Murine Mammary Carcinoma Exosomes Promote Tumor Growth by Suppression of NK Cell Function[1]

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Many tumor cells shed specialized membrane vesicles known as exosomes. In this study, we show that pretreatment of mice with exosomes produced by TS/A or 4T.1 murine mammary tumor cells resulted in accelerated growth of implanted tumor cells in both syngeneic BALB/c mice and nude mice. As implanted TS/A tumor cells grew more rapidly in mice that had been depleted of NK cells, we analyzed the effects of the tumor-derived exosomes on NK cells. The tumor-derived exosomes inhibit NK cell cytotoxic activity ex vivo and in vitro as demonstrated by chromium release assays. The treatment of mice with TS/A tumor exosomes also led to a reduction in the percentages of NK cells, as determined by FACS analysis, in the lungs and spleens. Key features of NK cell activity were inhibited, including release of perforin but not granzyme B, as well as the expression of cyclin D3 and activation of the Jak3-mediated pathways. Human tumor cell lines also were found to produce exosomes that were capable of inhibiting IL-2-stimulated NK cell proliferation. Exosomes produced by dendritic cells or B cells did not. The presentation of tumor Ags by exosomes is under consideration as a cancer vaccine strategy; however, we found that pretreatment of mice with tumor exosomes blunted the protective effect of syngeneic dendritic cells pulsed ex vivo with tumor exosomes. We propose that tumor exosomes contribute to the growth of tumors by blocking IL-2-mediated activation of NK cells and their cytotoxic response to tumor cells. ***The Journal of Immunology,*** **2006, 176: 1375–1385.**

The ability of tumor cells to evade or suppress an active immune response is considered to be a significant factor in the development of tumors and their progression, and may play a defining role in their response to immunotherapeutics. Several mechanisms may contribute to the ability of tumor cells to grow in the context of an active immune response, including evasion of the immune response by down-regulation of the expression of MHC molecules on their surface (1–3), inhibition of immune responses by the production of inhibitory cytokines such as TGF-β (4) and IL-10 (5), or secretion of specific factors that inhibit the cytotoxic activity of several types of hemopoietic cells (6, 7).

Many tumor cells, as well as hemopoietic and epithelial cells, produce exosomes (8–14). These are extracellular, membrane-bound vesicles that are heterogeneous in size (ranging from 60 to 100 nm). They are thought to be formed by inward budding of the limiting membrane into the lumen of endosomes creating multivesicular endosomes that are most likely released into the extracellular environment upon fusion with the plasma membrane. Their function is largely unknown, although they have been implicated in cell-to-cell signaling. In the immune system, they may be involved in presentation of Ags to T cells (15) and the exosomes released by tumor cells may promote the immune response to tumors by presentation of tumor Ags (2). This possibility is being explored with the hope of developing more effective immunotherapeutic strategies. However, it has been reported recently that breast tumor exosomes inhibit T cell activation (16).

In this study, we evaluated the effects of murine mammary tumor exosomes on NK cell activation. We found that murine mammary tumor exosomes inhibit NK cell cytolytic activity by selective reduction of the expression of perforin and prevention of entry of NK cells into the cell cycle through blockade of expression of Jak3 and cyclin D3. Treatment of mice with dendritic cells pulsed with TS/A tumor exosomes led to the stimulation of the host immune response and either a delay in growth of the implanted tumors or elimination of the tumor cells. However, these protective effects were attenuated if mice were pretreated with tumor exosomes. This finding may be of importance in the design of effective strategies that use dendritic cells for the treatment of cancer.

Materials and Methods

Animals

Adult female BALB/c mice and female BALB/c-background athymic nude mice (The Jackson Laboratory) were 6–8 wk old when used and housed in the Animal Care Facility at the University of Alabama at Birmingham (UAB). Mice were maintained on standard laboratory chow and water was supplied ad libitum. Ten to 15 mice were used in each experimental group.

Cell isolation and culture

The TS/A cell line, a moderately differentiated and immunogenic murine mammary adenocarcinoma of spontaneous BALB/c origin that is MHC class I^+ ($H\text{-}2D^d$, $H\text{-}2K^d$) was maintained in vitro at 37°C in a humidified 5% CO_2 atmosphere in air in complete medium (DMEM with 5% FCS) as described previously (17). YAC-1 cells (American Type Culture Collection (ATCC)) were maintained in RPMI 1640 medium supplemented with 10%

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FBS, 2 mM glutamine, 10 mM HEPES (pH 7.4), and antibiotics (100 U of penicillin/ml and 100 μg of streptomycin/ml) (16). A2058 melanoma cells and the MDA231 breast cancer cell line were obtained from ATCC and maintained in the ATCC-recommended medium supplemented with 10% FBS. 4T.1 murine mammary tumor cells were a gift from Dr. T. V. Strong (UAB), and were cultured in DMEM medium supplemented with 10% FBS.

Mature spleen NK cells were enriched using DX5-conjugated microbeads, as recommended by the manufacturer (Miltenyi Biotec), with a typical yield of >80% CD49b-positive NK cells. NK cells were cultured in IMDM supplemented with 50 μM 2-ME, 10% heat-inactivated FCS in the presence of rIL-2 (100 U/ml; R&D Systems) before use.

To purify splenic B cells, splenocytes isolated from the BALB/c mice were labeled with biotinylated anti-B220, captured using streptavidin-conjugated magnetic beads, and sorted with the use of MACS (Miltenyi Biotec). T cell populations were prepared using a T cell column loaded with Scrubbed Nylon fiber (Cellular Products) to remove the monocyte/macrophage population. Single-cell suspensions (1×10^6 cells) were then stimulated with anti-CD3 (100 ng/ml; clone 145-2C11) plus IL-2 (5 ng/ml; BioSource International) and cultured in RPM1 1640 medium containing 10% FCS, 50 μM 2-ME, and 25 mM HEPES buffer solution at 37°C in a 5% CO_2 incubator.

Bone marrow-derived dendritic cells were generated according to a procedure described previously (18). Briefly, bone marrow cells were flushed from femurs of mice, filtered through a nylon mesh, depleted of erythrocytes with lysis buffer (BioWhittaker) and plated onto bacteriological petri dishes (2×10^6 per 100-mm dish; 10 ml) in RPMI 1640 (Cellgro) containing heat-inactivated FCS (10% v/v), L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), nonessential amino acids (10 μl/ml), sodium pyruvate (1.0 mM), and 2-ME (50 μM), and supplemented with mouse GM-CSF (20 ng/ml; PeproTech). At day 3 of culture, an additional 10 ml of culture medium supplemented with mouse GM-CSF (20 ng/ml) was added. Cultures were fed fresh medium on day 5. At day 7 of culture, nonadherent cells were removed for analysis and inoculations. The resulting population consisted of 80–90% dendritic cells as determined by flow cytometry analysis of CD11c.

Isolation of leukocytes from lung and liver

Mice were perfused with PBS and heparin (75 U/ml) before removal of the lung and liver tissues. Lung tissue was minced and incubated with stirring in HBSS with 1.3 mM EDTA at 37°C for 30 min, followed by treatment with collagenase (150 U/ml; Sigma-Aldrich) in RPMI 1640 with 5% FCS at 37°C for 1 h. The resulting suspension was pelleted by centrifugation, resuspended in 44% Percoll (Pharmacia) layered on 67.5% Percoll, and centrifuged at $600 \times g$. The cells at the gradient interface were harvested and washed extensively before use. Liver tissue was mashed through a 70-μm strainer in HBSS with 2% FCS and 10 mM HEPES. The resulting suspension was centrifuged and the pellet was resuspended in a solution of 35% Percoll and heparin (200 U/ml) and centrifuged at $600 \times g$. Cells in the resulting pellet were treated with Tris-ammonium chloride to remove RBC, followed by extensive washes. Similarly, single-cell suspensions of spleen and lymph node cells were prepared by mashing the organs through a 70-μm strainer in HBSS with 2% FCS and 10 mM HEPES. The cells were then washed with PBS before use. The total numbers of cells in the suspension cultures was determined and the number of NK cells analyzed by staining with anti-DX5 and FACS analysis as described below.

