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Exosomes Represent an Immune Suppressive T Cell Checkpoint in Human Chronic Inflammatory Microenvironments.

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Abstract

Background: T cells present in chronic inflammatory tissues such as nasal polyps (from chronic rhinosinusitis patients) have been demonstrated to be hypo-responsive to activation via the TCR, similar to tumor-specific T cells in multiple different human tumor microenvironments. While immunosuppressive exosomes have been known to contribute to the failure of the tumor-associated T cells to respond optimally to activation stimuli, it is not known whether they play a similar role in chronic inflammatory microenvironments. In the current study, we investigate whether exosomes derived from chronic inflammatory microenvironments contribute to the immune suppression of T cells.

Methods: Exosomes were isolated by ultracentrifugation and characterized by size and composition using nanoparticle tracking analysis, scanning electron microscopy, antibody arrays and flow cytometry. Immunosuppressive ability of the exosomes was measured by quantifying its effect on activation of T cells, using nuclear translocation of NF κ B as an activation endpoint.

Results: Exosomes were isolated and characterized from two different types of human chronic inflammatory tissues - nasal polyps from chronic rhinosinusitis patients and synovial fluid from rheumatoid arthritis patients. These exosomes arrest the activation of T cells stimulated via the TCR. This immune suppression, like that which is seen in tumor microenvironments, is dependent in part upon a lipid, ganglioside GD3, which is expressed on the exosomal surface.

Conclusion: Immunosuppressive exosomes present in nonmalignant chronic inflammatory tissues represent a new T cell checkpoint, and potentially represent a novel therapeutic target to enhance the response to current therapies and prevent disease recurrences.

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DECLARATION OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

Keywords

Exosomes; Ganglioside GD3; T cells; nasal polyps; rheumatoid arthritis; synovial fluid; Immune checkpoint; chronic inflammation

INTRODUCTION

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease with unknown etiology, and its pathogenesis has not yet been well-defined (Newton and Ah-See, 2008). A better understanding of the cells and acellular components that contribute to the pathology of this disease would likely lead to improved prognostic indicators, to the design of novel therapeutic approaches and to the prediction of patient responses to therapy.

Early attempts to understand the pathogenesis of CRSwNP led investigators to quantify and identify by phenotype the inflammatory cells in polyp tissues. Multiple reports established that T lymphocytes constituted one of the dominant cell types. A comprehensive review of these studies concluded that the functional role of the T cells in the pathology of polyps was not established (Ryan and Davis, 2010). While circumstantial evidence suggested that bacterial antigen stimulation of T cells and defective regulatory T cells may contribute to the nasal polyp inflammation, further studies were required to determine if and how T cells were responsible for the polyp pathology (Ryan and Davis, 2010).

Subsequent studies showed that the CD3⁺ T cells in polyps are predominantly CD8⁺ with a minority of CD4⁺ T cells (Bernstein et al., 2004; Ickrath et al., 2017), and that the majority of these CD8⁺ T cells express CD44, CD45RO, CD28, and CXCR3 and are negative for CD62L and CD25 (Baba et al., 2015; Lehman et al., 2012; Pant et al., 2013; Sanchez-Segura et al., 1998). This phenotype is consistent with that of T cells called effector memory T cells, (T_{EM}) (Sallusto et al., 1999). The T_{EM} are a subset of long-lived cells that have previously encountered and are activated by their cognate antigen, and are typically found in the periphery at sites of chronic inflammation. This T cell subset has been found in human tumor microenvironments and has been shown to be hypo-responsive to activation via the T cell receptor (Agrawal et al., 1998; Broderick et al., 2006; Broderick et al., 2005; Simpson-Abelson et al., 2013). This led investigators to determine whether the T_{EM} from nasal polyps are similarly impaired in their ability to be activated. T cells derived from multiple different nasal polyp tissues were found to be hyporesponsive to activation while T cells derived from the peripheral blood of the nasal polyp patients responded normally to the same stimulation (Lehman et al., 2012).

The hypo-responsive state of T cells in the nasal polyp tissues has also been shown in T cells derived from another chronic inflammatory microenvironment i.e. T cells isolated from the synovial fluids of patients with rheumatoid arthritis (Broderick et al., 2006). Factors present in these chronic inflammatory microenvironments that contribute to the apparent T cell anergy have not yet been identified. However, extracellular vesicles identified as exosomes that are present in tumor microenvironments have been shown to suppress the activation of T cells and correlated with the hypo-responsiveness of the tumor associated T cells (Hong et

al., 2016; Kelleher et al., 2015; Keller et al., 2009; Muller et al., 2016; Roma-Rodrigues et al., 2014; Shenoy et al., 2018a; Szajnik et al., 2013; Whiteside, 2016; Xie et al., 2013).

We report here that extracellular vesicles are present in nasal polyp tissues and in the rheumatoid synovial fluid. These vesicles isolated from the two chronic inflammatory microenvironments have been characterized by size and composition and determined to be exosomes. They are shown to inhibit significantly the activation of T cells through the T cell receptor (TCR). The immune suppression is causally linked in part to a lipid - ganglioside GD3 that is expressed on the outer surface of the exosomes. We conclude that the immune suppressive exosomes represent a T cell checkpoint, and suggest that they may contribute to the hypo-responsiveness of the T cells in these inflammatory tissues. We discuss the possibility that the immune suppressive exosomes represent a potential therapeutic target for the treatment of nasal polyps and possibly other chronic inflammatory diseases.

MATERIALS AND METHODS

Specimens:

Nasal polyp tissues were received from the surgical suite at DeGraff Memorial Hospital, North Tonawanda, New York, from patients under the care of Dr. JM Bernstein, or from Buffalo ENT Specialists, Buffalo, New York. The polyps were from patients undergoing surgery for chronic hyperplastic rhinosinusitis with nasal polyposis. The tissue was placed and transported in RPMI-1640 medium with penicillin (20U/mL), streptomycin (20µg/mL) and fungizone (2µg/mL). Synovial fluid was obtained from patients with rheumatoid arthritis by arthrocentesis. Normal donor peripheral blood was provided by the Flow and Image Cytometry Facility at Roswell Park Cancer Institute (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 (Broderick et al., 2006; Simpson-Abelson et al., 2013). All specimens were obtained under sterile conditions and using Institutional Review Board (IRB) approved protocols (protocol number MODCR00003458). All patient information was de-identified in accordance to the IRB protocol.

