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Human Tumor-Derived Exosomes Down-Modulate NKG2D Expression¹

Aled Clayton,^{2*} J. Paul Mitchell,* Jacquelyn Court,[†] Seamus Linnane,[‡] Malcolm D. Mason,* and Zsuzsanna Tabi*

NKG2D is an activating receptor for NK, NKT, CD8⁺, and $\gamma\delta^+$ T cells, whose aberrant loss in cancer is a key mechanism of immune evasion. Soluble NKG2D ligands and growth factors, such as TGF β 1 emanating from tumors, are mechanisms for down-regulating NKG2D expression. Cancers thereby impair the capacity of lymphocytes to recognize and destroy them. In this study, we show that exosomes derived from cancer cells express ligands for NKG2D and express TGF β 1, and we investigate the impact of such exosomes on CD8⁺ T and NK cell NKG2D expression and on NKG2D-dependent functions. Exosomes produced by various cancer cell lines in vitro, or isolated from pleural effusions of mesothelioma patients triggered down-regulation of surface NKG2D expression by NK cells and CD8⁺ T cells. This decrease was rapid, sustained, and resulted from direct interactions between exosomes and NK cells or CD8⁺ T cells. Other markers (CD4, CD8, CD56, CD16, CD94, or CD69) remained unchanged, indicating the selectivity and nonactivatory nature of the response. Exosomal NKG2D ligands were partially responsible for this effect, as down-modulation of NKG2D was slightly attenuated in the presence of MICA-specific Ab. In contrast, TGF β 1-neutralizing Ab strongly abrogated NKG2D down-modulation, suggesting exosomally expressed TGF β as the principal mechanism. Lymphocyte effector function was impaired by pretreatment with tumor exosomes, as these cells exhibited poor NKG2D-dependent production of IFN- γ and poor NKG2D-dependent killing function. This hyporesponsiveness was evident even in the presence of IL-15, a strong inducer of NKG2D. Our data show that NKG2D is a likely physiological target for exosome-mediated immune evasion in cancer. *The Journal of Immunology*, 2008, 180: 7249–7258.

Exosomes are a population of nanometer-sized vesicles, actively secreted by diverse cell types. Originating within late endosomal compartments, as multivesicular bodies (1), or perhaps from endosome-like plasma membrane patches (2), their functions, once released into the extracellular milieu, remain a focus of interest, particularly in the context of immunology. Because exosomes express a phenotype similar to that of the cell of origin, they may, at least in part, harbor some of the functions associated with the parent cell. Dendritic cell-derived exosomes (3–6), for example, express high levels of MHC and costimulatory molecules, and can directly activate naive T lymphocyte responses (7). Exosomes from other cell types, such as cancer cells, are also capable of direct interactions with lymphocytes, but the immunological outcome(s) are somewhat contentious. Some reports detail lymphocyte activation following tumor-exosome interactions (8) while others describe immune-suppressive effects (9, 10). The discrepancies are almost certainly due to differences in the phenotype of the exosomes being studied.

Cancer cell exosomes, isolated from cell culture (11) or from in vivo sources such as malignant effusions (12, 13), exhibit the properties typical of exosomes of nonmalignant cells, including their small size (30–100-nm diameter), their capacity to float on sucrose gradients (at around 1.1–1.2 g/ml), and the expression of markers common to exosomes in general (e.g., MHC molecules, tetraspanins, and heat shock proteins (hsp)³). Cancer exosomes also express tumor-associated Ags, suggesting they may play some functions in cancer immunology, such as transport of Ags to dendritic cells, to initiate an antitumor immune response (11, 12).

As well as expression of tumor-associated Ags, tumor cells identify themselves to the immune system by displaying cell surface receptors that activate innate and/or adaptive immunity. A key example is the expression of MHC class-I related NKG2D ligands. These include members of the MICA and UL16-binding protein (ULBP) families, molecules that have highly restricted expression patterns in health, but are readily up-regulated following viral infection or genotoxic stress (14). Elevated levels of NKG2D ligands have been documented in a range of epithelial and other malignancies (15), and are important in tumor immune surveillance (16), essentially rendering cancer cells as attractive targets for NK cells (17), $\gamma\delta$ T cells (18), and in certain situations, $\alpha\beta$ CD8⁺ T cells (19) through NKG2D-mediated lymphocyte activation. In advanced malignancies, however, growth factors produced by cancer cells, such as TGF β 1 can systemically down-regulate NKG2D expression (20). In addition, proteolytic cleavage of NKG2D ligands from cancer cell surfaces leads to soluble ligands (sMICA, sMICB, and others (15, 21–23)) appearing in the circulation, which drive

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³ Abbreviations used in this paper: hsp, heat shock protein; s, soluble; PF, pleural fluid; MFI, mean fluorescence intensity; ULBP, UL16-binding protein.

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systemic impairment of NK (22) and CD8⁺ T cell (21, 22) functions. The principal mechanism for this is through binding to, and lasting down-regulation of, surface NKG2D, leading to a loss of this key NK/T cell activation trigger (21). Recently, sNKG2D ligands have also been shown to mediate NKG2D-independent immune-suppressive effects, suggesting that other, as yet unidentified, receptors for NKG2D ligands may exist (24). Membrane shedding of NKG2D ligands from tumor cells into the circulation is therefore an important systemic immune-evasion mechanism, but is also likely important in the local tumor microenvironment as poor expression of NKG2D ligands on the tumor surface correlates with reduced susceptibility to immune attack.

Proteolytic cleavage of surface receptors is not the only mode of eliminating membrane proteins from the cell. Exosomes, in certain scenarios, may constitute a major cellular mechanism for purging membrane molecules (25), as they are particularly enriched in transmembrane and GPI-anchored proteins (26). In addition, recent evidence suggests exosomes may have the capacity to deliver growth factors and subsequently modulate cellular responses (27, 28). In this report, we demonstrate that tumor-derived exosomes express NKG2D ligands, and can directly interact with NK cells and CD8⁺ T cells in a manner that is at least partly NKG2D dependent, but strongly dependent on exosomal TGFβ1. This interaction leads to a significant reduction in cell surface NKG2D expression, comparable to that achieved using high-dose soluble TGFβ1. Exosome-mediated down-modulation of NKG2D correlated with poor functional responses. The data implicate NKG2D as a target for exosome-mediated tumor immune evasion.

Materials and Methods

Cancer cell lines

The majority of experiments presented in this report were based upon a collection of mesothelioma cell lines, generated within the department from explanted mesothelioma tissue or cells isolated from pleural fluid (PF). Additional cell lines of prostate cancer (DU145, PC3) were obtained from American Type Culture Collection (ATCC). A B-lymphoblastoid cell line (IB4), an immortalized NK cell line (NKL), and noncancerous skin fibroblast lines (MRC5 and HFF) were obtained from the Medical Research Council (MRC) Cooperative, Cardiff University. For preparation of exosomes from normal blood lymphocytes, 250-ml bags of plasma-free, erythrocyte-poor leukocytes were obtained from the Welsh Blood Service, and maintained in culture with 20 U/ml IL-2 for 72 h. Cells were cultured in RPMI 1640 (Cambrex), supplemented with 1000 U/ml penicillin/streptomycin, 5 mM L-glutamine, and 5–10% FBS. For all experiments, FBS was rendered free of bovine exosomes by 18-h centrifugation at 100,000 × *g* at 4°C, and the supernatants were filtered through 0.2-μm and then 0.1-μm Vacuum filters (Millipore). Ethical approval for obtaining patient-derived materials, or blood from healthy volunteers was obtained from the Bro Taf local regional ethics committee.

