Extracellular Vesicles Present in Human Ovarian Tumor Microenvironments Induce a Phosphatidylserine-Dependent Arrest in the T-cell Signaling Cascade


Abstract

The identification of immunosuppressive factors within human tumor microenvironments, and the ability to block these factors, would be expected to enhance patients’ antitumor immune responses. We previously established that an unidentified factor, or factors, present in ovarian tumor ascites fluids reversibly inhibited the activation of T cells by arresting the T-cell signaling cascade. Ultracentrifugation of the tumor ascites fluid has now revealed a pellet that contains small extracellular vesicles (EV) with an average diameter of 80 nm. The T-cell arrest was determined to be causally linked to phosphatidylserine (PS) that is present on the outer leaflet of the vesicle bilayer, as a depletion of PS-expressing EV or a blockade of PS with anti-PS antibody significantly inhibits the vesicle-induced signaling arrest. The inhibitory EV were also isolated from solid tumor tissues. The presence of immunosuppressive vesicles in the microenvironments of ovarian tumors and our ability to block their inhibition of T-cell function represent a potential therapeutic target for patients with ovarian cancer. Cancer Immunol Res; 1–10. ©2015 AACR.

Introduction

Recent successes with clinical trials designed to enhance immune killing of tumors have led to an increased optimism and focus upon immunotherapy for cancer (1). For example, positive therapeutic effects in some cancer patients have been reported with the use of antibodies that block the inhibitory effects of PD-1/PD-L1 (2, 3) and CTLA-4 (4). In addition, dramatic and durable tumor reductions have been seen in some patients with hematologic malignancies with the adoptive transfer of tumor-specific chimeric antigen receptor (CAR) T cells (5, 6). Although these results are highly encouraging, many patients fail to exhibit significant reductions in tumor or tumor reductions are only short-lived. Ultimately, the success of these and other immunologic approaches to the treatment of cancer will depend on a more comprehensive understanding of multiple complex cellular and molecular mechanisms that contribute to the inhibition of antitumor immune responses (7).

T cells present within tumor microenvironments have been found to be hyporesponsive to activation via the T-cell receptor (TCR; refs. 8–12). Also, it has been reported that human T cells derived from peripheral blood (13) and tumor-specific CAR T cells (14) become functionally arrested following their entry into human tumor xenografts. Several immunologic checkpoints have been identified that may contribute to the loss of function of tumor-associated T cells (13). Investigations into such checkpoints have focused largely upon those that are mediated by regulatory T cells, myeloid-derived suppressor cells, immunosuppressive dendritic cells, immunoinhibitory receptors, and inhibitory factors, including TGFβ, prostaglandins, and adenosine (16). The mechanisms by which these different checkpoints suppress T-cell function in human cancer have not yet been completely determined. It is possible that some checkpoints are mediated by acellular factors that act directly upon T cells to induce a functional arrest.

We previously reported that activation of T cells derived from ovarian solid tumor tissues or patient tumor ascites fluids is rapidly and reversibly arrested by cell-free ovarian tumor ascites fluids (17). Tumor ascites fluids also reestablish anergy in ovarian tumor-associated T cells in which the TCR signaling arrest had been reversed (17). It was determined that the tumor...
ascites fluids blocked the activation of both NF-κB and NFAT in T cells receiving a TCR activation signal, and that the signaling arrest occurred at or just proximal to PLC-γ. On the basis of these earlier results, we concluded that a factor or factors present in tumor ascites fluids may represent yet another T-cell checkpoint. However, the factors present in the tumor ascites fluids responsible for the induction of the TCR signaling arrest were not identified (17).

We have now determined and report here that the T-cell inhibitory activity of tumor ascites fluids is mediated by very small, 50- to 120-nm, extracellular vesicles (EV) derived from ovarian tumor ascites fluids and from solid ovarian tumors, and that the TCR signaling arrest is dependent, in part, on a mechanism for the EV-induced TCR signaling arrest, and we suggest that EV present within ovarian tumor microenvironments represent a novel T-cell checkpoint and a potentially viable therapeutic target.

