Human Tumor-Released Microvesicles Promote the Differentiation of Myeloid Cells with Transforming Growth Factor-β–Mediated Suppressive Activity on T Lymphocytes

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Abstract

Human tumors constitutively release endosome-derived microvesicles, transporting a broad array of biologically active molecules with potential modulatory effects on different immune cells. Here, we report the first evidence that tumor-released microvesicles alter myeloid cell function by impairing monocyte differentiation into dendritic cells and promoting the generation of a myeloid immunosuppressive cell subset. $CD14^+$ monocytes isolated from healthy donors and differentiated with interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor in the presence of tumor-derived microvesicles turned into $HLA\text{-}DR^{-/low}$ cells, retaining CD14 expression and failing to up-regulate costimulatory molecules, such as CD80 and CD86. These phenotypic changes were paralleled by a significant release of different cytokines, including IL-6, tumor necrosis factor-α, and transforming growth factor-β (TGF-β), and a dose-dependent suppressive activity on activated T-cell–proliferation and cytolytic functions, which could be reversed by anti-TGF-β–neutralizing antibodies. Microvesicles isolated from plasma of advanced melanoma patients, but not from healthy donors, mediated comparable effects on $CD14^+$ monocytes, skewing their differentiation toward $CD14^+HLA\text{-}DR^{-/low}$ cells with TGF-β–mediated suppressive activity on T-cell–functions. Interestingly, a subset of TGF-β–secreting $CD14^+HLA\text{-}DR^-$ cells mediating suppressive activity on T lymphocytes was found to be significantly expanded in peripheral blood of melanoma patients compared with healthy donors. These data suggest the development in cancer patients of an immunosuppressive circuit by which tumors promote the generation of suppressive myeloid cells through the release of circulating microvesicles and without the need for cell-to-cell contact. Therapeutic interventions on the crucial steps of this pathway may contribute to restore tumor/immune system interactions favoring T-cell–mediated control of tumor growth in cancer patients. (Cancer Res 2006; 66(18): 9290-8)

Introduction

Tumor cells of different histotypes release intact vesicular membrane organelles known as microvesicles or exosomes (1–3) and characterized by a specific protein profile, reflecting the parental cell protein content and including molecules, such as HLA, tumor antigens, heat shock proteins, cytoskeleton components, adhesion factors, etc. (1, 2, 4–6).

Although the extracellular release of membrane vesicles has been characterized under specific physiologic conditions in different normal cell types (including blood, endothelial, and epithelial cells), the rate of shedding seems to be constitutive and markedly increased in most neoplastic cells (2, 7), thus suggesting a role of this phenomenon in malignant transformation and/or progression (8).

Growing interest has been focused on tumor-released microvesicles since the profusion of these phospholipid particles of 50 to 200 nm size in body fluids of cancer patients with advanced disease was shown (2, 3, 9, 10). In particular, due to microvesicle ability to transport a wide array of biologically active molecules involved in plasma membrane discharge, cell signaling, receptor transfer, and apoptosis, microvesicle shedding could be exploited by neoplastic cells not only to maintain their own growth and survival at the tumor site but also to deliver detrimental signals to the host's immune defenses independently from cell-to-cell contact.

We and others have shown that human cancer cells (including melanoma, colon carcinoma, head and neck, and ovary cancers) produce large amounts of microvesicles bearing proapoptotic molecules, such as Fas ligand (FasL) and tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL; refs. 1–3, 10). These membranous organelles, purified from tumor cell culture supernatants or from cancer patient sera, can induce apoptosis of activated tumor-specific T cells, thus impairing the ability of effector lymphocytes to exert their cytolytic activity on tumor targets. Cancer cells could hence use proapoptotic microvesicles to promote the establishment in the host of an immunoprivileged environment, as it occurs in different physiologic conditions requiring maintenance of immunotolerance, such as pregnancy, or during the downsizing of immune responses after antigen clearance (11–13). However, being T lymphocytes variably susceptible to apoptosis depending on their activation state (2), it would be more advantageous for cancer cells to interfere upstream with the development of antitumor immune responses through the direct impairment of T-cell–priming by dendritic cells. In fact, this cell population plays a key role in the modulation of adaptive immunity, switching on and off T-cell–responses depending on their functional state (14). Patients with different chronic diseases, including cancer, have been reported to display defects in dendritic cell activity (15) together with more generalized dysfunctions in the homeostasis of the myeloid compartment, leading to the *in vivo* accumulation of cells with suppressive effects on T lymphocytes (the so-called "myeloid suppressor cells"; ref. 16).

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Based on these evidences, we chose to investigate the effects of microvesicles released by human cancer cells on the process of monocyte differentiation into dendritic cells. Here, we report that microvesicles produced by human melanoma and colorectal carcinoma cells, both *in vitro* and *in vivo*, skew monocyte differentiation toward myeloid cells with altered phenotypic features and functions. These cells, which are expanded in peripheral blood of melanoma patients, exert suppressive activity on T cells mostly mediated by the microvesicle-induced release of transforming growth factor-β (TGF-β).

Materials and Methods

Healthy donor and melanoma patient samples. Peripheral blood mononuclear cells (PBMC) from 10 healthy donors and 16 American Joint Committee on Cancer stage IV melanoma patients were purified from peripheral blood by Ficoll gradient (Ficoll-Paque, Amersham Biosciences, Uppsala, Sweden). Patient characteristics were described elsewhere (17).

This study was approved by the Ethics Committee of the Istituto Nazionale Tumori of Milan and informed consent was obtained from each patient and healthy volunteer.

Monocyte isolation and differentiation into dendritic cells. Monocytes were immunosorted from healthy donor PBMC using human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting cell population was represented by >95% $CD14^+$ monocytes as assessed by flow cytometry. Immature dendritic cells were obtained by *in vitro* culture of $CD14^+$ cells (10^6 per well in 48-well plates) for 3 to 6 days in X-VIVO 15 (BioWhittaker, Walkersville, MD) supplemented with 50 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ) and 20 ng/mL interleukin (IL)-4 (Peprotech) in the presence or absence of amounts of microvesicles corresponding to ~30 μg protein/10^6 target cells. Control experiments were done with 30 μg bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO) or 0.02 μm latex beads (aldehyde/sulfate latex, Interfacial Dynamics Corp., Portland, OR) in a 1:8 monocyte/bead ratio. To purify $CD14^+HLA\text{-}DR^+$ and $CD14^+HLA\text{-}DR^-$ cells from the PBMC of melanoma patients, monocytes were first isolated by negative selection with Monocyte Negative Isolation kit (Dynal Biotech ASA, Oslo, Norway) and then positively sorted for HLA-DR expression on incubation with 5 μg/10^6 cells phycoerythrin (PE)–conjugated anti-human HLA-DR monoclonal antibody (mAb; BD PharMingen, San Jose, CA) and anti-PE microbeads (Miltenyi Biotec) according to the manufacturer's instruction. The purity of the immunosorted $CD14^+HLA\text{-}DR^+$ and $CD14^+HLA\text{-}DR^-$ cell populations was assessed by flow cytometry.