Histology and immunohistochemistry:

Nasal polyp tissues were fixed in 10% neutral-buffered formalin overnight and processed by the Histology Service Laboratory at University at Buffalo. Some tissues were stained with hematoxylin and eosin. Other tissues were stained with anti-human antibodies specific for the following leukocyte markers: CD20, CD138, CD3, and CD68. The Dako LSAB+ Peroxidase kit (Dako Corporation) was used for the immunohistochemical staining.

Processing nasal polyps for exosome isolation:

The polyps were transferred to wells of a 6-well tissue culture plate along with the media they were transported in, with fresh media added. The polyps were cut up into smaller pieces and incubated overnight at 37°C, 5% CO₂ to allow the exosomes to be released into the medium. After overnight incubation, the fluid (henceforth referred to as nasal polyp fluid) was harvested and then centrifuged at 300 x g for 10 minutes at room temperature to pellet

any cells or debris. The acellular fluid containing the exosomes was then cryopreserved at -80°C .

Isolation of exosomes:

Cryogenically preserved nasal polyp fluid or synovial fluid was thawed and diluted appropriately in RPMI-1640 + 2% human serum albumin (HSA) or phosphate buffered saline (PBS) before filtering through a $0.22\mu\text{m}$ PES filter (Millipore). The filtrate was then ultracentrifuged at $200,000\times g$ ($45,900\text{rpm}$ s) for 90 minutes at 4°C (with brake applied) in a Beckman Coulter Optima XPN-100 Ultracentrifuge using the SW55Ti swing rotor. The exosome pellets were resuspended in RPMI-1640 + 1% HSA (for functional experiments) or PBS (for biophysical characterization) and stored overnight at 4°C .

Scanning Electron Microscopy (SEM):

Exosomes were loaded onto a membrane scaffold comprised of $0.1\mu\text{m}$ nucleopore membrane (Whatman), which were then fixed with 2% glutaraldehyde at 4°C for 90 min. The fixative was washed off and the samples treated with 30%, 50%, 70%, 80%, 95% and 100% ethanol sequentially for 15 min each. The samples were then exchanged into 100% hexamethyldisilazane (HMDS) and air dried in a chemical fume hood. The specimens were coated with evaporated carbon and analyzed using Hitachi SU-70 Field Emission Scanning Electron Microscope (Hitachi), operated at 2.0 kV.

Size measurement of exosomes.

The size of exosomes was measured by nanoparticle tracking analysis (NTA) using a ZetaView PMX 110 (Particle Metrix GmbH). The exosomes were diluted appropriately to give counts in the linear range of the instrument. Instrument performance was checked daily using the 100 nm non-fluorescent beads using the ZetaView software (version 8.05.04) quality control suite. The camera settings included a sensitivity of 82, shutter speed of 150 and a frame rate 30 with applied post-acquisition parameters of minimum brightness 20, minimum size 10, maximum size 200 and trace length 15. Eleven positions were recorded for each sample with 2 cycles at each position. The data (as fcs files) were analyzed using FlowJo (Tree Star).

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 a ChemiDoc imager (BioRad).

Flow exometry:

Exosomes were attached to $100\mu\text{l}$ of aldehyde/sulfate latex beads ($4\mu\text{m}$; 4% w/v) and incubated overnight at 4°C on a rotator/mixer. Glycine was then added to a final concentration of 100 mM to saturate remaining free binding sites on the beads. The beads were then washed in PBS with 0.5% bovine serum albumin (BSA) and used for

immunofluor staining. Anti-phosphatidylserine (Upstate /EMD Millipore) and Anti-GD3 (Genetex) were used to detect the respective lipids in exosome samples, in combination with goat anti-mouse IgG Alexa 488 (Life Technologies) as a secondary and control antibody. Data were acquired using a LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star).

Activation of T cells with PMA and Ionomycin:

Normal donor peripheral blood leukocytes (NDPBL) (1ml at 10^6 cells/ml) were activated with 1.5 μ M Ionomycin (Sigma) and 20ng/ml PMA (Sigma) for 2 hours at 37°C, 5% CO₂ with or without exosomes as described previously (Simpson-Abelson et al., 2013).

T cell activation with antibodies to CD3 and CD28:

Antibodies were immobilized on maxisorb 12 $\times$ 75 mm tubes (Nunc) by incubating 0.1 μ g of purified anti-CD3 (Bio X Cell, clone OKT3) and 5 μ g of purified anti-CD28 (Invitrogen, clone 10F3) in 500 μ l 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 the anti-CD3/anti-CD28 coated tubes at 37°C/5% CO₂ with or without exosomes for the duration of activation. In antibody blocking experiments, exosomes were pre-treated with 10 μ g anti-PS antibody (Upstate/EMD Millipore) or 10 μ g anti-GD3 (Genetex) antibody for 1 hour at 37°C before adding them to the activation tubes.

Detection of NF κ B translocation following T cell activation:

After activation, the cells were attached to alcian blue coverslips prepared as previously reported (Sommer, 1977) in a humid chamber (10 min) and fixed in 2% formaldehyde in 1x PBS (40 min). The cells were then permeabilized and blocked with 30 μ g normal mouse IgG in 5% normal mouse serum in 1x PBS + 0.4% Triton X-100 (block/perm). This was followed by staining 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 μ g/mL goat anti-mouse IgG-Alexa Fluor 568 for 15 minutes. This was followed by staining with purified rabbit anti-human NF κ B p65 in NGS block/perm for 1 hour. After washing twice with NGS block, the cells were incubated with 2 μ g/mL goat anti-rabbit IgG-Alexa Fluor 488 in 100 μ L 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). Cells were then observed on a Zeiss LSM 510 Confocal Microscope with at least 400 CD3+ cells counted per condition.

