

ORIGINAL RESEARCH



Human tumor-derived exosomes (TEX) regulate Treg functions via cell surface signaling rather than uptake mechanisms

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ABSTRACT

Tumor-derived exosomes (TEX) are ubiquitously present in the tumor microenvironment and plasma of cancer patients. TEX carry a cargo of multiple stimulatory and inhibitory molecules and deliver them to recipient cells, serving as a communication network for the tumor. The mechanisms TEX use for delivering messages to recipient cells were evaluated using PKH26-labeled TEX produced by cultured human tumor cells, exosomes produced by dendritic cells-derived exosomes (DEX), or exosomes isolated from plasma of cancer patients (EXO). Human T-cell subsets, B cells, NK cells, and monocytes were co-incubated with TEX, DEX, or EXO and binding or internalization of labeled vesicles was evaluated by confocal microscopy and/or Amnis-based flow cytometry. Vesicle-induced Ca^{2+} influx in recipient T cells was monitored, and TEX-induced inosine production in Treg was determined by mass spectrometry. In contrast to B cells, NK cells or monocytes, conventional T cells did not internalize labeled vesicles. Minimal exosome uptake was only evident in Treg following prolonged co-incubation with TEX. All exosomes induced Ca^{2+} influx in T cells, with TEX and EXO isolated from cancer patients' plasma delivering the strongest, sustained signaling to Treg. Such sustained signaling resulted in the significant upregulation of the conversion of extracellular ATP to inosine (adenosine metabolite) by Treg, suggesting that TEX signaling could have functional consequences in these recipient cells. Thus, modulation of Treg suppressor functions by TEX is mediated by mechanisms dependent on cell surface signaling and does not require TEX internalization by recipient cells.

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Introduction

Exosomes are the smallest subset of extracellular vesicles, measuring 30–150 nm in diameter.^{1,2} Exosomes are produced by all cells and can be found in all body fluids.³ Their origin from the endocytic compartment of the parent cell sets them apart from other vesicles, which are either formed by “blebbing” of the surface membrane or represent remnants of apoptotic cells.⁴ The exosome biogenesis involves the fusion of multivesicular bodies enclosing pools of endosomal vesicles with the cell plasma membrane and the subsequent release of the vesicles into intercellular space.⁵ The exosome cargo of proteins, lipids, and glycans characteristically contains endocytic components, such as ALIX or TSG101 as well as tetraspanins (e.g., CD63 and CD81) commonly used as exosome “markers.”⁶

Exosomes are considered to be vehicles for information transfer between the parent and recipient cells. As such, exosomes carry and deliver messages from the parent to the recipient cell. While considerable knowledge has accumulated about the biogenesis of exosomes,^{7,8} relatively little is known about their uptake by recipient cells. It is known, however, that exosomes can interact with target cells by several different

mechanisms, ranging from receptor/ligand interactions to endocytosis [reviewed in ref. 9]. The mechanism used for the delivery of the exosome cargo and its uptake by recipient cells depends primarily on the nature of the recipient cell. Thus, cells equipped with the ability to phagocytose, such as macrophages, readily internalize exosomes, un-coat them and process their content, which results in alterations in the transcriptome and ultimately cellular functions.¹⁰ Non-phagocytic cells, such as subsets of T lymphocytes, which express receptors for ligands carried by exosomes, depend on specific receptor-ligand recognition for the implementation of exosome-delivered signals.¹¹ In this case, the molecular content of exosome membrane-bound cargo could serve as an “address,” and might play a role in directing exosomes to the appropriate recipient cells.

Cellular responses to the exosome-mediated information transfer involve phenotypic, functional, and genetic modifications in responding cells. We have previously reported that tumor-derived exosomes (called TEX) suppress functions of immune cells either directly by delivering suppressive signals/molecules to activated lymphocytes^{12–15} or indirectly by

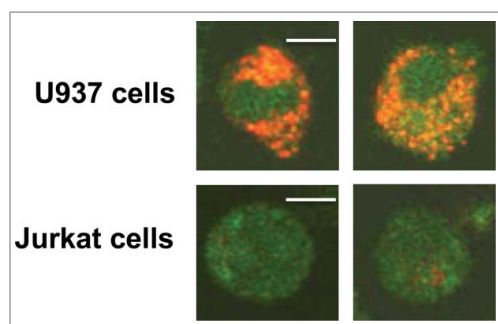


Figure 1. Differential uptake of PKH26-labeled TEX by U937 tumor cells and by Jurkat T cells. Representative images showing strong internalization of PKH26-labeled TEX by U937 cells (upper row) but no TEX uptake in CD8⁺ Jurkat cells (lower row). TEX were isolated from supernatants of PCI-13 tumor cells and labeled with the PKH26 dye as described in Methods. Cells co-incubated with 10 μg of TEX for 24 h in a 35 mm MatTek plate were examined in a confocal microscope. The bar = 5 μm.

promoting expansion and activity of regulatory T cells (Treg).¹⁶ However, mechanistic aspects of direct or indirect T cell interactions with TEX remain poorly understood. Specifically, it is not clear whether exosomes have to be internalized by T cells before inducing cellular changes or whether signals received upon exosome binding to the cell surface are sufficient for of T cell responses. Based on our preliminary (i.e., receptor-ligand signaling) data reported by Muller et al.,¹⁷ we hypothesized that negative signaling by TEX interacting with conventional CD8⁺ and CD4⁺ T cells depends on the receptor-ligand contact on the cell surface. In contrast, the upregulation of Treg functions might require TEX uptake and re-programming of cellular activities in Treg. To gain insights into exosome-T cell crosstalk, we co-incubated dye-labeled TEX with human T cells or other mononuclear immune cells and evaluated signal delivery via Ca²⁺ signaling and exosome uptake by the recipient cells. We report that T lymphocytes, unlike other mononuclear cells, do not internalize exosomes but rather respond to exosome signals delivered to the cell surface by Ca²⁺ influx followed by down-stream functional responses. Thus, functions of Treg as well as conventional T cells are regulated by TEX via receptor-ligand signaling on the cell surface rather than uptake mechanisms.

