8⁺ T cells is shown in (D). The expression of CD107a on CD3⁺CD8⁺ cells 6 hours after activation was measured by flow cytometry (E). The compiled mean $\pm$ SEM from three independent experiments is shown in (F). $n = 3$, *** $P < 0.001$

Fig. 4. Exosomes inhibit T cell proliferation in response to persistent stimulation

NDPBL were labeled with CellTrace Violet and either incubated in medium only (UN; filled histogram), or activated for 7 days with immobilized antibodies to CD3 and CD28 in the absence (Act; solid line) or presence (Act + Exo; dotted line) of exosomes derived from ovarian tumor ascites fluid. The proliferation of T cells was estimated by measuring dye dilution in live CD3⁺ T cells using flow cytometry. Representative dye dilution profiles are shown in (A). Proliferation index, which indicates the average number of divisions undergone by the cells, was calculated using ModFit Lt Software (B). $n = 3$, mean $\pm$ SEM.

*** $P < 0.001$

Fig. 5. Inhibition of T cell activation is associated with exosome binding

NDPBL were either incubated in medium only (UN), or activated for 2 hours with immobilized antibodies to CD3 and CD28 in the absence (Act) or presence (Act + Exo) of CellTrace Violet labeled exosomes derived from ovarian tumor ascites fluid. The expression of CD69 following overnight culture was measured by flow cytometry. Gating strategy used to define T cells not bound to exosomes (Exo⁻), showing intermediate binding to exosomes (Exo^{int}) and showing high binding to exosomes (Exo^{hi}) is shown in (A). Representative data for the expression of CD69 on Exo⁻, Exo^{int} or Exo^{hi} CD3⁺ cells is shown in (B). The compiled mean $\pm$ SEM from three independent experiments is shown in (C). $n = 3$, mean $\pm$ SEM. NS = Not significant; * $P < 0.05$

Fig. 6. T cells that internalize exosomes fail to translocate NFκB upon stimulation

NDPBL were either incubated in medium only (UN), or activated for 2 hours with immobilized antibodies to CD3 and CD28 in the absence (Act) or presence (Act + Exo) of PKH67 labeled exosomes derived from ovarian tumor ascites fluid. Activation was then determined by measuring NFκB translocation to the nucleus using imaging flow cytometry. Representative images of CD3⁺ T cells that internalized exosomes (white arrows) and did not translocate NFκB to the nucleus (marked by DAPI) are shown in (A). Nuclear translocation of NFκB in CD3⁺ T cells activated in the absence of exosomes (positive control) is shown in (B). Internalization score (ratio of the fluorescence intensity inside the cell to the intensity of the entire cell) for CD3 as well as exosomes is shown in (C). Higher score signifies greater concentration of intensity inside the cell. Cells with internalized signal typically have positive scores whereas cells with little internalization have negative scores. Cells with scores around 0 have a mix of internalization and membrane intensity. Similarity scores that indicate NFκB translocation to the nucleus are shown in (D). A negative similarity score in the unactivated group indicates absence of NFκB from the nucleus. Representative of 3 experiments. *** $P < 0.001$

Fig. 7. Exosome-mediated inhibition of T cells is reversible

NDPBL were activated with immobilized antibodies to CD3 and CD28 in the absence (Act) or presence (Act + Exo) of exosomes derived from ovarian tumor ascites fluid. These cells were then rested for 24 or 48 hours and reactivated in the absence of exosomes. Activation was then determined by monitoring NFκB translocation to the nucleus of CD3⁺ cells using fluorescence microscopy (A) or intracellular expression of IFNγ in CD4⁺ and CD8⁺ T cells using flow cytometry (B). The compiled mean ± SEM from three independent experiments is shown. NS = not significant; ** $P < 0.01$; *** $P < 0.001$