Tumor cell lines and microvesicle purification. The melanoma cell lines 501mel and 624.38mel were described previously (18, 19). The colorectal carcinoma cell line SW403 was purchased from the American Type Culture Collection (Manassas, VA), whereas the 1869col colorectal carcinoma line was kindly provided by Dr. Cristina Maccalli (Istituto Superiore di Sanità, Rome, Italy; ref. 20). Tumor lines were negative for *Mycoplasma* contamination as routinely tested by enzymatic assay (MycoAlert *Mycoplasma* Detection kit, Cambrex, Vervier, Belgium). Microvesicles were isolated by serial centrifugations (2) from supernatants of 72-hour confluent tumor cells cultured with 10% fetal bovine serum (FBS; Cambrex) deprived previously of bovine microvesicles by ultracentrifugation (90 minutes at 100,000 × *g*). The same protocol was used to purify microvesicles released by healthy donor macrophages (differentiated *in vitro* from 10^7 $CD14^+$ monocytes cultured for 1 week in RPMI 1640 supplemented with 30% FBS). Microvesicle purification from healthy donors (n = 10) and melanoma patients (n = 10) was obtained after 1:2 plasma dilution in PBS by sequential centrifugations (30 minutes at 2,000 × *g*, 45 minutes at 12,000 × *g*, and 2 hours at 110,000 × *g*). Microvesicle pellets were resuspended in PBS, filtered through a 0.22 μm filter (Stericup, Millipore Corp., Billerica, MA), and further centrifuged for 1 hour at 110,000 × *g*. After a final 1-hour wash at 100,000 × *g*, microvesicle pellets were recovered and resuspended in appropriate medium and proteins were quantified by Bradford assay (Bio-Rad Laboratories, München, Germany).

Analysis of apoptosis and phagocytic activity. Immunosorted $CD14^+$ cells and human Jurkat T cells (positive control) were stained with Annexin V/propidium iodide (PI; Bender MedSystems, Vienna, Austria) after a 24-hour incubation (10^6/mL) at 37°C with melanoma-derived and colorectal carcinoma–derived microvesicles (30 μg) or bioactive concentrations of recombinant FasL (SuperFasLigand, Alexis, Lausen, Switzerland) and TRAIL (R&D Systems, Minneapolis, MN) proteins. The percentage of apoptotic cells was then analyzed by FACSCalibur and the CellQuest software (Becton Dickinson, San Jose, CA). Phagocytic activity of monocyte-derived dendritic cells differentiated for 6 days in the presence or absence of melanoma-derived and colorectal carcinoma–derived microvesicles was assessed in terms of FITC-dextran internalization (1 mg/mL; Sigma-Aldrich) at 37°C or 4°C as specificity control. After a 30-minute incubation, cells were analyzed for green fluorescence emission using FACSCalibur and the CellQuest software.

Analysis of monocyte-microvesicle interaction. $CD14^+$ monocytes and melanoma-derived or colorectal carcinoma–derived microvesicles were stained with the membrane $PKH26_{green}$ and $PKH26_{red}$ fluorescent dyes, respectively (Sigma-Aldrich) and then coincubated at 37°C with microvesicles (30 μg protein/10^6 monocytes). Samples collected at different time points were analyzed by confocal microscopy in terms of transfer of the microvesicle-derived red fluorescence to the green fluorescent monocytes. Control experiments were done at 4°C on pretreatment of monocytes with 1 μg/mL cytochalasin D (Sigma-Aldrich) or in the presence of the Ca^{2+}-chelating agent EDTA (5 mmol/L). Images were obtained using a Radiance 2100 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA) equipped with a krypton/argon laser. Noise reduction was achieved by "Kalman filtering" during acquisition.

Flow cytometry and confocal microscopy. Differentiating monocytes were stained with the following mouse anti-human IgG mAbs (BD PharMingen): PE anti-CD14, PE anti-HLA-DR, PE anti-CD80, PE anti-CD86, FITC anti-CD40, FITC anti-HLA-DR, and APC-anti-CD14. In each experiment, IgG-matched isotypic controls were used. Events were gated according to light scatter properties, selecting monocyte cell population and excluding cell debris. To standardize the mean channel number values, instrument detector setup for FL-1 channel was set so that the FITC-Calibrite (Becton Dickinson) bead pick gave ~560 to 600 linear units in log-scale amplification. Data were acquired and analyzed by a FACSCalibur and the CellQuest software. The same samples were analyzed for surface and intracellular HLA-DR expression by confocal microscopy after culture in Permanox four-well chamber slides (Nunc, Rochester, NY), fixation in 4% buffered paraformaldehyde, and overnight incubation at 4°C with primary mouse anti-human anti-HLA-DR mAb (L243) in a 30% goat serum, 0.3% Triton X-100, 0.45 mol/L NaCl, and 0.02 mol/L phosphate buffer. After suitable washings, slides were incubated for 1 hour at room temperature with a secondary rhodamine-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Europe Ltd., Soham, United Kingdom). Images were obtained using a laser scanning confocal microscope (Radiance 2100) equipped with a krypton/argon laser. Noise reduction was achieved by "Kalman filtering" during acquisition.

Evaluation of suppressive activity of microvesicle-treated monocytes and $CD14^+HLA\text{-}DR^-$ cell subset on lymphocytes. Cytokine-treated monocytes differentiated in the presence or absence of microvesicles of different origin were irradiated and added to 10^5 allogeneic PBMC at different responder/stimulator ratios in round-bottomed 96-well plates (Costar-Corning, New York, NY) in X-VIVO 15. Suppressive activity was analyzed on autologous PBMC proliferation in response to 1 μg/mL OKT3 (anti-CD3 Orthoclone OKT3 agonist antibody, Ortho Biotech, Bridgewater, NJ) coated previously for 2 hours at 37°C in 96-well plates. For blocking experiments, anti-IL-6 (1 μg/mL; Abcam, Cambridge, United Kingdom), anti-TNF-α (2 μg/mL; Abcam), or anti-TGF-β–neutralizing antibody (1 μg/mL; R&D Systems) was added at the beginning of cocultures. The suppressive activity of $CD14^+HLA\text{-}DR^-$ cells isolated from melanoma patients (20×10^3 per well) was evaluated on $CD14^+$-depleted PBMC (10^5 per well) stimulated for 3 days with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/mL phytohemagglutinin (PHA; Sigma-Aldrich). T-cell–proliferation was assessed on day 4 by measuring [^{3}H]thymidine

incorporation (1 μCi/well; Amersham Biosciences) after 18-hour pulse using a liquid scintillation β-counter. Each assay was done in quadruplicates.

Cytokine quantification by cytokine bead array and ELISA. Supernatants from monocytes (as bulk or sorted subsets) were collected after 6-hour incubation at 37°C. Samples were tested for the presence of TNF-α, IL1β, IL-6, IL-10 and IL-12 by multiple cytokine analysis with cytokine bead array (CBA; human inflammation kit, Becton Dickinson), and, on acidification, for TGF-β_1 by ELISA (Amersham Biosciences).

Analysis of cytolytic molecules and IFN-γ intracellular expression by *in vitro* activated T cells. PBMC were cocultured for 24 hours at 37°C with autologous monocytes differentiated in the presence or absence of melanoma-derived and colorectal carcinoma–derived microvesicles (4:1, PBMC/monocyte ratio) in round-bottomed 96-well plates coated previously with anti-CD3 OKT3 agonist antibody. Blocking experiments were done using 1 μg/mL anti-TGF-β_1-neutralizing antibody. Nonadherent cells were then collected and surface stained with PerCP-conjugated anti-CD8 antibody (BD PharMingen). Cells were then fixed and permeabilized with Cytofix/Cytoperm kit (BD PharMingen), stained with FITC anti–granzyme B, PE anti-perforin, and APC anti-IFN-γ antibody (BD PharMingen), and evaluated by fluorescence-activated cell sorting (FACS) analysis.