Results

Co-incubation with TEX induces apoptosis of activated CD8⁺ T cells

Our previously reported and current data consistently show that activated human CD8⁺ T cell co-incubated with TEX bind AnnexinV and undergo apoptosis (Fig. S1). Interestingly, when we co-incubated CD8⁺ Jurkat cells (as surrogates for primary T cells) with the PKH26 dye-labeled TEX isolated from supernatants of tumor cell lines or plasma of patients with cancer for up to 24 h, we did not observe TEX uptake by these T cells (Fig. 1). No TEX were internalized even after prolonged co-incubation (up to 72 h). In contrast, a tumor cell (U937) strongly internalized the same TEX (Fig. 1). Staining intensity of tumor cells treated with trypsin after co-incubation with TEX was comparable to that of untreated cells, suggesting the TEX uptake and not just their binding to the cell surface. These studies suggested that TEX can induce functional changes (i.e., apoptosis) in CD8⁺ Jurkat cells without internalization.

Binding or internalization of TEX by human mononuclear cell subsets (MNC)

To further evaluate exosome interactions with the MNC subsets, we used the AMNIS image analysis system. Binding to or internalization of TEX, DEX, or EXO by immune cells was found to be time dependent, dose dependent, and recipient cell type dependent. As shown in Fig. 2A, the co-incubation of PKH26-labeled TEX with T cells did not lead to the uptake of TEX even after >72 h co-incubation. In contrast, monocytes readily internalized labeled TEX, as indicated by the cell images taken at 48 h (Fig. 2B).

In addition to monocytes, B cells and NK cells internalized TEX, as illustrated in Fig. 3A.

Differences in the exosome uptake at 24 h between T cells and the other MNC subsets were highly significant (Fig. 3A). Clearly, the uptake of exosomes by immune cells depended on the type of recipient immune cells: T cells did not internalize exosomes, while the other MNCs did.

To determine whether pre-activation of the recipient cells influences exosome uptake, we co-incubated resting or activated T cells with PKH26-labeled TEX or DEX. As shown in

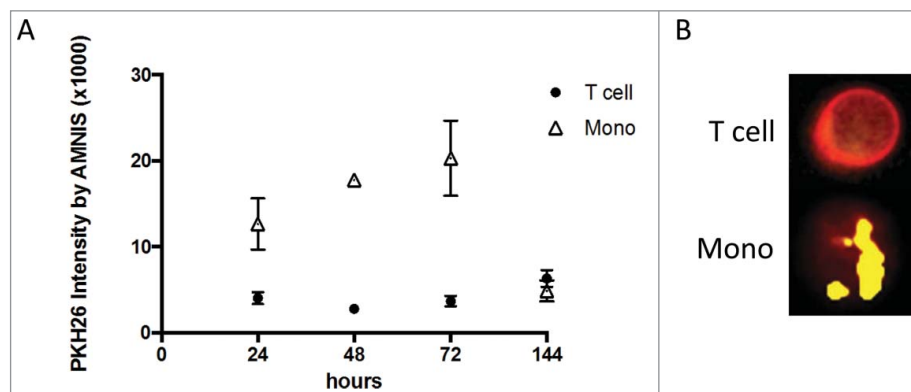


Figure 2. Differences in uptake of PKH26-labeled TEX cells and monocytes. (A) Time-dependent uptake of TEX by monocytes contrasts with the slower and lower TEX uptake by T cells. Amnis measurements of PKH26 intensity in cells were acquired after increasing co-incubation times. (B) Representative Amnis images show TEX uptake by monocytes vs. no uptake by T cells after 48 h of co-incubation.

