Human Tumor-Derived Exosomes Selectively Impair Lymphocyte Responses to Interleukin-2

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Abstract

Exosomes are nanometer-sized vesicles, secreted by normal and neoplastic cells. The outcome following interaction between the cellular immune system and cancer-derived exosomes is not well understood. Interleukin-2 (IL-2) is a key factor supporting expansion and differentiation of CTL and natural killer (NK) cells but can also support regulatory T cells and their suppressive functions. Our study examined whether tumor-derived exosomes could modify lymphocyte IL-2 responses. Proliferation of healthy donor peripheral blood lymphocytes in response to IL-2 was inhibited by tumor exosomes. In unfractionated lymphocytes, this effect was seen in all cell subsets. Separating $CD4^+$ T cells, $CD8^+$ T cells, and NK cells revealed that $CD8^+$ T-cell proliferation was not inhibited in the absence of $CD4^+$ T cells and that NK cell proliferation was only slightly impaired. Other exosome effects included selective impairment of IL-2–mediated CD25 up-regulation, affecting all but the $CD3^+CD8^-$ T-cell subset. IL-2–induced Foxp3 expression by $CD4^+CD25^+$ cells was not inhibited by tumor exosomes, and the suppressive function of $CD4^+CD25^+$ T cells was enhanced by exosomes. In contrast, exosomes directly inhibited NK cell killing function in a T-cell–independent manner. Analysis of tumor exosomes revealed membrane-associated transforming growth factor β_1 ($TGF\beta_1$), which contributed to the antiproliferative effects, shown by using neutralizing $TGF\beta_1$-specific antibody. The data show an exosome-mediated mechanism of skewing IL-2 responsiveness in favor of regulatory T cells and away from cytotoxic cells. This coordinated "double hit" to cellular immunity strongly implicates the role of exosomes in tumor immune evasion. [Cancer Res 2007;67(15):7458–66]

Introduction

Exosomes are a population of nanometer-sized vesicles, actively secreted by a diverse range of living cells, with physiologic functions that include immune modulation (1). Several reports document the immune-activating capacity of exosomes isolated from dendritic cells (2–5) and other cell types (6, 7), yet cells such as intestinal epithelia may produce tolerogenic exosomes (8).

The immunology of tumor exosomes is poorly understood, with reported activatory (9) and inhibitory effects (10); differences are probably dependent on the exosome phenotype. Exosomes are produced in advanced human malignancies, shown by the analysis of malignant effusions (11, 12), and likely enter the circulating exosome pool (13). However, patients with malignant tumor effusions do not spontaneously mount a protective antitumor immune response, suggesting that the immune system is either insufficiently activated or nonresponsive to tumor exosomes *in vivo*. Another possibility is that tumor exosomes actively impair immune responses, and this is the subject of the current report.

Interleukin-2 (IL-2) is of central importance in the homeostasis of lymphoid cells and in promoting the survival, proliferation, and functional differentiation of several lymphocyte subsets, including natural killer (NK) cells, $CD8^+$ T cells, and $CD4^+CD25^+$ regulatory T cells (Treg; refs. 14–16). Here, the responses of peripheral blood lymphocytes (PBL), obtained from healthy donors, to IL-2 in the absence or presence of tumor exosomes was compared. We show impaired lymphocyte proliferation in response to IL-2 in the presence of tumor exosomes. This inhibition requires the $CD4^+$ T-cell subset. We highlight a novel mechanism in which IL-2–mediated up-regulation of CD25 is selectively inhibited by tumor exosomes in NK cells and $CD8^+$ CTL but not $CD8^-$ T cells. The cytotoxic function of NK cells was impaired following tumor exosome treatment, but $CD4^+CD25^+$ Treg cells remained IL-2 responsive through induction of Foxp3 expression, and their inhibitory function was enhanced by tumor exosomes. Our data show that tumor exosomes express membrane-associated transforming growth factor β_1 ($TGF\beta_1$), which contributes to the antiproliferative effects. Exosomes may therefore be physiologically important for skewing immune responses away from cytotoxic effector mechanisms toward Treg cell responses, contributing to tumor immune escape.

Materials and Methods

Tumor cells and exosome purification. Data presented were obtained with tumor cell line–derived exosomes (mesothelioma cell line established within the department from pleural fluid; obtained from a patient with advanced pleural malignant mesothelioma). Additional tumor cell lines were used in some experiments, as stated, including prostate cancer cells (DU145 and PC3), the myelogenous leukemia line K562, the T-cell lymphoma Jurkat (from EACC), and the natural killer cell line NKL (ref. 17; a gift from Dr. E. Wang, Medical Biochemistry and Immunology, Cardiff University, Cardiff, United Kingdom). EBV-immortalized B cells were prepared as described (18). Cultures were confirmed free of *Mycoplasma* by monthly screening by PCR (purchased from Minerva Biolabs). Cells were maintained in RPMI 1640 supplemented with 10% exosome-depleted fetal bovine serum and antibiotics. Medium from confluent cultures (5–7 days) was depleted of cells and debris by centrifugation before ultracentrifugation (LE80K, Beckman Coulter) on a 30% sucrose/D_2O cushion (19). The cushion was collected, diluted in excess PBS, and exosomes were pelleted by ultracentrifugation. The protein content of each preparation was quantified using the bicinchoninic acid protein assay (Pierce).

Electron microscopy. A method similar to that previously reported (6) was used to visualize exosome preparations. Briefly, frozen exosomes were

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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thawed on ice and resuspended in 1% glutaraldehyde in PBS (pH 7.4). A 5-μL drop of the suspension was pipetted onto a pioloform-coated copper grid and allowed to stand for 5 min at room temperature. The grid was transferred to a 50-μL drop of double distilled water for 2 min and this process was repeated seven times for a total of eight water washes. The sample was stained on ice for 10 min with a 5-μL drop of 2% methyl cellulose containing 2% uranyl acetate. Excess fluid was removed with a piece of Whatman filter paper and allowed to air dry for 10 min before viewing by transmission electron microscopy (Philips EM 208, FEI).

Western blotting. Ten micrograms of whole-cell lysates were prepared as previously described (20), or 10 μg of purified exosomes were made to 40-μL final volume in sample buffer (6 mol/L urea, 2% SDS, 30% glycerol, 50 mmol/L Tris-HCl, and freshly added 5% v/v β2-mercaptoethanol). Samples were subjected to electrophoresis through 10% bis-acrylamide gels and transferred to polyvinylidene difluoride membranes. Following overnight blocking in 3% nonfat milk in 0.5% Tween 20 in PBS (PBS-T), primary antibody (added at 0.2–2 μg/mL) was added for 1 h, followed by three washes in PBS-T. Molecular weight markers (Cruz Marker, Santa Cruz Biotechnology, Inc.) and primary antibodies were detected using Cruz Marker–compatible goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) conjugated at 1:12,000 dilution in PBS-T for 30-min incubation followed by five washes in PBS-T. Bands were visualized using the ECL$^+$ system (GE Healthcare). The antibodies used included TGS101, lysosomal integral membrane protein 1 (LAMP-1), intercellular adhesion molecule (ICAM)-1, β_1-integrin, heat shock protein (hsp)-70, hsp90, Her-2/neu, histone H1, and calnexin-specific monoclonal antibodies (mAb; Santa Cruz Biotechnology). Anti-CD81 and anti-CD9 mAbs (Santa Cruz Biotechnology) were used on samples prepared with nonreducing conditions. Anti–MIC-A and anti–MIC-B were from R&D Systems. Anti-mesothelin and anti-acetylcholinesterase mAbs were from Abcam Plc. Anti–class I (clone HC10) was a gift from the Medical Research Council Co-operative, Cardiff University. Rat anti-grp94 (gp96; Stressgen) was detected using antirat IgG-HRP conjugated antibody (Dako) at 1:15,000 dilution in PBS-T. In this case, molecular weight markers (high-range rainbow markers) were from Bio-Rad.