Flow cytometry of cell lines

The cells used as a source of exosomes were subject to flow cytometric analysis, to ascertain patterns of NKG2D-ligand expression. Adherent cells were harvested without trypsin treatment, as we found this cleaved surface NKG2D ligands, giving false negative results. Adherent monolayers were washed three times in PBS, and were incubated on a shaking platform in 2 mM EDTA/PBS for 5 min. Harvested cells were incubated on ice, in 5 μg/ml primary Ab diluted in PBS for 40 min. Following two washes in PBS, secondary Ab (goat anti-mouse FITC conjugated) was added, and incubation on ice continued for 30 min. After washing, cells were analyzed on a FACScan flow cytometer (BD Biosciences). Primary Abs included anti-MICA, -MICB, -ULBP-1–3, or irrelevant isotype controls (from R&D Systems). As positive control for staining, anti-MHC class I (DakoCytomation) or anti-CD81 (Santa Cruz Biotechnology) was used.

Exosome purification

Exosomes were purified from cell-free medium of tumor cell cultures, or from PF of malignant mesothelioma patients, by ultracentrifugation on a sucrose/D₂O cushion as we (28–30) and others (12, 31) previously described. The method has been shown effective in eliminating membranous

fragments, apoptotic particles, and other cell debris of density >1.2 g/ml. Briefly, 50 ml of PF or 180 ml of culture medium (from 10 to 20 75-cm² flasks, following up to 7 days in culture) was subjected to serial centrifugation to remove cells and debris. Preclarified medium was underlaid with 30% sucrose/D₂O, and subjected to ultracentrifugation at 100,000 × *g* for 3 h. The exosomes, captured within the sucrose layer, were collected, diluted in at least a seven times volume of PBS and pelleted by ultracentrifugation at 100,000 × *g* for a further 2 h. The pellet was resuspended in 100–150 μl of PBS and stored at –80°C. Exosome protein content was determined using the BCA protein assay (Pierce).

Isolation of lymphocytes

PBMC of healthy donors were isolated by centrifugation on a Histopaque gradient (Sigma-Aldrich). To maximize the opportunity for exosomes to directly encounter lymphocytes rather than being taken up by phagocytes, cell preparations were enriched for lymphocytes by removing plastic adherent cells. The nonadherent fraction of PBL was collected and used in experiments. Purification of NK cells or CD8⁺ T cells was performed using immunomagnetic beads (from Miltenyi Biotec), according to the manufacturer's protocols.

Flow cytometry of lymphocytes

Flow cytometric analysis of exosome-treated lymphocytes was performed with a FACSCanto cytometer running FACSDiva software (BD Biosciences). Conjugated Abs used included: CD3-FITC (DakoCytomation), CD94-PE (clone HP-3D9), CD69-PE (clone FN50), CD56-PECy5 (clone B159), CD16-PECy5 (clone 3G8), CD8-PECy7 (clone RPA-T8), and NKG2D-allophycocyanin (clone 1D11), obtained from BD Biosciences. For the study of intracellular proteins, cells were fixed and permeabilized (using the IntraPrep fix/perm kit; Beckman Coulter), and stained with Abs against granzyme-B-PE (clone GB11), obtained from Serotec, and perforin-FITC (clone δG9), obtained from BD Biosciences.

Immunoblotting

Whole cell lysates, prepared as described (32), and purified exosome protein were solubilized by addition of 30% volume of 6 M urea, 50 mM Tris-HCl, 2% SDS, and 0.002% w/v bromophenol blue in the presence or absence of 5% 2-ME for reducing and nonreducing conditions, respectively. Samples were electrophoresed through 10% polyacrylamide gel, and transferred to polyvinylidene difluoride membranes which were blocked overnight in 3% w/v nonfat milk, 0.05% v/v Tween 20 in PBS. Primary Abs (below) were incubated for 1 h; following five washes, goat anti-mouse Ig-HRP conjugate, obtained from Santa Cruz Biotechnology (at 1/15,000 dilution) was added for 30 min. After five washes, bands were detected using the ECL⁺ system (Amersham/GE Healthcare). Primary Abs included: anti-TSG101, hsp90 (Santa Cruz Biotechnology), MICA and MICB (R&D Systems), mesothelin (Abcam), and MHC class I (MRC Cooperative, Cardiff University).

Exosome surface analysis

Purified exosome preparations were coupled to the surface of latex beads (surfactant-free, white, aldehyde-sulfate 4-μm beads; Interfacial Dynamics), and analyzed by indirect immunostaining and flow cytometry, as described (31, 33). Abs included mouse isotype controls (IgG2a and IgG2b), and mouse anti-human MICA, MICB, ULBP-1, ULBP-2 and ULBP-3 (R&D Systems), and anti-mesothelin (Abcam). As a positive control to confirm exosome binding to beads, anti-HLA-A/B/C (DakoCytomation) was used. The exosome-bead complexes were analyzed on a FACScan flow cytometer.

TGFβ1 ELISA

An ELISA duo-set kit for human TGFβ1 was purchased from R&D Systems, with subsequent assays performed as recommended by the manufacturer. Blocking, substrate, and stopping solutions were purchased from R&D Systems. High-binding microtiter ELISA strips were obtained from Greiner. Absorbance was measured with wavelength correction (A450–A540 nm) on a 3550 Microplate Reader (Bio-Rad).

Plate-bound NKG2D Ab, binding and cell activation assays

The well-characterized mouse anti-human NKG2D-activating Ab (clone 11D1) or an isotype-matched control (both functional grade; eBioscience) was added at 200 ng/well in 100 μl of PBS to high-binding microwell strips (Greiner). After overnight incubation at 4°C, wells were washed and blocked with 5% FBS/RPMI 1640 for at least 24 h at 4°C. To determine the capacity of PBL to bind to the wells, cells were pretreated with exosomes,

IL-15, or in combination for 48 h, before fluorescent labeling (with 5 μ M CFSE; Sigma-Aldrich), for 10 min at 37°C). Cells were subsequently added to anti-NKG2D or isotype Ab-coated strips. After incubation for 30 min at 37°C, nonadherent cells were washed away, and plate-bound cells were quantified by measuring fluorescence (Wallac Victor-3; PerkinElmer), and comparing readings to a standard curve of known cell numbers. To determine cell activation through NKG2D, PBL were pretreated with various combinations of exosomes or IL-15 (as detailed in figure legends), for 48 h, before adding to anti-NKG2D or isotype-coated strips. After 30 min, brefeldin A (Golgi Plug; BD Biosciences) was added, and incubation continued for a further 18 h. Cells were harvested, fixed, and permeabilized (IntraPrep; Beckman Coulter), and stained with IFN- γ -FITC and CD3-allophycocyanin.CY7 (BD Biosciences). The proportion of IFN- γ -positive cells was determined by flow cytometry, as described (34).