Flow cytometry

Dendritic cell surface expression of CD80, CD86, and CD11c (BD Pharmingen) and NK cell surface expression of DX5 (BD Pharmingen) and IL-2Rs, including α-, β-, and γ-chains, were determined by flow cytometry as described previously (18).

Preparation of exosomes

For preparation of exosomes, the TS/A, 4T.1, MDA231, and A2085 were cultured in vitro at 37°C in a humidified 5% CO_2 atmosphere in air in complete medium (DMEM with 5% FCS that had been ultracentrifuged for 16 h at $141{,}000 \times g_{max}$ to exclude bovine exosomes). The supernatants were harvested from these cells after 48 h in culture. The supernatants containing the exosomes produced from spleen B cells were harvested after 36 h culture in complete medium (RPMI 1640 with 10% FCS in which bovine exosomes have been depleted) in the presence of LPS (10 μg/ml). The supernatants containing the exosomes produced by dendritic cells were harvested from dendritic cells that had differentiated from bone marrow and cultured for 5 days. The exosomes were purified from the supernatants by differential centrifugation. In brief, cells were removed by centrifugation for 10 min at $200 \times g$. Supernatants were collected and centrifuged sequentially twice for 10 min at $500 \times g_{max}$, once for 15 min at $2{,}000 \times g_{max}$, once for 30 min at $10{,}000 \times g_{max}$, and once for 60 min at $70{,}000 \times g_{max}$ using an SW28 rotor (Beckman Instruments). Exosomes were pelleted at the final centrifugation step and were resuspended in PBS and repelleted at $70{,}000 \times g_{max}$. Repelleted exosomes were resuspended in 5 ml of 2.6 M sucrose, 20 mM Tris-HCl (pH 7.2), and floated into an overlaid linear sucrose gradient (2.0–0.25 M sucrose, 20 mM Tris-HCl (pH 7.2)) in an SW41 tube for 16 h at $270{,}000 \times g_{max}$ to remove nonmembranous protein (complexes). Gradient fractions (1 ml) were collected from the bottom of the tube and washed with PBS by centrifugation at $70{,}000 \times g_{max}$ for 1 h. Finally, the exosomes (fraction 3) were resuspended in PBS. The nonbanded fraction (fractions 6 and 7), which contains nonmembrane protein complexes, also was collected and concentrated using a protein concentrator with a 100-kDa cutoff (Amicon) for use as the exosome control (E-control)[3]. The protein content of the exosomes and control fractions was determined using a BCA protein assay kit (Bio-Rad). The aliquots were then stored at −80ºC before examination for the presence of exosomes by electron microscopy.

Tumor growth in vivo

Mice were injected s.c. in the right anterior mammary region with 0.1 ml of a single-cell suspension containing 1.2×10^5 TS/A or 4T.1 adenocarcinoma cells. Tumor size was measured weekly following tumor challenge using calipers. Tumor area was calculated by multiplying the length by the width of measured tumor which is presented as the mean ± SEM. For detection of disease progression and metastases, cytospin preparations of single-cell suspensions from lung and draining lymph nodes were prepared, fixed with methanol, and stained with eosin and methylene blue (Fisher). Although tumor cells appeared heterogeneous in size, they were easily differentiated as predominately larger cells with an elevated nuclear to cytoplasm ratio. Counts were performed on a total of 200–300 cells on coded slides.

NK cell depletion in vivo

NK cells were depleted by i.p. injection with 50 μl (in a total volume of 1 ml) of anti-asialo GM1 (ASGM1) antisera (Wako Pure Chemicals) or control rabbit IgG on days −2, +4, and +10 relative to tumor cell inoculation. Anti-ASGM1 Ab was purchased from WAKO Pure Chemicals. Control rabbit IgG was purchased from Sigma-Aldrich. Depletion of NK cells, but not of other cell populations including $CD8^+$, $CD4^+$, T cells, $F4/80^+$ macrophages, on treatment with anti-ASGM1 Ab was verified by flow cytometry.

NK cell cytotoxicity assay

Specific NK cell cytotoxic activities were determined using a standard 4-h chromium release assay as described previously (19). Briefly, spleen NK cells were plated in triplicate in 96-well U-bottom culture plates (Corning Glass) and cocultured for 4 h with sodium chromate-labeled (100 μCi; NEN) YAC-1 lymphoma cells (ATCC) or similarly labeled TS/A tumor cells. Supernatants were collected, radioactivity was measured, and the specific lysis was calculated according to the equation: percentage of specific cytotoxicity = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100. Maximum chromium release was determined from supernatants of lysed target cells incubated with Triton X-100 (5% v/v). Spontaneous release was determined from target cells incubated without added effector cells.

To determine the effects of TS/A exosomes on NK cell cytotoxic activity in vivo, 7-wk-old BALB/c mice (10 mice/group) were injected i.p. with either TS/A tumor exosomes or PBS or the nonmembrane protein complex (E-control). The mice were treated twice per week for 3 wk. After the treatments, mice were sacrificed and the cytotoxicity of NK cells to the YAC-1 cells was determined by a chromium release assay as described above. Also, the total number and percentages of NK cells in the liver, lung, spleen, and lymph nodes were determined by FACS analysis of the NK cell marker, DX5.

ATPLite assay

The possibility that the TS/A tumor cell exosomes exert a cytotoxic effect on the NK cells was determined directly by titration of the exosome cytotoxicity using an ATPLite assay. In brief, 1×10^4 primary NK cells were plated in 96-well plates and cultured as described above. The cells were treated with different amounts of TS/A exosomes or E-control for 24 h and

[3] Abbreviations used in this paper: E-control, exosome control; ASGM1, asialo GM1; cps, count per second.

then cytotoxicity was assessed using the ATPLite assay as described previously (18). In this assay, ATP is determined as light units measured as counts per second (cps) in a luminescence counter (Packard Instrument). The percentage of cytotoxicity is therefore expressed as (1 − cps sample/cps control) × 100.

Immunoblot analysis of perforin and granzyme B

Western blot analysis of proteins expressed in NK cells treated with tumor exosomes was conducted using a method as described previously (20). In brief, NK cell lysates were boiled in SDS sample buffer, and 50 μg of total protein was loaded into each well of an SDS-PAGE gel for separation by electrophoresis. The proteins were then transferred onto nitrocellulose membranes, and the blotted membranes blocked for 1 h with PBS-Tween 20 (0.25 M Tris (pH 7.5), PBS, 150 mM sodium chloride, and 0.2% Tween 20) containing 5% powdered skim milk. Blots were then probed overnight with 1 μg/ml of the indicated Ab, washed three times with PBS-Tween 20 and then probed for 1 h with the appropriate HRP-conjugated secondary Ab. After three washes with PBS-Tween 20, the protein bands on the blots were detected using an ECL Western blot detection system (Amersham Pharmacia Biotech).

RT-PCR

Total RNA was isolated using the TRIZOL reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. A total of 500 ng of RNA was reverse-transcribed using random primers (Boehringer Mannheim) and a Superscript II reverse transcription kit (Invitrogen Life Technologies) according to the manufacturer's instructions. A negative reverse transcription control in which water was substituted for RNA was included. Primer pairs were designed for mouse perforin and β-actin using Primer Express software (Applied Biosystems), and synthesized by Qiagen. The sequence of each primer pair was as follows: perforin, sense 5′-acacagaggttcctgaggcc-3′ and antisense primer 5′-gctccacagagcatgcttac-3′ based on the mouse perforin sequence (GenBank accession no. NM_011073) and β-actin, sense primer 5′-atggatgacgatatcgctgc-3′ and antisense primer 5′-cacactgtgc ccatctacga-3′ based on mouse β-actin sequence (GenBank accession no. NM_007393). After PCR amplification, products were separated by agarose gel electrophoresis.

