Secreted Protein Acidic and Rich in Cysteine Produced by Human Melanoma Cells Modulates Polymorphonuclear Leukocyte Recruitment and Antitumor Cytotoxic Capacity

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Abstract

The expression of secreted protein acidic and rich in cysteine (SPARC) has been associated with the malignant progression of different types of human cancer. SPARC was associated with tumor cell capacity to migrate and invade, although its precise role in tumor progression is still elusive. In the present study, we show that SPARC produced by melanoma cells modulates the antitumor activity of polymorphonuclear leukocytes (PMN). Administration to nude mice of human melanoma cells in which SPARC expression was transiently or stably knocked down by antisense RNA (SPARC-sup cells) promoted PMN recruitment and obliterated tumor growth even when SPARC-sup cells accounted for only 10% of injected malignant cells. In addition, SPARC-sup cells stimulated the *in vitro* migration and triggered the antimelanoma cytotoxic capacity of human PMN, an effect that was reverted in the presence of SPARC purified from melanoma cells or by reexpressing SPARC in SPARC-sup cells. Leukotrienes, interleukin 8, and growth-related oncogene, in combination with Fas ligand and interleukin 1, mediated SPARC effects. These data indicate that SPARC plays an essential role in tumor evasion from immune surveillance through the inhibition of the antitumor PMN activity. (Cancer Res 2005; 65(12): 5123-32)

Introduction

Secreted protein acidic and rich in cysteine (SPARC) is a secreted glycoprotein that has been implicated in tumor-host interactions by virtue of its ability to modulate cellular interaction with the extracellular matrix (1). SPARC interacts with several extracellular matrix components, binds and modulates the activity of specific growth factors, and regulates matrix metalloproteinase expression and activity (2). Moreover, SPARC expression is associated with tissue remodeling processes, like wound healing and angiogenesis, both of which include physiologic steps of invasive phenotypes (3). Several reports associated SPARC expression with the invasive and metastatic capacity of different human cancers although its precise role in tumor progression is still controversial (4–7). Suppression of SPARC expression in human melanoma cells abrogated their tumorigenic capacity (4). However, breast carcinoma and glioma cells engineered to express SPARC showed reduced tumor cell proliferation probably due to the capacity of SPARC to inhibit cell cycle (7) and SPARC expression in ovarian carcinoma cells impaired their tumorigenic potential (8). Interestingly, SPARC production by tumor stromal cells has been also associated with the neoplastic progression of tumors in which SPARC is hardly detected in the malignant cells themselves (9, 10).

The role of inflammatory cells in tumor progression is highly controversial. Inflammatory cells can promote tumor progression by degrading the extracellular matrix, activating tumor-associated fibroblasts, and enhancing angiogenesis (11). Conversely, inflammatory cells can be activated to eliminate tumor cells (12) or might release cytokines that activate professional antigen presenting cells that recruit specific immune effector cells with the capacity to complete tumor rejection (13). Only very recently, the role of polymorphonuclear leukocytes (PMN) in the immune surveillance against tumors has emerged. PMNs are the first line of defense against infection. They release soluble chemotactic factors and proteases that alter the microenvironment inducing extracellular matrix remodeling and recruitment of nonspecific and specific immune effector cells (11). Recent reports highlighted the participation of PMNs as direct effector cells in the immune surveillance against cancer (14).

In a previous work, we have shown that suppression of SPARC expression in human melanoma cells prevented tumor growth in nude mice in coincidence with the massive recruitment of PMN to the site of tumor cell injection (4). Here we show for the first time that stable and transient knockdown of SPARC expression in different human melanoma cell lines promoted the *in vivo* PMN recruitment and rejection of tumor cells in nude mice and triggered the *in vitro* migration and antitumor cytotoxic capacity of human PMN (hPMN). PMN activity was reverted in the presence of native SPARC and by reexpressing SPARC. We have identified chemotactic factors and molecules involved in apoptotic pathways that mediate SPARC effect both *in vitro* and *in vivo*. Overall, these results suggest that SPARC produced by malignant cells has an important role in the escape of tumor cells from antitumor immune surveillance by blocking PMN antitumor activity.

Materials and Methods

Cell lines, reagents, and antibodies. IIB-MEL-LES and A375N melanoma cell lines were grown in melanoma medium and IIB-MEL-J human melanoma cells were grown in melanoma medium containing 5 μg/L epidermal growth factor and 500 μg/L transferrin, supplemented with 10% fetal bovine serum (FBS) and antibiotics (4). Cultures were maintained at 37°C in a 5% CO_2 humidified incubator. L-1D and L-1E are stable cell clones selected in G418 (Invitrogen, Grand Island, NY) after transfection of IIB-MEL-LES cells with Rc/CMV vector (Invitrogen, San Diego, CA)

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

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carrying SPARC full-length cDNA cloned in antisense orientation (4). Clones were thawed from original stocks, selected in G418, and routinely checked for SPARC production. L-CMV and L-βgal cells were generated by stable transfection of IIB-MEL-LES cells with Rc/CMV vector alone or carrying the bacterial β-galactosidase gene, respectively.