Ab-mediated redirected killing assay

The murine Fc γ R⁺ mastocytoma cell line P815 (from ATCC) was used as targets in an Ab-redirected killing assay as previously described (35–37). Briefly, P815 cells were labeled at 37°C with ⁵¹Cr (28), for 1 h, and after three washes in medium, were incubated at room temperature for 20 min with mouse anti-human NKG2D-activating Ab (clone 5C6, functional grade; eBioscience), or an isotype-matched irrelevant control Ab. P815 cells were plated, without washing, at 2500 cells/well of a “U”-bottomed 96-well plate. Effector cells, PBL pretreated with IL-2 (100 U/ml), exosomes, or other factors for 72 h, were added at various E:T ratios, and incubated for 4 h. Released radioactivity in the supernatant was measured (Wallac, MicroBeta-3; PerkinElmer), and percent-specific lysis was calculated in the usual manner as described (28).

Statistics

Comparisons between exosome-treated and untreated cells were performed using paired *t* tests (two tails), calculated using Graph Pad Prism-4 (version 4.03) graphing and statistical software.

Results

Tumor exosomes carry NKG2D ligands and TGF β 1

This study was performed using a panel of tumor cell lines, and some non-tumor fibroblasts or peripheral blood cells as controls. We first examined the cellular expression of NKG2D ligands by flow cytometry (Fig. 1). This revealed variable and heterogeneous NKG2D-ligand expression across the tumor cell types, as expected, yet all tumor cells studied were positive for one or more ligands of NKG2D. Of these, a mesothelioma cell line expressed the highest levels of NKG2D ligand, and was hence used as a source of exosomes in much of the subsequent experiments. In contrast, fibroblasts (HFF) or PBLs did not stain positively for any NKG2D ligand, consistent with the premise that NKG2D-ligand expression is a feature of cancer cells, and not found constitutively on non-cancer cells. Exosomes, from various human-cultured cancer cells, were analyzed by electron microscopy, Western blotting and, following coupling to microbeads, by flow cytometry. Tumor exosomes appeared as nanovesicular structures (28), within the size range previously described for exosomes from other cellular sources, i.e., ~30- to 100-nm diameter. Analysis of tumor exosome phenotype revealed positive staining (by Western blot) for MHC class I and for a recognized marker of multivesicular bodies, TSG101 (Fig. 2A). The NKG2D ligands, MICA and MICB, were also exosomally expressed, and flow cytometric analyses of exosomes coupled to latex microbeads demonstrated expression of NKG2D ligands at the exosome surface (Fig. 2B). The expression patterns of exosomal NKG2D ligands match those of the cell of origin (Fig. 1). Tumor cells, therefore, express ligands of NKG2D on the surface of their secreted exosomes. Exosomes were also isolated from PF of three patients with malignant pleural mesothelioma, and were analyzed by Western blot (Fig. 2C) or flow cytometry (Fig. 2D) as above. Exosomes, purified from malignant effusions comprise a mixture of exosomes derived from tumor cells and nontumor cells *in vivo* (13), and given the complexity of PF, such preparations likely harbor some more nonexosomal con-

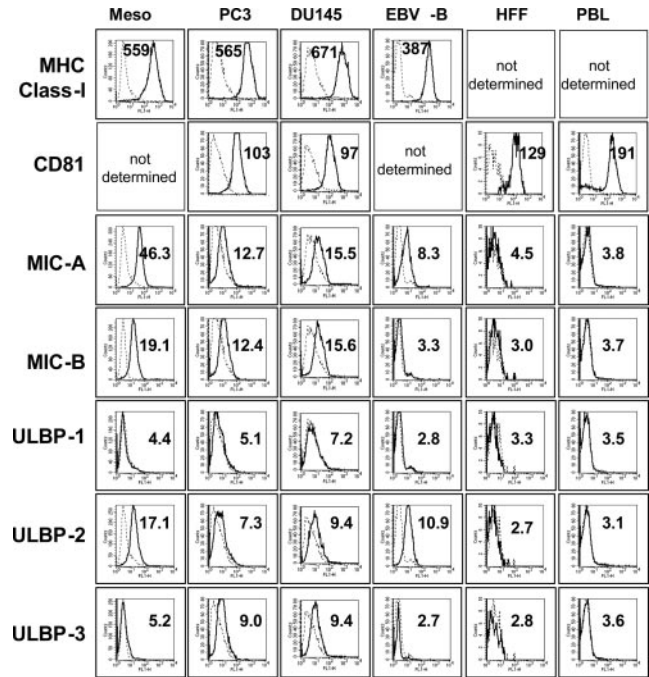


FIGURE 1. Flow cytometric characterization of NKG2D ligands expressed by tumor and nontumor cells. Tumor cell lines (Meso; mesothelioma, PC3 and Du145; prostate, EBV-B, EBV-transformed B-lymphoblastoid cells) or nontumor cells (HFF; skin fibroblasts; PBLs) were stained with various Abs against NKG2D ligands, or as positive controls with anti MHC class I and/or CD81 (solid line). Staining was compared with irrelevant isotype control Ab (dashed line). Numerical values indicating MFI of the histogram are shown.

taminants than exosomes purified from cell culture sources. Nevertheless, the exosome markers TSG101 and hsp90 were present in PF-derived exosome preparations (Fig. 2C), albeit at lower levels than exosomes from mesothelioma cell line cultures. The tumor-associated Ag, mesothelin, which is highly expressed by the majority of pleural mesothelioma, was present in exosomes from cultured mesothelioma cells, and detectable in one of two of the PF-exosome preparations tested. NKG2D ligands were also expressed by PF-derived exosomes (Fig. 2, C and D).

We previously reported that TGF β 1 expression by tumor exosomes (28) was involved in some of the immune-modulatory properties of tumor exosomes. To ascertain whether exosomes of PF origin also expressed TGF β 1, we performed ELISA on PF-exosome preparations from three patients, and demonstrated comparable levels of TGF β 1 found in exosomes derived from mesothelioma cultures (Fig. 2E).

Tumor exosomes trigger selective down-regulation of cell surface NKG2D

The expression of MICA and MICB by tumor exosomes suggested to us that perhaps exosomes may alter expression of NKG2D, as reported for tumor-derived soluble MICA/MICB (21). We investigated the effects of exosomes, secreted by several tumor cell types, on the phenotype of PBLs, obtained from healthy volunteers. Healthy donor PBL, treated with tumor exosomes or IL-15 or TGF β , as positive and negative controls, respectively, were analyzed by multicolor flow cytometry (the gating scheme is shown in Fig. 3A). Expression of NKG2D, CD94, or CD69 was measured. Tumor-exosome treatment resulted in a significant reduction in cell surface NKG2D expression by CD8⁺ T cells (Fig. 3B) and NK