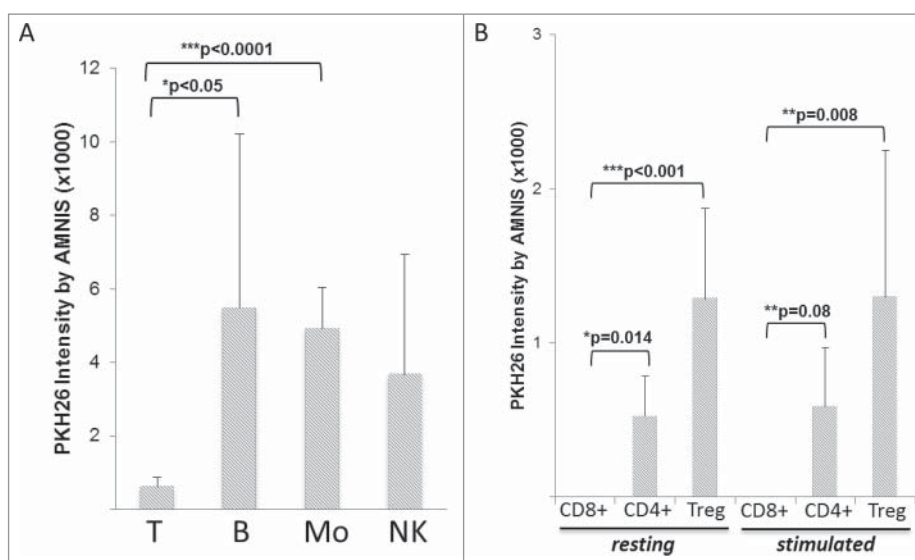


Figure 3. Amnis-generated data showing differences in TEX uptake by various human MNC subsets. (A) Various MNC subsets isolated from different ND were co-incubated with TEX for 24–48 h. T cells T show significantly lower TEX uptake compared with other MNC subsets. (B) Comparisons of TEX uptake by resting or activated CD8⁺ T cells, CD4⁺ T cells, or CD4⁺CD39⁺ Treg show a lack of TEX internalization by CD8⁺ T cells relative to low but significantly increased TEX uptake by Treg. T cell subsets were isolated from the peripheral blood of four different ND. In (A) and (B), the data are presented as mean levels of PKH26 intensity in recipient cells \pm SD. The *p* values denote significant differences.

Fig. S2A, the activation of the recipient T cells had no effect on the uptake of either TEX or DEX, which was equally low and not significantly different for these two exosome types. In contrast to T cells, resting or activated B cells, effectively internalized TEX or DEX, and the uptake by activated B cells was greater ($p = 0.03$) than that by resting B cells (Fig. S2B). Also, activated NK cells and monocytes internalized TEX or DEX with significantly greater efficiency ($p < 0.0001$) than activated recipient T cells (Fig. S2C). In aggregate, the Amnis-generated results showed that TEX and DEX are equally well internalized by MNC, except for T cells that did not internalize either. Pre-activation of recipient cells appears to improve the uptake of TEX and DEX by monocytes and NK cells as well as B cells.

Exosome interactions with Treg

We have previously reported that the co-incubation of CD4⁺CD25^{hi}CD39⁺ Treg with TEX or DEX induced changes in the transcriptome of the recipient cells.¹⁷ Therefore, it was of interest to determine whether Treg internalized any TEX or DEX relative to CD8⁺ or CD4⁺Tconv cells. As shown in Fig. 3B, pre-activated or resting CD8⁺ T cells did not take up labeled exosomes during 24 h co-incubation, with CD4⁺ Tconv cells demonstrating only weak positivity by Amnis, and Treg showing significantly better but still very low uptake at 24 h (compare with the uptake by other MNC in Fig. 3A). The activation of Treg via the T cell receptor (TcR) did not improve

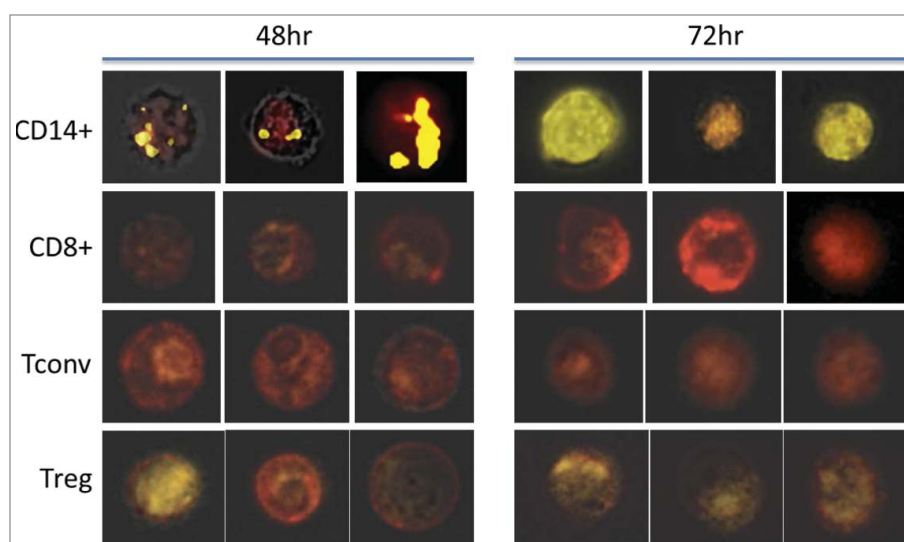


Figure 4. Amnis-generated representative images of recipient MNC co-incubated with PKH26-labeled TEX for 48 or 72 h. Immune cell subsets were isolated from healthy donors' plasma and analyzed by Amnis Image Stream as described in Methods. The presented images are representative results of four experiments performed with MNC of different donors and show results obtained by a triple overlay (PKH26-stain in yellow, surface stain in red, and a brightfield image) as described in Methods.

exosome uptake, and the uptake of EXO obtained from cancer patients' or normal control's (NC's) plasma was equivalent to that of TEX or DEX (data not shown). Fig. 4 presents representative Amnis images of the TEX uptake by monocytes and various T cell subsets following 48 h and 72 h co-incubation with labeled exosomes. The images clearly show that in comparison to negative CD8⁺T cells and CD4⁺Tconv, weak but detectable remnants of PKH26⁺ exosomes can be encountered in some but not all Treg. Thus, interactions of TEX, DEX, or EXO with T lymphocytes did not involve their internalization, except in the case of Treg, where the binding of exosomes to the cell surface was followed by weak and reluctant internalization.