Unless specified, the cell culture reagents were from Invitrogen (Grand Island, NY), and the chemicals were from Sigma (St. Louis, MO). MK-886 was purchased from Calbiochem (San Diego, CA). The leukotriene $B_4/C_4/D_4/E_4$ enzyme immunoassay system was from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Anti-Fas (B-G27) and anti–Fas ligand (NOK-1) monoclonal antibodies (mAb) and horseradish peroxidase–conjugated secondary antibody were from RDI (Flanders, NJ). Anti–interleukin (IL)-8, anti–growth-related oncogene (GRO)-α and anti-panGRO monoclonal antibodies, anti–IL-8 and anti–GRO-α polyclonal antibodies, and human recombinant IL-8 and GRO-α were from R&D Systems, Inc. (Minneapolis, MN). Anti-CD31 and anti-CD54 monoclonal antibodies were from the 6th International Workshop on human leukocyte differentiation antigens (HLDA6); anti-CD18 monoclonal antibody was from the HLDA3. Isotypic control mAbs and phycoerythrin-conjugated secondary antibodies were from DAKO A/S (Glostrup, Denmark).

Human SPARC was purified from A375N serum-free cell-conditioned medium (SF-CCM) conditioned for 24 hours, clarified by centrifugation, supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L EDTA, concentrated to 50 mL in an Amicon ultrafiltration cell with YM10 membrane, MW cutoff 10,000 (Millipore Corp., Billerica, MA), and dialyzed against 20 mmol/L Tris-HCl (pH 7.8). Samples were loaded on a HiTrap Q column (GE Healthcare, Piscataway, NJ) and eluted in a continuous salt gradient (100-800 mmol/L NaCl). After SPARC identification by Western blot, selected fractions were run in SDS-PAGE and those with purity higher than 90% were pooled, dialyzed against Tris 20 mmol/L, 150 mmol/L NaCl, pH 7.8, and run in a Superdex 200 column. The eluted fractions, showing 98% purity, were dialyzed against PBS, concentrated with a Centricon 10 (Millipore Corp., Billerica MA), sterilized by passing through a 0.22 mm pore filter, snap-frozen in liquid N_2, and stored at −80°C. Protein samples showed circular dichroism spectra similar to those reported for SPARC obtained from murine Engelbreth-Holm-Swarm tumors and were able to inhibit bovine aorta endothelial cell proliferation *in vitro* (15).[5]

Adenoviral constructs. A 1.7 kb *Sal*I fragment containing the coding sequence of human SPARC and a 527 bp *Sal*I fragment containing the bacterial β-galactosidase gene were cloned in pADPSY-LTRSVpolyA vector to generate adenoviral vectors carrying SPARC cDNA in sense (AdSPARC) and antisense (AdSP-AS) orientations or Adβgal, respectively. The 537 bp cDNA of IL-1 receptor antagonist was obtained by reverse transcription-PCR from lipopolysaccharide-stimulated rat spleen, cloned in pSP72 plasmid and subcloned in the same vector. Adenoviral particles were produced as described (16). The concentration of recombinant vector was expressed as 50% tissue culture infectious doses (TCID50) per milliliter (17).

***In vivo* assays.** Eight- to ten-week-old athymic N:NIH(S)-nu mice received s.c. injections of 5×10^6 melanoma cells in the left flank, in a total volume of 100 μL. Perpendicular diameters were used to determine tumor volume, as $d_1^2 \times d_2 / 2$, where d_1 is the smaller diameter and d_2 is the larger one. Mice harboring tumors greater than 2 cm^3 were considered not survivors and euthanized following institutional guidelines. Surviving mice were followed for 6 months.

For histologic analysis, the sites of injection were removed, paraffin embedded, cut, and stained with H&E. Immunohistochemical detection of PMN and macrophages was done on paraffin-embedded tissue with anti–Ly-6G antibody (RB6-8C5, BD PharMingen, San Diego, CA) and anti-F4/80 antibody (A3-1, Serotec, Oxford, United Kingdom), respectively. Samples were stained with Vectastain ABC kit (Vector Lab., Burlingame, CA) and counterstained with hematoxylin.

For functional blocking assays, melanoma cells were preincubated for 30 minutes with 2 μg antibody or 500 pmol MK886 in a final volume of 50 μL and injected in the left flank of nude mice. At the indicated times, the injection areas were removed, paraffin embedded, cut, and stained with H&E. PMNs were blindly evaluated by one of us (A.I.B., an anatomopathologist) using a light microscope under air and oil immersion at ×630 magnification, and confirmed in some cases by immunohistochemical detection in subsequent slides of the same area. Only clearly viable PMNs were quantified.

***In vitro* assays with human leukocytes.** PMN, lymphocytes, and monocytes were isolated as described (18). Briefly, leukocytes obtained from venous blood of healthy human volunteers were resuspended in Tyrode's solution containing 10% autologous platelet-poor plasma supplemented Percoll (Pharmacia Fine Chemicals, Dorval, PQ). Six different Percoll cushions were layered in a 15 mL conical tube followed by centrifugation at $400 \times g$ (25 minutes at 22°C). Harvested bands were washed twice with Tyroyd's solution. PMN, monocytes, and lymphocytes were recovered with >90% purity and >95% viability as assessed by trypan blue exclusion. PMNs were identified by nuclear morphology following crystal violet staining; monocytes were identified by anti-CD14 flow cytometry; and lymphocytes were identified by anti-CD3 flow cytometry.