Microvesicle scanning electron microscopy and Western blot analysis. Microvesicles isolated from plasma of melanoma patients were resuspended in ethanol 100%, laid on cover glasses, and then sputter coated with gold by Edwards S150A (BOC Edwards, Crawley, United Kingdom) for scanning electron microscopy. Samples were examined by Philips XL-40 scanning electron microscope (FEI, Eindhoven, the Netherlands) with a cold cathode field emission gun at an accelerating voltage of 30 kV. All images were recorded using the secondary electron detector. For Western blot analysis, equal amounts of proteins obtained from the plasma-derived microvesicle lysates of healthy donors or stage IV melanoma patients were separated by gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were incubated with mAb specific for gp100 (DAKOCytomation, Glostrup, Denmark) and LAMP-2 (BD PharMingen) molecules. Antibody binding was detected by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL).

Statistical analysis. Statistical analysis was done by *t* test for paired samples, with exception of data reported in Fig. 6*A*, which were analyzed by Dunnett's test. $P < 0.05$ was considered as statistically significant.

Results

CD14$^+$ monocytes are resistant to apoptosis induced by melanoma and colorectal carcinoma–released microvesicles. As we showed previously that human tumors, such as melanoma and colorectal carcinoma, release endosome-derived microvesicles able to induce FasL- and TRAIL-mediated apoptosis in activated T cells (1, 2), herein we first evaluated the proapoptotic activity exerted by microvesicles on monocytes, representing the circulating precursors of dendritic cells and macrophages. To this aim, CD14$^+$ cells were immunosorted from peripheral blood of healthy donors and tested for sensitivity to apoptosis induced by microvesicles purified from melanoma (501mel and 624.38mel) and colorectal carcinoma (1869col and SW403) cell line supernatants. As reported in Fig. 1*A*, no significant apoptosis could be detected in monocytes incubated with microvesicles derived from any of the four tumor cell lines tested. In contrast, and according to our previous reports (1, 2), the same microvesicle preparations induced apoptosis in activated T cells (Jurkat). Interestingly, CD14$^+$ monocytes were also resistant to apoptosis induced by human recombinant FasL and TRAIL proteins (Fig. 1*A*) despite the expression of their cognate receptors Fas and TRAIL receptors 1 and 2, respectively (data not shown).

Interaction with tumor-derived microvesicles impair CD14$^+$ monocytes differentiation into dendritic cells and promote the generation of CD14$^+$HLA-DR$^{-/low}$ cells. We then analyzed

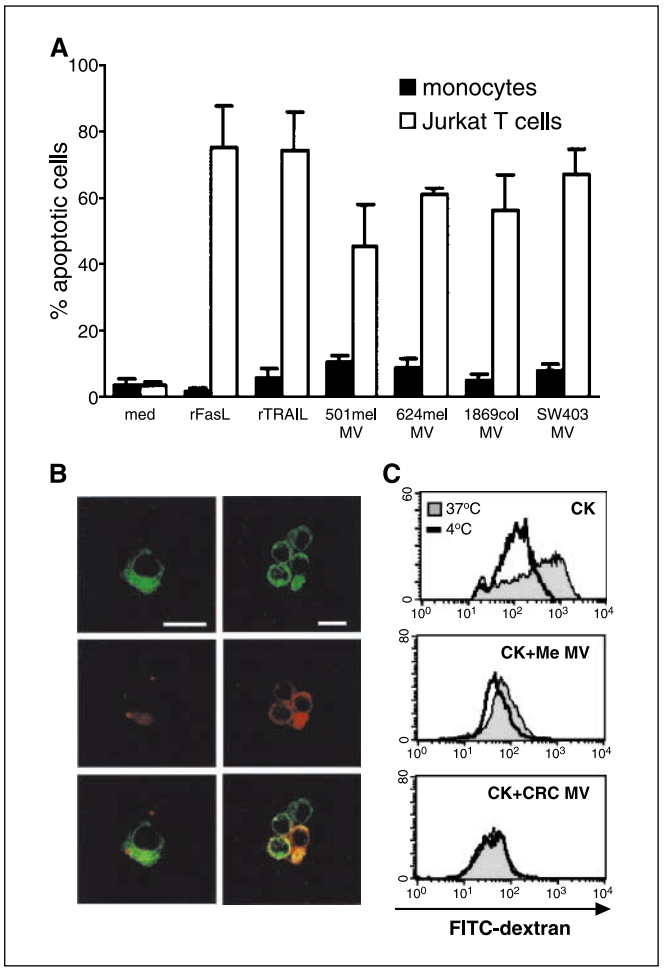

Figure 1. *A,* resistance of monocytes to apoptosis induced by tumor-derived microvesicles (*MV*) or recombinant FasL and TRAIL. Jurkat cells and CD14$^+$ monocytes, immunosorted from healthy donor PBMC, were incubated with bioactive concentrations of recombinant FasL and TRAIL or microvesicles purified from tumor cell line supernatants (melanoma: 501mel and 624.38mel, colorectal carcinoma: SW403 and 1869col). Cells were analyzed at 24 hours for their susceptibility to apoptosis through Annexin V/PI staining. *B,* tumor-derived microvesicle-monocyte interaction. PKH26$_{green}$-stained monocytes were incubated at 37°C with PKH26$_{red}$-labeled colorectal carcinoma–released microvesicles; reaction was stopped at different time points and preparations were analyzed by confocal microscopy (*left,* 30 minutes; *right,* 1 hour). Bar, 10 μm. *C,* tumor-derived microvesicles inhibit the acquisition of the phagocytic ability by differentiating monocytes. CD14$^+$ monocytes from healthy donors were treated for 6 days with IL-4 and GM-CSF [cytokines (*CK*)] in the presence of melanoma (*Me*)–derived or colorectal carcinoma (*CRC*)–derived microvesicles (*middle* and *bottom*, respectively). Control experiments included monocytes treated with cytokines without microvesicle addition (*top*). Phagocytic activity was tested in terms of internalization of FITC-dextran at 37°C or 4°C as specificity control. Representative of at least three independent experiments.

the possible interaction occurring between tumor-derived microvesicles and monocytes and the consequent effects on immature dendritic cell differentiation. Figure 1*B* (*left*) shows that tumor-derived microvesicles were captured by monocytes after a short-term incubation (10-30 minutes) as detected by confocal microscopy done with PKH67$_{green}$-labeled monocytes and PKH26$_{red}$-labeled colorectal carcinoma–derived microvesicles. This contact consisted in microvesicle-monocyte membrane interaction as indicated by the diffusion of stained microvesicle-membrane components within monocyte cell surface evidenced at longer incubation times (Fig. 1*B, right*). Similar data were obtained with melanoma-derived microvesicles (data not shown). Even if this

phenomenon was not synchronized in every single cell analyzed, the whole monocyte population acquired microvesicle-derived red fluorescence staining after 2 hours (data not shown). Microvesicle uptake was not modified by the actin inhibitor cytochalasin D at 4°C, whereas it was inhibited by the Ca^{2+}-chelating agent EDTA, thus confirming the fusogenic nature of microvesicle-monocyte interaction and excluding the involvement of a phagocytosis-mediated mechanism (data not shown).