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 nasal polyp-derived exosomes in tubes that were coated with immobilized antibodies to human CD3 (Bio X Cell, clone OKT3) and CD28 for 7 days. On day 7, the cells were labeled with PE-conjugated anti-human CD3 (Invitrogen; clone UCHT1). Sytox Red (ThermoFisher Scientific) 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. Data analysis and proliferation modeling was done using FlowJo software (Tree Star Inc.).

Calculation of % inhibition and % reversal:

These were calculated using the formulae:

$$\% \text{ Inhibition} = [1 - (\% \text{ activation with exosomes} / \% \text{ activation without exosomes})] \times 100$$

$$\% \text{ Reversal} = [1 - (\% \text{ inhibition in test group} / \% \text{ inhibition in control group})] \times 100$$

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) while *p 0.05; **p 0.01 and ***p 0.001 were considered significant.

RESULTS

Histopathology and Immunohistochemistry of Nasal Polyp Tissues

Nasal polyps are benign growths resulting from a rapid proliferation of the upper airway epithelium and other associated cells in the lateral wall of the nose (Newton and Ah-See, 2008; Ryan and Davis, 2010). All of the cellular and molecular factors that are responsible for the development, progression and failure to resolve the polyps in humans have not yet been determined. The polyps arise from a persistent inflammation associated with microbial infections, asthma, rhinitis, and cystic fibrosis (Bernstein et al., 2006). The histopathology of nasal polyps includes the hyperplasia of the mucosal epithelium allergic, infiltration of multiple different inflammatory cell types, mucin producing goblet cells and submucosal glands (Bernstein et al., 2009). Multiple inflammatory cells and many of the factors they produce have been identified and associated with the pathology observed in this condition (Bernstein et al., 2004; Ickrath et al., 2017; Sanchez-Segura et al., 1998). However, the mechanisms and complex interactions of the cells and subcellular components that contribute to nasal polyposis, and its failure to be resolved normally or by therapeutic intervention are largely unknown. To address these issues, we have chosen to investigate the interaction of immune cells with acellular factors present in the polyp microenvironment and to assess the consequences of this interaction with respect to cell function.

We began this study by histologically and immunohistochemically characterizing the microenvironment of nasal polyp tissues derived anonymously from patients undergoing an elective surgery to remove the pathologic tissues. These tissues were provided to us sterile, under an IRB approved protocol, and were used in our subsequent studies that are reported here. The histology of the tissues confirmed the diagnosis of nasal polyps by revealing a characteristic display of chronic inflammatory cells, with hyperplasia of the nasal epithelium, and submucosal mucin producing glands. In the H&E stained sections of these fixed tissues, we observed florid accumulations of leukocytes in the submucosa (Fig 1A) with multiple clusters of eosinophils (Fig 1F). Immunohistochemistry revealed the presence of CD3+ T-cells, CD20+ B-cells, and CD138+ plasma cells (Fig 1 B, C and D). The

presence of large numbers of Periodic Acid-Schiff (PAS)+ mucinous submucosal glands is consistent with the diagnosis of nasal polyposis (Fig 1G). Other cell types found in these polyp tissues included macrophages, mast cells and fibroblasts (data not shown).

Because the T-cells are consistently present and are one of the most prominent immune cell types in the polyp tissues, we have focused our attention to study the function of these cells and their possible role in the pathogenesis of nasal polyps. Because activated T-cells are able to kill cells directly as well as indirectly by producing inflammatory cytokines, it seemed reasonable to postulate that they were simply contributing to the tissue damage and edema seen in polyps. We and others had also suggested that T-cells could be contributing to the recruitment of many of the inflammatory cells by the release of multiple chemokines from the polyp-associated T-cells.

However, when T-cells derived from nasal polyps were tested for their response to activation via the T-cell receptor, it was established that both CD4+ and CD8+ T-cells were profoundly hypo-responsive to an activation stimulus (Lehman et al., 2012). These T-cells had a phenotype of effector memory cells and their anergy was manifested by a blockade in the activation signaling cascade before or just after diacylglycerol (Lehman et al., 2012). Similar hypo-responsiveness has now been demonstrated in the T-cells derived from the chronic inflammatory microenvironments of tumor tissues and this T cell arrest was postulated to result from the phosphorylation of diacylglycerol into an inactive phosphatidic acid by diacylglycerol kinase (Kelleher et al., 2015).

In view of these findings, it was necessary to determine why the T-cells in nasal polyps and other chronic inflammatory tissues were hypo-responsive to activation. It was also necessary to revise our early ideas about the role of these T-cells in the generation and progression of nasal polyps and to establish a role for T-cells in other chronic inflammatory tissues.

Isolation and Characterization of Extracellular Vesicles from Nasal Polyp Tissues

Multiple reports have documented that extracellular vesicles (exosomes) derived from mouse and human tumor microenvironments suppress T-cells (Hong et al., 2016; Kelleher et al., 2015; Keller et al., 2009; Muller et al., 2016; Roma-Rodrigues et al., 2014; Shenoy et al., 2018a; Shenoy et al., 2018b; Szajnik et al., 2013; Whiteside, 2016; Xie et al., 2013). The tumor-associated exosomes bind to and internalize into T cells. The exosome-induced suppression occurs rapidly, is reversible, and results in directly arresting the activation of CD4+ and CD8+ T-cells stimulated via the T-cell receptor with antibodies or antigen (Kelleher et al., 2015; Shenoy et al., 2018a; Shenoy et al., 2018b).

These findings led us to investigate the possibility that exosomes may also be present in nasal polyp tissues and that they are contributing to the hypo-responsiveness of the polyp-associated T-cells. Sterile nasal polyp tissues, obtained as fresh surgical samples, were incubated overnight in complete medium. Cell free tissue culture supernatant fluids were filtered and centrifuged at low and high speeds and ultra-centrifuged. The re-suspended pellets from the ultra-centrifuged sample were processed for analysis by scanning electron microscopy (SEM) as we have previously reported for the isolation of vesicles from tumor tissues (Kelleher et al., 2015; Shenoy et al., 2018a).