NK, T, and B cell proliferation assay

NK, T, and B cell proliferation was determined using a standard tritium incorporation assay as described previously (21). In brief, NK cells (1×10^4/well in 96-well plates) were stimulated with IL-2 (100 U/ml) with or without tumor exosomes for various periods of time. T cells were stimulated with an anti-CD3 (clone, 100 ng/ml) plus IL-2 (5 ng/ml) as described previously (21) and B cells were stimulated with LPS (10 μg/ml; Sigma-Aldrich) with or without TS/A exosomes for various periods of time. The cells were then pulsed with 1 μCi of tritium-labeled thymidine (NEN) harvested after 14 h onto glass-fiber mats, and the incorporation of tritium was determined by scintillation counting.

Internalization of exosomes by NKs and stability of exosomes in NK cells

The PKH67 kit was used to label the TS/A tumor exosomes according to the instruction manual (Sigma-Aldrich). In brief, 1 μg of TS/A tumor exosomes in 100 μl of PBS was resuspended in 1 ml of diluent C, then mixed rapidly with a freshly prepared PKH67 solution in diluent C (final concentration during labeling step: $5 \times 10-6$ M) and incubated for exactly 3 min to ensure homogeneous staining. The labeling step was stopped by addition of an equal volume of FBS for 1 min, followed by an equal volume of complete DMEM medium. After three washes in PBS by ultracentrifugation, the exosomes were resuspended in 200 μl of complete culture medium containing FBS, and concentrated to 100 μl using an Amicon concentrator with a 100-kDa molecular mass cut-off. The flow-through (100 μl) was used as a control to determine the effects of any free fluorescent dye present in the medium used for suspension of the exosomes. All of the staining procedures were conducted at room temperature. The labeled TS/A exosomes or the flow-through were then mixed with 5×10^6 spleen NK cells and incubated at 37°C for 30 min. The uptake of exosomes by NK cells was stopped by washing in cold PBS, followed by either fixation in 3% formaldehyde for determination of percentages of TS/A exosome-transduced NK cells using a Leica TCS-NT confocal microscope (Leica Microsystems), or retention in culture in IMDM for determination of the stability of the transfected exosomes in the NK cells. For calculation of the percentage of TS/A exosome-positive NK cells, five fields were selected randomly and the total number of NK cells as well as the number of PKH67 green fluorescent-positive NK cells were counted. The percentage of TS/A exosome-positive NK cells was then determined by calculating the average number of green fluorescent-positive NK cells in five fields divided by the average of the total number of NK cells counted in these five fields.

Statistics

All results are expressed as the mean ± SD. The statistical significance was assessed using one-way ANOVA with Bonferroni correction.

Results

Tumor-derived exosomes promote tumor growth

To characterize the effects of tumor-derived exosomes on tumor cell growth, we pretreated syngeneic BALB/c mice with sucrose-gradient purified exosomes from two murine mammary tumor cell lines, TS/A and 4T.1, and evaluated the effect of the exosomes on the growth of implanted TS/A tumor cells. The effects were compared with those elicited by an exosome-depleted fraction (E-controls) or PBS. We found that the growth rate of the implanted TS/A tumor cells was significantly greater in the mice pretreated with exosomes than in the mice pretreated with either the E-control fraction ($p < 0.001$) or PBS ($p < 0.001$). The growth of the implanted tumor cells in the pretreated mice was rapid and progressive with development of tumor necrosis and metastases by day 35 (Fig. 1*A*) for mice pretreated with TS/A exosomes and day 30 (Fig. 1*D*) for mice pretreated with 4T.1 exosomes. At these time points, the protocol limits on tumor size and animal condition were reached, necessitating the sacrifice of the mice. In BALB/c mice that had been pretreated with the E-control fraction or PBS, the implanted tumors typically required 50 days to attain a similar size (Fig. 1, *A* and *D*). Similar results were obtained when mice were challenged with 4T.1 tumor cells (data not shown).

In BALB/c-background nude mice, the effects of pretreatment with tumor-derived exosomes on tumor growth were even more dramatic (Fig. 1, *B* and *E*). This enhancement of the effect in nude mice, in which the NK cell response is functional but the T cell responses are not, led us to reason that the tumor-derived exosomes may act, at least in part, by suppressing the cytotoxic activity of NK cells. Indeed, in vivo NK cell depletion of BALB/c mice led to faster growth of implanted TS/A tumor cells in the anti-ASGM1 antisera-treated mice than in control Ab-treated mice (Fig. 1*G*). Similarly, depletion of NK cells in nude mice led to accelerated tumor growth (Fig. 1*H*).

Tumor-derived exosomes inhibit the cytolytic activity of NK cells

To determine the effect of the tumor-derived exosomes on the cytolytic activity of NK cells, DX5-positive NK cells were isolated from the spleens of mice that had been treated with exosomes and their cytotoxic activity was determined in vitro using a standard chromium release assay with YAC-1 target cells. BALB/c mice were injected i.p. with purified tumor exosomes produced by either the TS/A (Fig. 1*C*), or the 4T.1 murine mammary tumor cell lines (Fig. 1*F*) suspended in PBS, or the exosome-depleted E-control fractions or PBS twice weekly for 3 wk as described above. We found that the DX5-positive cells isolated from the mice treated with the tumor-derived exosomes exhibited significantly lower cytotoxicity activity than did the DX5-positive cells isolated from the mice injected with the E-control fractions or PBS (Fig. 1, *C* and *F*). These data indicate that NK cell cytotoxicity is impaired in mice treated with TS/A exosomes.

Pretreatment with TS/A tumor exosomes results in a reduction in the total number and percentages of NK cells

The exosomes could modulate NK cell activity through several different mechanisms, including an exosome-mediated reduction in the numbers of NK cells and exosome-mediated interference