Materials and Methods

Specimens

Ovarian tumor ascites fluids were received from the Roswell Park Cancer Institute (RPCI; Buffalo, NY) Tissue Procurement Facility. Experiments were done using ascites fluids that had been stored at −80°C before being thawed. Fresh ascites and EV isolated from the unfrozen ascites fluids have also been tested and shown to have no difference in their inhibitory activity. All ascites fluids were depleted of cells by centrifugation and passed through a 0.22-μm Millipore filter. Normal donor peripheral blood was provided by the Flow and Image Cytometry Facility at RPCI.

Delipidation of tumor ascites fluid

The tumor ascites fluid was treated with 800 μg/mL pronase (EMD Millipore) overnight at room temperature. The reaction was stopped by putting into a boiling-water bath for 10 minutes. The ascites fluid was then added to 2 volumes of methanol-diethyl ether (20:80 v/v) in an extraction funnel. The funnel was rotated end over end at 30 rpm for 2 hours, and then centrifuged at 1,500 rpm for 2 minutes to separate the aqueous and organic phases. The aqueous phase was isolated by careful suction with a needle and syringe and the residual methanol was washed out with 2 volumes of diethyl ether. The aqueous phase was then placed in a rotavap at 37°C for 5 to 10 minutes to fully remove the residual organics.

Isolation and quantification of EV

Undiluted cell supernatant fluids from patient-derived solid tumors or 50% diluted ascites fluids (with RPMI-1640 +2% HSA) were ultracentrifuged at 200,000 × g for 90 minutes. The pellets were resuspended in RPMI-1640 +1% HSA. Protein quantification of EV suspended in HBSS was determined by the BCA assay (18).

Immunofluorescence staining of human T cells for CD4+ and CD8+ for isolation by flow cytometry

Human NDPBL were thawed in complete medium (RPMI-1640 + heat-inactivated 10% fetal bovine serum). The cells (8 × 10⁶ cells) were washed and resuspended in 1× PBS and added to a sterile 12 × 75 polystyrene capped tube. The cells were pelleted and then resuspended in the residual fluid. The cells were blocked with NMIgG (50 μL of 3 mg/mL stock) for 10 minutes and then stained with 100 μL of anti-human CD4-APC and 100 μL of anti-human CD8-PE (BD Biosciences) for 20 minutes. The cells were washed with 1× PBS and brought up to 2 × 10⁶ cells/mL in 1× PBS for isolation by flow cytometry using a FACSAria III cell sorter (BD Biosciences). After the sort, the cells were allowed to recover overnight at 37°C, 5% CO₂ in complete medium.

T-cell activation

Human NDPBL or sorted T cells were activated for 2 hours at 37°C with immobilized anti-huCD3/CD28 with or without liposomes, anti-PS antibody, 100 μmol/L diacylglycerol kinase (DGK) inhibitors I [R59022] and II [R59949] (Santa Cruz Biotechnology Inc.), 50% tumor ascites fluid, 50% delipidated ovarian ascites fluid, or exosomes derived from tumor ascites fluids or solid ovarian tumors. When the effect of anti-PS antibody on EV inhibition was determined, the EV were incubated with 10 μg/mL of clone 1H6 anti-PS antibody (Upstate-Cell Signaling Solutions) for 1 hour at 37°C and diluted to 1× for the activation step. This antibody has been shown to be specific for PS and did not show cross-reactivity with other phospholipids (19). PS on the EV was also blocked using 10 μg/mL of Annexin V (BD Biosciences). The percentage of activated T cells was determined by monitoring the translocation of NF-κB from the cytosol into the nucleus using fluorescence confocal microscopy as previously reported (17).