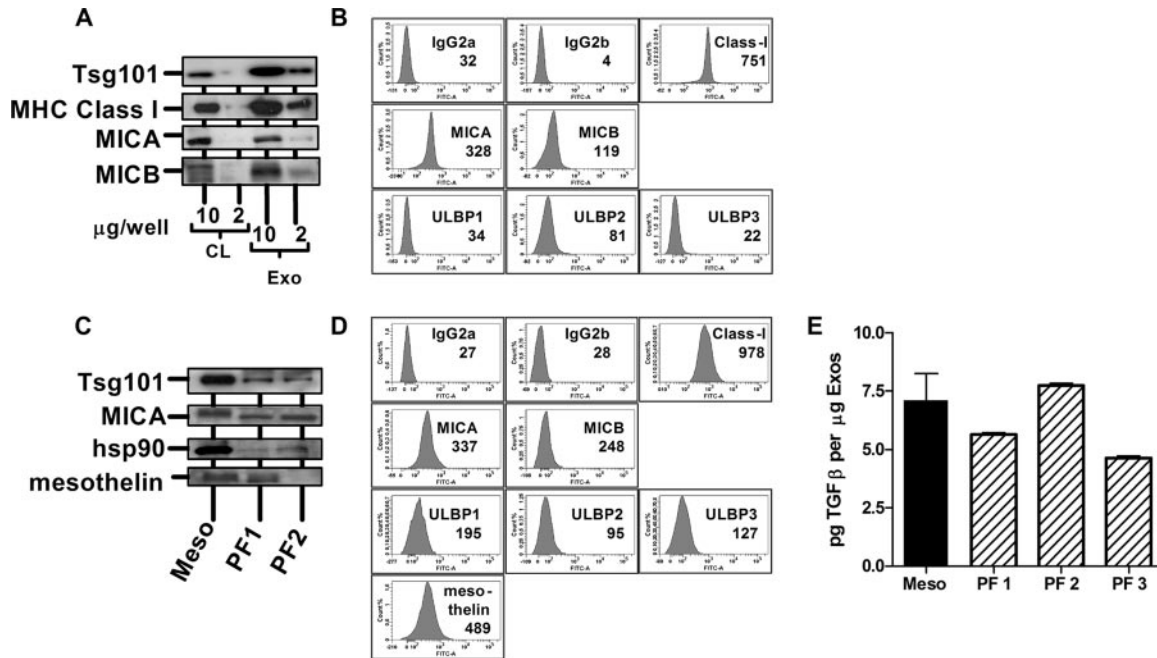


FIGURE 2. Characterization of tumor exosomes. Whole cell lysates of a mesothelioma cell line, or purified exosomes from this line were analyzed by Western blot (2 or 10 $\mu\text{g}/\text{well}$), demonstrating expression of typical exosome markers including MHC class I and TSG101. Expression of the MHC-like molecules MICA and MICB was also evident (A). These exosomes were coupled to the surface of aldehyde-sulfate latex beads, and after blocking were stained with primary Ab (as indicated) followed by goat anti-mouse IgG-FITC. Beads were analyzed by flow cytometry, demonstrating exosome surface expression of class I, MICA, MICB, and ULBP2. MFI values are shown (B). Similarly, 7 μg of these exosomes (Meso) were compared with 7 μg of exosomes purified from malignant mesothelioma PF (from two patients, designated PF1 and PF2) by Western blot (C). Exosomes from PF1 were further analyzed by flow cytometry, as above (D). TGF β 1 ELISA analysis of exosomes from cultured mesothelioma cell exosomes (Meso, mean of five preparations) or from three PF exosome preparations (PF1, PF2, and PF3, each measured in triplicate) by ELISA, indicating comparable levels of TGF β 1 (E).

cells (Fig. 3C) by 48 h (shown). This down-modulation was evident both in terms of reducing the proportion of NKG2D-positive cells, and also the level of expression (indicated by a decrease in the mean fluorescence intensity (MFI)). This phenotypic alteration was specific, as tumor-exosome treatment did not alter expression of CD3, CD4, CD8, CD56, or CD16. Furthermore, the level of CD94, a molecule that is also expressed on a subset of NK cells and CD8⁺ T cells and found in association with NKG2 receptors, remained unchanged following tumor exosome treatment (Fig. 3, B and C, line graphs). To determine whether NKG2D down-modulation resulted in cell activation, we also studied the expression of the activation marker CD69. Tumor exosome treatment did not induce up-regulation of CD69 (Fig. 3, B and C, line graphs). Experimental controls (IL-15 and TGF β 1) demonstrated that CD94 or CD69 expression was responsive to suitable stimuli in these experiments (Fig. 3, B and C, columns). Similarly, there was no exosome-mediated change in constitutive perforin or granzyme B expression (data not shown). Performing these experiments on immunomagnetically isolated CD8⁺ T cells or NK cells, or by using the NK cell line (NKL) revealed that exosomes mediate these effects directly, and do not require the activity of CD4⁺ T cells, or dendritic cells (data not shown). Studies comparing fixed cells with fixed and permeabilized cells revealed that while surface NKG2D was decreased following exosome treatment, the total cellular NKG2D was largely stable, suggesting internalization of NKG2D from the surface following exosome interaction (data not shown). Our data demonstrate that tumor exosomes trigger a selective decrease in cell surface NKG2D, without concomitant cellular activation.

Analysis of NKG2D down-regulation over a 4-day period revealed a continuous decrease in the proportion of NKG2D-positive

CD8⁺T cells, still decreasing at 96 h, with the greatest fall within the initial 12–24 h (Fig. 4A). Similarly, NK cells reduced expression levels of NKG2D up to \sim 48 h, but this appeared to recover thereafter, although at these latter time points the absence of exogenously added NK-survival factors such as IL-2 in these experiments meant that the numbers of remaining NK cells were low (<400 events in the NK cell flow cytometry gate) (Fig. 4B). In essence, therefore, a single treatment with tumor exosomes can impair NKG2D expression for at least 3 days (NK cells) or longer (CD8⁺ T cells).

Reduced NKG2D surface expression occurs with tumor but not with non-tumor exosomes, and is at least in part due to exosomal MIC expression

We next tested whether the down-modulation of NKG2D was dependent on exosome phenotype, by comparing exosomes isolated from different cellular sources. In multiple experiments, PBL from several healthy donors were treated with exosomes isolated from NKG2D ligand-positive tumor cells, including a mesothelioma cell line, or exosomes purified from a mesothelioma patient's PF, prostate cell lines (PC3 or DU145), EBV-B lymphoblastoid cells (IB4), or from noncancerous cells, including normal skin fibroblasts (HFF), or from cultured PBMC. The tumor cells were confirmed positive, by flow cytometry, for expression of NKG2D ligands (various expression patterns of MICA, MICB, ULBP-1, ULBP-2, or ULBP-3), while the non-tumor cells were negative for NKG2D ligands (Fig. 1). Tumor exosomes were capable of driving down the expression of NKG2D in both NK and CD8⁺ T cell subsets (Fig. 5). In contrast, exosomes from 72-h cultures of PBMC had no effect on NKG2D expression, and exosomes from fibroblasts had