Isolation and analysis of lymphocytes. PBL from healthy donors, purified by Histopaque (Sigma), were subjected to 2-h adhesion to plastic to remove adherent monocytes. The nonadherent cells were used in subsequent experiments. Multicolor flow cytometry (FACSCanto, Becton Dickinson) running FACSDiva software was done with antibodies from Becton Dickinson: CD3-allophycocyanin (APC), CD8-phycoerythrin (PE)-Cy7, CD4-APC-Cy7, CD56-PE-Cy5, and CD16-PE-Cy5 (used together) and CD25-FITC. Foxp3 analysis was done using eBioscience kit following the manufacturer's instructions. NK cells were defined by the lack of CD3 expression and as CD56$^+$CD16$^-$, CD56$^+$CD16$^+$, or CD56$^-$CD16$^+$. Purification of lymphocyte subsets was done using CD4$^+$ or CD8$^+$ T-cell negative isolation kits (Miltenyi Biotec) following the manufacturer's instructions. CD56$^+$ NK cells were purified first by depleting CD3$^+$ T cells, followed by positive isolation of CD56$^+$ cells (Miltenyi Biotec).

Lymphocyte proliferation and cytotoxicity assay. Lymphocytes were treated with recombinant human IL-2 (0–500 units/mL; Proleukin) or phytohemagglutinin (PHA; Sigma) to trigger proliferation. In some experiments, TGFβ_1 (from Peprotech) was added as an inhibitor of proliferation/cytotoxicity. Proliferating cells in 96-well plates were pulsed with 1 μCi/well of [^{3}H]thymidine for the final 18 h of cultures before harvesting and measuring [^{3}H]thymidine incorporation (Microbeta-3, Perkin-Elmer). For NK cytotoxicity assays, NK cells were enriched (using anti-CD3 antibody–coated Dynal beads), and the remaining population (confirmed as >79% CD3$^-$ by flow cytometry) was used in standard 4-h ^{51}Cr release assays with K562 cells as targets.

Treg cell isolation and *in vitro* suppression assay. Healthy donor PBL (50×10^6) were subjected to CD4$^+$CD25$^+$ isolation using a Treg isolation kit (Miltenyi Biotec) following the manufacturer's instructions. Fractions before and after isolation were compared by flow cytometry, staining with anti–CD4-APCCy7 (Becton Dickinson) and anti–CD25-PE (Miltenyi Biotec), to assess purity. The CD4$^+$CD25$^+$ fraction was left untreated for 24 h or treated with IL-2 (250 units/mL) and/or exosomes (10 μg/10^5 cells). The cells were washed in 10× volume (in culture medium) to remove IL-2 and exosomes and added back (at 2×10^3, 10×10^3, or 50×10^3 per well) to unfractionated PBL from the same donor (100×10^3 per well). Proliferation was then initiated with 1 μg/mL PHA (Sigma) and, after 3 days, [^{3}H]thymidine incorporation was measured.

ELISA for IL-10 and TGFβ_1. ELISA development reagents (duo-set kit) for human IL-10 and human TGFβ_1 were purchased from R&D Systems, with subsequent assays done as recommended by the manufacturer. Blocking, substrate, and stopping solutions were purchased from R&D Systems. High-binding microtitre ELISA strips were from Greiner. In TGFβ_1 colocalization experiments, antihuman CD81 mAb (carrier protein free; Becton Dickinson) was substituted for the TGFβ_1 capture antibody, with the remainder of the assay done exactly as for the TGFβ_1 ELISA. Absorbance was measured with wavelength correction (A_{450}–A_{540} nm) on a 3550 Microplate Reader (Bio-Rad).

TGFβ_1 neutralization. Lymphocytes were treated with IL-2 (250 units/mL) to trigger proliferation in the presence or absence of recombinant human TGFβ_1 (5 ng/mL) or tumor exosomes (10 μg/10^5 cells). In some wells, increasing concentrations (up to 10 μg/mL) of a well-characterized TGFβ_1 neutralizing antibody (clone 141322, R&D Systems; refs. 21, 22) were added. After 3 days, the degree of proliferation was assessed by measuring [^{3}H]thymidine incorporation.

Statistical analysis. All data are represented as mean ± SE. Comparisons between exosome-treated and nontreated groups were done using paired *t* tests, calculated using graphing and statistical software Prism 4 (version 4.03) from GraphPad.

Results

Tumor exosome characterization. Purification of exosomes derived from cultured cancer cells (of various types) revealed small (30–100 nm in diameter) vesicular structures, typical of exosomes in general (2, 6, 23), by electron microscopy (Supplementary Fig. S1*A*). Preparations were free of contaminating membranous fragments or electron dense apoptotic bodies (as previously described; ref. 24). The molecular phenotype of the exosomes was also typical of exosomes from other sources. Comparing whole-cell lysates with exosome preparations revealed an absence of endoplasmic reticulum–resident proteins (calnexin and grp94) or nuclear proteins (histone H1) from the exosomes. Yet, exosomes were relatively enriched in the multivesicular body marker TSG101 and the GPI-anchored protein acetyl-cholinesterase (Supplementary Fig. S1*B*). Exosomes also stained positively for CD9, CD81, LAMP-1, glyceraldehyde-3-phosphate dehydrogenase, hsp70, hsp90, β_1-integrin, and ICAM-1. Tumor-associated antigens, such as Her-2/neu and mesothelin, and MHC molecules including members of the MICA-family were also exosomally expressed (Supplementary Fig. S1*C*).

The effect of tumor exosomes on IL-2–mediated lymphocyte proliferation. We first investigated the effect of IL-2 on lymphocyte proliferation in the presence or absence of tumor exosomes. In the presence of exosomes, IL-2–mediated proliferation was severely impaired, measured by a marked inhibition of [^{3}H]thymidine incorporation, after only 48 h following IL-2 treatment (Supplementary Fig. S2*A*). CFSE labeling and multicolor flow cytometry revealed that the antiproliferative effect of tumor exosomes affected CD3$^+$CD8$^+$, CD3$^+$CD8$^-$, and CD3$^-$ lymphocytes (not shown). Flow cytometry also revealed an exosome-driven impairment in morphologic changes (assessed by increased forward scatter/side scatter) associated with IL-2–driven lymphocyte blast formation (Supplementary Fig. S2*B* and *C*). We conclude that tumor exosomes strongly impair proliferative responses to IL-2 in all lymphocyte populations.