Immunofluorescence staining of human T cells for confocal imaging

After activation, cells were attached to Alcian blue coverslips in a humid chamber (10 minutes) and fixed in 2% formaldehyde in 1× PBS (40 minutes), the cells were permeabilized and blocked with 30 μg of NMIgG in 5% normal mouse serum in 1× PBS + 0.4% Triton X-100. The cells were then stained for intracellular CD3 (mouse anti-human CD3-Alexa Fluor-647; BD Biosciences) for 20 minutes. After washing once with NGS block (5% normal goat serum in 1× PBS), the cells were blocked with 30 μg of NGLgG in NGS + 0.4% Triton X-100 (NGS block/perm) for 10 minutes. After blotting off the NGLgG, the cells were incubated with 2 μg/mL purified rabbit anti-human NF-κB p65 (Santa Cruz Biotechnology, Inc.) 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 (Life Technologies) in 100 μL of NGS block/perm for 30 minutes. The cells were washed twice with NGS block and twice with 1× PBS before mounting the coverslips on glass slides with Vectashield Mounting Medium (Vector Laboratories). Cells were then observed on a Zeiss LSM 510 Confocal Microscope with at least 100 CD3+ cells counted per condition.

Immunophenotyping of EV

An 18-parameter LSR Fortessa (BD Biosciences) was used to measure phenotypic characteristics of ascites-derived EV and...
engineered liposomes. The cytometer was mechanically and electronically adapted in order to optimize the detection of submicron particles (See Supplementary Methods).

For the immunophenotyping of EV, PE–Annexin V–labeled or unlabeled EV were separately incubated with saturating amounts of BV421-conjugated isotype control, BV421-conjugated anti–CD90, BV421-conjugated anti–CD326, or BV510-conjugated anti–CD45, BV421-conjugated anti–CD41, BV510-conjugated anti–CD71, FTC-conjugated anti–CD235a (all from BD Biosciences), or PE-conjugated MHC class I (Caltag) for 30 minutes at room temperature in the dark.

For the analysis of flow cytometric data, WinList software (version 7.1.1; Verity Software House) was used, with no gating strategy applied to the raw data files. Background fluorescence intensity was measured from a sample that contained microparticles that were not labeled with fluorescently conjugated reagents. Events that were brighter than this background level of fluorescence intensity were considered to be positive for the marker of interest.

Depletion of PS-expressing EV
A total of 50 μg of anti-PS antibody (Upstate–Cell Signaling Solutions) or isotype control (mouse IgG; Caltag) was conjugated to 5 mg Dynabeads M-280 Tosylactivated (Life Technologies) according to the manufacturer’s instructions. The conjugated beads were incubated with 0.34 mg of ovarian tumor ascites fluid–derived EV with tilting and rotation for 1 hour at 4°C to capture PS-expressing EV. The unbound EV (not expressing PS) were separated from the EV-bead complex using a magnet (BD Biosciences).

Preparation of PS liposomes
The PS-containing liposomes were prepared using thin film method as described previously (20). Briefly, chloroform solutions of phosphatidylcholine (PC) and brain PS were mixed in a molar ratio of 70:30 in a glass tube and the solvent was evaporated using a roto evaporator. The thin film thus obtained was further flushed with nitrogen to ensure that there was no residual solvent. The dried film was then resuspended with 1× PBS and incubated at 37°C for 5 minutes to form multilamellar vesicles (MLV). The vesicles were sonicated for 5 minutes and were then extruded using 80-nm polycarbonate filter multiple times to form small unilamellar vesicles (ULV).

Statistical analysis
All statistical analyses were calculated using SigmaPlot 12 (Systat Software). Differences between groups were considered statistically significant if P < 0.05.

Results
Inhibitory factor present in ovarian tumor ascites fluids is a lipid
We previously established that T cells present in ovarian tumor ascites fluids were hyporesponsive to activation and that this was due to an unidentified factor or factors present in the tumor ascites fluid (17). The ascites fluids also induced a rapid and reversible arrest in the T-cell signaling cascade that occurred downstream of diacylglycerol (DAG; ref. 17). It was determined that the inhibitory activity in the tumor ascites fluid was heat stable (30 minutes in a boiling-water bath) and resistant to proteolytic digestion with pronase. On the basis of these findings, we considered that lipids may contribute to the TCR signaling arrest.