The SEM of the extracellular vesicles isolated from the nasal polyps, revealed small (50–100nm) homogeneous spherical extracellular vesicles (Fig. 2A). The size of the individual vesicles was confirmed by nanoparticle tracking analysis (NTA) using an instrument called ZetaView (Particle Metrix GmbH). The NTA determined that the vesicles had a median diameter of 99.9nm with a range of 64–161nm (Fig. 2B).

Nasal polyp vesicles assayed for protein composition identified markers typically found in a subset of extracellular vesicles called exosomes (Thery et al., 2006). Immunoblots of frozen and thawed vesicles derived from nasal polyp tissues established the presence of a tetraspanin (CD81) and several other proteins often associated with exosomes i.e. TSG101, EpCAM, Annexin V, and ALIX, (Fig 2C)..

Based upon their size, morphology, and composition, we conclude that the large number of vesicles isolated from fresh nasal polyp tissues are very similar to extracellular vesicles called exosomes isolated from tumor tissues (Shenoy et al., 2018b).

Nasal Polyp-derived Exosomes Arrest Activation of T Cells

To determine if the nasal polyp vesicles, like the tumor-associated vesicles, are also immune suppressive, we next investigated their ability to arrest the activation of T-cells. We established that when T cells are incubated with nasal polyp-derived exosomes during a brief (2h) period of stimulation with immobilized antibodies to CD3 and CD28, their activation endpoint (nuclear translocation of NFκB) was significantly suppressed (Fig, 3A). These experiments were performed using exosomes derived from seven different patients diagnosed with chronic rhinosinusitis with nasal polyposis. The composite data of these experiments clearly demonstrate a significant and consistent suppression of the T cell activation by the nasal polyp-derived exosomes (Fig. 3A).

Exosomes Isolated from Nasal Polyp Tissues Do Not Inhibit PMA and Ionomycin-Mediated Activation of T cells

To determine if the polyp-derived exosome-mediated inhibition was associated with an arrest of the activation of the T cells that was dependent upon the signaling via the T cell receptor (TCR) cascade, the effect of the exosomes was monitored on T cells that were activated by phorbol 12-myristate 13 acetate (PMA), a diacylglycerol (DAG) analog and the Ca ionophore Ionomycin (I). The PMA and I stimulus bypasses the classical TCR cascade that is activated by the cross-linking of CD3 and CD28 (Kelleher et al., 2015). As shown in Fig.3B exosomes derived from three different nasal polyp patients did not inhibit the T cells activated by PMA and I. The exosomes derived from these same patients significantly inhibited the activation of T cells (up to 80%) when stimulated with immobilized antibodies to CD3 and CD28.

Nasal Polyp-derived Exosomes Inhibit T cell Proliferation

Having demonstrated that exosomes derived from nasal polyp tissues suppress an early activation endpoint (nuclear translocation of NFκB) of T cells, we next investigated their effect on a late/downstream T cell activation endpoint i.e. proliferation. For this, T cells were 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 CellTrace Violet (CTV) labeled T cells (Fig. 4A). The data were analyzed using a proliferation modeling platform, which estimates the percentage of cells in each generation of cell division (Fig. 4B). As expected, T cells persistently stimulated through the TCR in the absence of exosomes proliferated significantly as seen from the dye dilution profile as well as cell yields (Fig. 4A, D) and had a higher proportion of cells in the later generations (Fig. 4B). In contrast, T cells that were persistently stimulated in the presence of exosomes led to a lower percentage of their cells in the later generations of division, suggesting a proliferation arrest (Fig 4A-B). This cohort also had much lower cell yields, comparable to the unstimulated cells (Fig. 4D). Not surprisingly, the percentage of viable T cells correlated with better activation, and was therefore higher in the absence of exosomes (Fig 4C). We conclude that exosomes suppress the proliferation of T cells in response to persistent stimulation.

Immune Suppressive Nasal Polyp-derived Exosomes Express Two Lipids on Their Surface That Have Been Causally Linked to the Immune Suppression.

T cell suppression induced by exosomes has been causally linked to two lipids, phosphatidylserine (PS) (Kelleher et al., 2015) and the ganglioside GD3 (Shenoy et al., 2018a) that are expressed on the surface of exosomes derived from human tumors. To determine if the exosomes derived from NP also express one or both of these lipids, the exosomes were first attached to latex beads and the exosome-bound beads were stained with either anti-PS or anti-GD3 antibodies. Flow cytometric analysis revealed the both PS (MFI 2891) and ganglioside GD3 (MFI 853) were expressed on the surface of the NP exosomes (Fig. 5 A and B).

Isolation and Characterization of Extracellular Vesicles from Synovial Fluid Derived from Rheumatoid Arthritic Joints

Similar to the T cells isolated from nasal polyp tissues, the T cells present in the synovial fluids of patients with rheumatoid arthritis were also determined to be hypo-responsive to activation (Broderick et al., 2006). The anergy of T cells present in the synovial fluids led us to determine if immune suppressive extracellular vesicles were also present in this other chronic inflammatory microenvironment that could be contributing to the T cell anergy.

To address this question, we attempted to isolate and characterize extracellular vesicles from the synovial fluid derived from rheumatoid arthritic joints using ultracentrifugation. The re-suspended pellets from the ultra-centrifuged sample were processed for analysis of the vesicles composition, size, and the surface expression of two lipids, PS and ganglioside GD3, which have been causally linked to exosome-mediated inhibition of T cells.

The vesicles present in the synovial fluid strongly expressed CD63, a tetraspanin associated with exosomes (Thery et al., 2006) (Fig 6A) and the vesicles had a medium diameter of 86.6nm with a range of 52–154nm (Fig.6B). It was also determined that the synovial fluid vesicles expressed both PS and ganglioside GD3 on their surface (Fig 6C and D).

We conclude that the vesicles isolated from the synovial fluid are exosomes and very similar to the exosomes isolated from nasal polyp tissues.

Exosomes Isolated from Synovial Fluid Derived from Arthritic Joints Arrest Activation of T Cells

To determine if the synovial fluid exosomes, like the nasal polyp exosomes, are also immune suppressive, we next investigated their ability to arrest the activation of T-cells. Exosomes derived from the synovial fluid significantly inhibited the activation of T cells stimulated via the TCR by immobilized antibodies to CD3 and CD28 (Fig.7). As was found with the nasal polyp exosomes, the synovial fluid exosomes did not inhibit the activation of T cells stimulated with PMA and I (Fig.7).