$CD14^+$ monocytes were then treated with IL-4 and GM-CSF in the presence or absence of melanoma-derived and colorectal carcinoma–derived microvesicles, and their differentiation into immature dendritic cells was monitored as phagocytic ability and immunophenotypical modifications. The presence of tumor-released microvesicles impaired the ability of differentiating monocytes to acquire phagocytic activity as expected in immature dendritic cells. In fact, whereas the majority of cells were able to internalize FITC-dextran in microvesicle-untreated samples, monocytes differentiated in the presence of either melanoma-derived or colorectal carcinoma–derived microvesicles were unable to uptake this substrate (Fig. 1*C*). In addition, in microvesicle-treated monocytes, the down-regulation of CD14 surface expression, which physiologically occurs when differentiation into immature dendritic cells is complete, was inhibited in a consistent percentage of cells (Fig. 2*A, left*). This seemed to be a persisting effect, as it could be detected even 6 days after microvesicle treatment (Fig. 2*A, middle*). The dependence of this effect on the tumor origin of microvesicles was shown by the evidence that $CD14^+$ cells treated under the same conditions with microvesicles purified from supernatants of human macrophages underwent physiologic CD14 down-modulation (Fig. 2*A, right*). Further control experiments, in which $CD14^+$ cells were treated with comparable amounts of BSA or with latex beads, showed a normal loss of CD14 expression (data not shown), thus excluding that the inhibitory effect of tumor-microvesicle on dendritic cell

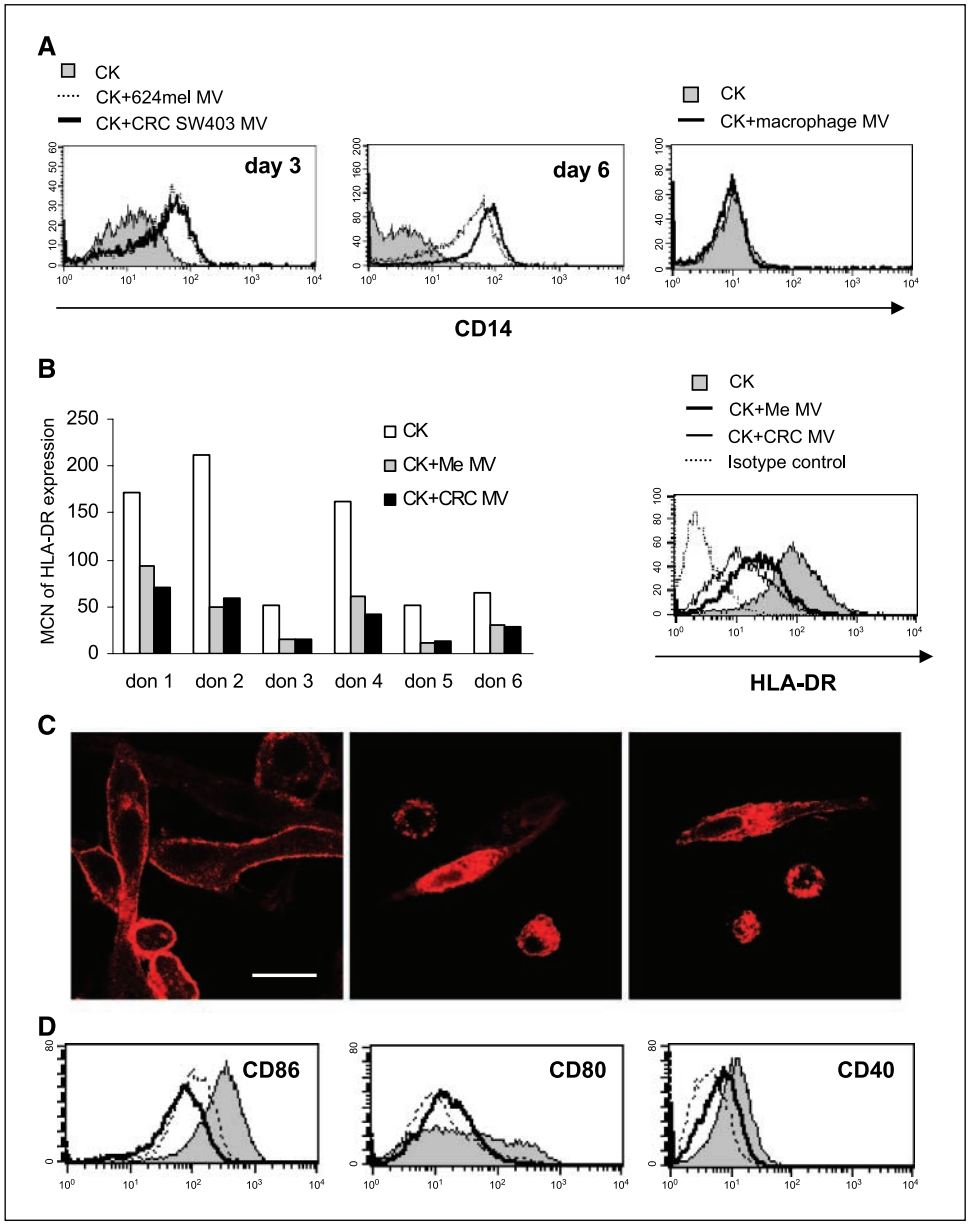

Figure 2. *A,* tumor-derived microvesicles inhibit the down-regulation of CD14 surface expression during dendritic cell differentiation. Monocytes, treated for 3 or 6 days with IL-4 and GM-CSF (cytokines, *CK*) in the presence or absence of melanoma-derived and colorectal carcinoma–derived microvesicles, were analyzed for CD14 expression by FACS analysis (*left*). Control experiments were done incubating monocytes for 3 days with cytokines in the presence or absence of equal amounts of macrophage-released microvesicles (*right*). *B,* tumor-derived microvesicles inhibit the up-regulation of HLA-DR molecule surface expression during dendritic cell differentiation. Monocytes were incubated for 3 days with cytokines in the presence or absence of melanoma and colorectal carcinoma–released microvesicles and then analyzed for HLA-DR surface expression by FACS-Calibur and CellQuest analysis. Data obtained with monocytes from six different healthy donors (*left*) and a representative histogram plot (*right*). $P < 0.05$, statistically significant differences in HLA-DR expression between microvesicle-untreated and treated samples (Student's *t* test). *C,* untreated (*left*), melanoma-derived microvesicle-treated (*middle*), and colorectal carcinoma–derived microvesicle-treated (*right*) monocytes were permeabilized, stained for HLA-DR expression with a red fluorescent-specific antibody, and analyzed by confocal microscopy. Bar, 10 μm. *D,* tumor-derived microvesicles impair up-regulation of activatory and costimulatory molecules during dendritic cell differentiation. Untreated (*gray area*) and melanoma-treated (*dot line*) or colorectal carcinoma–treated (*bold line*) monocytes were analyzed after 3-day culture for the expression of the indicated markers by FACSCalibur and CellQuest analysis. Representative of at least three independent experiments.

differentiation could be due to endocytic machinery engulfment on microvesicle treatment.

Tumor-derived microvesicles promote the generation of CD14$^+$HLA-DR$^{-/low}$ cells. In addition to CD14 retention, monocytes differentiated into dendritic cells in the presence of tumor-derived microvesicles displayed a marked decrease of HLA-DR expression intensity on cell surface (Fig. 2*B*) and a reproducible reduction in the percentage of HLA-DR$^+$ cells (from 91.7 ± 4.7% in untreated cells to 38.7 ± 18.6% in microvesicle-treated cells, $n = 6$). This phenomenon was confirmed by confocal microscopy showing that microvesicle-treated monocytes failed to transport HLA-DR molecules to the cell surface and retained the fluorescent signal into cytoplasmic vesicular organelles (Fig. 2*C, middle* and *right*), whereas untreated cells (Fig. 2*C, left*) transferred most of HLA-DR intracellular pool to the cell membrane. Moreover, microvesicle treatment induced marked modifications in differentiating monocyte morphology in terms of reduced membrane spreading on the plastic support and retention of a spherical shape (Fig. 2*C*).