Exosomes induce Ca²⁺ influx in T cells

The data we previously reported showed that TEX induced significant changes in the phenotype and functions of T lymphocytes, including Treg.¹⁵ The Amnis uptake data for TEX described above suggest that these phenotypic and functional changes in T cells are not accompanied by TEX internalization. Therefore, we considered the possibility that signals delivered by TEX to the T cell surface are responsible for the observed effects. Given that Ca²⁺ signaling is essential for T cell activation and T-cell-dependent immune responses,¹⁸ we next sought to determine whether co-incubation of T cells with exosomes induced Ca²⁺ influx in recipient cells. We measured the strength of Ca²⁺ flux induced by TEX or EXO in various T cell subsets by confocal microscopy after labeling T cells with the Fluo-3 dye. As expected, Ca²⁺ flux in T cells was time dependent. Fig. 5A illustrates results of confocal microscopy for T cells co-incubated with TEX or DEX for various time periods. Ca²⁺ influx induced by DEX appears weaker and is already extinguished by 10 min relative to TEX-induced flux. EXO isolated from the plasma of a cancer patient induced stronger and longer lasting flux than TEX, while EXO isolated from NC's plasma were negative. Among T cells, CD4⁺CD39⁺ Treg were preferentially targeted by TEX, showing the strongest and sustained Ca²⁺ influx within the first 20 min of co-incubation with TEX (Fig. 5B). DEX co-incubated with Treg induced only a very weak signal that remained flat during 20 min co-incubation (Fig. 5B). Furthermore, we compared Ca²⁺ flux in conventional CD4⁺T cells co-incubated with EXO obtained from the plasma of head and neck squamous cell carcinoma (HNSCC) patients (*n* = 3) with active disease (AD) vs. EXO obtained from the plasma of NDs (*n* = 3). Representative data (Fig. 5C) indicate that the EXO of patients with cancer induced Ca²⁺ flux and signaling in T cells, while the EXO of ND did not, similar to what we saw with EXO isolated from the plasma of NC in Fig. 5A. These data are consistent with our previous reports on effects of exosomes on activated immune cells, with a caveat that TEX-induced signaling leads to activation of inhibitory pathways in these T cells, while EXO from NC's plasma do not induce immunosuppression.¹²

TEX induce functional alterations in T cells

In experiments performed in parallel with Ca²⁺ flux measurements, we co-incubated CD4⁺CD39⁺ Treg or CD4⁺ T cells with TEX in the presence of exogenous ATP for 16 h.

Production of purines by the cells ± TEX was measured by mass spectrometry (Fig. 6A). In addition, PCI-13 cells (the TEX parent cells) were co-incubated with T cells (Fig. 6B). The data showed that after 16 h of co-incubation, adenosine is consumed and inosine is the main product of enzymatic ATP hydrolysis. Co-incubation with TEX induced significant (*p* < 0.0074) upregulation of inosine production in Treg but not in CD4⁺ T cells, probably because of large differences among the three cell donors (Fig 6B). These data are consistent with the results of Ca²⁺ experiments. Interestingly, PCI-13 cells did not significantly upregulate inosine production, suggesting that TEX deliver a stronger signal to recipient T cells than to the parent tumor cells. Overall, the data indicate that in T cells, signaling by TEX leads to detectable, biologically relevant changes in functions of recipient cells.

Discussion

Exosomes are considered to be information-transfer vehicles that deliver messages from the parent to recipient cells located within or outside of the microenvironment. The mechanisms used for directing exosome trafficking and message delivery and the role of exosomes in inducing phenotypic and functional changes in recipient cells are not yet understood. Evidence has emerged that TEX exert inhibitory effects on immune cells.^{15,19,20} TEX, which are ubiquitous in the tumor microenvironment, carry a cargo of suppressive and stimulatory molecules¹⁵ and, upon contacting the surface of immune cells, might suppress functions of some cells, while upregulating functions of others. Specifically, based on our previous data,^{14,16} we assumed that Treg would respond to stimulatory signals, while CD8⁺ effector T cells would be suppressed by interactions with TEX. Furthermore, it is likely that TEX and exosomes produced by normal cells, e.g., DEX, which carry molecularly distinct cargos, as we previously reported,¹⁷ will exert different effects upon co-incubation with human T cell subsets.