Together, these results establish for the first time that exosome-like extracellular vesicles present in this chronic inflammatory microenvironment are immune suppressive, and are likely contributing to the hypo-responsiveness previously reported with nasal polyp-associated T cells (Lehman et al., 2012), as well as the anergy of T cells present in other chronic inflammatory tissues including the synovial fluid of patients with rheumatoid arthritis (Broderick et al., 2005).

Exosome-Induced Arrest of T Cell Activation Is Partly Rescued By Antibody-Mediated Blockade of Immune Suppressive Lipids On Their Surface

Since the synovial fluid exosomes expressed the two lipids previously determined to be causally linked to the immune suppression in tumor associated exosomes it was of interest to determine if an antibody blockade of PS or GD3 similarly suppressed the exosome-mediated T cell inhibition. Antibody to PS only partially but significantly decreased (reversed) the exosomal arrest of T cell activation, and antibody to GD3 blocked the exosome arrest of the T cells to a greater extent (Fig.7).

DISCUSSION

We previously reported that T cells with an effector memory phenotype in nasal polyps are hypo-responsive to activation via the TCR (Lehman et al., 2012). Currently, very little is known about the role of T cells in the pathogenesis of nasal polyps and how the dysfunction of these T cells possibly contribute to the disease progression and the resistance to treatment. Impaired TCR signaling and downstream effector functions of T cells have been observed in T cells derived from other chronic inflammatory viral infections and tumor tissues (Barber et al., 2006; Broderick et al., 2006; Mueller and Ahmed, 2009; Peggs et al., 2008; Simpson-Abelson et al., 2013). The dysfunction of T cells in these tissues has been linked to the upregulation of multiple inhibitory receptors including PD-1, CTLA-4, LAG3 and CD180 that result from the chronic antigenic stimulation of the T cells (Barber et al., 2006; Blackburn et al., 2009; Crawford and Wherry, 2009; Fourcade et al., 2012; Jiang et al., 2015; Jin et al., 2010; Joller et al., 2011; Wherry, 2011). However, we have not consistently identified any of these inhibitory molecules on the T cells from nasal polyps (Lehman et al., 2012) and therefore consider this to be an unlikely explanation for their dysfunction in nasal polyps.

We report here that exosomes isolated from both nasal polyp tissues and from the synovial fluid of patients with rheumatoid arthritis act directly on T cells to arrest their activation. We

suggest that these immune suppressive exosomes contribute to the hypo-responsiveness of T cells in the microenvironment of these two chronic inflammatory tissues. While the ideal control for nasal polyp exosomes would have been exosomes from non-inflammatory tissue such as healthy nasal mucosae, healthy tissue from patients/healthy donors is rarely, if ever surgically removed making these controls impossible to include.

We propose that the hypo-responsiveness of the T cells in nasal polyp tissues could result from the persistent activation by antigen stimulation resulting from one or more as yet unidentified pathogenic microbes (virus, bacteria or fungal) or possible self- antigens that provoke the generation of the immune suppressive exosomes. Consistent with this possibility, persistent antigen stimulation has been reported to result in T cell dysfunction in several chronic viral (Feunou et al., 2003; Hu et al., 1996; Jelley-Gibbs et al., 2005; Letvin and Walker, 2003) and mycobacterial infections (Dagur et al., 2010) that were not associated with inhibitory receptors. Evidence that both class I and II HLA are linked to nasal polyposis suggests that the immune suppressive exosomes and T cell arrest could also be driven by a persistent stimulation by a self- antigen.

We show here for the first time, that exosomes are an immune suppressive T cell checkpoint in two different chronic inflammatory microenvironments. The presence of immune suppressive exosomes in the microenvironment of the chronic inflammatory tissues is considered significantly important for at least two reasons. First, there could be a beneficial effect. The exosome-mediated arrest of T cells would prevent significant tissue damage resulting from an uncontrolled T cell proliferation and an associated overproduction of inflammatory cytokines. However, it is also possible that the exosome-mediated T-cell function arrest would have a very untoward impact upon the pathogenesis and enhance the progression of the disease, which might explain why polyps continue to recur following multiple unsuccessful therapeutic approaches (Bernstein et al., 2004; Ickrath et al., 2017; Newton and Ah-See, 2008; Ryan and Davis, 2010; Sanchez-Segura et al., 1998). The loss of function of T cells specific for microbial pathogens, which may be responsible for driving the pathology of nasal polyps, might explain why nasal polyps and other chronic inflammatory diseases fail to be resolved.

With the recognition of immune suppressive exosomes and their possible role in the pathogenesis of nasal polyps and other chronic inflammatory diseases, it will be possible and important to design and test novel therapeutic strategies to block exosomes, and to reverse the suppression of the quiescent T cells. By re-activating microbe-specific T cells, we may be able to rid the tissues of causative pathogenic microbes thereby reversing the inflammation that is driven by innate and adaptive immune responses to the unidentified pathogens. Similar approaches have been taken to re-activate hypo-responsive T cells in tumor microenvironments that have led to several successful immune based therapies for cancer (Oiseth and Aziz, 2017). Additionally, immune suppressive exosomes have been suggested to represent a potential new approach for the treatment of other inflammatory diseases and autoimmune diseases including arthritis (Anel et al., 2019; Yang and Robbins, 2012).

The finding that the activation with PMA, a DAG analogue, overcomes the exosome-mediated inhibition is considered to be important for at least two reasons. First it validates that exosomes are not toxic since they do not inhibit T cell response to an alternative stimulus. Second, these results suggest that exosomes may inhibit by a mechanism that blocks DAG in the activation cascade. The inactivation of DAG has been reported to occur in anergic T cells resulting from a phosphorylation of DAG into an inactive phosphatidic acid (Olenchok et al., 2006; Zhong et al., 2003). Supporting this as a possible mechanism of inhibition by exosomes, we have reported that two inhibitors of diacylglycerol kinase (DGK) partially block the immune suppressive effects of exosomes derived from ovarian tumors (Kelleher et al., 2015).