Together with the down-modulation of HLA-DR molecules, microvesicle-treated monocytes displayed a significant decrease in the expression of costimulatory (CD80 and CD86) and activatory (CD40) molecules (Fig. 2*D*).

Monocytes differentiated in the presence of tumor-released microvesicles exert TGF-β–mediated suppressive activity on T-cell–function. Based on the phenotypic alterations mentioned above, we evaluated whether monocytes differentiated in the presence of tumor-derived microvesicles exerted a reduced stimulatory capacity on allogeneic HLA-mismatched PBMC proliferation. Indeed, monocytes differentiated in the presence of melanoma or colorectal carcinoma–released microvesicles exerted no stimulatory effects on allogeneic PBMC proliferation compared with untreated monocytes (Fig. 3*A*). In addition, microvesicle-treated monocytes acquired a strong and dose-dependent suppressive activity on autologous T-cell–proliferation in response to T-cell–receptor (TCR) triggering, which was instead potentiated by the presence of microvesicle-untreated cells (Fig. 3*B*).

The potential role of soluble factors in the immunosuppressive activity observed was investigated through the analysis of the cytokine profile released by differentiating monocytes on incubation with tumor-derived microvesicles. Although no significant release of IL-1β and IL-12 was observed, a slight enhancement of IL-10 together with a marked induction of TNF-α, IL-6, and TGF-β secretion was detected in microvesicle-treated monocytes compared with untreated cells (Fig. 3*C*).

The potential role of these cytokines in the suppressive activity of microvesicle-treated monocytes was investigated through the use of neutralizing mAbs. Interestingly, lymphocyte proliferation on TCR triggering was completely restored by TGF-β neutralization, whereas no boost was induced targeting TNF-α and IL-6 (Fig. 4*A*).

Based on recent report showing TGF-β–mediated impairment of CD8$^+$ lytic functions (21), we investigated the effect of microvesicle-treated monocytes on the expression of granzyme B, perforin, and IFN-γ cytolytic molecules by CD8$^+$ T cells on TCR stimulation. Microvesicle-treated monocytes impaired the cytolytic potential of activated CD8$^+$ lymphocytes by significantly reducing the intracellular accumulation of granzyme B, perforin, and IFN-γ compared with CD8$^+$ T cells cultured with untreated monocytes (Fig. 4*B*). This effect was mainly mediated by TGF-β released by microvesicle-treated monocytes, as the expression of

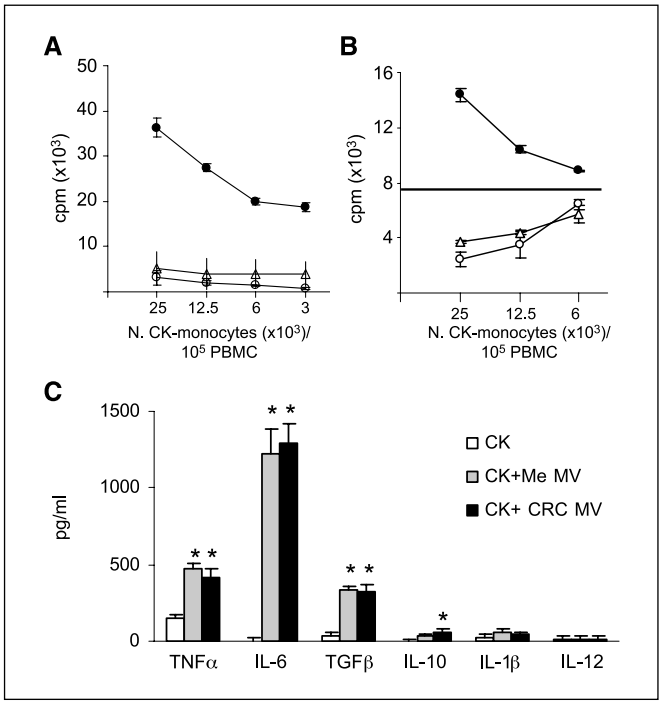

Figure 3. *A,* tumor microvesicle-treated monocytes lose their allostimulatory activity. Untreated (●), melanoma-derived microvesicle-treated (○), and colorectal carcinoma–derived microvesicle-treated (△) monocytes at the third day of differentiation with cytokines were incubated for 3 days with allogeneic HLA-mismatched PBMC at the indicated stimulator/target ratios. *B,* tumor microvesicle-treated monocytes exert suppressive effects on T-cell–proliferation in response to TCR triggering. Untreated (●), melanoma-derived microvesicle-treated (○), and colorectal carcinoma–derived microvesicle treated (△) monocytes after a 3-day culture were incubated with autologous OKT3-stimulated PBMC at the indicated stimulator/target ratios. OKT3-stimulated PBMC alone were included as control (*continuous line*). T-cell–proliferation (*A* and *B*) was assessed on day 4 by measuring [^{3}H]thymidine incorporation after a 18-hour pulse. Each assay was done in quadruplicates. $P < 0.05$, statistically significant differences between microvesicle-treated and untreated samples. *C,* cytokine profile of untreated and microvesicle-treated differentiating monocytes. The indicated cytokines were quantified in cell culture supernatants of cytokine-treated monocytes (10^6/mL) incubated or not with tumor-derived microvesicles for 6 hours at 37°C. Cytokines were detected by CBA (TNF-α, IL-6, IL-10, IL-1β, and IL-12) or by ELISA (TGF-β1). Assays were done in duplicates. *, $P < 0.05$, compared with control samples treated with cytokines in the absence of microvesicles. Representative of at least three independent experiments.

these molecules was completely restored by the anti-TGF-β–neutralizing antibody (Fig. 4*B*). Microvesicle-treated monocytes exerted their suppressive activity also on the cytotoxic function of tumor-specific T cells, impairing MelanA/MART-1-specific T-cell–ability to mobilize CD107a molecules on degranulation in response to MART-1$^+$ melanoma cells (22) in a TGF-β–dependent manner (data not shown).

No up-regulated arginase activity, a hallmark of myeloid suppressor cells (MSC) in tumor-bearing mice (23, 24), was detected in microvesicle-treated monocytes compared with untreated cells, and no overcome of their suppressive activity on T-cell–proliferation was observed in the presence of arginase inhibitors (see Supplementary Data).