Previously, we and others have shown that TEX-induced immunosuppression involves signaling of FasL⁺ exosomes via CD95 (Fas) receptor on activated CD8⁺ T cells.^{13,21} Other receptor-ligand interactions (e.g., TRAIL/TRAILR, TGF-β/TGF-βR, or ADO/A_{2A}R) are also implicated in TEX-mediated suppression in the tumor microenvironment.^{17,22,23} The data suggest that TEX convey inhibitory signals to T cells by delivering ligands tethered to the exosome membrane to the cognate receptors on immune cells. We and others have observed that phenotypic and functional changes brought about by TEX interacting with T cells result in transcriptional alterations of multiple immunoregulatory genes.¹⁷ As exosome cargos contain mRNAs and miRNAs,^{24,25} transfer of genetic materials from the parent to recipient cells could be the mechanism responsible for the observed effects. Indeed, evidence for transfer of genes by exosomes has been forthcoming from numerous studies involving different cell types.²⁶⁻²⁸ Exosomes transferring their contents to recipient cells must bind, fuse with the surface membrane, and unload their cargo inside the recipient cell. Several mechanisms have been proposed for this type of information transfer, largely based on the use of inhibitors able to partially (but almost never completely) block the

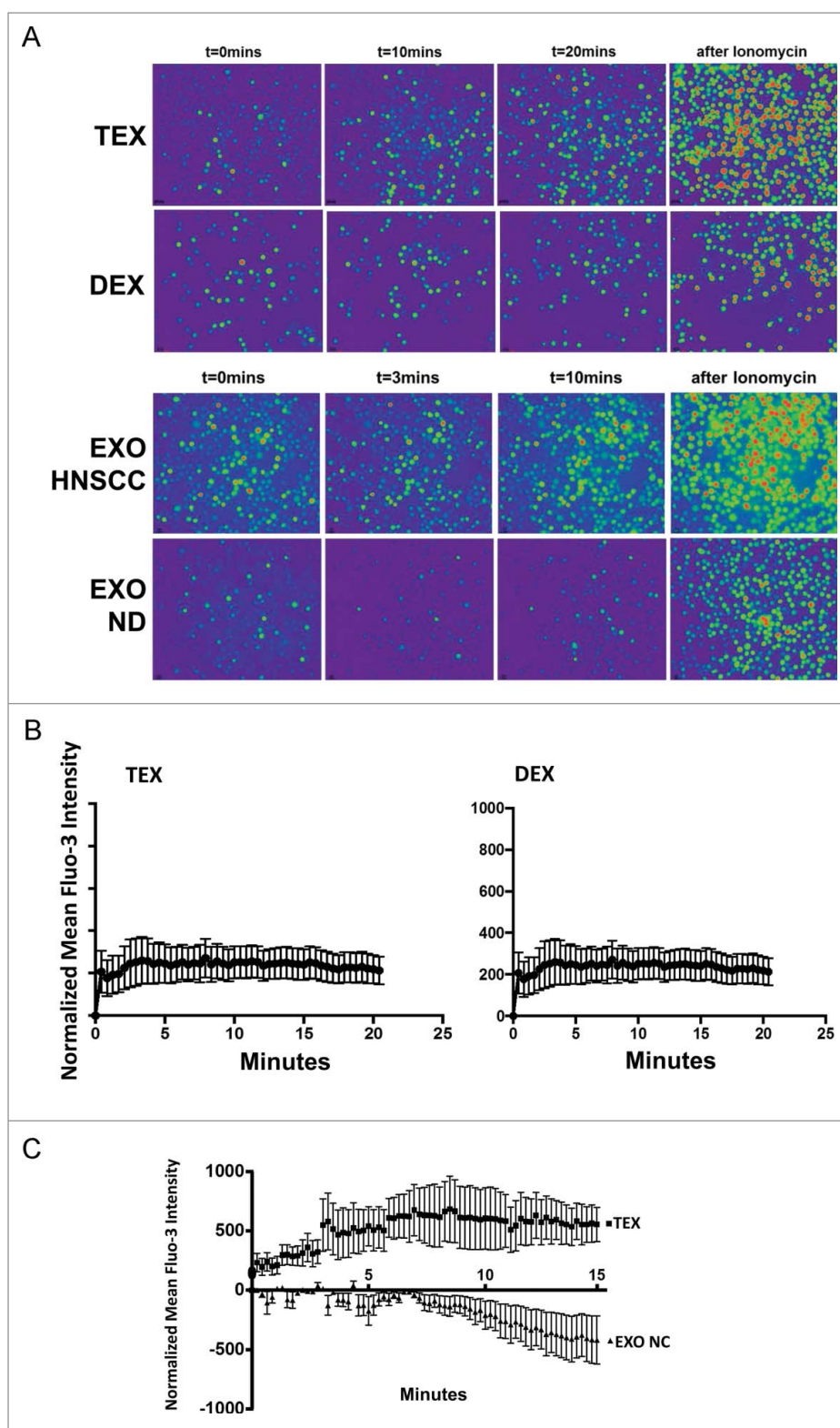


Figure 5. Ca^{2+} flux in human T cells co-incubated with TEX, DEX, or EXO. In (A), T cells co-incubated with exosomes were labeled with Fluo-3 and examined in a Nikon A1 microscope as indicated in Methods. Ionomycin, which induces strong Ca^{2+} flux, was used as a positive control. T cells were imaged at different times after Fura-2 addition. The *upper panel* compares effects of TEX and DEX. The *lower panel* compares effects of EXO isolated from plasma of a representative HNSCC patient vs. EXO isolated from ND's plasma. In (B), Fluo-3 intensity levels in T cells co-incubated with TEX or DEX. The data are from a representative experiment of 3 performed with T cells of different donors. Note the strong and prolonged signal in T cells co-incubated with TEX and the absence of signaling in T cells co-incubated with DEX. In C, Fluo-3 intensity levels in CD4^{+} T conv cells co-incubated with EXO from the plasma of HNSCC patient with active disease (AD) or with EXO from ND's plasma. Note the strong, prolonged signal induced by patients' EXO vs. an absence of signal with ND's EXO. Shown are representative Ca^{2+} flux data obtained from four to six experiments performed with T cells of different subjects.