Several recent papers have identified exosomes in patients with nasal polyps that have been shown to have a significant impact on the generation, progression and assay of this disease (Lasser et al., 2016; Mueller et al., 2019; Mueller et al., 2018; Nocera et al., 2017; Zhang et al., 2018). The T-cell arrest by the immune suppressive exosomes that we report here, and in tumor microenvironments, are causally linked to lipids on the surface of the exosomes (Kelleher et al., 2015; Shenoy et al., 2018a; Shenoy et al., 2018b). In contrast, other nasal polyp exosome functions have been correlated with proteins present within the exosomes. For example, nasal exosomes containing p-glycoprotein (that drives type-2 helper T cell inflammation) were shown to rapidly mediate the intraepithelial transfer of this bioactive protein into mucosal epithelial cells (Nocera et al., 2017). A proteomic analysis of bioactive molecules present in nasal exosomes revealed a unique signature for nasal polyps (including cystatin SN, peroxiredoxin-5 and glycoprotein VI) that was responsible for altered coagulation (Mueller et al., 2018), and led to a noninvasive test for nasal polyposis (Mueller et al., 2019). Nasal polyp exosomes containing two proteinases (a disintegrin and metalloprotease 10) were reported to promote angiogenesis and vascular permeability (Zhang et al., 2018).

We conclude that exosomes present in nasal polyp tissues are heterogeneous and may contribute in multiple ways to the pathogenesis and to the monitoring of this chronic inflammatory disease. It is interesting to note here that Chronic Rhinosinusitis without Nasal Polyps (CRSsNP) is another chronic inflammatory disease that has a completely different immunological signature (Cho et al., 2016). The comparison of exosomes derived from CRSwNP and CRSsNP in terms of composition and function may reveal additional mechanisms that underlie their pathology. Our results and those discussed here suggest strongly that nasal polyp exosomes represent potential therapeutic targets for the treatment of nasal polyps and possibly other chronic inflammatory diseases.

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REFERENCES

- Agrawal S, Marquet J, Delfau-Larue MH, Copie-Bergman C, Jouault H, Reyes F, Bensussan A, Farcet JP. 1998 CD3 hyporesponsiveness and in vitro apoptosis are features of T cells from both malignant and nonmalignant secondary lymphoid organs. *J Clin Invest* 102:1715–23. [PubMed: 9802885]
- Anel A, Gallego-Lleyda A, de Miguel D, Naval J, Martinez-Lostao L. 2019 Role of Exosomes in the Regulation of T-cell Mediated Immune Responses and in Autoimmune Disease. *Cells* 8.
- Baba S, Kagoya R, Kondo K, Suzukawa M, Ohta K, Yamasoba T. 2015 T-cell phenotypes in chronic rhinosinusitis with nasal polyps in Japanese patients. *Allergy Asthma Clin Immunol* 11:33. [PubMed: 26594227]
- Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. 2006 Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682–7. [PubMed: 16382236]
- Bernstein JM, Ballou M, Rich G, Allen C, Swanson M, Dmochowski J. 2004 Lymphocyte subpopulations and cytokines in nasal polyps: is there a local immune system in the nasal polyp? *Otolaryngol Head Neck Surg* 130:526–35. [PubMed: 15138416]
- Bernstein JM, Broderick L, Parsons RR, Bankert RB. 2006 Human nasal polyp microenvironment maintained in viable and functional states as xenografts in SCID mice. *Ann Otol Rhinol Laryngol* 115:65–73. [PubMed: 16466102]
- Bernstein JM, Brooks SP, Lehman HK, Pope L, Sands A, Shultz LD, Bankert RB. 2009 Human nasal polyp microenvironments maintained in a viable and functional state as xenografts in NOD-scid IL2rgamma(null) mice. *Ann Otol Rhinol Laryngol* 118:866–75. [PubMed: 20112521]
- Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DA, Wherry EJ. 2009 Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10:29–37. [PubMed: 19043418]
- Broderick L, Brooks SP, Takita H, Baer AN, Bernstein JM, Bankert RB. 2006 IL-12 reverses anergy to T cell receptor triggering in human lung tumor-associated memory T cells. *Clin Immunol* 118:159–69. [PubMed: 16271513]
- Broderick L, Yokota SJ, Reineke J, Mathiowitz E, Stewart CC, Barcos M, Kelleher RJ Jr., Bankert RB. 2005 Human CD4+ effector memory T cells persisting in the microenvironment of lung cancer xenografts are activated by local delivery of IL-12 to proliferate, produce IFN-gamma, and eradicate tumor cells. *J Immunol* 174:898–906. [PubMed: 15634912]
- Cho SH, Kim DW, Gevaert P. 2016 Chronic Rhinosinusitis without Nasal Polyps. *J Allergy Clin Immunol Pract* 4:575–82. [PubMed: 27393771]
- Crawford A, Wherry EJ. 2009 The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr Opin Immunol* 21:179–86. [PubMed: 19264470]
- Dagur PK, Sharma B, Kumar G, Khan NA, Katoch VM, Sengupta U, Joshi B. 2010 Mycobacterial antigen(s) induce anergy by altering TCR- and TCR/CD28-induced signalling events: insights into T-cell unresponsiveness in leprosy. *Mol Immunol* 47:943–52. [PubMed: 20018378]
- Feunou P, Poulin L, Habran C, Le Moine A, Goldman M, Braun MY. 2003 CD4+CD25+ and CD4+CD25- T cells act respectively as inducer and effector T suppressor cells in superantigen-induced tolerance. *J Immunol* 171:3475–84. [PubMed: 14500643]
- Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Olive D, Kuchroo V, Zarour HM. 2012 CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* 72:887–96. [PubMed: 22205715]
- Hong CS, Funk S, Muller L, Boyiadzis M, Whiteside TL. 2016 Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. *J Extracell Vesicles* 5:29289. [PubMed: 27018366]
- Hu HL, Cornwell WD, Rogers TJ, Lin YS. 1996 In vivo analysis of a superantigen-induced T cell suppressor factor. *Cell Immunol* 167:285–92. [PubMed: 8603438]