For migration assays, 1.25×10^5 PMNs or 2.5×10^5 monocytes or lymphocytes were seeded on 3 μm pore size Transwells inserts (Corning Costar, Cambridge, MA) and allowed to migrate for 45 minutes toward SF-CCM supplemented with 5 mg/mL human serum albumin (UNC hemoderivados, Córdoba, Argentina). PMN migration in the presence of 10 nmol/L formyl-Met-Leu-Phe was used as a positive control. Migrated cells were counted with a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ). For neutralization assays, 10 μg/mL each of anti–IL-8, anti-GRO, and anti–Fas ligand (FasL) antibodies were added to SF-CCM 30 minutes before the chemotaxis assay. For blocking leukotriene production, cells were preincubated overnight in melanoma media supplemented with 1 μmol/L MK886 followed by the preparation of SF-CCM containing 1 μmol/L MK886. For PMN migration assays toward melanoma cells, malignant cells were grown in 24-well plates and the culture supernatant was replaced with SF-CCM 1 hour before the assay. Isolated PMNs were seeded on 3 μm pore size transwells and allowed to migrate toward melanoma cells for 90 minutes. The number of migrated PMN was estimated by myeloperoxidase activity.

For adhesion analysis, hPMNs were incubated in 200 μCi ^{51}Cr–containing PBS for 30 minutes. After washing, ^{51}Cr-labeled hPMNs were incubated with melanoma cells in serum-free medium supplemented with 5 mg/mL human serum albumin. After 45 minutes, nonadherent PMNs were removed by washing with PBS supplemented with Ca^{2+} and Mg^{2+}. The fraction of adherent PMN was obtained by using the following formula: 100 × aC / tC, where aC and tC are the ^{51}Cr counts corresponding to adherent and total PMN, respectively. For blocking adhesion, PMNs and melanoma cells were separately preincubated, each with 20 μg/mL antibody for 30 minutes, followed by incubation with the same amount of antibody during the adhesion assay. Expression of CD18 at the cell surface of hPMN and of CD31 and CD54 in both hPMNs and melanoma cells were confirmed by flow cytometry.

PMN azurofilic granule exocytosis was evaluated by myeloperoxidase release. Freshly isolated hPMN, preincubated for 5 minutes with 500 ng/mL cytochalasin B, were coincubated with melanoma CCM for 30 minutes and myeloperoxidase activity in the supernatant was quantified as described (19).

For cytotoxic assays, trypsinized melanoma cells were labeled with 200 μCi ^{51}Cr. hPMN samples were cocultured with labeled melanoma cells for 40 hours in 5% FBS–supplemented medium. At the end of the incubation period, the supernatant was harvested and cells were lysed with 0.3 N NaOH. Specific lysis, expressed as percentage, was estimated from the radioactivity present in the different fractions with the following formula: 100 × (tCs × sCi / tCi − sCs) / (tCs − sCs), where sCi and sCs are the ^{51}Cr counts of supernatants that correspond to treatment i and spontaneous ^{51}Cr release control, respectively, and tCi and tCs are the total counts (supernatant plus cell lysate) for treatment i and spontaneous release control, respectively.

For PMN apoptosis determination, 5×10^5 hPMNs were incubated for 16 hours in 100 μL of 5% FBS–supplemented melanoma medium alone

[5] F. Prada et al., manuscript in preparation.

(basal treatment) or containing 500 nmol/L human SPARC. In parallel experiments, 5×10^5 hPMNs were coincubated with 2×10^4 melanoma cells in 100 μL of 5% FBS–supplemented melanoma medium for 16 hours. PMN apoptosis was then evaluated by three different methods: (*a*) quantification of metabolically active apoptotic PMNs by flow cytometry after annexin V plus propidium iodide staining (annexin V:FITC apoptosis detection kit I, BD PharMingen); (*b*) flow cytometry of propidium iodide–stained ethanol-fixed cells (20); and (*c*) counting of metabolically active apoptotic PMNs in hemocytometer by inspection of their nuclear morphology after acrydine orange plus ethidium bromide staining (20).

ELISA assays and fluorescence-activated cell sorting analysis. IL-8 and GRO-α levels in SF-CCM were determined by ELISA following the instructions of the manufacturer (R&D Systems). For fluorescence-activated cell sorting analysis (FACS), subconfluent melanoma cells were detached with 1 mmol/L EDTA–supplemented PBS and labeled with anti-Fas, anti-FasL mAbs, and isotypic control mAbs, followed by phycoerythrin-conjugated secondary antibody in 0.01% NaN_3–supplemented PBS. FACS analysis was done in a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA).

Statistical analysis. Significance levels for orthogonal comparisons were estimated by one-way ANOVA. Analysis of data from several independent experiments was carried out with a mixed effects ANOVA, taking the different experiments as random effects. Significance of multiple comparisons was estimated by Newman-Keuls test.