Ovarian tumor ascites fluids were pronase-treated, boiled, and delipidated by extracting with diethyl ether. As expected, the vast majority of lipid material was retained in the organic fraction as visualized by multiple bands (Supplementary Fig. S1). The aqueous fraction retained only a small quantity of a single band, which was tentatively identified as a water-soluble disialoganglioside. The aqueous (delipidated) phase was assayed for its ability to inhibit the induction of the T-cell signaling arrest. The activation of T cells in a mixed population of peripheral blood leukocytes following their incubation with immobilized anti-CD3 and CD28 was monitored by immunofluorescence confocal microscopy for the translocation of NF-κB into the nucleus. T-cell activation (translocation of NF-κB) was significantly inhibited by the tumor ascites fluid prior to delipidation, but this inhibition was largely eliminated in the delipidated tumor ascites fluid (Fig. 1A).

To identify possible lipids that could be responsible for the T-cell signaling arrest, tumor ascites fluids were extracted with hexane and isopropyl alcohol and the lipids present in the organic phase analyzed and identified by thin-layer chromatography
Tumor ascites fluids (TLC). We identified both nonpolar lipids (cholesterol esters, triglycerides, and free fatty acids) and polar lipids. A two-dimensional TLC analysis of the polar lipids revealed the presence of the following phospholipids: lysophosphatidylcholine (LPC), sphingomyelin (SM), PC, phosphatidylinositol (PI), cardiolipin (CL), and PS.

**PS induces a signaling arrest in T cells**

PS is known to play important roles in many biologic processes resulting in anti-inflammatory effects. For example, exposure of PS on the outer leaflet of apoptotic lymphocytes induces their phagocytosis by macrophages, leading to an active suppression of their inflammatory mediators (21). We have shown that a normally immunogenic protein is converted into a tolerogen when used together with PS (20). Others have reported that PS significantly inhibits adaptive immune responses by a variety of different (as of yet poorly defined) mechanisms (22). In view of these findings, PS was tested for its ability to inhibit T-cell activation.

Because of the relative insolubility of PS in water, we began by formulating PS-containing liposomes. As the liposomes formulated with 100% PS aggregated in the cation-containing buffers required for our activation experiments, we prepared liposomes formulated with 30% PS and 70% PC that we found remained in suspension in the calcium-containing activation buffer. Control liposomes were formulated with 100% PC and did not aggregate in the activation buffer. Peripheral blood T cells were activated in the presence of either the PS/PC or PC-only liposomes. As shown in Fig. 1B, the PS/PC liposomes significantly inhibited T-cell activation, whereas PC-only liposomes failed to inhibit the activation. Also, it was established that the PS/PC liposomes inhibited T-cell activation in a dose-dependent fashion (Supplementary Fig. S2). We conclude that PS has the capacity to induce the same signature TCR signaling arrest that we have observed previously with tumor ascites fluids (17).

**Tumor ascites fluid acts directly upon CD4+ and CD8+ T cells to induce the TCR signaling arrest**

Because our evaluations of T-cell activation were made on mixed populations of leukocytes, it was possible that the T-cell signaling arrest was mediated by other cell types. To address this question, CD4+ and CD8+ cells were isolated from NDPBL and sorted by flow cytometry (Supplementary Fig. S3). It was determined that the tumor ascites fluid inhibited the isolated CD4+ and CD8+ T cells to the same degree as that which was observed with the unsorted PBL (Fig. 2). Because no other cells were present in the sorted population, we conclude that the tumor ascites fluids directly inhibit TCR signaling of both CD4+ and CD8+ T cells.

**Small 50- to 120-nm EV are present in ovarian tumor ascites fluids**

The presence of PS in the tumor ascites fluids, and the ability of PS-containing liposomes to induce the signature TCR signaling arrest, led us to explore the possibility that PS was one of the inhibitory factors present in the tumor ascites and that it was present in lipoprotein complexes, micelles, large EV (apoptotic bodies), uni- or multilamellar liposome-like bodies, or very small extracellular microsicles (possibly exosomes). If it were present within EV (large or small), one would expect to be able to recover them from the pellet following the ultracentrifugation of the tumor ascites fluids that had been subjected to low-speed centrifugation and 0.2-μm filtration.

Following ultracentrifugation of these tumor ascites fluids, an examination of the resultant pellet by quasi-elastic light scatter analysis revealed the presence of homogenous (by size and lamellarity) EV with an average diameter of 80 nm (Supplementary Fig. S4A). A broad phase transition from solid to fluid phase, centered around 37°C, was observed (Supplementary Fig. S4B), indicating that the EV were surrounded by a lipid bilayer. The size and lipid bilayer composition of the isolated vesicles were confirmed by transmission electron microscopy (Supplementary Fig. S4A, inset).