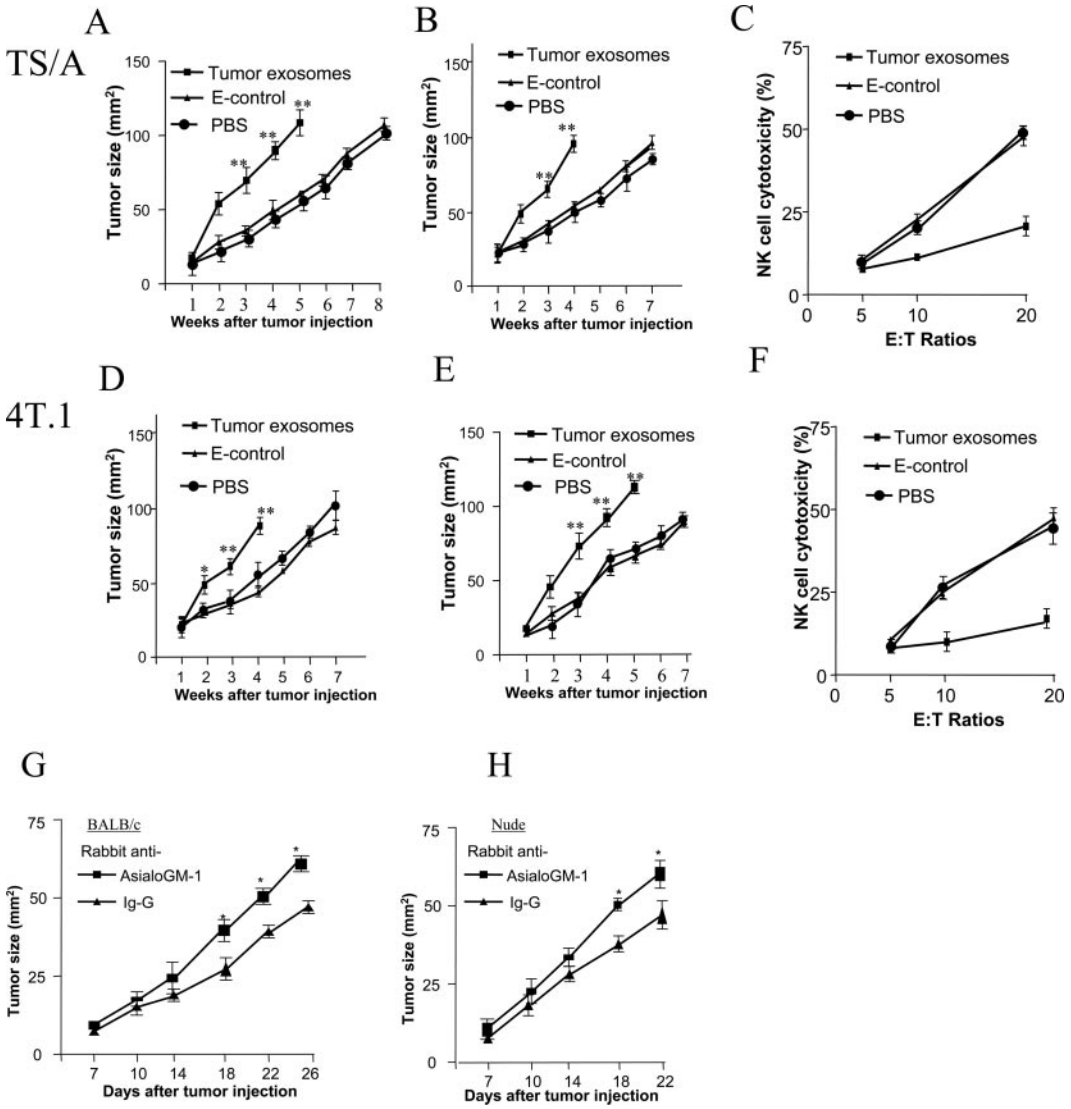

FIGURE 1. Tumor cell exosomes promote tumor growth and inhibit the cytolytic activity of NK cells. BALB/c (*A*, *C*, *D*, and *F*) or BALB/c-background athymic nude (*B* and *E*) mice were injected i.p. with sucrose-gradient purified TS/A exosomes (*A* and *B*) or 4T.1 exosomes (*D* and *E*) (10 μg in 100 μl of PBS for each mouse with 15 mice/group) twice weekly for 3 wk. Control mice were injected with the exosome-depleted fraction obtained from the sucrose density gradient (E-control) or PBS. Ten mice of each group were injected s.c. with TS/A murine mammary tumor cells (1.2×10^5 cells/flank in 50 μl of PBS), and the size of the implanted tumors was measured weekly. Each point represents the mean size and SD of the 10 mice from each group. The asterisks above the curve indicate treatment groups of mice that were significantly different from the group of mice that did not receive exosomes: *, $p < 0.05$; **, $p < 0.01$. Five of the BALB/c mice treated with TS/A tumor exosomes (*C*) or 4T.1 (*F*) tumor exosomes were sacrificed, without tumor challenge, and the NK cells purified from the spleens using flow cytometry sorting of DX5-positive cells, which were stimulated with a rIL-2 (100 U/ml) for 24 h. The stimulated NK cells were then added to chromium-labeled YAC-1 cells at various effector-target ratios as indicated. The amount of chromium released into the supernatant was determined, and the percent-specific release was calculated. Data are representative of at least two independent experiments. The effect of NK cells on TS/A tumor cell growth was determined by depletion of NK cells in BALB/c mice (*G*) or nude mice (*H*), in which, depletions of NK cells in vivo began before mice were challenged with TS/A tumor cells (1.2×10^5 cells/flank in 50 μl of PBS). Depletion was conducted by i.p. injection of anti-ASGM1 antisera on days -2, $+4$, and $+10$ relative to TS/A tumor cell inoculation, with i.p. injection of rabbit IgG as a control. The growth of the implanted tumor was measured throughout a 26-day period. Each point represents the mean of average areas of tumors of 10 mice.

with the normal functional activity of the NK cells. To determine the effects of the tumor-derived exosomes on NK cells, 10 BALB/c mice were pretreated with either TS/A exosomes, or E-control, using a protocol identical to that described above. Ten mice were sacrificed at days 3 and 8 after cessation of treatment. The total numbers and percentages of NK cells in single-cell suspensions of the lungs, liver, lymph nodes, and spleens were determined using FACS analysis of DX5 staining as described in *Materials and Methods* and the legend of Fig. 2*A*. There was a significant reduction in the number and percentages of NK cells in the lung, but not in the liver and lymph nodes, at day 3 after treatment of mice with TS/A exosomes, but not with E-control (Fig. 2*B*). Interestingly, in the exosome-treated mice, the total number of splenocytes as well as the total number of NK cells exhibited an increase (Fig. 2*B*) although the percentage of spleen NK cells was decreased (Fig. 2*A*). At day 8 after the treatments, the same patterns of changes were observed as those observed on day 3 (data not shown). These data suggest that a reduction in the percentages of NK cells contributes to the TS/A-exosome-mediated reduction in NK cell cytotoxic activity in vivo.

To determine whether the effect of the tumor-derived exosomes on the cytotoxic activity of NK cells is a direct effect, we isolated

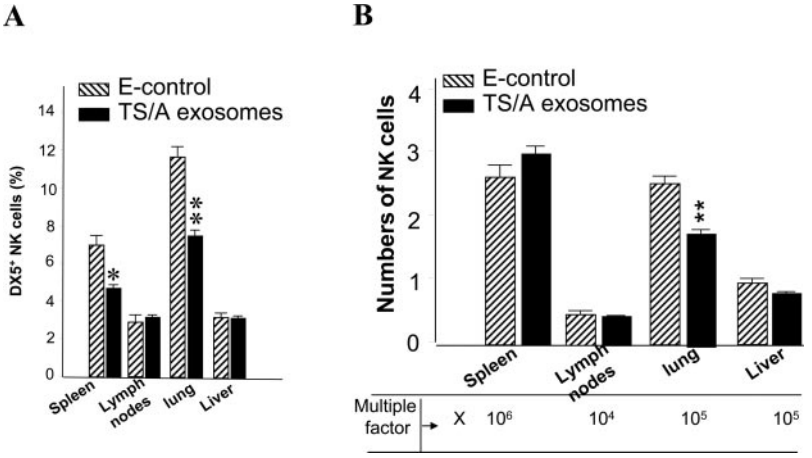

FIGURE 2. TS/A tumor exosome treatment leads to the reduction of the percentage of NK cells in vivo. Seven-week-old BALB/c mice were treated with either TS/A exosomes (10 μg/mouse) or E-control as described in the legend of Fig. 1. The treated mice were sacrificed and the total number and percentages of NK cells in the lung, spleen, liver, and inguinal lymph nodes were determined. Leukocytes in the liver and lung were isolated as described in *Materials and Methods*, and the total number of leukocytes per organ was calculated from hemocytometer counts. The total numbers of cells in the suspensions of spleen and inguinal lymph nodes were calculated similarly. The percentages of DX5-positive NK cells were determined by FACS analysis as described in *Materials and Methods* (*A*). The absolute number of NK cells in each organ were calculated by multiplying the total number of cells in the organ by the percentage of DX5-positive cells determined by FACS analysis (*B*). Each bar is representative of the mean of 10 mice analyzed.

primary NK cells from the spleens of 2-mo-old BALB/c mice and incubated them with different concentrations of TS/A tumor exosomes for 24 h in the presence of recombinant mouse IL-2 (100 U/ml). The NK cells were then washed with PBS three times to remove unbound exosomes before the cells were used in a standard in vitro chromium release assay. The addition of tumor-derived exosomes strongly inhibited the NK cell-mediated killing of YAC-1 target cells (Fig. 3*A*). At a 20:1 E:T ratio, there was a 10.3- $\pm$ 1.2-fold reduction in the cytotoxic activity of the exosome-treated NK cells as compared with the NK cells that had been treated with PBS as a control. Similarly, the NK cell-mediated killing of TS/A tumor cells was inhibited (Fig. 3*B*). As these results could simply reflect a toxic effect, we determined the viability of NK cells treated with TS/A exosomes in vitro using an ATPLite assay 24 h after the NK cells were treated with either TS/A exosomes or E-control. The results revealed that there was no significant differences in the results of the ATPLite assay for the NK cells treated with TS/A exosomes or E-control over 24 h ($p > 0.05$; Fig. 3*C*). These data suggest that the viability of the NK cells is not affected by TS/A exosomes.