Microvesicles isolated from plasma of melanoma patients skew monocyte differentiation toward TGF-β–secreting CD14$^+$HLA-DR$^{-/low}$ myeloid cells with suppressive activity. In line with previous studies showing the presence of exosome-like microvesicles in different biological fluids from tumor patients

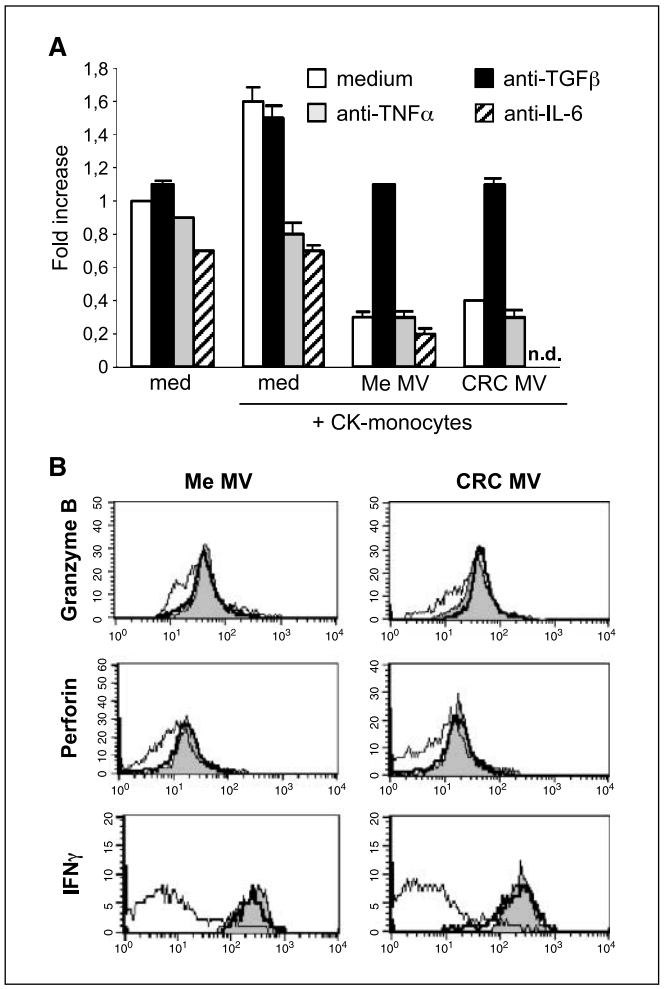

Figure 4. *A,* TGF-β secreted by microvesicle-treated differentiating monocytes is responsible for the suppressive effect exerted on T-cell–proliferation. Untreated, melanoma-derived, and colorectal carcinoma–derived microvesicle-treated monocytes at the third day of differentiation with cytokines were incubated for 3 days with autologous OKT3-stimulated PBMC (1:4 stimulator/target ratio) in the presence or absence of saturating concentrations of anti-TNF-α, anti-IL-6, or anti-TGF-β–neutralizing antibody. OKT3-stimulated PBMC alone were included as control. T-cell–proliferation was assessed on day 4 by measuring [^{3}H]thymidine incorporation after a 18-hour pulse. Each assay was done in quadruplicates. Data are fold increase normalized on counts/min (cpm) measured in samples containing responder PBMC alone (fold increase = 1). $P < 0.05$, statistically significant differences between microvesicle-treated and untreated samples. *B,* microvesicle-treated differentiating monocytes impair the expression of cytolytic molecules and IFN-γ by CD8$^+$ T cells on TCR stimulation in a TGF-β–dependent manner. OKT3-stimulated T cells cocultured with untreated (*gray area*), melanoma-derived or colorectal carcinoma–derived microvesicle treated (*thin line*) differentiating monocytes for 24 hours, in the presence (*bold line*) or absence of anti-TGF-β–neutralizing antibody, were analyzed for the intracellular expression of Granzyme B, Perforin and IFN-γ (gated on CD8$^+$ cells) by flow cytometry. Representative of at least three independent experiments.

(2, 3, 9, 10) and from healthy donors (25), we isolated microvesicles from plasma of stage IV melanoma patients, where scanning electron microscopy evidenced purified vesicular structures dimensionally compatible with exosomes (diameter, ≤200 nm; Fig. 5*A*). Microvesicle samples from both melanoma patients and healthy donors expressed LAMP-2, a lysosomal-associated membrane protein usually found in microvesicles and exosomes (25), as shown by Western blotting (Fig. 5*B*). Additionally, microvesicles from melanoma patients, but not from healthy volunteers, expressed the melanoma antigen gp100, thus confirming the presence of tumor-derived microvesicles in the blood of cancer patients (Fig. 5*B*).

To verify whether patient-derived microvesicles reproduced the effects observed with microvesicles derived from tumor cell lines, CD14$^+$ monocytes were treated with IL-4 and GM-CSF in the presence or absence of equal amounts of microvesicles derived from melanoma patients or healthy donors. The presence of microvesicles from melanoma patients inhibited CD14 down-regulation, which was not observed by treating monocytes with microvesicles from healthy donors (Fig. 5*C, left*). Moreover, monocytes treated with patient-derived microvesicles displayed a marked reduction in the percentage of HLA-DR$^+$ cells, due to a considerable loss of this molecule expression on cell surface (Fig. 5*C, right*), undetectable instead in monocytes treated with microvesicles from healthy donors. In response to melanoma patient microvesicles, monocytes released higher amounts of TGF-β compared with healthy donors [933.5 ± 219.8 (n = 5) versus 253.5 ± 163.2 pg/mL (n = 3), respectively].

Consistent with monocytes differentiated using tumor cell-derived microvesicles, CD14$^+$ cells treated with microvesicles from melanoma patients significantly impaired T-cell–proliferation in a TGF-β–dependent manner (Fig. 5*D*). On the contrary, a pronounced stimulatory effect was observed with monocytes treated with healthy donor microvesicles, most likely attributable to their origin from immune cells, such as dendritic cells and B lymphocytes (26).

TGF-β–secreting CD14$^+$HLA-DR$^-$ cells with immunosuppressive activity are expanded in peripheral blood of melanoma patients. To evaluate the *in vivo* relevance of our findings, we searched for CD14$^+$HLA-DR$^-$ cells in PBMC from stage IV melanoma patients (n = 16) and healthy donors (n = 10). A statistically significant ($P < 0.005$) expansion of this cell subset was detected in patient PBMC (8.4 ± 8.2%) compared with healthy volunteers (0.8 ± 1.0%; Fig. 6*A*). Thus, CD14$^+$HLA-DR$^-$ cells were immunosorted from patient PBMC (Fig. 6*B*) and evaluated for spontaneous release of TGF-β and suppressive activity. CD14$^+$HLA-DR$^-$ cells spontaneously secreted TGF-β *ex vivo*, although this cytokine was barely detectable in supernatants from the CD14$^+$HLA-DR$^+$ cell subset (Fig. 6*C*). Moreover, the addition of CD14$^+$HLA-DR$^-$ cells to CD14$^+$-depleted PBMC resulted in a significant inhibition of lymphocyte proliferation in response to mitogenic stimuli (Fig. 6*D*), hence confirming the immunosuppressive potential of this cell subset in melanoma patients.

Discussion

In the present study, we identified a new pathway by which human tumor cells, through the release of membrane vesicles, skew monocyte differentiation into dendritic cells toward the generation of myeloid cells with suppressive activity on T cells. Indeed, a consistent percentage of monocytes treated with tumor-released microvesicles in the presence of GM-CSF and IL-4 retained CD14, failed to up-regulate costimulatory molecules CD80 and CD86, and reduced surface expression of HLA class II molecules. The resulting CD14$^+$HLA-DR$^-$ phenotype was associated with suppressive activity on different T-cell–functions in response to TCR triggering, including proliferation, expression of cytotoxic molecules, and IFN-γ release. This effect was consistent with the rapid production by microvesicle-treated monocytes of bioactive TGF-β, in turn responsible for the suppressive effects on T cells. Comparable phenotypic and functional effects on monocyte differentiation

were achieved with microvesicles purified from the plasma of melanoma patients but not from healthy donors. In addition, TGF-β–secreting $CD14^+HLA\text{-}DR^-$ cells with suppressive activity on autologous T-cell–proliferation were significantly expanded in the same set of patients but barely detectable in healthy volunteers. Hence, these data suggest that the release of vesicular structures may represent a novel pathway enabling tumor cells to modulate host environment for surviving and progressing *in vivo*.