Results

Knockdown of SPARC expression in human melanoma cells enhances polymorphonuclear leukocyte recruitment *in vivo* and promotes rejection of human melanoma cells: kinetic study. First, we did a kinetic study of the evolvement of the host response following the injection of a cell clone (L-1D) that produced on average 20% or less SPARC than control L-CMV cells (Fig. 1*A*). Eight hours after injection, we observed a similar inflammatory response in both L-1D and L-CMV injection sites, which was probably the result of host response to the xenogeneic transplant (data not shown). However, 24 hours was enough to observe clear histologic differences between samples. Indeed, mice injected with L-CMV cells showed tumor nodules with a conspicuous PMN infiltrate (Fig. 1*B*, *inset* and data not shown). These tumors continued growing for over 12 days showing an increased extracellular matrix layer and only a scant PMN infiltrate (Fig. 1*C* and *D* and data not shown). Conversely, injection of L-1D cells at 24 to 72 hours resulted in an increased inflammatory infiltrate compared with L-CMV cells (Fig. 3*A*). This inflammatory infiltrate was composed mainly of PMNs that invaded the entire area of tumor cell injection with no evidence of viable tumor cells after 72 hours (Fig. 1*E* and *F*). Starting from day 5 up to day 12, macrophages were seen to invade the site of injection (Fig. 1*G-I*, *insets*). At day 7 fibroblasts were also present (Fig. 1*H*). The area was completely cleared at day 12 when fibrotic tissue replaced the area of tumor injection (Fig. 1*I*, *inset*).

To confirm that PMN recruitment is under the control of SPARC, we transiently blocked SPARC expression in a second human melanoma cell line, A375N. This cell line, which produced 5-fold the amount of SPARC produced by L-CMV cells, showed up to 90% reduction in secreted SPARC compared with Adβgal-transduced cells after transduction with AdSP-AS (Fig. 1*J* and *K*). Reduction in SPARC levels was steady for 3 days.[5] A375N cells transduced *ex vivo* by AdSP-AS induced a strong PMN infiltrate at 72 hours compared with control cells transduced with Adβgal, with only few viable tumor cells and both viable and damaged PMN sparse in all the tumor areas (Fig. 1*L-O*). Moreover, AdSP-AS–transduced A375N cells showed reduced *in vivo* growth compared with control cells (Fig. 1*P*). Similar results were obtained with another human melanoma cell line, IIB-MEL-J (data not shown). The whole data indicates that knockdown of SPARC expression in melanoma cells promotes a persistent PMN infiltrate associated with tumor rejection.

SPARC knockdown in melanoma cells promotes an increased migration of human polymorphonuclear leukocytes *in vitro*. Next, we sought to establish whether knockdown of SPARC expression was directly affecting PMN migration *in vitro*. In addition to L-1D, we have also used the L-1E cell clone which expressed on average 40% of SPARC levels observed in L-CMV cells (Fig. 1*A*). L-1D and L-1E cells (collectively referred to as SP-AS cells) were conditioned in serum-free medium for 1 hour as a chemotactic stimulus for PMN. Such a short conditioning period was sufficient to stimulate 2- to 3-fold the transmigration of mouse PMN (mPMN) compared with L-CMV cells (Fig. 2*A*). Similar results were obtained when SF-CCM conditioned for 16 hours was used as a chemotactic source (data not shown).

To further confirm SPARC effects, we used hPMN. In 59% of the samples (13 of 22), SF-CCM obtained from SP-AS cells induced on average a 2-fold increase in migration of hPMNs compared with L-CMV cells (Fig. 2*B*). hPMNs obtained from the other nine donors were equally attracted by both cell types (not shown). The chemotactic capacity of the CCM was specific for PMN because L-CMV and SP-AS CCM had no effect on human lymphocyte and monocyte transmigration capacity (Fig. 2*C* and *D*). Thus, the effect that SPARC knockdown showed on transmigration assays *in vitro* supported the likelihood that SPARC produced by the malignant cells modulated the *in vivo* PMN recruitment.

Specific chemotactic factors mediate SPARC effect *in vivo* and *in vitro*. We next attempted to identify the chemotactic factors that might be responsible for stimulating PMN recruitment *in vivo* and transmigration *in vitro* following knockdown of SPARC expression. IL-8 and GRO-α are likely candidates because they are produced by human melanoma and tumor stroma cells acting both as autocrine growth factors for melanoma cells and chemoattractants of PMN (21, 22). SP-AS cells produced higher levels of IL-8 than L-CMV cells (1,060 ± 120 pg/10^6 cells in L-1E, 1,750 ± 4.37 pg/10^6 cells in L-1D, and 345 ± 10.8 pg/10^6 cells in L-CMV cells, mean ± SE from four experiments; $P < 0.01$, when L-1E and L-1D were compared versus L-CMV). In addition, GRO levels were undetectable in L-CMV cells, whereas L-1E produced as much as 120 ± 19 pg/10^6 cells and L-1D cells produced 60 ± 7 pg/10^6 cells. Despite this increased production, incubation of L-1D cells with neutralizing antibodies against IL-8 and GRO did not block L-1D–mediated mPMN recruitment *in vivo* and inhibited only by 22% hPMN migration *in vitro* (Fig 3*A* and *B*). These antibodies were physiologically active because they completely blocked the *in vitro* hPMN migration induced by L-CMV cells (Fig. 3*B*) and did not affect hPMN migration induced by formyl-Met-Leu-Phe (data not shown).