- Ickrath P, Kleinsasser N, Ding X, Ginzkey C, Beyersdorf N, Hagen R, Kerkau T, Hackenberg S. 2017 Characterization of T-cell subpopulations in patients with chronic rhinosinusitis with nasal polyposis. *Allergy Rhinol (Providence)* 8:139–47. [PubMed: 29070271]
- Jelley-Gibbs DM, Dibble JP, Filipson S, Haynes L, Kemp RA, Swain SL. 2005 Repeated stimulation of CD4 effector T cells can limit their protective function. *J Exp Med* 201:1101–12. [PubMed: 15795235]
- Jiang Y, Li Y, Zhu B. 2015 T-cell exhaustion in the tumor microenvironment. *Cell Death Dis* 6:e1792.
- Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, Freeman GJ, Kuchroo VK, Ahmed R. 2010 Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 107:14733–8. [PubMed: 20679213]
- Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, Sharpe AH, Kuchroo VK. 2011 Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 186:1338–42. [PubMed: 21199897]
- Kelleher RJ Jr., Balu-Iyer S, Loyall J, Sacca AJ, Shenoy GN, Peng P, Iyer V, Fathallah AM, Berenson CS, Wallace PK, Tario J, Odunsi K, Bankert RB. 2015 Extracellular Vesicles Present in Human Ovarian Tumor Microenvironments Induce a Phosphatidylserine-Dependent Arrest in the T-cell Signaling Cascade. *Cancer Immunol Res* 3:1269–78. [PubMed: 26112921]
- Keller S, Konig AK, Marme F, Runz S, Wolterink S, Koensgen D, Mustea A, Sehouli J, Altevogt P. 2009 Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett* 278:73–81. [PubMed: 19188015]
- Lasser C, O'Neil SE, Shelke GV, Sihlbom C, Hansson SF, Gho YS, Lundback B, Lotvall J. 2016 Exosomes in the nose induce immune cell trafficking and harbour an altered protein cargo in chronic airway inflammation. *J Transl Med* 14:181. [PubMed: 27320496]
- Lehman HK, Simpson-Abelson MR, Conway TF Jr., Kelleher RJ Jr., Bernstein JM, Bankert RB. 2012 Memory T cells in the chronic inflammatory microenvironment of nasal polyposis are hyporesponsive to signaling through the T cell receptor. *J Assoc Res Otolaryngol* 13:423–35. [PubMed: 22310933]
- Letvin NL, Walker BD. 2003 Immunopathogenesis and immunotherapy in AIDS virus infections. *Nat Med* 9:861–6. [PubMed: 12835706]
- Mueller SK, Nocera AL, Dillon ST, Gu X, Wendler O, Otu HH, Libermann TA, Bleier BS. 2019 Noninvasive exosomal proteomic biosignatures, including cystatin SN, peroxiredoxin-5, and glycoprotein VI, accurately predict chronic rhinosinusitis with nasal polyps. *Int Forum Allergy Rhinol* 9:177–86. [PubMed: 30485711]
- Mueller SK, Nocera AL, Dillon ST, Wu D, Libermann TA, Bleier BS. 2018 Highly multiplexed proteomic analysis reveals significant tissue and exosomal coagulation pathway derangement in chronic rhinosinusitis with nasal polyps. *Int Forum Allergy Rhinol* 8:1438–44. [PubMed: 30091854]
- Mueller SN, Ahmed R. 2009 High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 106:8623–8. [PubMed: 19433785]
- Muller L, Mitsushashi M, Simms P, Gooding WE, Whiteside TL. 2016 Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. *Sci Rep* 6:20254. [PubMed: 26842680]
- Newton JR, Ah-See KW. 2008 A review of nasal polyposis. *Ther Clin Risk Manag* 4:507–12. [PubMed: 18728843]
- Nocera AL, Miyake MM, Seifert P, Han X, Bleier BS. 2017 Exosomes mediate interepithelial transfer of functional P-glycoprotein in chronic rhinosinusitis with nasal polyps. *Laryngoscope* 127:E295–E300. [PubMed: 28485529]
- Oiseth SJ, Aziz MS. 2017 Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead. *Journal of Cancer Metastasis and Treatment* 3:250.
- Olenchock BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, Zhong XP. 2006 Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat Immunol* 7:1174–81. [PubMed: 17028587]