The results here described contribute to highlight the intricate tumor-host interactions, which, in spite of the expression of tumor-associated antigens and the existence of tumor-specific T cells, may render immune responses ineffective in tumor growth control even after stimulation with cancer vaccines. Indeed, although evidences support a potential role of the immune system in containing tumor development (and thus promoting a more favorable prognosis) in the initial phases of neoplastic disease (27), it is undoubted that, thanks to mechanisms not completely defined, tumors can progressively remodel the host environment, shutting off potential interferences with their own survival *in vivo*. This process could occur even in those tumors (such as melanoma) in which recall responses against certain tumor antigens are apparently detectable *ex vivo* (28) but most likely as mere indicators of tumor burden rather than effective mediators of antitumor immunity (29). In the context of tumor immune escape strategies, many studies have focused on the ability of cancer cells to eliminate, damage, or anergize T-cell–functions (30). However, the mechanisms exploited by growing tumors to dampen the initial phases of the immune response involving the APC compartment are still poorly defined. Nonetheless, several reports showed the existence in cancer patients of marked systemic defects involving APC, including dendritic cells, as a general consequence of abnormal myeloid differentiation and activation (15). In particular, tumor cells are known to promote the expansion of a myeloid cell population with negative regulatory effect on T-cell–functions, known as MSC (16). These cells, although extensively characterized in tumor-bearing mice, where they display a conserved $Gr1^+CD11b^+$ phenotype and common suppressive mechanisms, still need to be defined in the human setting (16). Nevertheless, several findings support the occurrence of aberrant myeloid differentiation in cancer patients, with defects involving decreased frequencies of mature immunocompetent dendritic cells, accumulation of HLA class II^{low} immature dendritic cells and expansion of immature myeloid cells (15). In particular, the accumulation of $CD14^+$ monocytes expressing reduced levels of HLA-DR and costimulatory molecules was described by Schadendorf et al. in peripheral blood monocytes from melanoma patients compared with healthy controls in association with disease progression (31).

Thus, we could speculate that the $CD14^+HLA\text{-}DR^{-/low}$ cell subset we identified as the result of monocyte interaction with tumor-released microvesicles may represent a component of the MSC population. Although the contribution of arginase activity, a hallmark of MSC in tumor-bearing animals (23) and selected human tumor histologies (such as renal carcinoma; ref. 16), could not be detected, we identified TGF-β as the soluble factor responsible for the immunosuppressive activity of microvesicle-treated monocytes. This cytokine, exerting a well-defined role in

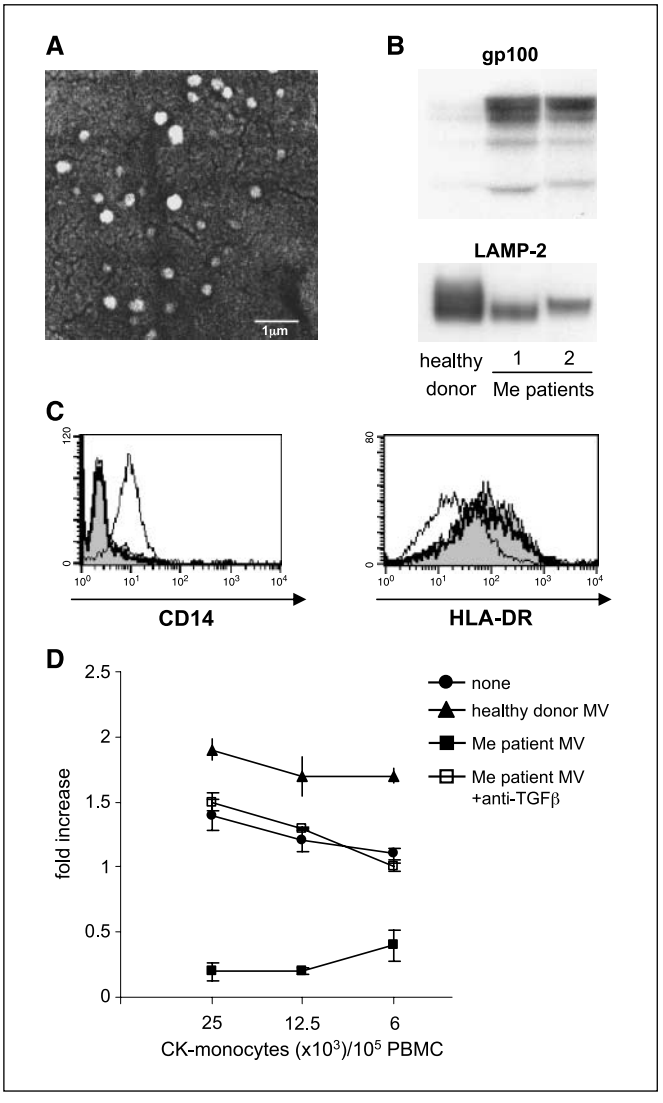

Figure 5. *A* and *B*, tumor-derived microvesicles are present in the plasma of advanced melanoma patients. Microvesicles were isolated from plasma of stage IV melanoma patients by serial centrifugations and analyzed by scanning electron microscopy. *A,* representative case. *B,* equal amounts of microvesicles isolated from healthy donors and melanoma patients were lysed and analyzed for the expression of gp100 and LAMP-2 by Western blot. *Top,* bands corresponding to gp100 protein migration at different molecular weights representing the glycosylated protein (100 kDa) and its fragments (45 and 64 kDa). Whereas the tumor antigen gp100 was detected in lysates of microvesicles derived from melanoma patients but not from the healthy donor, LAMP-2 molecules (*bottom*) were detected in both patient and healthy donor samples at 120 kDa, corresponding to the predicted molecular weight of this protein, thus confirming the presence of microvesicles in these samples. *C,* microvesicles isolated from plasma of melanoma patients impair CD14 down-regulation and HLA-DR expression during dendritic cell differentiation. $CD14^+$ monocytes from healthy donors were treated for 3 days with differentiating cytokines (IL-4 and GM-CSF; *gray area*) in the presence or absence of equal amounts of microvesicles purified from the plasma of stage IV melanoma patients (*thin line*) or healthy donors (*bold line*) and subsequently analyzed for the surface expression of CD14 (*left*) and HLA-DR (*right*) molecules by FACS analysis. *D,* monocytes differentiated into dendritic cells in the presence of microvesicles from plasma of melanoma patients acquire TGF-β–mediated suppressive activity on T-cell–proliferation. Untreated, melanoma patient microvesicle-treated, and healthy donor microvesicle-treated monocytes at the third day of differentiation with cytokines were incubated for 3 days with autologous OKT3-stimulated PBMC at the indicated stimulator/target ratios in the presence or absence of saturating concentrations of anti-TGF-β–neutralizing antibody. OKT3-stimulated PBMC alone were included as control. T-cell–proliferation was assessed on day 4 by measuring [^{3}H]thymidine incorporation after a 18-hour pulse. Data are fold increase normalized on cpm measured in samples containing responder PBMC alone (fold increase = 1). Each assay was done in quadruplicates. $P < 0.05$, statistically significant differences between microvesicle-treated and untreated samples. Representative of at least three independent experiments.