**EV isolated from ovarian tumor ascites fluids induce the signature TCR signaling arrest in peripheral blood T cells**

As shown in Fig. 3A, a 2-fold dilution of the EV in the resuspended pellet (equivalent to 50% of the tumor ascites fluid) inhibited the activation of the T cells, slightly less than that observed in 50% tumor ascites fluid. The EV-induced inhibition has been repeated eight times with EV derived from different tumor ascites fluids and found to induce significant inhibition of T-cell activation. The readout for T-cell activation shown in Figs. 1–6 is the translocation of NF-κB from the cytosol into the nucleus. We have seen very similar ascites and EV-induced inhibition of T-cell activation using other readout systems, including the upregulation of CD69 (Supplementary Fig. S5), upregulation of CD25 (data not shown), and a translocation of CD107a from the cytosol to the plasma membrane (data not shown). We have consistently observed, with the ultracentrifugation of several different tumor ascites fluids, that the inhibitory activity in the supernatant fluids was significantly reduced or completely eliminated (Fig. 3B). As shown in Fig. 3C, the EV inhibited the T-cell signaling in a dose-dependent fashion. We further establish that the EV, like the tumor ascites fluids, are acting directly on CD4+ and CD8+ T cells to arrest the T-cell signaling (Supplementary Fig. S6) and that EV do not inhibit the activation of the T cells with PMA and ionomycin (Fig. 3D). This is consistent with the possibility that
the EV are acting at or just upstream of DAG. As we have previously established with the tumor ascites fluids (17), the inhibition of the T-cell signaling is reversible because T cells exposed to EV fully recover their activation potential following an overnight incubation without EV (data not shown).

**PS is expressed on the surface of the tumor-associated EV**

EV isolated from ovarian tumor ascites fluid were stained with fluorescently labeled Annexin V and assayed using an LSR Fortessa flow cytometer that was configured to resolve and quantify the very small EV for evidence of Annexin V binding. A portion (23%–26%) of the EV bound the fluorescently labeled Annexin V (Fig. 4B). The binding was shown to be specific, as it was completely inhibited by unlabeled Annexin V, but was not inhibited by bovine serum albumin (Fig. 4C and D).

**EV are derived from tumor cells and tumor-associated fibroblasts, leukocytes, and erythrocytes**

Phenotypic analysis with fluorescently labeled antibodies identified EV derived from ovarian tumor ascites fluid that expressed a tumor marker (CD326; EpCAM), a fibroblast marker (CD90), a leukocyte marker (CD45), and a portion of each of these microvesicles also expressed PS on the surface (by binding Annexin V; Fig. 4E). Other cell markers found on the EV surfaces include an erythrocyte marker (CD235a; glycophorin), a marker of most nucleated cells (class I MHC), a platelet marker (CD41), and a marker for proliferating cells that could include both tumor cells and tumor-associated cells (CD71; transferrin receptor; Fig. 4F).

**Inhibitory activity of EV derived from ovarian tumor ascites fluids and from solid ovarian tumor tissues is blocked by anti-PS antibody and by a depletion of the PS-expressing subset of EV**

As PS-containing liposomes were found to inhibit T-cell activation (Fig. 1B) and because a portion of the EV express PS on their surface (Fig. 4A–D), we predicted that PS plays a significant role in the EV-induced signaling arrest of T cells. Consistent with this prediction, it was determined that the inhibitory activity that is observed with EV derived from ovarian tumor ascites fluids was blocked by anti-PS antibody (Fig. 5A). Another approach to blocking PS on the EV was to incubate EV with Annexin V. It was established in three separate experiments that Annexin V blocked the EV-induced T-cell activation inhibition by 52%/C6.8%.