The antiproliferative exosome effects are mediated principally by the CD4$^+$ T-cell subset. We next studied whether the antiproliferative effect of exosomes was retained on fractionating peripheral blood lymphocytes into various subpopulations. Using immunomagnetic beads, CD4$^+$ T cells, CD8$^+$ T cells, and CD56$^+$ NK cells were purified and stimulated with IL-2 in the presence or absence of tumor exosomes. The proliferative response was assessed after 3 days (Fig. 1). Tumor exosomes strongly inhibited IL-2–mediated lymphocyte proliferation in unfractionated cells (>67% inhibition at 250 units/mL IL-2 in the experiment shown; Fig. 1*A*). The antiproliferative effect remained evident in the purified CD4$^+$ T-cell population (50% inhibition; Fig. 1*B*). Whereas purified CD8$^+$ T cells responded strongly to IL-2, there was no exosome-mediated inhibition of this proliferative response (Fig. 1*C*), and the proliferation of CD56$^+$ NK cells was only weakly inhibited by tumor exosomes (24% inhibition; Fig. 1*D*). The CD56$^+$ NK cell fraction, however, contained ~10% non-CD56$^+$ cells, which may account for the partial antiproliferative exosome effect. We therefore repeated the experiments using the NK cell line NKL (Fig. 1*E*). Although tumor exosomes exerted an antiproliferative effect on NKL cells, this was weak and was undetectable at IL-2 doses of ≥200 units/mL. In contrast, a CD4$^+$ T-cell line, Jurkat, showed high sensitivity to the antiproliferative effects of tumor exosomes (Fig. 1*F*). In none of the studies was there evidence of apoptotic death mediated by exosomes or by IL-2 (assessed by Annexin V binding; data not shown). We conclude, therefore, that it is principally through the CD4$^+$ T-cell population that the antiproliferative effect of exosomes is mediated, as CD56$^+$ cells are only weekly inhibited, and the IL-2–driven proliferation of CD8$^+$ T cells is not impaired by direct interactions with tumor exosomes.

The effect of exosomes on CD25 expression. We hypothesized that inhibition of IL-2–driven proliferation may be due to exosome-mediated impairment of the IL-2 receptor. We examined possible changes in the expression of the IL-2Rα chain (CD25),

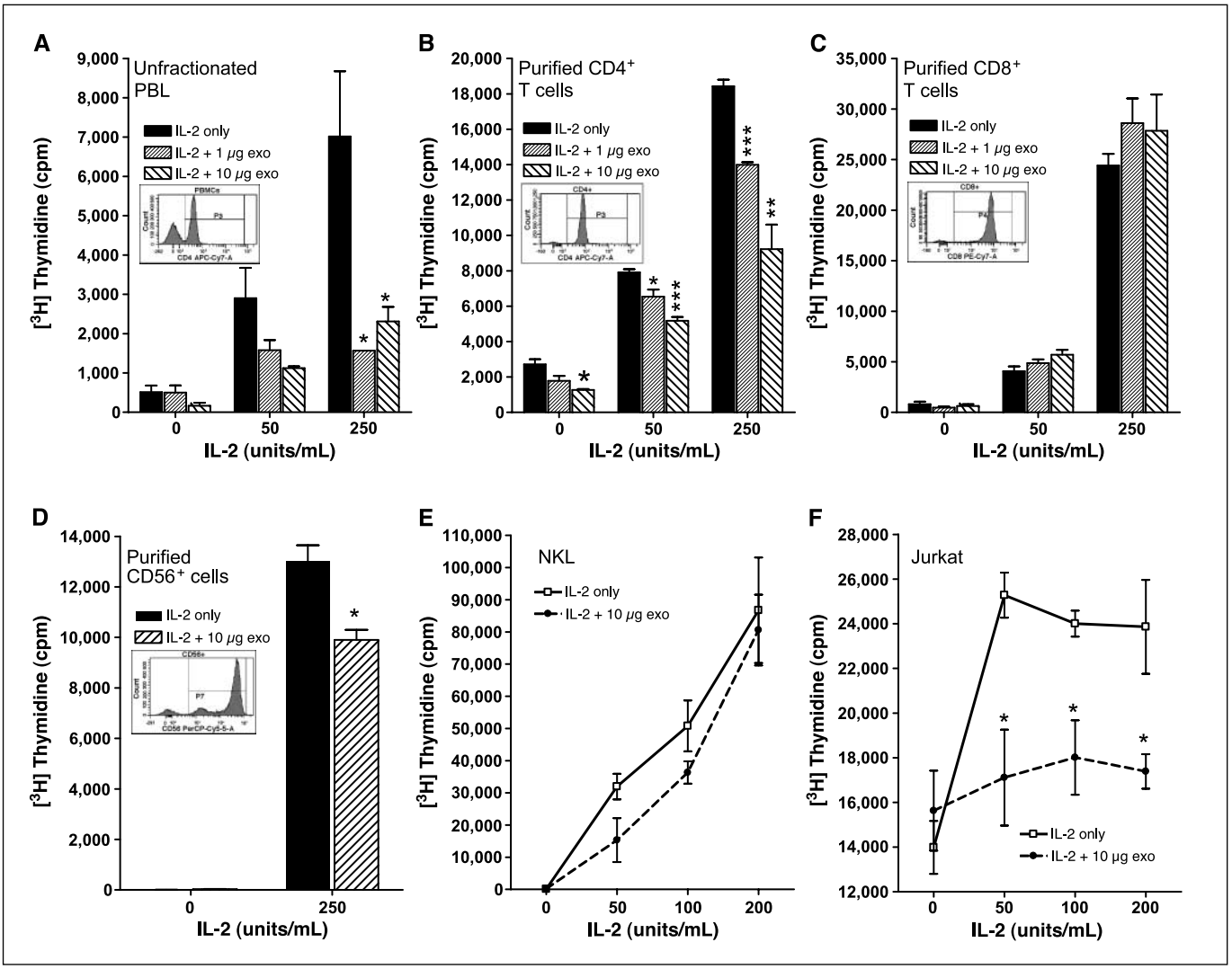

Figure 1. The antiproliferative exosome effects are mediated principally by CD4$^+$ T cells. Healthy donor PBL were left unfractionated (*A*) or purified into CD4$^+$ T-cell (*B*), CD8$^+$ T-cell (*C*), or CD3$^-$CD56$^+$ NK cell subsets. Cells (10^5 per well) were treated with IL-2 [0, 50, or 250 units/mL (*A–C*) or, due to limiting cell numbers, 0 and 250 units/mL (*D*)] in the presence or absence of tumor exosomes. Proliferation was assessed by measuring [^{3}H]thymidine incorporation at day 3. *Columns,* mean of triplicates; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Lymphocyte subset purity, assessed by flow cytometry (*histogram insets*), was 59.3% CD4$^+$ T cells (*A*), 95.2% CD4$^+$ T cells (*B*), 95.4% CD8$^+$ T cells (*C*), and 89.8% NK Cells (*D*). Representative of two experiments done with PBL from different donors. *E* and *F,* two cell lines (NKL and Jurkat, respectively) were treated in a similar manner.