Tumor exosomes selectively modulate the expression of cytolytic effector molecules in NK cells

The effect of in vitro coculture of the tumor exosomes on the expression of the cytotoxicity effector molecules of NK cells, perforin, and granzyme B was determined by immunoblot analysis. We found that the expression of perforin in IL-2-stimulated NK cells was reduced dramatically and in a dose-dependent manner on treatment with the TS/A tumor-derived exosomes (Fig. 4*A*), whereas the expression of granzyme B was unaffected.

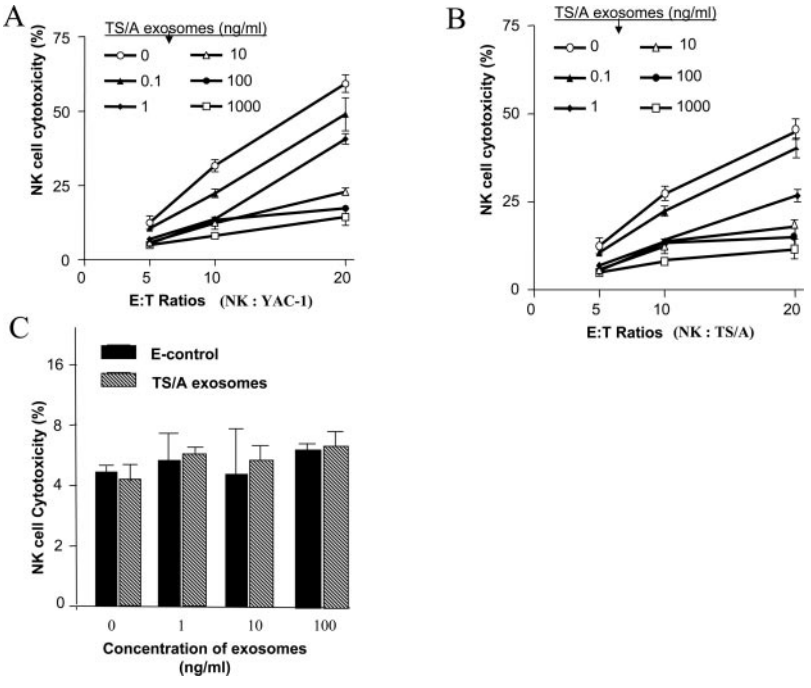

FIGURE 3. TS/A tumor exosome treatment suppresses the cytolytic activity of NK cells in vitro. $DX5^+$ NK cells from the spleens of untreated BALB/c mice were stimulated with IL-2 (100 U/ml) in the presence of different concentrations of TS/A exosomes for 24 h and then added to chromium-labeled YAC-1 cells (*A*) or TS/A tumor cells (*B*) at varying effector/target ratios. The amount of ^{51}Cr released into the supernatant was determined 4 h later, and the percentage of specific release determined. To determine the effects of TS/A tumor exosomes on the viability of NK cells, spleen DX5-positive NK cells were stimulated with IL-2 (100 U/ml) in the presence of either TS/A exosomes or E-control for 24 h. The viability of NK cells was then analyzed using the ATPlite assay (*C*) as described previously (18). The data are representative of three independent experiments.

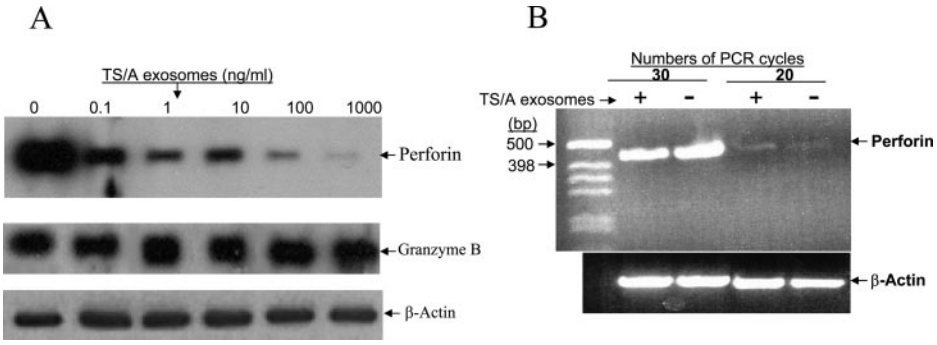

FIGURE 4. Tumor exosome-mediated inhibition of the cytolytic activity of NK cells is associated with a reduction in the expression of perforin in the NK cells. Spleen DX5$^+$ NK cells were stimulated with IL-2 (100 U/ml) in the presence of various amounts of TS/A exosomes for 24 h. A total of 50 μg of total protein lysates was used for Western blot analysis of the expression of perforin and granzyme B (*A*) using a method as described in *Materials and Methods* or total RNAs were extracted and used for RT-PCR analysis of expression of the perforin gene (*B*). The data are representative of three independent experiments. β-Actin served as an internal control to confirm equivalent loading.

To further determine whether the TS/A-exosome-mediated reduction of perforin is regulated at the transcriptional or posttranscriptional level, RT-PCR analysis was performed. The pretreatment of the NK cells with TS/A exosomes did not affect the expression of perforin mRNA in comparison with NK cells treated with PBS. This result was not due to the amount of mRNA used for the RT-PCR analysis as there were no differences in the amount of β-actin amplified (Fig. 4*B*).

Tumor exosomes selectively affect proliferation signals in the NK cells

Our finding of an absence of an increase in the levels of ATPLite after 15 h of culture of NK cells with tumor-derived exosomes (Fig. 3*C*) suggested that the treatment of the NK cells with TS/A exosomes may lead to inhibition of proliferation. We therefore determined the effect of the tumor exosomes on IL-2-dependent NK cell proliferation. Spleen NK cells isolated from BALB/c mice were cultured in medium containing IL-2 with or without exosomes for 24 h, and the IL-2-induced NK cell proliferation was assessed over the next 3 days by a standard tritiated-thymidine incorporation assay. The proliferation of NK cells in response to IL-2 was inhibited significantly by the tumor-derived exosomes (Fig. 5*A*). Flow cytometric analysis revealed that the tumor exosomes did not significantly affect the expression of the IL-2R by NK cells (data not shown), indicating that tumor exosome-treated NK cells retain their ability to bind IL-2.

To clarify whether the exosome-mediated inhibition of NK cell proliferation is a peculiarity of exosomes derived from TS/A tumor cells, tumor exosomes produced by different types of tumor cells were used for treatment of NK cells using the same protocol. The results indicated that the exosome-mediated inhibition of NK cell activation is not restricted to exosomes produced by TS/A tumors, but also can be mediated by exosomes produced by other tumor cells, including the MDA231 human breast tumor cell line, the A2058 human melanoma cell line, and the 4T.1 murine mammary carcinoma (Fig. 5*B*). The exosomes isolated from these tumor cell