Because T cells derived from solid tumor tissues, like those isolated from the tumor ascites fluids, have been shown to have a reversible TCR signaling arrest (8, 11), we further predicted that inhibitory EV would be present within solid tumor microenvironments. To address this possibility, tissues derived from solid ovarian tumors were mechanically disrupted into viable cell suspensions and the tissue suspensions subjected to the same isolation protocol that was used to isolate EV from ovarian tumor ascites fluid. We determined that EV were present in the ultracentrifuged pellet, and that these EV derived from the solid tumors similarly inhibited the activation of T cells (Fig. 5B). Also, as we found with the ascites fluid-derived EV, the inhibitory activity induced by the solid tumor-derived EV was also blocked by the addition of anti-PS antibody (Fig. 5B).
These data establish that tumor-associated immunoinhibitory EV exist in both fluid and solid ovarian tumor microenvironments. The blockade of the inhibitory activity with anti-PS antibody suggests that PS is causally linked to the microvesicle-induced T-cell signaling arrest. Consistent with this possibility, we determined that the depletion of the PS-expressing subset of EV using anti-PS antibody-coated magnetic beads resulted in the loss of the EV-induced inhibition of T-cell function (Fig. 5C).

Inhibition of DGK blocks the T-cell inhibitory effect of tumor ascites fluids and tumor-associated EV

Our discovery that the tumor ascites fluid–induced TCR signaling arrest (17) and the inhibition with EV (Fig. 3D) are overcome by the addition of a DAG analogue (PMA; ref. 17), and the findings of others that the inactivation of DAG is observed in anergic T cells (23) suggest that EV interacting with T cells may lead to an inactivation of DAG. The inactivation of DAG has been reported to occur in anergic T cells resulting from a phosphorylation of DAG into the inactive phosphatidic acid, a reaction that is mediated by DGK (24). We hypothesized that the inhibitory activity of tumor ascites fluids and EV on fully functional T cells derived from normal donor PBL was mediated by the same or a similar mechanism that is observed in anergic T cells. Consistent with this hypothesis, we found that two DGK inhibitors (DGKi) [R59949 and R59022] reverse the tumor ascites-induced inhibition of the TCR signaling arrest (Fig. 6A) and significantly blocked the EV-induced inhibitory activity (Fig. 6B).

We conclude that the induction of the TCR signaling arrest by PS present in tumor ascites fluids and PS-expressing EV derived from the tumor ascites fluids results from an inactivation of DAG that is mediated by DGK.

Discussion

The release of very small EV by viable tumor cells was initially reported over three decades ago (25). We subsequently reported that EV are released from normal as well as neoplastic cells and characterized these vesicles ultrastructurally and biochemically (26). We report here that EV present in the ascites fluids and solid tumors of ovarian cancer patients induce a rapid and reversible arrest in the TCR signaling cascade of CD4+ and CD8+ T cells that is dependent in part on PS. In addition, our data support a likely mechanism by which this signaling arrest is achieved.
The lamellarity, nanometer size, and presence of PS on the outer leaflet of the EV that we report here are similar to that which has been reported for EV called exosomes isolated from bodily fluids (27). However, further characterization of our EV will be needed to determine whether they conform to the current definition of exosomes as defined by the International Society for EV (27). Others have reported finding PS-exosomes in ovarian cancer patients’ tumor ascites fluids and blood and have suggested that the exosomes may play a role in tumor progression (28).

Previous reports have suggested that EV isolated from tumor microenvironments suppress antitumor responses indirectly by augmenting the function or preventing the apoptosis of T regulatory cells, generating myeloid-derived suppressor cells, and blocking the maturation of dendritic cells and macrophages (29–33). However, our findings establish that the EV derived from ovarian tumor ascites fluids and solid tumors are acting directly upon T cells to arrest their function. It has been postulated by others that tumor EV/exosomes may modulate lymphocyte function directly by mimicking activation-induced cell death (34, 35) by the induction of apoptosis resulting from suppression of CD3-ζ chain, or through the expression of apoptosis-inducing ligands such as Fasl, PDL, and TRAIL on the surface of the vesicles/exosomes (36). However, all of these suggested mechanisms would result in a rather slow and nonreversible inhibition of T-cell function. We present evidence that is consistent with a mechanism that is compatible with the initial rapid and reversible T-cell inhibition that we observe with the EV. Although the T-cell inhibition that we have observed here in vitro with EV is reversible, it is possible that T cells that may be chronically exposed to EV in vivo could ultimately become irreversibly inhibited. It has been suggested that the T-cell exhaustion that arises in chronic infections and cancer progresses from a reversible to a nonreversible T-cell arrest that coincides with the accumulation of the number and different types of checkpoint molecules on the cell surface (37).