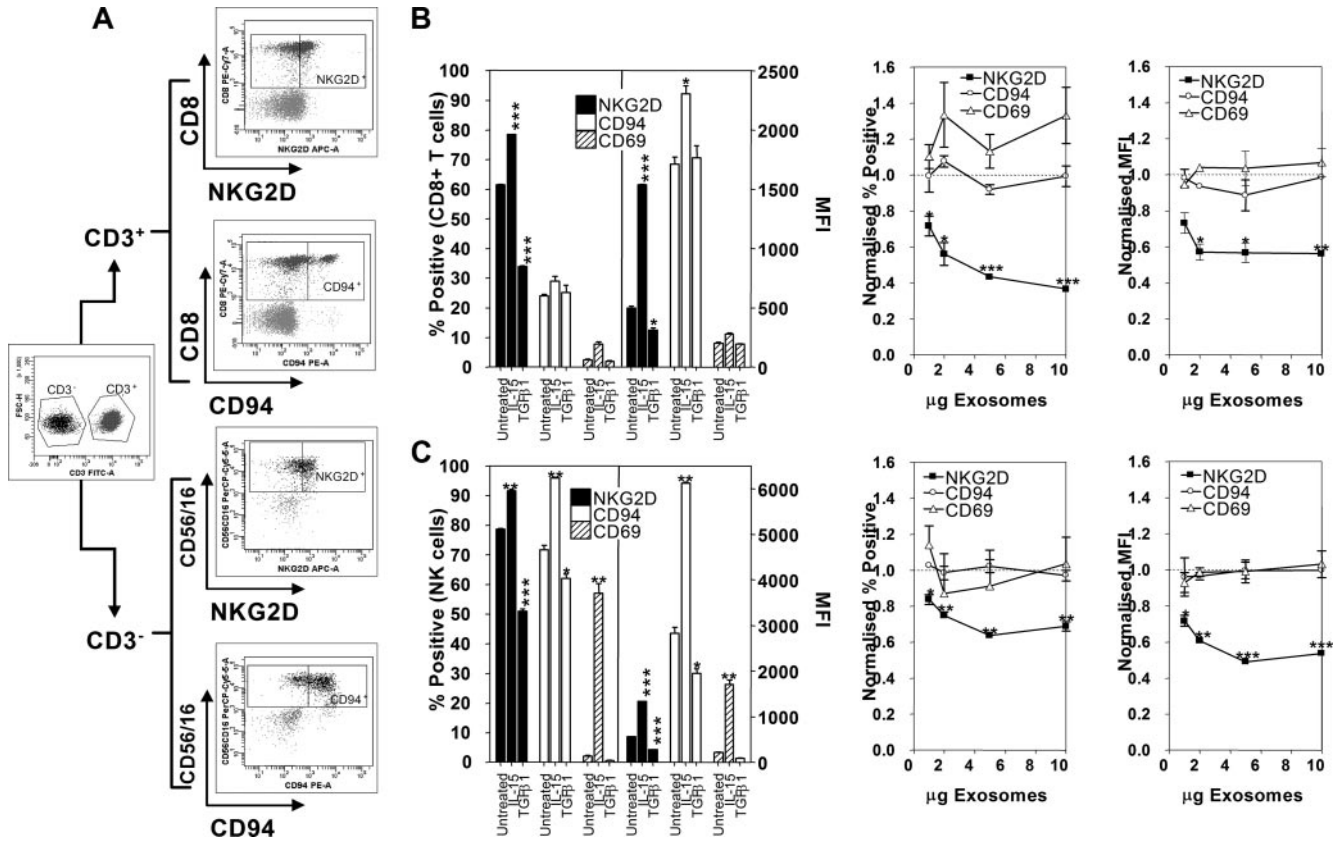


FIGURE 3. Tumor exosomes trigger down-modulation of cell surface NKG2D. Schematic representation of the multicolor flow cytometry gating scheme used to analyze lymphocyte NKG2D and CD94 or CD69 expression (A). Exosomes purified from a mesothelioma cell line were added at doses (1–10 $\mu\text{g}/10^5$ PBL) to fresh healthy donor PBL and after 48-h incubation, flow cytometry was performed to determine levels of expression of these markers, by CD8⁺ T cells (B) or NK cells (C). Data show the proportion of positive cells and MFI as indicated. The bar graph (left) shows constitutive levels, and levels following IL-15 or TGF β 1 treatment (both at 50 ng/ml). The line graphs (right) show expression relative to untreated PBL (normalized to a value of 1). Graphs show mean \pm SE, of triplicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The data are representative of over 10 experiments with various tumor cell type-derived exosomes, and different healthy donors.

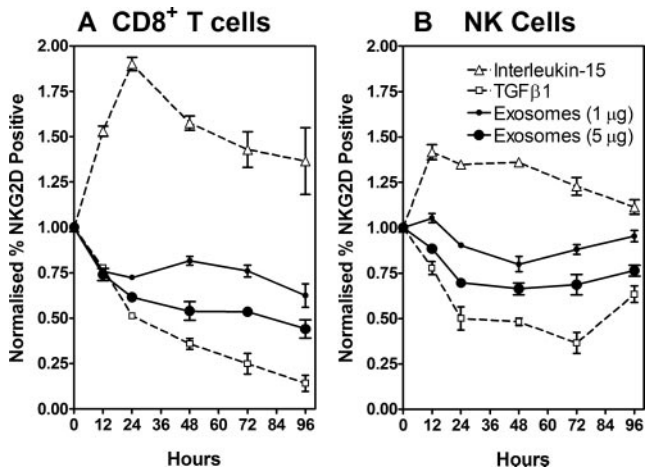


FIGURE 4. Kinetics of exosome-mediated down-regulation of NKG2D. IL-15 or TGF β 1 (50 ng/ml each) or mesothelioma exosomes (1 or 5 μg) was added to 10^5 fresh healthy donor PBL, and changes in proportion of NKG2D-positive CD8⁺ T cells (A) and NK cells (B) was monitored by multicolor flow cytometry immediately, and thereafter at indicated times up to 96 h. Graph shows the percentage of NKG2D-positive cells relative to untreated (normalized to a value of 1), for each time point. The results were obtained from three experiments (mean \pm SE, $n = 3$) conducted using different donors and different preparations of mesothelioma exosomes on separate occasions.

a weak (CD8⁺ T cells) or no effect (NK cells) on NKG2D expression (Fig. 5). Exosomes isolated from PF (obtained from three patients with malignant mesothelioma) were also able to significantly down-regulate NKG2D expression. Thus, exosomes directly taken from the tumor environment can also exhibit these effects. The data indicate that the decrease in CD8⁺ T or NK cell NKG2D is exosome phenotype dependent, and occurs with tumor but not with nontumor exosomes.

To determine whether exosomal NKG2D-ligand expression was involved in this down-regulation, we performed similar assays using mesothelioma cell line-derived exosomes which strongly express surface MICA (see Fig. 2, A and B) in the presence of neutralizing MICA-specific Ab, or isotype-matched control Ab (Fig. 6). NKG2D levels following treatment of PBL with soluble TGF β 1 was not affected by the presence of MICA-specific Ab as expected. In contrast, the down-modulation of NKG2D mediated by exosome treatment was inhibited by anti-MICA, but not isotype-control Ab, inhibiting the exosome effect by 46 and 41%, for CD8⁺ T cell, and NK cell subsets, respectively (Fig. 6). Increasing Ab dose beyond 5 $\mu\text{g}/\text{ml}$ did not increase the blocking effect (data not shown).

We also examined whether TGF β 1, expressed by these exosomes (Fig. 2E) (28), also played a role in down-regulating NKG2D. In this case, the reduction in NKG2D levels following treatment of PBL with soluble TGF β 1 was strongly inhibited in the presence of neutralizing TGF β 1-specific (but not control) Ab, demonstrating the efficacy of the Ab in blocking TGF β 1 effects.