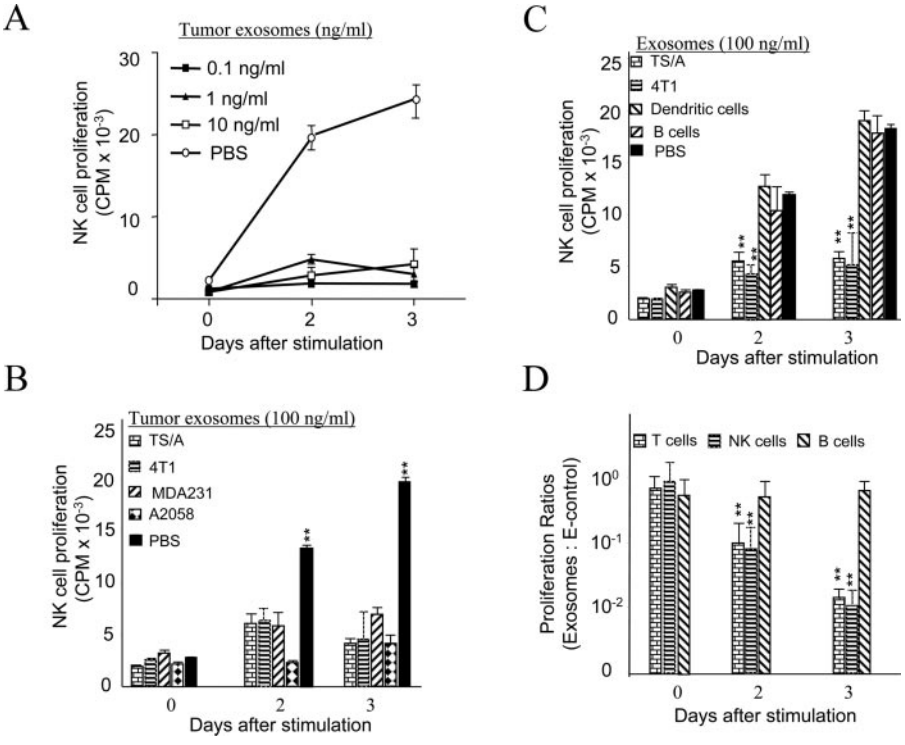

FIGURE 5. TS/A tumor exosomes prevent NK cell growth. Spleen DX5-positive NK cells were cultured in IL-2 (100 U/ml) with varying concentrations of TS/A tumor exosomes (*A*) or with exosomes (100 ng/ml) isolated from different types of tumor cells (*B*) or with exosomes (100 ng/ml) isolated from dendritic cells differentiated from bone marrow and LPS (10 μg/ml)-stimulated spleen mouse B cells (*C*) for 24 h. Subsequently, the IL-2-stimulated NK cell-proliferative response was measured daily for 3 days using a tritiated-thymidine uptake assay after a 16-h pulse of tritiated thymidine. The radioactivity was measured using a scintillation counter. The effects of TS/A exosomes on the growth of T cells, NK cells, and B cells also were determined using a thymidine incorporation assay. In brief, spleen NK cells, T cells, and B cells were cultured in the presence of IL-2 (100 U/ml), anti-CD3 Ab (100 ng/ml) plus IL-2 (5 ng/ml), and LPS (10 μg/ml), respectively, with TS/A exosomes (100 ng/ml) or E-control (100 ng/ml) for 3 days. Cell proliferation was measured daily using the protocol identical to that described above. The ratios of [^{3}H]thymidine incorporation of the cells treated with TS/A exosomes vs E-control were calculated (*D*). Data represent the means and SDs from triplicate cultures.

lines also inhibit the proliferation of a human 92 NK cell line in response to IL-2 significantly (data not shown).

We then tested the ability of exosomes produced by nontumor cells to inhibit NK cell proliferation, and found that exosomes produced by primary dendritic cells or a primary spleen B cell did not inhibit NK cell proliferation ($p > 0.05$; Fig. 5*C*). To more fully explore the effects of TS/A tumor exosomes on the proliferation of cells other than NK cells, the changes in the tritiated thymidine incorporation of spleen T cells, B cells, and NK cells after exosome treatment were calculated and expressed as a ratio of the changes induced by treatment with the E-control fraction. The results indicated that TS/A exosomes inhibit proliferation of both NK cells and T cells (Fig. 5*D*) and confirmed that there was no significant effect on B cell proliferation ($p > 0.05$; Fig. 5*D*) at days 2 and 3 after treatment. Induction of IFN-γ in IL-2-stimulated NK cells or anti-CD3 plus IL-2-stimulated T cells also was inhibited (data not shown), suggesting that the activation of NK cell and T cells is inhibited by TS/A exosomes.

The generation of the proliferative response by the IL-2R complex is known to involve two primary signals. One of these leads to the activation of the p42/p44-MAPK (also known as ERK) and PI3K signaling pathways. The second involves Jak3-mediated activation of the transcription factor, Stat5. We found that the activity of p42/p44 and of Akt, a substrate of PI3K, in NK cells, was unaffected by treatment with the tumor-derived exosomes (data not shown). In contrast, the expression of Jak3 (Fig. 6*A*, *top panel*), but not Jak1 (Fig. 6*A*, *third panel* from the *top*), was inhibited by the tumor-derived exosomes. This inhibition of Jak3 expression was further demonstrated by the finding of a reduction in the levels of phosphorylated Stat5, which is one of Jak3 substrates (Fig. 6*A*, *second panel* from the *top*). The inhibition of phosphorylation of Stat5 was dependent on the concentration of tumor-derived exosomes, and the differences in the amount of phosphorylated Stat5 among samples were not attributable to differences in sample loading as equivalent levels of β-actin were detected (Fig. 6*A*, *bottom panel*). In addition, we found that expression of cyclin D3 (Fig. 6*B*, *top panel*), but not cyclin D1 (Fig. 6*B*, *second panel*), was inhibited in NK cells treated with tumor-derived exosomes and there was a reduction in the levels of phosphorylated Rb, which is a substrate of cyclin D3 (Fig. 6*B*, *third panel* from the *top*) but not total Rb, which was dependent on the concentration of tumor-derived exosomes (Fig. 6*B*, *third panel*).

The relatively long-term (3-day) effect of TS/A exosomes on NK cell proliferation raised the issue of the nature of the interaction of the TS/A tumor exosomes with the NK cells. Spleen NK cells were incubated with PKH67-labeled TS/A exosomes (100 ng/ml) or the labeled exosome sample that had been depleted of exosomes using the 100-kDa cut-off filter, and then washed with PBS. The numbers of green fluorescent-positive NK cells were then counted at different time points using a fluorescent microscope. Green fluorescent-positive cells were observed indicating that the exosomes can effectively fuse with the NK cell membrane and enter the NK cells. The green fluorescent-positive cells could be seen within 30 min, and the intensity of fluorescent dye in the cells peaked at 3 h (Fig. 7*A*) after addition of the TS/A exosomes, and had declined by 48 h. By day 3, TS/A exosome-labeled with PKH67 were no longer detectable. The percentages of TS/A exosome-positive NK cells are summarized as shown in Fig. 7*B*. The lack of staining of the NK cells when the exosome-depleted fraction (the flow-through) was used indicated that the staining of the NK cells could be attributed to the uptake of the exosomes (Fig. 7*B*).

Effects of tumor exosomes on dendritic cells

As exosomes have been implicated in tumor Ag presentation (2), we determined the effect of the tumor exosomes on maturation of dendritic cells. Bone marrow cells were isolated from 2-mo-old BALB/c mice and stimulated with GM-CSF for 5 days before addition of the tumor exosomes and stimulation with LPS. FACS analysis was then used to identify $CD11c^+CD80^+CD86^+$ DC cells. Somewhat to our surprise, the addition of the tumor-derived exosomes had no effect on dendritic cell maturation (Fig. 8*A*). There was a 28.8 $\pm$ 2.2% increase in $CD11c^+CD80^+$, and a 22.6 $\pm$ 1.4% increase in $CD11c^+CD86^+$ DC cells after treatment with LPS in the presence TS/A exosomes, with very similar results being obtained in the control cultures. Moreover, the production of IL-12, as determined by ELISA of the culture supernatants (data not shown), was unaffected. These results suggest that although the tumor-derived exosomes inhibit NK cells, they do not affect the dendritic cells that are involved in Ag presentation.

Other investigators have reported that dendritic cells pulsed with TS/A tumor exosomes are immunogenic and contribute to the exosome-mediated inhibition of tumor growth when administered prophylactically (2). Our results suggest that the effectiveness of such an approach may be compromised by existing disease. We therefore tested directly whether pretreatment of mice with tumor exosomes affects dendritic cell cancer immune therapy. Tumor exosomes (10 μg in 100 μl of PBS), or the exosome diluent alone (PBS), were injected i.p. into 7-wk-old BALB/c female mice twice a week for 3 wk. Then, 1×10^6 syngeneic BM-dendritic cells ($H\text{-}2^d$) loaded with TS/A tumor-derived exosomes (1 μg) were injected once into the lateral flank. The mice were challenged with TS/A tumor cells (1.2×10^5) and the growth of the implanted tumor was measured weekly. In the mice that had been pretreated