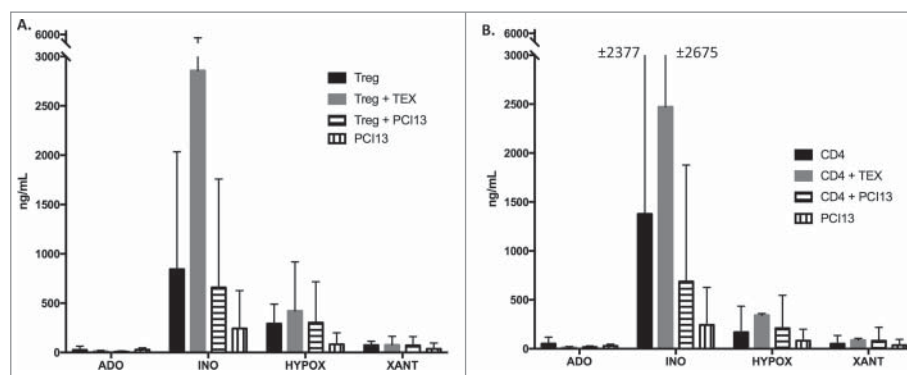


Figure 6. Mass spectrometry for purine derivatives of adenosine in co-cultures of TEX with Treg or CD4⁺ T cells. TEX were co-incubated with T cells or PCI-13 tumor cells in the presence of exogenous ATP for 16 h. T cells were isolated from three different donors. Cells and supernatants were collected and prepared for measurements of purines by mass spectrometry as described in Methods. In (A), TEX but not the parent PCI-13 tumor cells induced a large increase in inosine production by CD4⁺CD39⁺ Treg. In (B), TEX co-incubated with CD4⁺T cells also induced the upregulation of inosine production, although there were large differences among cells of different donors. The data are means \pm SEM from experiments performed with T cells of three different donors.

uptake.⁹ While endocytosis, a mechanistically complex and varied process of internalization, appears to be a major method of exosome entry into recipient cells, both the origin of exosomes and the cell type receiving them might be of critical importance for productive information transfer.²⁹

The mechanisms of information transfer and delivery by TEX to immune cells are critical for understanding the crosstalk between the tumor and the host immune system. Here, we show that signals delivered by TEX to the cell surface are sufficient for eliciting changes in the phenotypic or functional profile of recipient T cells and do not require TEX internalization by these cells. Among MNC, T cells alone did not internalize TEX, DEX, or EXO even following activation via the TcR. Their responses largely depended on surface signaling as indicated by Ca²⁺ influx, which was especially strong with TEX, weaker with DEX and EXO of cancer patients and negative with EXO isolated from NC's plasma. In contrast to CD8⁺ and CD4⁺ conv T cells, Treg showed the evidence of weak but positive uptake of TEX after prolonged (48 h) exposure to TEX. Importantly, TEX also induced strong and sustained Ca²⁺ signal in Treg, while in Treg co-incubated with DEX, the Ca²⁺ signal was weak and flat. This observation suggests that the exosomal cargo, most likely the presence of relevant ligands in the exosomal membrane, determines the strength of signals that translate downstream into functional alterations in T cells. Several examples of ligands carried by exosomes (or produced by enzymatic cleavage of exosome membrane proteins) interacting with receptors and inducing downstream signaling effects have been reported. For example, stimulation by exosomes of the MAPK pathway leading to altered activity in recipient cells has been reported.³⁰ Importantly, in our hands, only TEX induced sustained Ca²⁺ influx in Treg, while EXO from NC's plasma did not. This confirms that in cancer patients, TEX represent a highly efficient signaling system programmed by the parent tumor cell to crosstalk with Treg that regulate antitumor immune responses.

To demonstrate that TEX-delivered signals alter the functional repertoire of the immune cells expressing receptors for the cognate signals, we focused on the adenosine pathway. T cells are known to express the A_{2A}R and respond to adenosine by cAMP upregulation, which results in alterations in their

functions, as we and others have shown.³¹ More recently, it has been reported that inosine, a metabolite of adenosine, also binds to and signals via A_{2A}R.³² Furthermore, inosine sustains prolonged A_{2A}R signaling long after its more potent predecessor, adenosine, is degraded, thus maintaining the immunomodulatory role of the A_{2A}R.³² Our previous observations that CD39⁺CD73⁺ TEX signaling via the A_{2A}R rapidly induce adenosine production by Treg²² are now extended to illustrate that *prolonged* exposure to TEX upregulates the production of inosine in Treg. This may be an example of TEX-mediated “A to I editing,” which is now thought to play an important role in a variety of physiologically relevant cell functions.³³ It also appears that A_{2A}R-mediated signals delivered to Treg by TEX were significantly more effective in stimulating inosine production than were the TEX parent cells, emphasizing the importance of exosomes in mediating intercellular communication. The ability of TEX to regulate suppressive activities of Treg at a distance, using receptors present on the recipient cell surface, represents an efficient and so far underestimated mechanism of tumor-induced immune suppression operating in cancer.