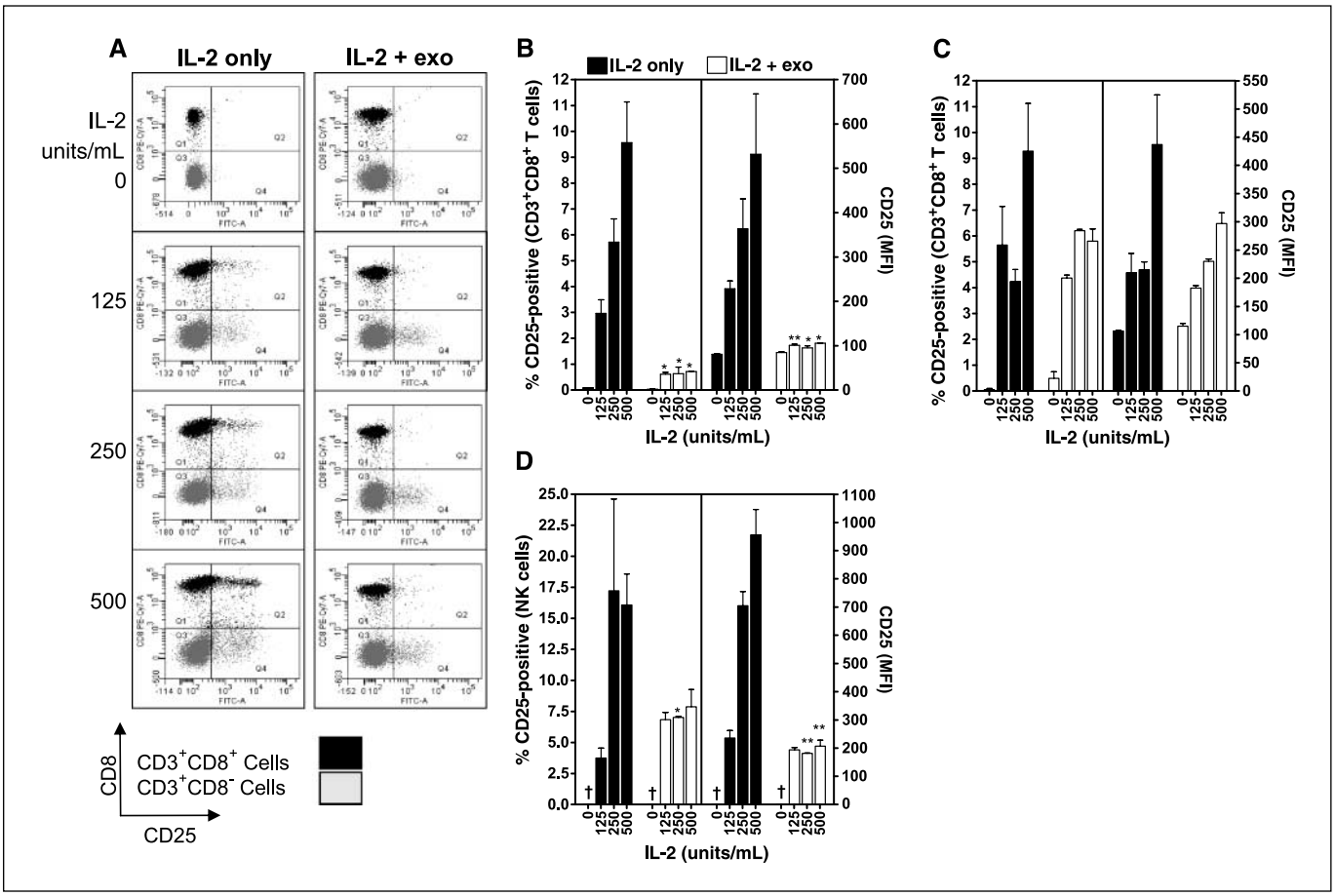

Figure 2. Tumor exosomes selectively impair up-regulation of CD25. Lymphocytes were examined for the expression of CD25 6 d after treatment with IL-2 in the presence or absence of tumor exosomes. *A,* dot plot representation of CD25 expression of $CD3^+$ gated cells. *B* to *D,* quantification of changes in the proportion of CD25-positive $CD8^+$ (*B*), $CD8^-$ (*C*), and NK cells (*D*), and CD25 expression levels [mean fluorescence intensity (*MFI*)]. *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. †, in the absence of IL-2, there were <100 events in the NK cell gate. Representative of three experiments done with different donors.

which is required for the formation of the high-affinity receptor complex. In unfractionated PBL, IL-2 stimulated a dose-dependent elevation in the proportion of CD25-positive $CD8^+$ T cells, $CD8^-$ T cells, and NK cells. In the presence of tumor exosomes, however, this induction was significantly inhibited, but in a cell type–specific manner as it was not seen in $CD3^+CD8^-$ T cells (Fig. 2). This observation shows that the expression of the high-affinity IL-2 receptor is selectively impaired on cytotoxic effector cells, remaining readily inducible on the Treg cell–containing population, suggesting that Treg cells may selectively remain functional in the presence of tumor exosomes.

Tumor exosomes do not impair the function of Treg cells. To address whether IL-2–dependent responses, other than proliferation, were retained by the $CD3^+CD8^-$ population, we examined the effect of IL-2 on the expression of the forkhead transcription factor Foxp3 (14), exclusively expressed by Treg cells (25). Following 18-h stimulation with IL-2, there was a 4-fold increase in the proportion of Foxp3-positive $CD3^+CD4^+$ cells. Importantly, however, there was no exosome-mediated impairment of Foxp3 induction (Fig. 3*A* and *C*), indicating that this cell population remained sensitive to proliferation-independent IL-2 effects. Yet, in the same samples, IL-2–mediated elevation of CD25 expression was inhibited by exosomes, but again only in the $CD4^-$ population (Fig. 3*B* and *D*). Interestingly, addition of exosomes alone (in the absence of IL-2) was sufficient to significantly increase the proportion of $Foxp3^+$ (but not $CD25^+$) $CD4^+$ cells, showing tumor exosomes as a stimulus in their own right for inducing a Treg cell phenotype. We next tested whether the ability of Treg cells to suppress immune responses was affected by exposure to tumor exosomes. $CD4^+CD25^+$ Treg cells were purified from fresh PBL (Fig. 4*A*) and were left untreated for 24 h or activated with IL-2 and/or tumor exosomes to elevate Foxp3 expression. The cells were subsequently washed, to remove IL-2 and exosomes, before adding at increasing numbers (2,000–50,000 per well) to 10^5 unfractionated lymphocytes. Proliferation was initiated by addition of PHA (1 μg/mL) and, 3 days later, measured by $[^3H]$thymidine incorporation. In the absence of PHA, there was minimal background cell division (107 ± 38 cpm) on day 3, whereas PHA stimulated a strong proliferative response (10,461 ± 403 cpm). Purified Treg cells did not proliferate in response to PHA treatment (37 ± 5 cpm). Adding increasing numbers of Treg cells to PHA-stimulated autologous PBL showed their capacity to inhibit proliferation. Of the conditions used to preactivate Treg cells, it was clear that a combination of IL-2 with tumor exosomes significantly enhanced their inhibitory capacity (67 ± 4% inhibition of proliferation) at 1:10 Treg/PBL ratio (Fig. 4*B*). Treatment of Treg cells with IL-2 alone (40 ± 3%) or with exosomes alone (41 ± 14%) both stimulated inhibitory