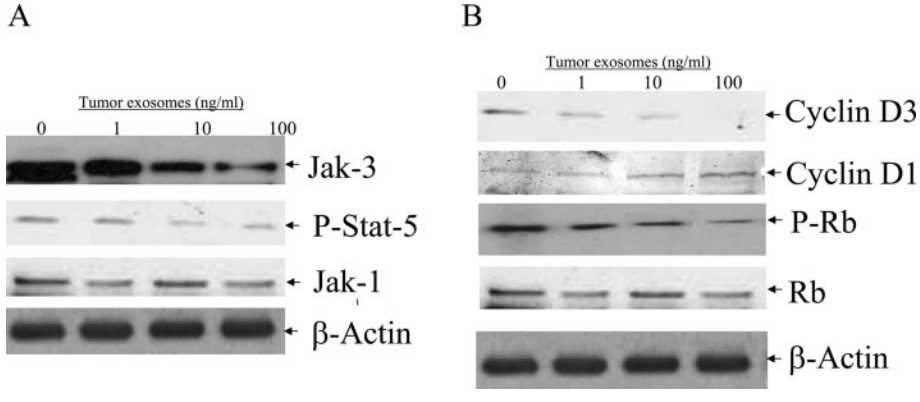

FIGURE 6. TS/A tumor exosomes block the activation of Jak3 and cyclin D3 in IL-2-stimulated NK cells treated with TS/A exosomes. Spleen DX5-positive NK cells were cultured and treated as described in the legend of Fig. 5 for 24 h. NK cells were lysed in protein lysis buffer after 72 h of culture. A total of 50 μg of total protein from each lysate was resolved on a 10% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane and the blots were probed with the Abs for detection of either Jak activation (*A*) or cell cycling (*B*). The data are representative of three independent experiments. β-Actin served as an internal control to confirm equivalent loading.

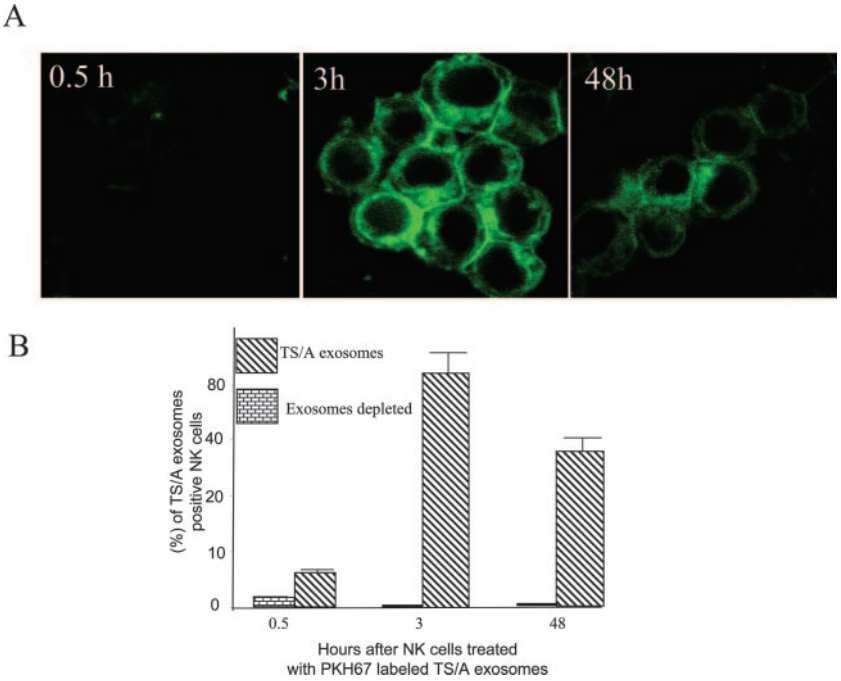

FIGURE 7. TS/A exosomes are taken up and remain stable in NK cells. TS/A exosomes were labeled with PKH67 dye as described in *Materials and Methods*. Spleen NK cells (1×10^4) were purified with DX5-conjugated microbeads, and stimulated with IL-2 (100 u/ml) in the presence of TS/A exosomes (100 ng/ml). The NK cells treated with TS/A exosomes, or the exosome-depleted fraction (the flow-through), were then harvested at different times and unbound exosomes were removed by three washes with PBS. The cells were cytospun onto poly-L-lysine-coated slides and green fluorescent-labeled NK cells were quantified by counting the total number vs green fluorescent-positive NK cells using a Leica confocal microscope (original magnification, ×60) (*A*). The percentage of green fluorescent-positive NK cells were calculated and expressed as the percentage of TS/A exosome-positive NK cells = average numbers of green fluorescent-positive NK cells/average numbers of NK cells both counted in five fields × 100. Data are representative of four independent experiments (*B*).

with tumor exosomes, the tumors started to grow 1 wk after inoculation and enlarged markedly after 3 wk regardless of whether the mice were immunized subsequently with dendritic cells pulsed with tumor exosomes or with PBS (Fig. 8*B*). In contrast, in mice that had not been pretreated with tumor-derived exosomes, the administration of dendritic cells pulsed with tumor exosomes, but not dendritic cells alone, resulted in the development of smaller tumors, with some tumors being rejected completely (2 of 15 mice by the end of the experiment), significantly.

Discussion

In this study, we show for the first time the pre-eminent role of tumor-derived exosomes in the promotion of growth of implanted tumors. Compelling evidence of tumor-derived exosome-mediated inhibition of the NK cell immune response was provided in three different settings: more rapid growth of the implanted tumors in mice that were pretreated with tumor-derived exosomes, potent inhibition of the cytotoxic activity of NK cells both in vivo and in vitro, and inhibition of IL-2-stimulated NK cell growth signaling.

We have demonstrated that breast cancer cells can communicate with NK cells through the production of exosomes by the tumor cells that are able to inhibit NK cell activation and promote tumor growth. Tumor immunosuppression of NK cell activity and the role of NK cells in tumor rejection have been well-documented. In patients with cancer, NK cell activities have been shown to be impaired as assessed by the reduced ex vivo functionality of the NK cells (22–24). Apparently disease-free patients with functional peripheral blood NK cells have a significantly longer metastasis-free survival time than those with low NK cell activity (25, 26). Our data and the data of other investigators also support the concept that NK cells play a role in the rejection of both TS/A and 4.T1 murine mammary tumor cells in BALB/c mice (27, 28). This concept is further supported by our finding that depletion of NK cells by anti-NK cell Ab, but not control Ab, resulted in the acceleration of the growth of the implanted tumor cells.

The activation of the NK cells can be blocked by binding of MHC class I molecules to NK cell inhibitory receptors or the prevention of activation of cytolytic pathways that mediate the release of the effector molecules. When NK cell inhibitory receptors bind to MHC class I molecules, the effector functions of the NK cells, including cytotoxicity and cytokine production, are blocked. Inhibitory receptors specific for MHC class I or MHC class I-related molecules can provide protection for target cells that express normal levels of class I molecules on their surface (29–31). Inhibition of the activation of the tumor cytolytic pathway seems to play a more important role in cancer development, however. Studies of gene-disrupted mice indicate that perforin is crucial for NK cell cytotoxicity (32). Perforin plays an important role in NK cell-mediated suppression of tumor initiation and metastasis (33–35). Cytokines activate NK cells to destroy a variety of tumors in a perforin-dependent manner (34, 36). Our results also support the concept that tumor exosome-mediated inhibition of NK cell function acts through the regulation of perforin expression, rather than through the interaction of MHC class I molecules with NK cell inhibitory receptors as neither dendritic cell nor B cell exosomes, which are both known to express high levels of MHC class I molecules on their cell surface, exerted an immunosuppressive effect on the NK cells. Further, our RT-PCR results indicate that treatment with TS/A tumor exosomes did not elicit a change in the levels of perforin mRNA in the NK cells, suggesting that the regulation of perforin expression most likely occurs posttranscriptionally. It will be of interest to further dissect the mechanisms underlying the tumor exosome regulation of the stability or function of perforin at the posttranscriptional level.

The expression of FasL has been found in tumors of different origin and reported to be correlated with metastatic spread and poor prognosis (26–29). However, the results of the ATPLite assay suggest that tumor exosomes do not affect the viability of spleen NK cells. Western blot analyses indicate that FasL and TRAIL are not expressed on TS/A exosomes (data not shown), indicating that exosome-mediated induction of apoptosis of NK cells is unlikely to play a major role in this particular model.