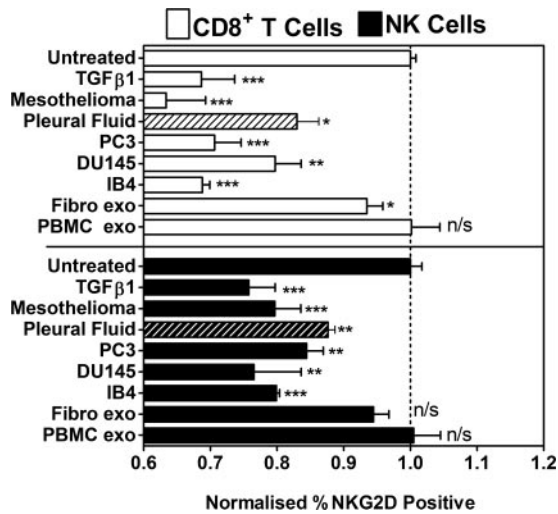


FIGURE 5. Exosome-mediated NKG2D down-regulation is dependent on the exosome phenotype. Fresh healthy donor PBL were left untreated, or were treated with TGF β 1 ($n = 8$) or 10 μ g of exosomes isolated from various cell types, including mesothelioma ($n = 8$), prostate cancer PC3 ($n = 2$), and DU145 ($n = 2$), B-lymphoblastoid cell line IB4 ($n = 2$), HFF fibroblasts ($n = 3$), and from 72-h lymphocyte cultures ($n = 2$) as indicated, of n experiments performed on different occasions with different blood donors. The effect of exosomes isolated from cancer patients PF is shown as ▨, showing average response measured from the same blood donor, with exosomes from three patients. Following 48-h incubation, NKG2D expression was measured by flow cytometry. Graph shows the percentage of NKG2D-positive cells relative to untreated (mean + SE of n experiments): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.01$, n/s = not significant).

The exosome effect on NKG2D expression was very strongly inhibited by TGF β 1-neutralizing Ab, with 89 and 90% inhibition of the exosome effect, for CD8 $^+$ T cell and NK cell subsets, respec-

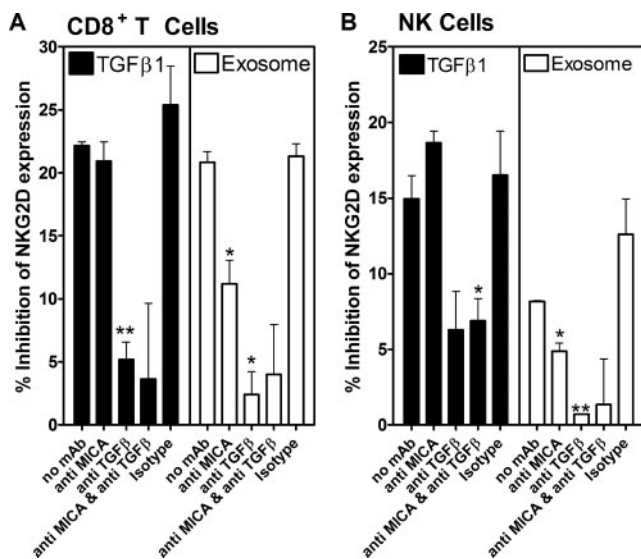


FIGURE 6. Down-regulation of NKG2D is driven by exosomal TGF β 1, and partially by MICA. Fresh healthy donor PBL were treated with TGF β 1 (10 ng/ml) or with mesothelioma exosomes (10 μ g/10 5 cells), in the presence of neutralizing Abs against human MICA, anti-TGF β 1 Ab, or with isotype-matched control Ab (all at 5 μ g/ml), as indicated. Expression of NKG2D by CD8 $^+$ T cells (A) and NK cells (B) was assessed by flow cytometry. Graphs show mean + SE, of triplicate measurements; **, $p < 0.01$), and is representative of two experiments performed with different donors.

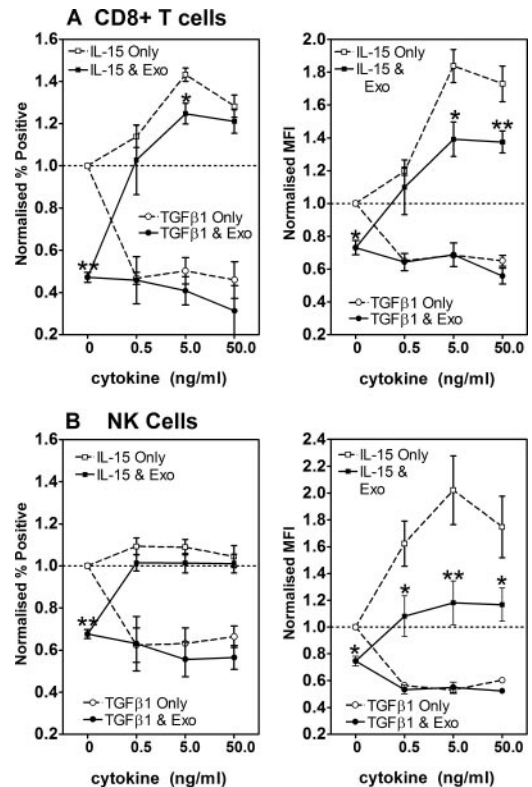


FIGURE 7. IL-15 counters exosome down-regulation of NKG2D. PBL obtained from three healthy donors, were treated with cytokines (IL-15 or TGF β 1, 0–50 ng/ml) in the absence or presence of mesothelioma exosomes (10 μ g/10 5 PBL), and at 48 h, expression of NKG2D in CD8 $^+$ T cells (A) and NK cells (B) was measured by flow cytometry. Graphs show the percentage of NKG2D-positive cells (left) or NKG2D expression levels (MFI) (right) relative to untreated PBL (normalized to a value of 1). (mean \pm SE, $n = 3$; *, $p < 0.05$; **, $p < 0.01$).

tively (Fig. 6). Combining both Abs had no further restorative effect. The experiments confirm that exosomal expression of NKG2D ligands is a contributory factor in down-regulating NKG2D; however, neutralizing TGF β 1 activity strongly protected against exosomal NKG2D down-regulation, suggesting this as the principal mode for NKG2D down-regulation.

IL-15-mediated up-regulation of NKG2D is impaired by exosomes

To better understand the potency of exosome-mediated down-regulation of NKG2D, we questioned whether the effect could be prevented in the presence of inflammatory cytokines, in particular IL-15, as enhancement of NKG2D-dependent tumor immunity by IL-15 was recently demonstrated in a murine cancer model (38). We also examined whether exosome-mediated impairment of NKG2D was further enhanced in the presence of exogenously added TGF β 1. Healthy donor PBL were treated with various doses of IL-15 or TGF β 1 in the presence or absence of tumor exosomes and, following 48-h incubation, NKG2D surface expression was measured by flow cytometry. In the absence of cytokines, exosomes alone mediated a significant reduction in NKG2D expression as expected (Fig. 7). Adding 0.5 ng/ml IL-15, however, was sufficient to abolish the effect of exosomes, restoring NKG2D to untreated levels. Importantly, however, increasing the IL-15 dose further, in the presence of exosomes, resulted in relatively poor up-regulation of NKG2D, compared with IL-15 alone indicating that even with high-dose IL-15 (at 50 ng/ml) the cellular response