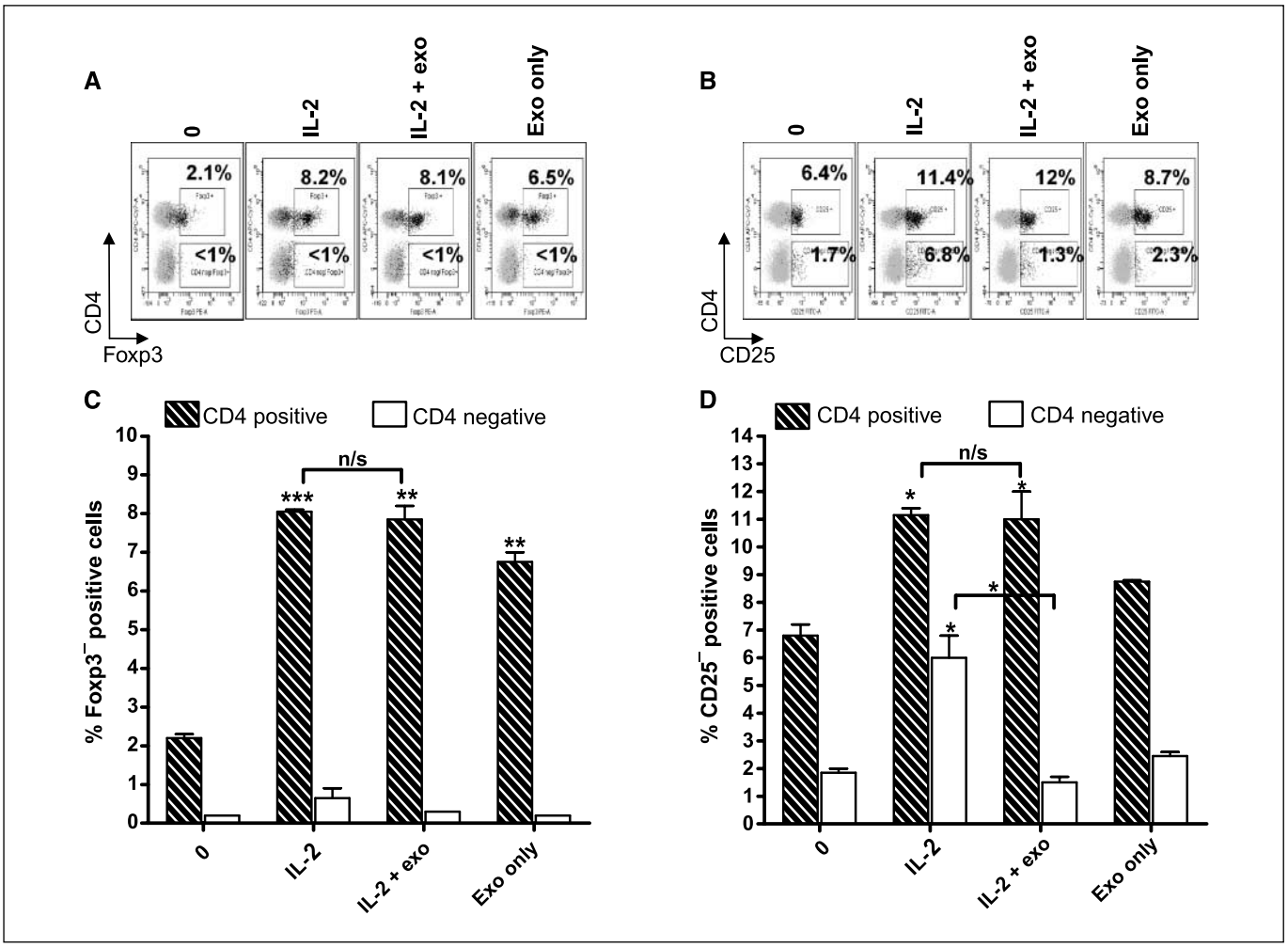

Figure 3. IL-2–mediated induction of Foxp3 is not impaired by tumor exosomes. Analysis of Foxp3 (*A* and *C*) or CD25 (*B* and *D*) expression after 18-h stimulation with IL-2 (250 units/mL) in the presence or absence of tumor exosomes (10 μg/10^5 PBL). The IL-2–driven induction of Foxp3 expression is not inhibited by tumor exosomes. Similarly, CD25 expression is unhindered by exosomes in the $CD4^+$ cells, but significant CD25 impairment is evident in the $CD4^-$ cells. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative of three experiments done with different donors.

functions above that of untreated Treg cells (25 ± 5%). Tumor exosomes, therefore, when combined with IL-2, strongly enhanced the inhibitory functions of Treg cells, and tumor exosomes were a stimulus in their own right, boosting the suppressive function of Treg cells.

Tumor exosomes impair cytotoxic cell functions. The consequence of exposure to tumor exosomes on NK cell cytolytic function was next examined. In the absence of exosomes, activation of purified (T-cell depleted) NK cells with 200 units/mL IL-2 for 3 days led to efficient cytotoxic killing of K562 cells. Adding exosomes with IL-2, however, resulted in a significant and dose-dependent reduction in killing (Fig. 5*A*), comparable to that achieved by high-dose (50 ng/mL) $TGF\beta_1$. This inhibition could not simply be explained by differences in NK cell numbers after 3-day proliferation with IL-2 because cell numbers were equalized through matched effector/target cell ratios for each group. Furthermore, the depletion of T cells suggests a direct exosome-mediated inhibition of NK cell killing capacity, which is unlikely to involve Treg cell–suppressive effects. These experiments have also been successfully reproduced using the NK cell line NKL (Fig. 5*B*), further confirming a Treg cell–independent mechanism for impaired NK killing.

Tumor exosomes express $TGF\beta_1$. To better understand the molecular mechanisms underlying the inhibitory effects of tumor exosomes, we questioned whether immunosuppressive cytokines were present in our exosome preparations. We focused on the possible expression of IL-10 and $TGF\beta_1$ by tumor exosomes because these factors are strongly implicated in immune evasion mechanisms and may be responsible for the antiproliferative (or anticytotoxic) effects observed in our study. IL-10 and $TGF\beta_1$ ELISAs were done in parallel on the same samples of exosomes derived from tumor cells. Whereas IL-10 was consistently absent, $TGF\beta_1$ was measurable in mesothelioma exosomes (Supplementary Fig. S3*A*) and in exosomes isolated from other cancer cell types (Supplementary Fig. S3*B*). The average $TGF\beta_1$ content for mesothelioma-derived exosomes was 7.12 ± 1.14 pg $TGF\beta_1$/μg exosomes (mean ± SE of five preparations). To ascertain whether $TGF\beta_1$ was present as a soluble contaminant or associated with the exosome membrane, we took two approaches: First, freshly prepared exosomes were split into three aliquots. One aliquot (labeled original) was used to assess starting $TGF\beta_1$ quantity. An equal aliquot was resuspended in >300× volume of PBS and pelleted at 100,000 × g to wash away any soluble $TGF\beta_1$. The final aliquot was