Our results also indicate that the inhibition of NK cell proliferation is not limited to exosomes produced by TS/A tumor cells and that exosomes produced by other breast cancer tumor cells lines, and also a melanoma cell line, are inhibitory. This effect does seem to be limited to exosomes produced by tumors, however, as the exosomes produced by nontumor cells, including primary dendritic

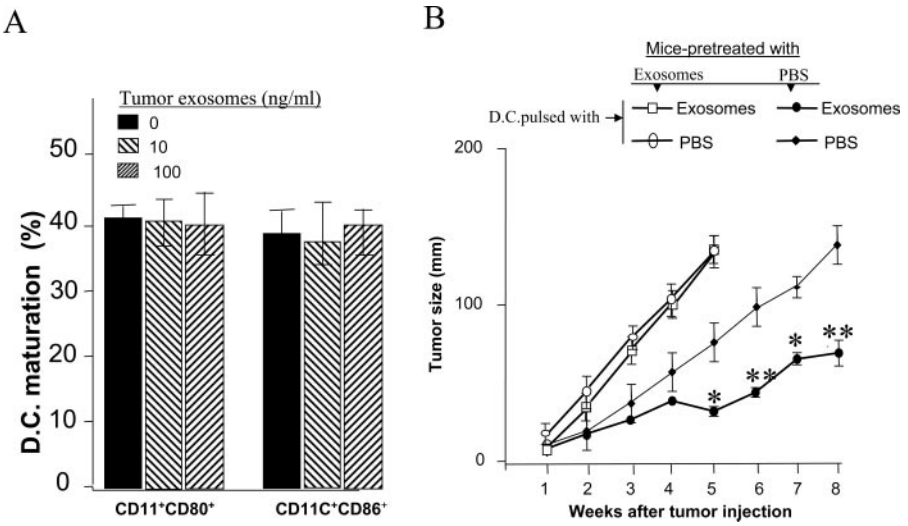

FIGURE 8. TS/A tumor exosomes inhibit TS/A exosome-pulsed dendritic cell therapy, and TS/A exosomes do not affect dendritic cell maturation. Dendritic cells isolated from the bone marrow of BALB/c mice were cultured in RPMI 1640 containing GM-SCF (20 ng/ml) for 5 days, and subsequently stimulated with LPS (1 μg/ml) for 16 h in the presence of different concentrations of TS/A exosomes. The expression of $CD11C^+$ maturation markers including CD80 and CD86 was then evaluated by FACS analysis and the percentages of double-positive dendritic cells, including $CD11c^+CD80^+$ or $CD11^+CD86^+$ cells were calculated (*A*). Results are representative of three experiments. Pretreatment of mice with TS/A tumor exosomes abolishes the protective effect generated by TS/A exosome-pulsed DC therapy (*B*). BALB/c mice were injected i.p. with TS/A exosomes (10 μg in 100 μl of PBS/mouse) or PBS twice weekly for 3 wk. Mice were then challenged s.c. with TS/A tumor cells (1.2×10^5 cells/mouse). The tumor size was measured weekly. Each point represents the mean tumor size ± SD of 10 mice from each group. Statistical significance was determined by ANOVA. *, $p < 0.05$; **, $p < 0.01$.

cells and primary spleen B cells, did not inhibit NK cell activation. Our data suggest that tumor exosomes express specific molecule(s) that mediate the inhibition of NK cell activation. The identification of these molecules will be of importance in the future design of successful cancer immune therapy. Numerous results from other investigators indicate that tumor-derived factors cause immunosuppression of both NK cells and T cells (37–42). Our results indicate that TS/A tumor exosomes can inhibit both T cell and NK cell activation. Taken together with the lack of inhibition of primary B cells or dendritic cells, these results suggest that the inhibitory effect of TS/A tumor exosomes is specific for certain types of cells and, perhaps, has specificity for IL-2-stimulated signaling.

Our in vitro results that indicated tumor exosome-mediated inhibition of NK cell proliferation were in agreement with the reduction in the percentages of NK cells resident in the lung and spleen after treatment with tumor exosomes. This selective reduction of the percentages of NK cells in the lung and spleen may be dependent on the routes of TS/A exosome trafficking in vivo. It is not know whether the trafficking routes of exosomes produced by tumor cells in vivo are the same as those that occur after i.p. injection, as used in these experiments. Interestingly, the total numbers, but not the percentages, of NK cells in the spleen were increased slightly after TS/A exosome treatment. However, the tumor-specific cytotoxicity of these spleen NK cells was reduced. These data suggest the biologic activity of the spleen NK cells may be impaired after TS/A exosome treatment.

Other investigators have reported that TS/A exosomes might induce an adaptive immune response (43). Although this was not the focus of the current study, ELISA analysis of sera collected from mice treated with TS/A exosomes, two doses per week for 3 wk did not indicate detectable Ab with specificity for the TS/A exosomes (data not shown). It should be noted, however, that in the current study, the exosomes were injected i.p. This route was chosen based the reports that tumor exosomes isolated from the peritoneal cavity of patients with cancer exhibit strong inhibitory activity against T cell activation (44).

Our data show that TS/A tumor-derived exosomes promote tumor growth through specific mechanisms (e.g., selective inhibition of Jak3 in the absence of an effect on Jak1) rather than through generalized down-regulation of NK cell activity The exosomes appear to act, however, by simultaneous blockade of several pathways. The ability of the tumor exosomes to inhibit perforin expression is consistent with the current model of NK cell-mediated control of tumor growth. The key role of perforin in NK cell-mediated control of tumor growth in vivo has been firmly established in a number of mouse experimental tumor models. Perforin-deficient mice have been observed to display increased susceptibility to many chemical or viral-induced tumors, and are significantly less proficient than wild-type mice in preventing the metastasis of tumor cells to the lung (36, 45, 46). The expression of perforin is regulated by IL-2 signaling through activation of Jak3/Stat5 (47, 48). Our Western blot analysis showed that tumor exosomes suppressed expression of Jak3, but not Jak1, and that the tyrosine phosphorylation status of Stat5 was altered in IL-2-stimulated NK cells. It is well-established that IL-2-induced activation of Jak3 kinases is associated with tyrosine phosphorylation and activation of Stat5 (49). Thus, the reduction in the pool of phosphorylated Stat5 further supports the concept that the tumor exosomes target the Jak3 expression pathway in the NK cells. These findings suggest that the tumor exosomes may suppress the expression of perforin through inhibition of Jak3-dependent signaling pathways, and that this may be a mechanism by which tumor exosomes exert their immunosuppressive effects.

Our data suggest that the tumor-derived exosomes not only inhibit the induction of perforin of IL-2-stimulated NK cells, but also inhibit IL-2-stimulated NK cell growth. This effect was found to be associated with the expression of cyclin D3, which plays a critical role in the G_1-S transition in lymphocytes. The effects of cyclin D3 on this transition are known to involve inactivation of the Rb family of proteins by direct phosphorylation (50). Our data show that the ability of exosomes produced by tumor cells to reduce the

expression of cyclin D3 is correlated with decreased phosphorylation of Rb, which further confirms that the tumor-derived exosomes target specific pathways in the NK cells.

Other investigators have demonstrated that tumor-derived exosomes can be used as cancer vaccines (2, 51). Using a similar strategy, we also found that priming of mice with bone marrow dendritic cells pulsed with tumor exosomes stimulates the host immune response against the growth of implanted TS/A tumors. As other investigators data suggest, BALB/c mice primed with dendritic cells pulsed with TS/A exosomes also exhibit a delay of 4T.1 murine mammary tumor growth (data not shown), suggesting that shared tumor Ags may be processed by dendritic cells. The tumor exosomes did not affect DC maturation, however. Notably, the ability of the tumor exosomes to stimulate the immune response was abolished when the mice were pretreated with tumor exosomes. We speculate that the dampening of the DC immune therapeutic effect by tumor exosomes may be associated with the blockade of the activation of the effector cells, including NK cells, suggesting that tumor exosome-mediated immune suppression could result in the failure of DC cancer immune therapy. Identification of the components of the tumor-derived exosomes that cause immune suppression would greatly benefit the development of effective DC-based cancer immune therapy.

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Disclosures

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