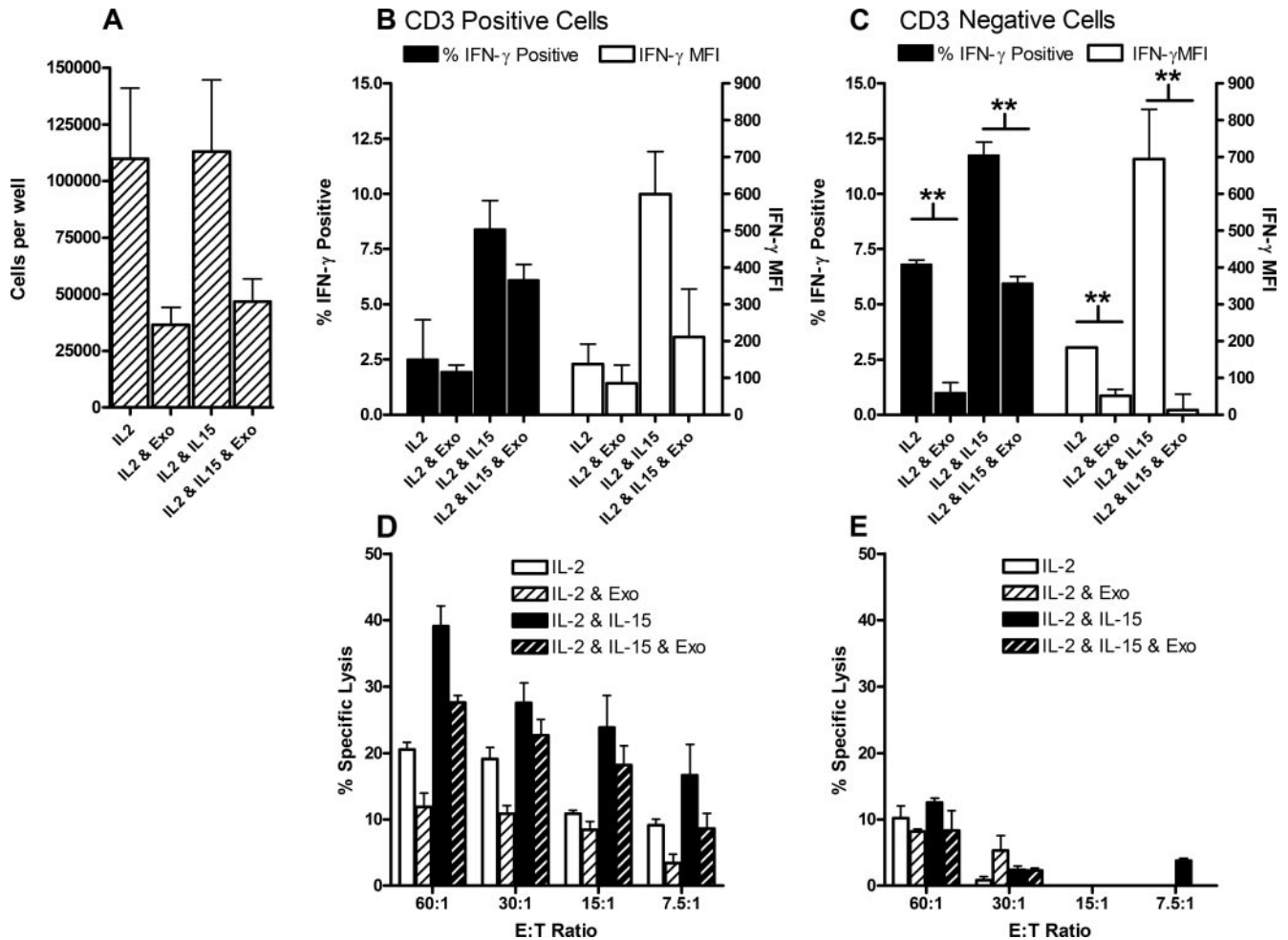


FIGURE 8. Tumor exosomes impair NKG2D-mediated lymphocyte activation. Fresh healthy donor PBL, were treated with mesothelioma exosomes and/or IL-15 (10 ng/ml) in the presence of IL-2 (100U/ml). After 48 h, PBL were washed and labeled with CFSE, and added to NKG2D Ab, or isotype control Ab-coated plates (3.5×10^5 cells/well). After incubation for 30 min, wells were washed three times, and fluorescence was measured to determine number of adhering cells. Graph shows mean \pm SE of triplicates, with isotype control subtracted (A). In a similar manner, PBL pretreated in parallel were incubated on Ab-coated plates in the presence of brefeldin A, and following 18 h of incubation, the production of IFN- γ was determined by flow cytometry. Graphs of mean \pm SE of triplicate measurements, with isotype control subtracted, show the proportion of positive cells (■) or MFI (□), gating on CD3-positive cells (B) or CD3-negative cells (C). PBL pretreated similarly, but for 72 h, were tested for their ability to kill murine Fc γ R⁺ P815-mastocytoma cells, in the presence of 2.5 μ g/ml NKG2D-agonist Ab (5C6) (D) or isotype-matched control Ab (E). Graphs of mean \pm SE of triplicate measurements.

to IL-15 is rendered suboptimal in the presence of tumor exosomes (Fig. 7). Combining tumor exosomes with TGF β 1 did not convincingly decrease NKG2D expression further, suggesting that the TGF β 1-dependent NKG2D down-regulation pathway was maximally activated by exosomes alone.

Lymphocyte activation through NKG2D is impaired following exosome treatment

We next investigated the consequences of diminished NKG2D expression on effector cell functions. Because exosomes express a complex array of receptors, some of which may interfere with effector functions in an NKG2D-independent manner (9, 10, 28), it was important to use a functional assay that was unequivocally reliant on stimulation through NKG2D. We have previously, for example, demonstrated diminished killing of tumor cells by tumor exosome-treated NK cells (28), yet the contribution of NKG2D down-modulation to such effects is unknown. To ascertain the functional importance of NKG2D down-modulation, we used two approaches, including activation of NKG2D-positive lymphocytes by plate-immobilized NKG2D-agonistic Ab. We also tested the

cytolytic capacity of PBL, in an Ab-redirected killing assay against Fc γ R⁺ P815 target cells, as described (35–37).

PBL were treated with IL-2 (100 U/ml) with or without combinations of exosomes and IL-15. After 48 h, the capacity of the cells to bind to anti-NKG2D-coated microtiter plates was examined. To do this, before adding to the plates, PBL were fluorescently labeled with CFSE. Following adherence for 30 min at 37°C, nonadherent cells were washed away, and the number of remaining cells was determined by measuring fluorescence, and comparing these values to a standard curve of known cell numbers. Treatment of PBL with exosomes for 48 h reduced the binding to anti-NKG2D-coated plates by around 50%. Treatment with IL-15 (10 ng/ml) enhanced NKG2D-dependent binding, and this was impaired when pretreatment was with both IL-15 and exosomes (Fig. 8A). In a similar fashion, PBL pretreated in the same way, were added to anti-NKG2D-coated plates, in the presence of brefeldin A. After 18 h, the proportion of IFN- γ -producing cells was determined by flow cytometry. The capacity to become activated by plate-immobilized agonistic Ab followed the same pattern as for the binding assay, where exosomes were generally

inhibitory, and capable of impairing the enhanced response mediated by IL-15. This was particularly true for the CD3⁻ (NK cell rich) subset (Fig. 8C), while exosome inhibition apparent on IL-15-treated CD3⁺ cells did not reach statistical significance (Fig. 8B).

For Ab-redirected lysis assays, PBL were primed with IL-2, and treated with various combinations of tumor exosomes, and IL-15 for 72 h. These were added to chromium-labeled P815 target cells, in the presence of NKG2D agonist or control Ab, and the degree of specific lysis was determined as for standard 4-h chromium release assays. NKG2D-dependent killing was reduced by ~50% by exosome pretreatment (at an E:T ratio of 60:1) (Fig. 8D). Although this was well-restored by IL-15 treatment, the killing function remained below that achieved with IL-15 treatment alone. Together, these experiments highlight that the down-regulation of NKG2D mediated by tumor exosomes negatively modifies effector functions that rely on NKG2D stimulus.