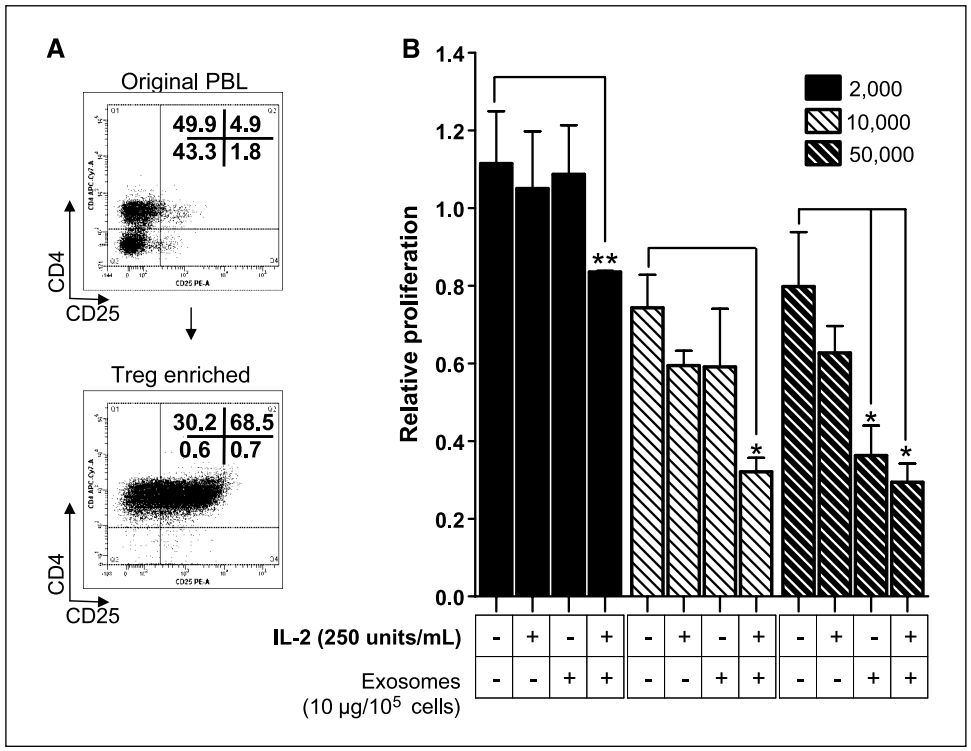

Figure 4. Treg cells retain inhibitory functions after tumor exosome treatment. $CD4^+CD25^+$ Treg cells were enriched (using Miltenyi Treg isolation kit) to 68.5% (*A*) before 24-h incubation alone, with IL-2 (200 units/mL), combination of IL-2 and exosomes (10 μg/10^5 Treg), or exosomes alone. Treg cells were washed in 10× volume with RPMI 1640 to remove traces of IL-2 and exosomes before adding (at 2×10^3–50×10^3 per well) to autologous nonadherent PBL (10^5 per well) in 96-well plates. PHA (1 μg/mL final) was added and proliferation measured after 3 d. Graph depicts proliferation relative to that of PHA-treated cells in the absence of Treg cell addition (set to a value of 1). *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. Representative of two experiments done with different donors (*B*).

incubated at room temperature for 2 h with 2,000 pg/mL recombinant human $TGF\beta_1$ before washing. Supplementary Fig. S3*C* reveals that washing exosomes in vast excess volume of PBS had little effect on $TGF\beta_1$ levels. Similarly, exogenously added soluble $TGF\beta_1$ did not increase exosomal $TGF\beta_1$ levels, suggesting that $TGF\beta_1$ was unlikely present as a soluble contaminant in the exosome preparations. In addition, from the TGF spiking experiment, it seems that either $TGF\beta_1$ is unable to passively adsorb onto the

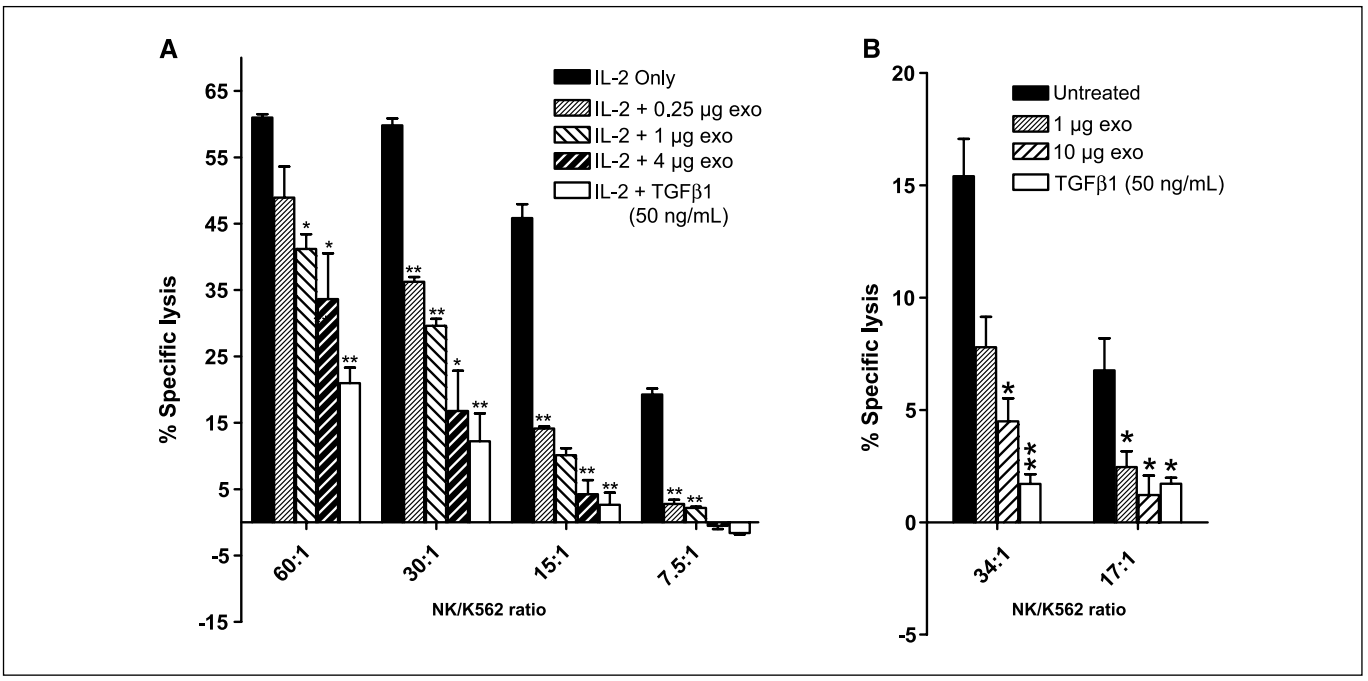

Figure 5. Tumor exosomes inhibit NK cell killing function. NK cell–enriched lymphocytes were treated with tumor exosomes (0.25–4 μg/10^5 PBL) or $TGF\beta_1$ in the presence of 200 units/mL IL-2, and on day 4, the capacity to kill K562 target cells was measured by standard chromium release assay. Graph depicts percent specific K562 lysis at various effector/target cell ratios, as indicated. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. Representative of three experiments done with different donors (*A*). Similarly, the cytolytic function of the NKL cell line was tested (*B*).

exosome membrane under these conditions or available binding sites on the exosome surface were already occupied with TGFβ$_1$. We also did a variation of the ELISA technique, where microtitre plates were coated with either TGFβ$_1$ capture antibody or anti-CD81 mAb (to bind exosomes to the plate based on their expression of CD81). Following sample addition and subsequent washing steps, TGFβ$_1$ content was assessed exactly as done for the TGFβ$_1$ ELISA (Supplementary Fig. S3*D*). Results revealed that the CD81 antibody–coated wells were unable to bind soluble TGFβ1, yet a signal was measured in wells containing exosomes, showing colocalization of CD81 with TGFβ$_1$. We conclude that mesothelioma and other tumor cell exosomes express membrane-associated TGFβ$_1$.