Materials and methods

Peripheral blood specimens

Buffy coats obtained from healthy volunteers and used for isolations of human leukocyte subsets were purchased from the Central Blood Bank of Pittsburgh. Fresh specimens of venous peripheral blood were obtained from patients with HNSCC or NC, all of whom signed the informed consent approved by the institutional IRB (IRB #991206). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque, washed in the RPMI medium and immediately used for experiments.

Exosome isolation

Exosomes were isolated from: (a) supernatants of the HNSCC cell line, PCI-13, maintained in a long-term culture.³⁴ This cell line served as a source of pure TEX; (b) the plasma of patients with cancer [HNSCC or acute myelogenous leukemia (AML)]

and the plasma of NC as described below. These plasma-derived exosomes are referred to as *EXO*; (c) supernatants of human dendritic cells (DC) cultured from monocytes isolated from PBMC by adherence to plastic and incubated in the presence of IL-4 and GM-CSF³⁵ for 4 d. These DC supernatants were used as a source of DC-derived exosomes (*DEX*). The DC cultured from plastic-adherent monocytes were >90% CD40⁺CD83⁺CD86⁺DR⁺ by flow cytometry. The media used for cell cultures contained FCS that was ultracentrifuged at 100,000×g for 3 h to deplete it of bovine exosomes.

The method for exosome isolation was described previously by us.³⁶ Briefly, differential centrifugation (1,000×g for 10 min and 2,000×g for 10 min at 4°C and then 10,000×g for 30 min at 4°C) was followed by ultrafiltration (0.22 μm filter; Millipore, Billerica, MA, USA) and then by size-exclusion chromatography (SEC) on mini-columns packed with Sepharose 2B (Sigma-Aldrich, St. Louis, MO, USA). The exclusion-volume fractions were collected and concentrated by Vivaspın 500 centrifugal concentrator (Sartorius Corp., New York, NY). The exosome fractions isolated from plasma contained a mix of vesicles originating from different parent cells, although in patients with cancer, these fractions were variably enriched in TEX.

Characterization of isolated exosomes

Protein concentrations of exosome fractions were determined using a BCA Protein Assay kit as recommended by the manufacturer (Pierce, Thermo Scientific, Rockford, IL 61105, USA). The isolated exosomes were evaluated for morphology by transmission electron microscopy (TEM), particle distribution and size in a NanoSight instrument and biologic activity by flow cytometry to demonstrate their ability to downregulate NKG2D expression in isolated human NK cells as described previously.³⁶ TEM of isolated exosomes was performed at the Center for Biologic Imaging at the University of Pittsburgh. Briefly, freshly isolated exosomes were put on a copper grid coated with 0.125% Formvar in chloroform. The grids were stained with 1% (v/v) uranyl acetate in ddH₂O, and the exosome samples were examined immediately. A JEM 1011 transmission electron microscope was used for imaging.

Exosome labeling with the PKH-26 dye

Isolated exosomes (up to 200 μg protein) were added to wells containing 2 mL of PKH26 (10⁻⁶ M in diluent buffer) and after 60 sec the staining reaction was stopped by adding 7 mL of PBS. Exosomes were pelleted by ultracentrifugation at 100,000×g for 2 h. They were then re-suspended in PBS at the concentration of ~1 μg protein/μL and used for co-incubation with immune cells.

Isolation of human mononuclear cell subsets

T cell subsets were isolated from human PBMC via an immunoaffinity-based capture procedure, using Miltenyi beads as described previously.³⁷ Briefly, before immunocapture, mononuclear cells were plated on Ab-coated plates to collect CD14⁺ monocytes. Negative selection was used to

recover CD19⁺ B cells, CD4⁺, or CD8⁺ T cells or CD3-CD56⁺CD16⁺ NK cells as needed. To isolate Treg, negatively selected CD4⁺ T cells were separated by positive selection using anti-CD39 Ab-coated Miltenyi beads into CD4⁺CD39⁺ Treg and CD4⁺CD39^{neg} T effector cells. The purity of the isolated cell subsets was determined by flow cytometry. The isolated T cell subsets were either directly used for experiments (resting T cells) or activated by incubation in the presence of anti-CD3/anti-CD28 antibody (Ab)-coated beads and IL-2 (150 U/mL) for 4 h or overnight, depending on the experiment. To confirm activation, cells were harvested, stained for CD69, and the frequency of CD69⁺ T cells as well as CD69 expression (MFI) on the cell surface were determined by flow cytometry.³⁶ The MFI values were converted into MESF units, based on fluorescent intensity curves generated with calibration beads.

Flow cytometry of PBMC

The phenotype of freshly-isolated primary mononuclear cell subsets was evaluated by multiparameter flow cytometry. Cells were incubated in the dark for 20 min at room temperature (RT) with a panel of labeled monoclonal antibodies (mAbs): anti-CD4-eFluor 450 (48-0048-42, eBioscience, CA, USA), CD8-ECD (6604728, Beckman Coulter, Brea, CA, USA), CD39-PC7 (25-0399-42, eBioscience); CD20-PE (IM1451U, Beckman Coulter); CD56-PE (IM2073U, Beckman Coulter); CD14-FITC (IM0645U, Beckman Coulter); CD16-FITC (IM0814U, Beckman Coulter); CD25-APC/Cy7 (302613, BioLegend, San Diego, CA, USA) and Foxp3-APC (17-4776-41, eBioscience). Isotype controls were used for each experiment. After incubation, cells were again washed, re-suspended in flow buffer and analyzed using a Gallios flow cytometer (Beckman Coulter). At least 5 × 10⁴ events were collected, and the data were analyzed using Kaluza software (Beckman Coulter). For fluorescence quantitation standardized fluorochrome microspheres were used as recommended by the company (QuantumTM FITC-5 MESF, 555, Bangs Laboratories, Fishers, Indiana, USA).