The findings that the inhibition of DGK completely blocks the tumor ascites fluid-induced TCR signaling arrest and partially blocks the EV-induced T-cell inhibition are consistent with the notion that the arrest in the activation of T cells is mediated by a DGK phosphorylation of DAG, converting it into the inactive phosphatidic acid. This mechanism accounts for both the rapid and reversible inhibition seen with the ascites fluid and the EV derived from ascites fluids, and for our finding that a DAG analogue (PMA) bypasses the inhibition induced by tumor ascites fluid (17) and by the EV derived from ascites fluids (Fig. 3D). The regulation of DAG by DGK has been shown by others to be critical in determining whether activation or anergy ensues after TCR stimulation (23, 24, 38, 39). The finding that an antibody to PS significantly blocks the inhibitory activity of both tumor ascites fluid and the tumor-associated EV establishes a role for this phospholipid in the inhibitory process. This causal link of the inhibitory activity of tumor-associated EV to PS was further documented by our finding that the depletion of the PS-positive EV significantly reduced the inhibitory activity of the EV. Others have reported that PS enhances the metabolic activity of DGK (40). Thus, PS on the tumor-associated EV may work in a similar fashion to enhance DGK activity resulting in the TCR signaling arrest.

The ability of PS liposomes to induce the TCR signaling arrest suggests that PS, itself, has the capacity to modulate the T-cell function. This is significant as others have suggested that PS may participate only as a way for EV (exosomes) to bind to and deliver...
their immune regulatory molecules to PS-binding target cells (28, 41). The presence of PS on the surface of the EV suggests that the inhibitory process may begin with the binding of PS-expressing EV to a PS receptor on T cells. The binding of PS by T cells to a PS receptor has been previously reported by others and shown to result in the inhibition of immune responses in vitro (22). An immunomodulatory capability of PS to convert a known immunogen into a tolerogen has been reported (20).

The presence of the immunosuppressive EV in both tumor ascites fluid and solid ovarian tumor tissues suggest that they may contribute to the hyporesponsiveness of tumor-associated T cells that has been previously reported (8, 10–12, 17, 42, 43). The anergy and hyporesponsiveness of T cells present in ovarian tumor microenvironments is known to be reversed when these cells are removed from the tumor microenvironment (17). The ability to block or reverse the EV-induced T-cell arrest with anti-PS antibodies or with DGKi represents two potential approaches that could be exploited therapeutically to enhance patients’ T-cell responses to their tumor.

A report showing that regulatory T cells secrete microvesicles/exosomes that are capable of suppressing cytotoxic T lymphocyte–mediated immunity against B16 melanoma (44), represents an elegant and intriguing report on the growing inventory of examples of tumor-associated EV that regulate antitumor immune responses. We recognize that lipids other than PS have been shown to modulate T-cell function and immunity. Polyunsaturated fatty acids (PUFA) were shown to directly modify T-cell signaling proteins (45) and to inhibit T-cell signaling by displacing Lck, Fyn, and LAT from detergent resistant to detergent sensitive fractions (46, 47). Elevated levels of gangliosides from renal cell carcinoma patients have been linked to tumor-associated T-cell dysfunction possibly by inducing apoptosis (48, 49) or by promoting immune deviation in favor of Th2 T-cell responses (50). In view of our inability to completely block the EV-induced inhibition of T cells signaling with antibodies to PS and with the depletion of PS-expressing EV, it is possible that one or more other lipids may act in concert or synergistically with the PS exosomes to suppress T-cell functions.

We conclude that by targeting and eliminating the immunosuppressive tumor-associated PS-containing EV, it will be possible to enhance patients’ antitumor immunity by reversing the TCR signaling arrest of T cells in the tumor and by preventing the arrest of T cells that enter the tumor microenvironment.

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