Exosomal TGFβ$_1$ contributes to antiproliferative effects and is more potent than soluble TGFβ$_1$. We investigated whether exosomally expressed TGFβ$_1$ was functional in mediating the antiproliferative effects of tumor exosomes. Lymphocytes were treated with IL-2 (250 units/mL) in the presence of soluble TGFβ$_1$ (5 ng/mL) or tumor exosomes (10 μg/10^5 PBL). Both conditions resulted in a significant impairment of IL-2–stimulated proliferation. Addition of TGFβ neutralizing antibody (but not irrelevant antibody, not shown) inhibited the antiproliferative effect of soluble TGFβ$_1$ and exosomes in a dose-dependent manner (Fig. 6*A*), showing that TGFβ$_1$ expressed by exosomes was indeed involved in the antiproliferative effect. When comparing the relative potency of soluble TGFβ$_1$ with exosomally delivered TGFβ$_1$ (where 1 μg exosomes = 7 pg TGFβ$_1$ measured by ELISA), it was evident that exosomal TGFβ$_1$ achieved a potent inhibitory effect at significantly lower doses than soluble TGFβ$_1$ (Fig. 6*B*).

Discussion

The priming of cytotoxic NK and CD8$^+$ T cells in readiness for killing function is driven by IL-2 but, paradoxically, Treg cells and their immune suppressive functions are also supported by IL-2 (15). Evidence from the clinic has shown that, in certain cancers, administering IL-2 as therapy can boost Treg cells and contribute to tumor immune escape (16). Our study investigated whether exosomes produced by cancer cells were involved in modulating T-cell and NK cell responses to IL-2. Tumor exosomes had the capacity to strongly inhibit IL-2–driven lymphocyte proliferation, preventing blast formation, in all subpopulations. On isolation of lymphocyte subsets, however, it became clear that although NK cell proliferation was weekly impaired following direct interactions with exosomes, the principal antiproliferative effect is mediated through the CD4$^+$ T-cell subset, as CD8$^+$ T cells were impervious to the antiproliferative effects of tumor exosomes in the absence of CD4$^+$ T cells.

When we investigated more subtle aspects of this phenomenon, we discovered a unique selectivity in this exosome effect. IL-2 induces the expression of the high-affinity IL-2Rα chain (CD25), and this occurs in CD3$^+$CD8$^+$ T cells, CD3$^+$CD8$^-$ T cells, and CD3$^-$CD56$^+$/16$^+$ NK cell subpopulations. However, in the presence of tumor exosomes, this CD25 induction is markedly inhibited in all but the CD3$^+$CD8$^-$ T cells. Further phenotypic analysis revealed that in the presence of tumor exosomes, the Treg cell–containing population remained capable of responding to IL-2, exemplified by induced Foxp3 expression. The Treg cell–mediated inhibition of proliferation was enhanced by tumor exosomes and further boosted when added in conjunction with IL-2. In contrast, freshly isolated NK cells activated with IL-2 exhibited strong cytotoxicity against K562 targets, but this function was significantly impaired following tumor exosome treatment through a mechanism independent of Treg cells. Our data therefore identify a novel mechanism by which tumors may drive immune responses away from cytotoxic effector mechanisms while not impairing, but supporting, Treg cell activities.

The exosomes in our study were from cultured mesothelioma cell lines, isolated from tissue samples or pleural fluid taken from patients with advanced malignant pleural mesothelioma. These exosomes may therefore represent an extreme tumor exosome type, being derived from a highly aggressive cancer type, with patient's survival after diagnosis typically ~9 to 12 months (26). Yet the exosome morphology was similar to those from other (tumor and nontumor) sources, and they expressed numerous markers typical of exosomes in general (27) as well as tumor-associated proteins such as Her-2/neu (11, 23), mesothelin (28), and MICA/B (29). In this sense, therefore, these are unremarkable tumor exosomes, and it may be that our observation is equally applicable across multiple tumor cell types. We have indeed observed the inhibition of lymphocyte proliferation (stimulated by various nonphysiologic

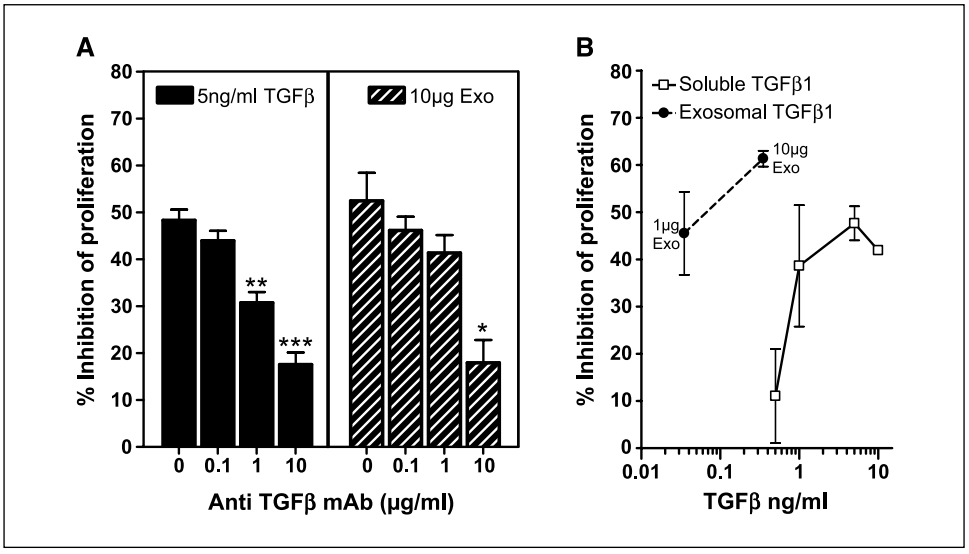

Figure 6. Exosomal-TGFβ$_1$ is functional and potent. *A,* lymphocyte proliferation, triggered with 250 units/mL IL-2, was inhibited by ~50% after the addition of soluble TGFβ$_1$ (5 ng/mL) or tumor exosomes (10 μg/10^5 PBL). Adding TGFβ neutralizing antibody (0.1–10 μg/mL) restored the proliferative response in a dose-dependent manner. Results of 3-d [^{3}H]thymidine incorporation assay, presented as percent inhibition of the IL-2 response. *Columns,* mean (n = 3); *bars,* SE. *B,* exosomes (1 or 10 μg/10^5 PBL) or TGFβ$_1$ (0.5–10 ng/mL) was added to IL-2–treated (50 units/mL) lymphocytes and proliferation assessed 3 d later. Percent inhibition of the IL-2–mediated proliferation response against TGFβ$_1$ dose, assuming 1 μg exosomes = 7 pg TGFβ$_1$. *Points,* mean (n = 3); *bars,* SE.

mitogens such as PHA and CD3/CD28 antibody–coated beads) using exosomes isolated from mesothelioma and other tumor types: breast carcinoma (T47D), prostate cancer (DU145 and PC3), and an EBV-immortalized B cells.[3] Whether such diverse exosome types can selectively impair IL-2 responses, as we have shown here, remains to be determined, and this is the subject of ongoing investigations in our laboratory. Other researchers have also previously described the inhibition of lymphocyte proliferation by exosomes. For example, EBV-encoded latent membrane protein-1 is expressed by exosomes secreted by EBV-immortalized B lymphocytes. These exosomes were able to impair proliferative responses to PHA and CD3/CD28 beads in a manner that may have been latent membrane protein-1 dependent (30). This molecule is not expressed by non-EBV malignancies and is therefore not sufficient to explain what seems to be an antiproliferative tumor exosome effect in general.