Leukotrienes are also potent chemotactic factors for PMN and are produced by human melanoma cells (23). Efforts to quantify the amount of leukotrienes produced by melanoma cells were unsuccessful because the amount was below the sensitivity of the assay (see Materials and Methods for details). To test leukotriene involvement, we treated melanoma cells with MK886, a specific inhibitor of 5-lipoxygenase activating protein, which is an essential protein in leukotriene production (24). MK886 treatment reduced by almost 40% the capacity of L-1D cells to

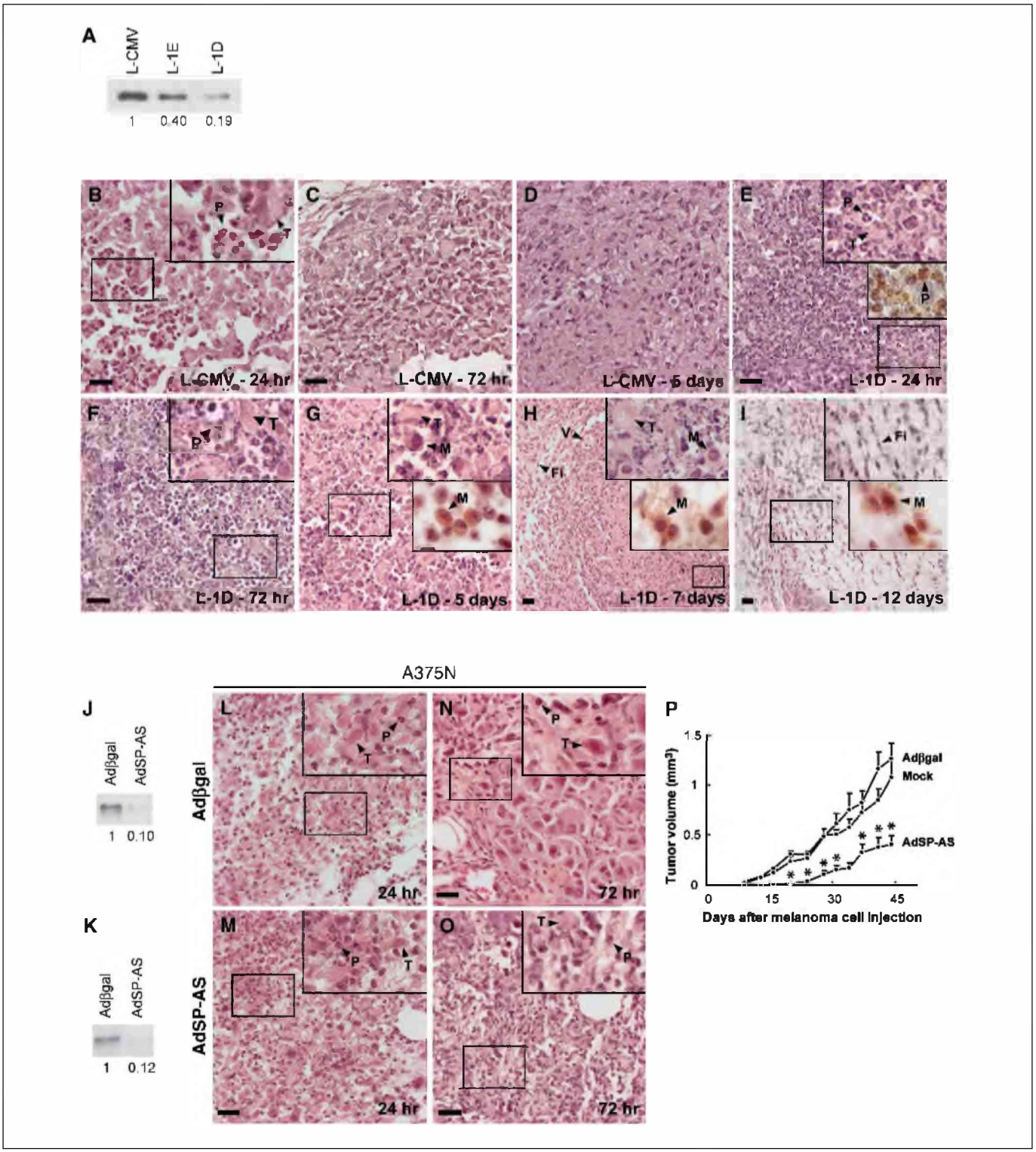

Figure 1. Kinetic analysis of the host inflammatory response following injection of nude mice with melanoma cells in which SPARC expression was knocked down. *A*, immunoblot of SPARC in the cell-conditioned media of L-CMV, L-1E, and L-1D cells in 1 μg of total protein. *B* to *I*, microphotographs corresponding to the area of injection of control L-CMV cells at 24 hours (*B*), 72 hours (*C*), and 5 days postinjection (*D*); L-1D cells 24 hours (*E*), 72 hours (*F*), 5 days (*G*), 7 days (*H*). and 12 days after cell injection (*I*). *E*, *inset*, anti-PMN (Ly-6G antigen) immunostaining; *G* to *I*, *insets*, antimacrophage (F4/80 antigen) immunostainings. *J* and *K*, immunoblots of SPARC in 1 μg total protein from cell-conditioned media of melanoma cells transduced with the different adenoviral vectors. *L* to *O*, A375N human melanoma cells were transduced *ex vivo* with 5×10^8 TCID50/mL particles of Adβgal and AdSP-AS adenoviral vectors 24 hours before s.c. injection in nude mice. Microphotographs correspond to the injection sites that were removed for histologic analysis at 24 hours for Adβgal (*L*) and AdSP-AS–transduced (*M*) cells, and at 72 hours postinjection for Adβgal (*N*) and AdSP-AS–transduced (*O*) cells. *Fi*, fibroblast; *M*, macrophage; *P*, PMN; *T*, melanoma cell; *V*, blood vessel. Bar, 10 μm. *P*, *in vivo* growth of A375N human melanoma cells. A375N cells were transduced *ex vivo* with 5×10^8 TCID50/mL particles of Adβgal and AdSP-AS adenoviral vectors. After 24 hours, untransduced (mock control) and adenovirus-transduced cells were injected s.c. in nude mice. *Points*, average tumor volume of eight mice; *bars*, SE. Representative of two experiments. *, $P < 0.01$.