Discussion

The role of tumor-derived exosomes in cancer immunology is a controversial subject, with apparent conflicting reports in the literature regarding activatory or inhibitory effects. Many research groups have demonstrated abundant quantity of cancer exosomes present in malignant effusions (10, 12, 39). In experimental models, cancer ascites derived exosomes may be therapeutically useful as a source of multiple tumor rejection Ags, for efficient delivery to dendritic cells, and subsequent activation of antitumor cytotoxic T cell responses (11, 12, 40). Whether tumor exosomes function predominantly in this manner, during the onset and development of cancer *in vivo*, remains unclear. The very existence of advancing, ascites producing malignancy suggests that this mechanism, should it naturally occur, is insufficiently protective. In the absence of *in vitro*-enriched dendritic cell populations used for such studies, exosomes are more likely to encounter and directly interact with lymphocytes *in situ*, and there is growing evidence demonstrating that such interactions can occur, with diverse functional consequences. Exosomes from some tumor cell types may interact directly with NK cells leading to enhanced NK cell migration and cytotoxicity (8). This effect, however, is not ubiquitous across all cancer-exosome types, and may be limited to those exosomes bearing surface-hsp70 molecules (8). Exosomes isolated from other cancer types may exert inhibitory effects upon lymphocyte interactions, interfering with proliferative responses, for example (28, 41, 42), and/or impairing cytotoxic functions (42). Other inhibitory effects may include down-regulation of the TCR complex (10), or FasL-dependent exosome-mediated T cell apoptosis (9). Such discrepancies in outcome, and the variety of mechanisms responsible, should not be surprising, given the differences in cancer cell types (and therefore exosome phenotypes), and the immunological settings which have been investigated to date.

Our experience of studying interactions between cancer exosomes and immune effector cells (28), leads us to the view that such exosomes are immune inhibitory, rather than activatory. This report provides further evidence to support this, highlighting exosomally driven down-modulation of NKG2D expression as an important mechanism by which diverse tumor exosomes may suppress this key tumor cell recognition and lymphocyte activation pathway. Demonstrating that *ex vivo* exosomes (purified from tumor-associated PF) were also capable of NKG2D down-modulation confirmed this effect to be physiologically relevant, and not merely a phenomenon associated with cultured tumor cells. Although our main focus here was pleural malignant mesothelioma,

we also showed deficient NKG2D expression following PBL treatment with exosomes from prostate, breast (data not shown), and B-lymphoblastoid cell lines, and not with nontumor cell exosomes, demonstrating that it was the exosome phenotype that was directing the response, and that the effect is applicable across widely differing cancer types.

The expression of NKG2D ligands is a common characteristic of these cancer cell lines. Ab-blocking experiments revealed that down-regulation of NKG2D was partly dependent on exosomally expressed MICA. However, the degree of blocking achieved using MICA-specific Abs was modest, yet consistently greater than any isotype-matched Ab effect. This may be explained by other potential NKG2D ligands present on the surface of tumor exosomes, such as MICB, that contribute toward the overall down-modulation of NKG2D. Yet, in numerous experiments (data not shown) adding combinations of other NKG2D-ligand specific Abs did not convincingly demonstrate an enhanced inhibitory effect, but this may simply be due to the poor blocking characteristics of the Abs presently available. In contrast, incubating a TGFβ1-blocking Ab together with exosomes almost completely abrogated the reduction in surface NKG2D. This strong neutralizing effect, may have masked the more subtle inhibition achieved with MICA Ab, when both anti-TGFβ and -MICA were used in combination, and hence we saw no additive or synergistic effect using both Abs together.

The data suggest that NKG2D down-modulation is due to direct exosomal delivery of TGFβ1 to CD8⁺ T cell or NK cell subsets, as we recently demonstrated this phenomenon in the context of lymphocyte proliferation (28). How precisely TGFβ1 and NKG2D ligands, delivered by exosomes, cooperate to modulate NKG2D expression requires clarification. Clearly, the dominant effect is due to exosomal TGFβ1, as addition of exogenous TGFβ1 together with tumor exosomes does not further decrease NKG2D expression, so that this pathway is maximally activated by exosomes alone. In addition, PBL treated with soluble TGFβ1 exhibit very similar responses to those reported here for tumor exosomes, in that other NK surface markers (CD94/NKG2A, CD44, CD16, 2B4, and CD56) are not affected by TGFβ1, with the effect selectively focused on NKG2D (20). Although it is tempting to speculate that MICA (or related molecules) may assist in targeting tumor exosomes to NKG2D-positive CD8⁺ T cells and NK cells, to deliver TGFβ1 in a cell-type selective manner, it is important to acknowledge that NKG2D-negative lymphocytes (e.g., CD4⁺ T cells (10) or CD4⁺CD25⁺ T regulatory cells (28)) can also interact with, and be influenced by, tumor exosomes. Interestingly, however, NKG2D may not be the sole receptor for members of the MICA and ULBP families, because soluble NKG2D ligands can exert antiproliferative effects on murine CD4⁺ T cells, a phenomenon confirmed in NKG2D^{-/-} mice (24). A detailed molecular examination of tumor-exosome interactions with lymphocytes is now required, and this can only be satisfactorily achieved when alternate receptors for MICA/ULBP molecules have been identified.

What is certainly clear, however, is that NKG2D-ligand positive tumor-exosome interaction with lymphocytes does not lead to the activation of CD8⁺ T cells or NK cells, as there was no proliferation of either subset following exosome treatment (data not shown), and no change in surface markers associated with cell activation, such as CD69. The constitutive levels of granzyme B and perforin, also remained unaltered by tumor exosomes, indicating not only a lack of cell activation here (i.e., no induction of these molecules), but also that tumor exosomes were not able to act as decoy target cells, and deplete effector lymphocytes of their

constitutive lytic machinery. The data point to a nonactivating cellular response, following direct interactions between exosomes and NK cells or CD8⁺ T cells.

NKG2D plays a key cell-activating function for NK cells, in response to virus-infected or tumor cell targets. NKG2D is also important for CD8⁺ T cells, behaving as a coreceptor (in concert with signals from the TCR), or as a TCR-independent activator in long-term strongly activated T cells (43). Our study, therefore, suggests that the capacity for NKG2D-dependent cell activation becomes impaired in the presence of tumor exosomes, potentially leading to systemic NK and CD8⁺ T cell dysfunction similar to that documented for soluble tumor-derived NKG2D ligands (21, 22). We demonstrated that NKG2D-dependent production of IFN- γ and also NKG2D-dependent cytotoxicity became impaired if PBL were pretreated with tumor exosomes. Furthermore, IL-15, a recognized potent positive modulator of NKG2D-dependent responses of NK cells in particular, is rendered poorly activating in the presence of tumor exosomes. Such exosomes, therefore, can strongly impair NKG2D function, and inhibit the effects of some factors that would otherwise enhance NKG2D-dependent responses.

In cancer patients, disease-associated soluble NKG2D ligands in plasma (15, 21, 22) and/or high systemic TGF β 1 levels (20) are established mechanisms suppressing the NKG2D-dependent pathway for tumor cell recognition. Our report has highlighted a novel combination of these mechanisms, in the form of a secreted nanovesicle, which suppresses NKG2D-surface expression and subsequent lymphocyte functions, even in the presence of the inflammatory cytokine IL-15. Overcoming complex tumor-immune suppressive mechanisms is a major challenge in designing effective immunotherapeutic strategies. We suggest efforts should focus not only on soluble molecules emanating from cancer cells, but also be broadened to encompass exosome-driven suppressive mechanisms.

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Disclosures

The authors have no financial conflict of interest.

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