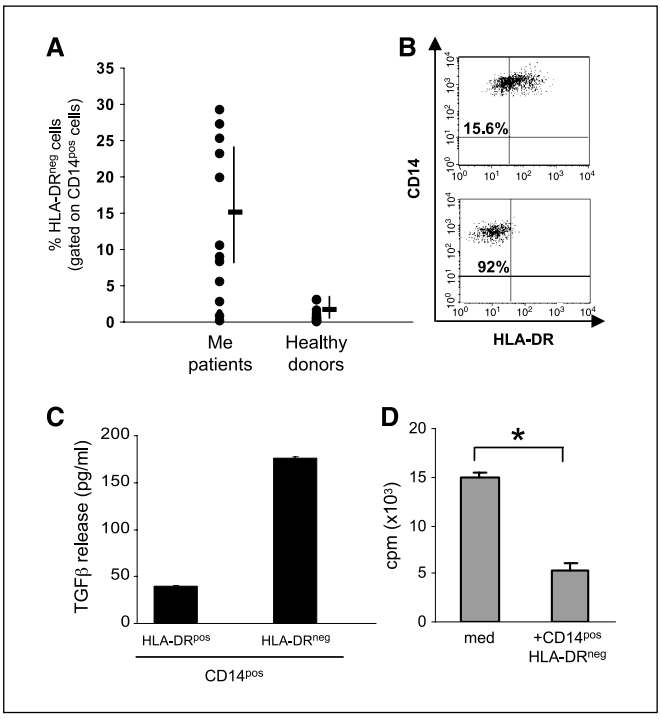

Figure 6. *A,* expansion of $CD14^+HLA\text{-}DR^-$ cells in the peripheral blood of advanced melanoma patients. PBMC of 10 healthy donors and 16 stage IV melanoma patients were stained for CD14 and HLA-DR surface expression. Data were analyzed by FACSCalibur and CellQuest. *Points,* mean; *bars,* SD. $P < 0.05$, statistically significant differences between healthy donors and melanoma patients (Dunnett's test). *B,* immunosorting of $CD14^+HLA\text{-}DR^-$ cells from the PBMC of advanced melanoma patients. Monocytes were isolated from PBMC of advanced melanoma patients by negative immunoselection. The resulting monocytic population was further stained with anti-HLA-DR antibody to purify $HLA\text{-}DR^-$ and $HLA\text{-}DR^{high}$ cell subsets. A dot blot showing the purity of immunosorted $CD14^+HLA\text{-}DR^-$ cell subset in a representative case. *C,* TGF-β release by melanoma patient PBMC and immunosorted $CD14^+HLA\text{-}DR^-$ and $CD14^+HLA\text{-}DR^+$ cell subsets. Spontaneous TGF-β release was quantified by ELISA in cell culture supernatants of melanoma patient PBMC (10^6/mL) or immunosorted $CD14^+HLA\text{-}DR^-$ and $CD14^+HLA\text{-}DR^+$ cell subsets (10^5/mL) after 6-hour incubation in X-VIVO 15 at 37°C. The assay was done in duplicates. *D,* inhibition of T-cell–proliferation mediated by $CD14^+HLA\text{-}DR^-$ cells isolated from advanced melanoma patient PBMC. Suppressive activity of $CD14^+HLA\text{-}DR^-$ was assessed by evaluating PHA/PMA-induced proliferation of CD14-depleted PBMC (100×10^3 per well) from melanoma patients in the presence or absence of autologous $CD14^+HLA\text{-}DR^-$ (20×10^3 per well). Lymphocyte proliferation was assessed on day 4 by measuring $[^3H]$thymidine incorporation after a 18-hour pulse. Each assay was done in quadruplicates. *, $P < 0.05$, compared with CD14-depleted PBMC (Student's *t* test). Representative of at least three independent experiments.

immunosuppression through mechanisms ranging from direct effects on T-cell–proliferative and cytolytic potential (21) to the activation of regulatory T cells (32), has been recently identified as the key player of MSC function in defined tumor murine models (33).

The multiple defects in myeloid differentiation and function detected systemically in cancer patients support the hypothesis that circulating factors, able to reach these cells in different tissue compartments, might be involved. Herein, we show that tumor-released microvesicles may play a role in this process. Microvesicles are membrane organelles shed from the cell surface under different physiologic and pathologic conditions by a large array of cells (26). Thanks to their ability to circulate and transport a broad protein spectrum from the membrane and cytoplasmic repertoire of the cell (34), microvesicles may act as important mediators for intercellular cross-talk and molecule delivery. Indeed, since their discovery (35), growing evidence has been collected about the presence of microvesicular organelles in different biological fluids of both cancer patients and healthy individuals (2, 3, 9, 10). The association between microvesicle release and tumor progression, suggested by microvesicle abundance in the body fluids of patients with advanced neoplastic disease, has been recently hypothesized in several studies showing the microvesicle ability to transport bioactive molecules potentially involved in immunosuppressive circuits, such as FasL and TRAIL (1–3, 10). Evidence of the possible detrimental effect of tumor-shed microvesicles on the APC compartment was collected in murine models by Taylor et al., reporting the inhibitory activity of membrane-derived vesicles released by melanoma metastatic variants on MHC class II surface expression in macrophages (36). However, the potential interference of tumor-released microvesicles with APC function has been thus far poorly investigated, being recent studies mainly focused on the use of tumor-derived microvesicles as vehicles for tumor antigen cross-presentation by APC (34). Although the possibility to induce effective immunoprotection in tumor-bearing mice vaccinated with tumor-derived exosomes has been reported (37), these results have not been thus far confirmed in human setting, where the clinical application of microvesicles as cancer vaccines has been mainly focused on dendritic cells instead of tumor-derived exosomes (38).

Secretion of microvesicles capable of skewing monocyte differentiation toward a suppressive phenotype seemed to be a tumor-related phenomenon, as microvesicles released by macrophages, used as normal counterpart, allowed the physiologic differentiation of monocytes into immature dendritic cells. Although it would be interesting to analyze the immunomodulatory effects of microvesicles released by melanocytes, representing melanoma nontransformed histologic counterpart, the lack of FasL expression in these normal cells (39) suggests that production of immunosuppressive microvesicles might be a selective feature of transformed tissues. Tumor specificity of this phenomenon was further evaluated using exosome-like membrane vesicles purified from plasma of both healthy donors and melanoma patients, where the presence of circulating tumor-released microvesicles was proven in terms of expression of the melanoma antigen gp100 (see Fig. 5*B*). Although tumor cells may not be the only source of microvesicles collected from patients' plasma, the detrimental effect on differentiating monocytes was exclusively induced by microvesicle preparations from melanoma patients and not from healthy donors, thus suggesting that the presence of a growing tumor is linked to the microvesicle-induced suppressive effect.

The mechanism by which microvesicle-treated monocytes may deliver their suppressive signals to antitumor T-cell–effectors without creating systemic immune suppression in the host is presently unknown, being still undefined the pathway used by MSC to expressly target tumor-specific immune responses (16). However, it could be hypothesized that $CD14^+HLA\text{-}DR^-$ cells may cross-present antigenic determinants contained in tumor-microvesicle (37) to specific $CD8^+$ T cells through their conserved expression of HLA class I molecules and then selectively deliver TGF-β–mediated suppressive signals.

In conclusion, our data suggest the possible development in cancer patients of a circuit primed by tumor cells to create an immunotolerant environment, through the active release of membrane vesicles, able to redirect normal differentiation of circulating monocytes into a $CD14^+$ immature myeloid population with a TGF-β–mediated suppressive activity on T-cell–proliferation

and function. Although further studies are needed to clearly depict the molecular mechanisms involved in the dysregulation of monocyte differentiation by tumor-derived microvesicles, our observations may offer new tools for prognostic and therapeutic strategies in cancer patients.

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