recruit PMN to the site of injection *in vivo* compared with vehicle-treated L-1D cells at 24 hours (Fig. 3*A*). MK886 was also effective as a single agent at 72 hours, and its combination with anti–IL-8 and anti-GRO antibodies completely abrogated PMN recruitment induced by L-1D cells (Fig. 3*A*). In *in vitro* studies, incubation of L-1D cells with either of the reagents separately did not significantly affect hPMN transmigration capacity (Fig. 3*B*). However, combinatorial treatment with MK886, anti–IL-8, and anti-GRO antibodies inhibited by almost 70% L-1D capacity to induce transmigration of hPMNs in three of five samples (Fig. 3*B*) and was partially effective with PMNs from the other two donors (data not shown). From the whole data, it can be concluded that mPMN recruitment *in vivo* and hPMN migration *in vitro* that occurs as a result of knockdown of SPARC expression are mediated, at least in part, by leukotrienes, IL-8, and GRO.

Role of Fas ligand and interleukin 1 as mediators of SPARC effects. The role of FasL in immune surveillance is highly controversial (25). Previous evidence showed that tumor cells expressing FasL are rejected by challenged mice through a PMN-mediated mechanism (25). Moreover, soluble FasL was shown to be chemotactic for human and murine PMNs (26). Flow cytometry analysis showed no changes in the expression levels of Fas following knockdown of SPARC expression. However, FasL was detected at the cell surface in the two SP-AS clones tested, but not in control L-CMV cells (Fig. 3*C*). Interestingly, injection of L-1D cells treated with anti-FasL neutralizing antibody had no effect on PMN recruitment at 24 hours (Fig. 3*A*, 24 hours). However, a dramatic reduction in PMN recruitment was observed 72 hours postinjection, suggesting a role of FasL in the persistence of the PMN infiltrate (Fig. 3*A*, 72 hours). IL-1 was shown to be a chemotactic molecule for PMN, and it was involved in PMN recruitment induced by ectopic FasL expression in tumor cells (27). Transient expression of the IL-1 receptor antagonist in L-1D cells had no effect at 24 hours but inhibited by almost 80% the *in vivo* PMN recruitment induced by L-1D cells at 72 hours compared with control Adβgal-transduced L-1D cells (Fig. 3*D*). In coincidence with the lack of involvement of FasL *in vivo* at 24 hours, incubation of L-1D cells with neutralizing anti-FasL antibody had no effect on hPMN migration *in vitro* (Fig 3*B*). Overall, these data indicate that the persistence of PMN recruitment to the site of injection of SPARC-sup cells requires the involvement of FasL and IL-1 at a later stage.