Co-incubation of resting or activated PBMC subsets with PKH26-labeled exosomes

Isolated subsets of PBMC (lymphocyte subsets or monocytes) were used either without or with *in vitro* activation. T lymphocytes were incubated in a medium containing 150 I U/mL of IL-2 (Miltenyi Biotech Inc.,) and 150 μL of microbeads coated with anti-CD3/CD28 Abs (Miltenyi). B cells were activated using CD40L and IL-4 as described previously.³⁸ Monocytes were activated with 10 ng/mL of LPS (Sigma Aldrich, St. Louis). All cells were activated for 24 h. Pre-activated or resting cells were seeded in wells of a 96-well plate at the cell concentration of 1 × 10⁶ per mL in triplicate. The PKH26-labeled exosomes (50–100 μg protein) were added to each well and the plate was incubated for various time periods from 4 to 72 h at 37°C. Control wells contained cells but no exosomes. Following extensive washing in PBS to remove exosomes, cells were examined by confocal microscopy or stained with labeled Abs for Amnis image cytometry.

AMNIS image cytometry

Following co-incubation with PKH26-labeled exosomes cells was harvested, washed, and stained for surface markers with antibodies specific for CD4-BY421, CD8-ALEXA488, CD39-APC, CD19-BY421 or CD14-APC as described above. Image analysis was performed using an Amnis cytometer. Cells were first visualized in a bright field, identified as T cells (CD4⁺, CD8⁺, CD39⁺), B cells (CD19⁺), or monocytes (CD14⁺) based on surface staining and observed for the presence of intracytoplasmic PKH26. The cell images were merged to confirm uptake of PKH26 by individual cells. Exosome binding to immune cells and their uptake by the cells were monitored over time. The IDEAS software was used to evaluate exosome uptake by cells based on the number of PKH-26⁺ cells and overall PKH-26 fluorescence intensity.

Confocal microscopy

Human cell lines U937 (purchased from ATCC) and CD8⁺Jurkat cells (a gift from Dr. H. Rabinowich, U. of Pittsburgh) were maintained in cultures of RPMI medium supplemented with 10% (v/v) fetal bovine serum that was pre-centrifuged at 100,000× g for 3 h to remove nanovesicles. Cells were harvested while in the log phase of growth and labeled with CFSE as described previously.³⁵ Exosomes were isolated from plasma of patients with AML as described by us³⁶ and labeled with PKH26 (see above). Exosomes (10 μg protein) were added to 50 × 10⁴ cells in a 35 mm culture dish, and co-cultures were incubated at 37°C in the atmosphere of 5% CO₂ in air for 24 h. Confocal microscopy was performed using a Nikon A1 microscope at the Center of Bioimaging, University of Pittsburgh.

To demonstrate that exosomes are internalized and not just bound to the cell surface, labeled cells were treated with trypsin before microscopy and images of untreated vs. trypsin-treated cells were compared.

Image analysis for Ca²⁺ flux

Freshly isolated T cells (200 × 10³ cells/50 μL HBSS (with calcium and magnesium, no phenol red) medium; Thermo Fisher Scientific, Waltham, MA 02451) were stained with Fluo-3 AM (diluted 1: 200; Sigma-Aldrich, St. Louis, MO 63178) for 20 min. Cells were centrifuged at 300×g for 5 min, resuspended in 2 mL medium, placed in a Matek dish and incubated at 37°C in an atmosphere of 5% CO₂ in air for 15 min. The dish then was placed on the microscope table (preheated at 37°C) and after establishing the settings, continuous readings were taken for 30 min using a Nikon A1 microscope. While the first cell images were taken TEX, DEX, or EXO were added. The acquired image data were extracted processed using the Imaris Image software (Bitplane, Concord, MA). Cells were defined as spots (min. *n* = 500) and cell movements were tracked over time (max. move = 2 cm). The mean intensity was measured for each cell over each time point. The data were normalized, averaged and the results plotted in Prism (GraphPad Software, Inc., La Jolla, CA 92037).

Co-incubation of T cells with TEX and mass spectrometry

Aliquots of CD4⁺ T cells or CD4⁺CD39⁺ Treg isolated from PBMC of ND as described above were co-incubated with TEX (10 μg protein added to 10⁵ cells/well in 96-well plates) or PCI-13 cells in the presence of exogenous ATP (20 μM) for 16 h. Control wells contained T cells alone or tumor cells alone. All experiments were performed in triplicate. Cells and supernatants were collected, centrifuged twice, and boiled for 5 min to inactivate ADO-degrading enzymes, and stored at −80°C for subsequent analyses. Purine nucleosides were measured using liquid chromatography-tandem mass spectrometry as described previously by us.^{2,22}

Statistics

Results are shown as mean values. Significance (*p*-value <0.05) was determined using Wilcoxon's signed rank test. Prism (GraphPad Software, Inc., La Jolla, CA 92037 USA) was used to perform the statistical analysis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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