Suppressive factors such as IL-10 or TGFβ are produced by many tumor types and may mediate antiproliferative and anticytotoxic effects similar to those we observed for tumor exosomes. When we examined the possible presence of these cytokines in our exosome preparations, we found that IL-10 was not present at detectable levels but $TGF\beta_1$ was consistently found in tumor exosomes (from multiple tumor types). Further studies showed that exosomes express a membrane-associated form of $TGF\beta_1$ that could not be removed by ultracentrifugation washing steps. This raised the possibility that tumor exosomes were mediating at least some of their effects through delivery of $TGF\beta_1$ to lymphocytes. Administering a well-characterized $TGF\beta_1$ neutralizing antibody to IL-2– and exosome-treated lymphocytes partially restored proliferative capacity, revealing that exosomal $TGF\beta_1$ was indeed functional and thus contributes to the inhibitory effect.

The amount of $TGF\beta_1$ present in mesothelioma-derived exosomes was very low, at 7 pg/μg of exosomes. In our experiments, therefore, the effective dose of exosomal $TGF\beta_1$ with which lymphocytes were treated was 350 pg/mL. When we compared the effects of soluble $TGF\beta_1$ on lymphocyte proliferation, it was clear that this dose was not sufficient to suppress IL-2–mediated proliferative responses. In fact, 0.35 pg/mL exosomal $TGF\beta_1$ administered to lymphocytes stimulated with 50 units/mL IL-2 mediated an antiproliferative effect comparable to 5,000 pg/mL soluble $TGF\beta_1$. This suggested that $TGF\beta_1$ presented in the form of exosomes was ~1,400 times more potent than the soluble form under these conditions. Differences in the cellular response to soluble $TGF\beta_1$ were also evident, such as a general impairment of CD25 (IL-2Rα) expression (not shown) rather than the cell type–selective effect obtained with exosomes. Enhanced potency of membrane versus soluble $TGF\beta_1$ in other systems has previously been described (31). It may arise from sustained signaling by membrane TGFβ resulting in distinctive intracellular signaling events (such as Notch1 activation), which do not occur with soluble TGFβ. It is possible that exosomal $TGF\beta_1$ achieves similar sustained signaling effects that explain their relative functional potency. The role of other exosomal molecules in assisting the delivery of $TGF\beta_1$ to lymphocytes and in directing the cell type selectivity, which we report here, clearly warrants further investigations. Furthermore, the nature of $TGF\beta_1$ anchorage to the exosome membrane remains unknown, and the possible involvement of latent associated peptide (32, 33) or β-glycan (34, 35), as in other systems, requires clarification.

Previous reports have also shown that exosome-NK cell (9) and -T-cell (36, 37) interactions are possible, with outcomes dependent on the exosome phenotype. Although antigen presenting cell–derived exosomes may directly activate T-cell responses (36), direct interactions between T cells and tumor exosomes seem to be inhibitory. For example, Andreola et al. (37) have suggested that Fas ligand–positive exosomes may directly deliver death signals to activated (Fas-positive) T cells. They suggest this as a possible mechanism by which tumors delete tumor-specific T cells *in vivo*, and similar studies have also been published more recently (38). Other reports suggest that tumor exosomes are responsible for defective signaling responses in T cells by down-modulating CD3-ζ and Janus-activated kinase 3, resulting in apoptosis (39), although many of these studies relied on immortalized T-cell lines (Jurkat cells), rather than freshly isolated T cells, and very high doses of exosomes (400 μg/mL). In none of the experiments presented in this report, or in allied investigations within our laboratory, was there evidence of exosome-mediated loss in $CD4^+$ or $CD8^+$ T cells, indicating that Fas ligand–driven T-cell death is not a universal consequence of tumor exosome interaction with T cells.

A recent report by Gastapar et al. (9) highlighted a role for hsp70 expression by tumor exosome in their interactions with NK cells. Their experimental system involved colorectal cancer cell lines, producing either hsp70 surface positive exosomes or a subline (otherwise identical) producing hsp70 surface negative exosomes. The former exosomes were potent at activating the migratory and cytolytic functions of NK cells, whereas the hsp70 negative exosomes remained nonactivating. Thus, even with such a closely controlled experimental system, differences in the phenotype of the exosomes were absolutely key to their immunologic effects. Although the mesothelioma-derived exosomes used in our study were hsp70 positive (by Western blot), it does not necessarily follow that hsp70 was present at the exosome surface, as we showed for other exosome types, even after robust cell stress (20). The fact that mesothelioma-derived exosomes conspicuously impaired the killing function of fresh NK cells and an NK cell line suggests that either the exosomes are hsp70 surface negative or this potential activatory mechanism is overwhelmed by inhibitory processes occurring in parallel.

Several studies have shown activation of immune responses by tumor exosomes, as prophylactic agents (40) or as therapeutic vaccines in murine cancer models (23) and in humans (11, 41), usually by loading them onto enriched dendritic cell populations. By binding and endocytosing exosomes, dendritic cells may cross-present exosome-delivered antigens to T cells and stimulate antitumor immunity (11, 23). Much of the literature relating to tumor exosome vaccines, therefore, are in reality dendritic cell–based vaccines with exosomes acting as an antigen source. Liu et al. (10) recently showed that pretreating mice with tumor exosomes before vaccination with tumor exosome–pulsed dendritic cells was counterproductive, resulting in accelerated growth of explanted tumors through a mechanism involving exosome-mediated impairment of NK cell functions (10). Their report showed that murine tumor exosomes were capable of inhibiting IL-2–induced proliferation of NK cells and of inhibiting NK cell cytolytic function (10). Our study supports such findings and confirms that these effects are also observed with human tumor exosomes in the absence of dendritic cells as carriers for exosomes.

[3] Our unpublished observations.

To date, however, Treg cells as potential exosome-reactive cells have not been described, and the capacity of tumor exosomes to inhibit cytotoxic effector cells while in parallel supporting Treg cell phenotype and function was hitherto unknown. These exosome effects, which are at least in part due to membrane TGFβ_1 expression, provide a coordinated "double hit" to cellular immunity. Tumor exosomes are likely to represent an important mechanism contributing to immune evasion in cancer.

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