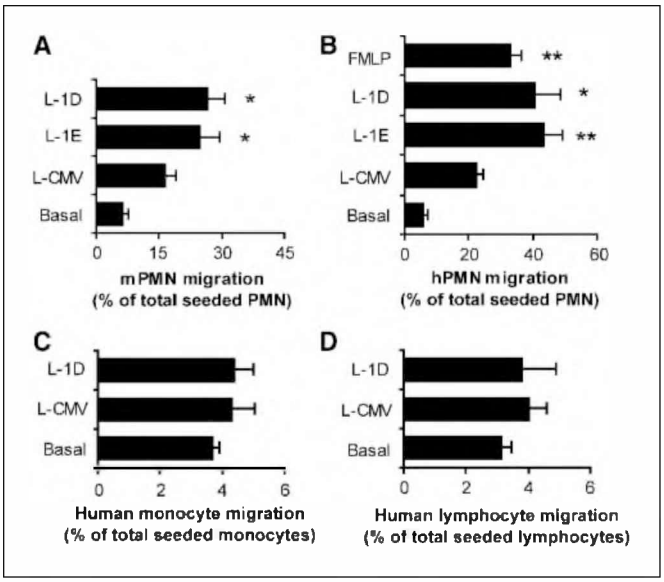

Figure 2. *In vitro* leukocyte migration induced by melanoma cells. *A*, mPMN migration towards melanoma cells with their CCM. Migrated PMNs were quantified by their myeloperoxidase activity. *Columns*, average percentage of PMN migration from two independent experiments; *bars*, SE. *, $P < 0.01$. *B*, hPMN migration induced by melanoma cell–conditioned media. Migrated PMNs were quantified by absolute flow cytometry. *Columns*, average percentage of PMN migration obtained from 13 selected donors; *bars*, SE. *, $P < 0.05$; **, $P < 0.0001$. *C* and *D*, human monocyte (*C*) and lymphocyte (*D*) migration induced by melanoma cell–conditioned media. Migrated leukocytes were quantified by absolute flow cytometry. *Columns*, average percentage of migration; *bars*, SE. Representative of two experiments.

***In vivo* coadministration of melanoma cells with knocked down SPARC expression with nonengineered melanoma cells promotes complete tumor rejection.** Our initial *in vivo* studies suggested that SPARC knockdown in melanoma cells promoted not only the recruitment of PMN but also triggered their antitumor cytotoxic capacity. This effect was potent enough to induce rejection of a mix of tumor cells composed of L-1D cells coinjected with nonengineered human melanoma cells. Indeed, nude mice xenografted with ratios of 1:1, 1:0.67, and 1:0.25 (control cells: L-1D cells) showed no tumor growth (Table 1). Even at 1:0.1 ratio, only 22% of mice (2 of 7) showed tumor growth for 2 weeks, which then ceased growing and remained stable until the end of the experiment (Table 1). Rejection of L-1D + L-βgal cell growth was accompanied by the massive recruitment of PMNs similar to what has been previously described in Fig. 1*E* and *F* (Supplementary Fig. S1*C* and *D*). No viable tumor cells were observed after 5 days (data not shown). On the other hand, L-CMV + L-βgal site of injection showed a PMN infiltrated similarly to what has been described in Fig. 1*B* and *C* (data not shown). This bystander effect was not cell line specific because tumor growth was significantly reduced in mice xenografted with a 1:1 ratio of another human melanoma cell line IIB-MEL-J to L-1D cells (Table 1). Rejection of this cell combination was also associated with the massive recruitment of PMNs (Supplementary Fig. S2). From these studies, it can be concluded that knockdown of SPARC expression in malignant cells is sufficiently potent to stimulate the elimination of bystander nonmodified cells, even when L-1D cells accounted for only 10% of injected cells. Interestingly, SPARC produced by the bystander cells was unable to revert the effect.

Modulation of SPARC levels in human melanoma cells regulates the antitumor cytotoxic capacity of human polymorphonuclear leukocytes *in vitro*. Finally, we did *in vitro* studies to confirm that SPARC produced by melanoma cells modulates hPMN antitumor cytotoxic activity. First, we observed an increased capacity of hPMN to adhere to either of the SP-AS cell clones compared with control L-CMV cells (Supplementary Fig. S3*A*). This binding was almost completely abrogated in the presence of anti-CD18 antibody and partially inhibited in the presence of anti-CD54 and anti-CD31 antibodies (data not shown). In addition, SP-AS CCM induced a strong PMN azurofilic granule exocytosis, indicating increased activation of hPMN compared with L-CMV cells (Supplementary Fig. 3*B*). Mostly important, hPMNs exerted a potent cytotoxic activity against SP-AS cells, but not against control L-CMV or parental IIB-MEL-LES cells (Fig. 4*A* and data not shown). In line with this, transient knockdown of SPARC expression in A375N human melanoma cells following *ex vivo* transduction with AdSP-AS also triggered the antitumor cytotoxic capacity of hPMN, whereas