- Pant H, Hughes A, Miljkovic D, Schembri M, Wormald P, Macardle P, Grose R, Zola H, Krumbiegel D. 2013 Accumulation of effector memory CD8+ T cells in nasal polyps. *Am J Rhinol Allergy* 27:e117–26. [PubMed: 24119592]
- Peggs KS, Quezada SA, Allison JP. 2008 Cell intrinsic mechanisms of T-cell inhibition and application to cancer therapy. *Immunol Rev* 224:141–65. [PubMed: 18759925]
- Roma-Rodrigues C, Fernandes AR, Baptista PV. 2014 Exosome in tumour microenvironment: overview of the crosstalk between normal and cancer cells. *Biomed Res Int* 2014:179486.
- Ryan MW, Davis LS. 2010 T cells in chronic rhinosinusitis with nasal polyposis. *Curr Opin Otolaryngol Head Neck Surg* 18:200–5. [PubMed: 20182357]
- Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. 1999 Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708–12. [PubMed: 10537110]
- Sanchez-Segura A, Brieva JA, Rodriguez C. 1998 T lymphocytes that infiltrate nasal polyps have a specialized phenotype and produce a mixed TH1/TH2 pattern of cytokines. *J Allergy Clin Immunol* 102:953–60. [PubMed: 9847436]
- Shenoy GN, Loyall J, Berenson CS, Kelleher RJ Jr., Iyer V, Balu-Iyer SV, Odunsi K, Bankert RB. 2018a Sialic Acid-Dependent Inhibition of T Cells by Exosomal Ganglioside GD3 in Ovarian Tumor Microenvironments. *J Immunol* 201:3750–8. [PubMed: 30446565]
- Shenoy GN, Loyall J, Maguire O, Iyer V, Kelleher RJ Jr., Minderman H, Wallace PK, Odunsi K, Balu-Iyer SV, Bankert RB. 2018b Exosomes Associated with Human Ovarian Tumors Harbor a Reversible Checkpoint of T-cell Responses. *Cancer Immunol Res* 6:236–47. [PubMed: 29301753]
- Simpson-Abelson MR, Loyall JL, Lehman HK, Barnas JL, Minderman H, O'Loughlin KL, Wallace PK, George TC, Peng P, Kelleher RJ Jr., Odunsi K, Bankert RB. 2013 Human ovarian tumor ascites fluids rapidly and reversibly inhibit T cell receptor-induced NF-kappaB and NFAT signaling in tumor-associated T cells. *Cancer Immun* 13:14. [PubMed: 23882159]
- Sommer JR. 1977 To Cationize Glass. *Journal of Cell Biology* 75:A245–A.
- Szajnik M, Derbis M, Lach M, Patalas P, Michalak M, Drzewiecka H, Szpurek D, Nowakowski A, Spaczynski M, Baranowski W, Whiteside TL. 2013 Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy. *Gynecol Obstet (Sunnyvale)* Suppl 4:3. [PubMed: 24466501]
- Thery C, Amigorena S, Raposo G, Clayton A. 2006 Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* Chapter 3:Unit 3 22.
- Wherry EJ. 2011 T cell exhaustion. *Nat Immunol* 12:492–9. [PubMed: 21739672]
- Whiteside TL. 2016 Exosomes and tumor-mediated immune suppression. *J Clin Invest* 126:1216–23. [PubMed: 26927673]
- Xie Y, Zhang X, Zhao T, Li W, Xiang J. 2013 Natural CD8(+)25(+) regulatory T cell-secreted exosomes capable of suppressing cytotoxic T lymphocyte-mediated immunity against B16 melanoma. *Biochem Biophys Res Commun* 438:152–5. [PubMed: 23876314]
- Yang C, Robbins PD. 2012 Immunosuppressive exosomes: a new approach for treating arthritis. *Int J Rheumatol* 2012:573528.
- Zhang W, Zhang J, Cheng L, Ni H, You B, Shan Y, Bao L, Wu D, Zhang T, Yue H, Chen J. 2018 A disintegrin and metalloprotease 10-containing exosomes derived from nasal polyps promote angiogenesis and vascular permeability. *Mol Med Rep* 17:5921–7. [PubMed: 29484441]
- Zhong XP, Hainey EA, Olenchock BA, Jordan MS, Maltzman JS, Nichols KE, Shen H, Koretzky GA. 2003 Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat Immunol* 4:882–90. [PubMed: 12883552]

Figure 1.

Histology and Immunohistochemistry of nasal polyp tissues: (A) Hematoxylin and eosin (H&E) staining of leukocytes in the submucosa 400X. (B) CD3 staining of T cells 400X. (C) CD20 staining of B cells. (D) CD 138 staining of plasma and plasmacytoid cells 400X. (E) Control staining with secondary antibody only 400X. (F) H&E staining of cells showing clusters of eosinophils, 400X. (G) PAS staining of mucinous submucosal glands 400X.

Figure 2.

Characterization of exosomes isolated from nasal polyps. (A) Scanning electron microscopy reveals exosomes (white arrows) with their respective sizes indicated. (B) Size distribution of the exosomes was determined using nanoparticle tracking analysis and revealed a median size of 100 nm. (C) The vesicle composition was determined using an Exosome Antibody Array. Dark spots indicate presence of the marked protein. Absence of a spot for GM130 indicates absence of cellular contaminants in the preparation.

Figure 3.

Nasal polyp-derived exosomes arrest the activation of T cells through the TCR. **(A)** 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 nasal polyps. The number in parenthesis represents percentage inhibition. **(B)** NDPBL were either left unactivated (UN), or activated for 2 hours with PMA and ionomycin in the absence (PMA/Iono) or presence (PMA/Iono + Exo) of exosomes derived from nasal polyps. Activation was determined by counting the number of CD3+ cells with nuclear NF- κ B using confocal microscopy. n = 6 for (A) and n = 3 for (B).

Figure 4.

Nasal polyp-derived exosomes inhibit T cell proliferation. NDPBL were labeled with CellTrace Violet and either incubated in medium only (Unact; 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 nasal polyps. The proliferation of T cells was estimated by measuring dye dilution in live CD3+ T cells using flow cytometry. (A) Representative dye dilution profiles for live T cells (Sytox Red- CD3+) from each cohort on day 7. (B) The percentage of live T cells in each generation of cell division obtained by proliferation modeling. (C) Percentage of live (Sytox Red-) and dead (Sytox Red+) CD3+ cells on day 7. (D) Total cell yields on day 7 normalized to input and expressed as fold change. Compiled data shown as Mean $\pm$ SEM (n = 3).

Figure 5.

Nasal polyp-derived exosomes express immunosuppressive lipids on their surface. **(A-B)** Exosomes were attached to latex beads and either left unstained (filled histogram), labeled with secondary Ab only (dotted line), or with the respective anti-lipid antibody (solid line). Data were acquired using a flow cytometer (LSR Fortessa, BD Biosciences).

Figure 6.

Characterization of exosomes isolated from synovial fluid of rheumatoid arthritis patients.

(**A**) The presence of exosomes was determined by demonstrating the presence of the exosomal marker CD63 using an Exosome Antibody Array. (**B**) Size distribution of the vesicles was determined using nanoparticle tracking analysis and revealed a median size of 86.6 nm. (**C-D**) Exosomes were attached to latex beads and either left unstained (filled histogram), labeled with secondary Ab only (dotted line), or with the respective anti-lipid antibody (solid line). Data were acquired using a flow cytometer.

Figure 7.

Antibodies to PS and ganglioside GD3 knock down exosome-induced T cell arrest. NDPBL were either left unactivated or activated under different conditions with or without exosomes and anti-PS or anti-GD3 antibodies as indicated. Activation was determined by counting the number of CD3+ cells with nuclear NF-kB using confocal microscopy. Percentage inhibition is shown in parentheses on the bars. Compiled data (Mean $\pm$ SEM) from three experiments are shown.