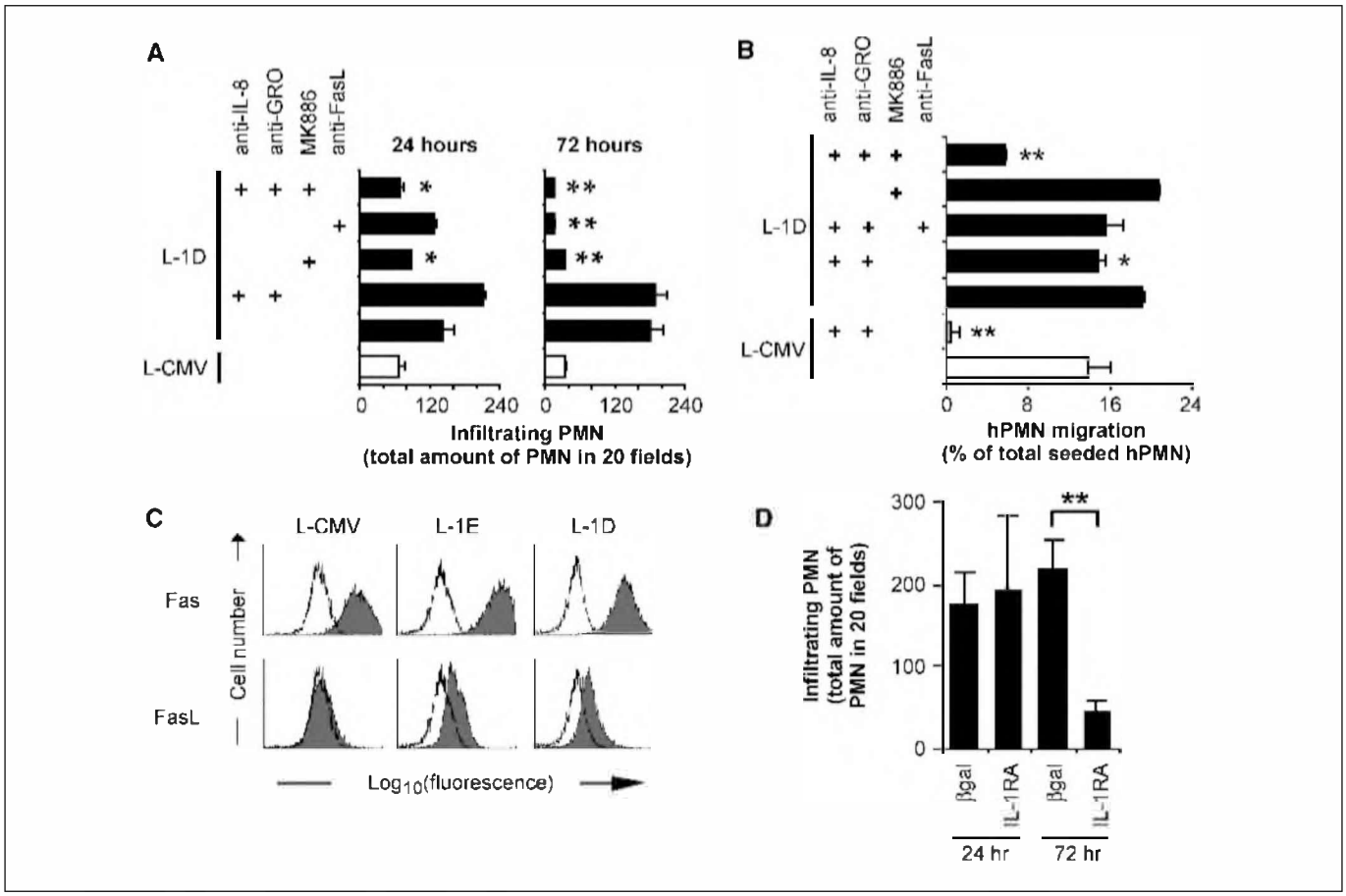

Figure 3. Factors involved in PMN recruitment. *A*, effect of IL-8, GRO. FasL, and leukotriene neutralization on *in vivo* mPMN recruitment at 24 and 72 hours after melanoma cell injection. *Columns*, mean number of recruited PMN of four mice; *bars*, SE. *, $P < 0.05$; **, $P < 0.001$. Only clearly viable PMNs were counted. *B*, inhibition of hPMN migration after treatment of melanoma cell–conditioned media obtained from L-CMV and L-1D cells with anti–IL-8, anti-GRO, and anti-FasL neutralizing antibodies and MK886. *Columns*, average migration as percentage of total PMN; *bars*, SE. The data for L-1D cells correspond to a representative experiment of three with similar results, done with PMN obtained from selected donors. The data for L-CMV cells correspond to a representative experiment done with samples from five donors. *, $P < 0.05$; **, $P < 0.01$, ANOVA for each antibody or inhibitor treatment compared with the untreated cell-conditioned media. *C*, expression of Fas and FasL at the cell surface of melanoma cells. FACS analysis was done as described in Materials and Methods with specific antibodies (*shaded histogram*) or isotypic control antibodies (*white histogram*). *D*, transient expression of IL-1 receptor antagonist (*IL-1RA*) in L-1D cells inhibited mPMN recruitment *in vivo*. L-1D cells were transduced with an adenoviral dose of 5×10^8 TCID50/mL, 24 hours before injection in mice. *Columns*, mean number of recruited PMN of four mice; *bars*, SE. **, $P < 0.001$.

control cells transduced with Adβgal had no effect (Fig. 4*B*). In close coincidence with the *in vivo* data showing rejection of bystander nonengineered melanoma cells, hPMNs were able to kill parental IIB-MEL-LES and control L-CMV cells when these cells were mixed with L-1D cells even at a ratio of 4:1 (Fig. 4*C* and data not shown). This bystander effect was only observed in the presence of L-1D cells, whereas L-1D–derived conditioned media or extracellular matrix had no effect (data not shown). This result indicates that hPMN must directly interact with L-1D cells to trigger their cytotoxic capacity against control cells.

To further confirm that the cytotoxic activity of hPMN is under SPARC control, we transiently reexpressed SPARC in L-1D cells using an AdSPARC vector. Treatment of L-1D cells with AdSPARC at 5×10^8 TCID50/mL, which transduced on average 55% of L-1D cells, reverted hPMN-mediated L-1D lysis by almost 50% compared with Adβgal-transduced cells (Fig. 5*A*). Lack of complete reversion was probably due to the stimulation of hPMN cytotoxicity by non-transduced cells. In coincidence, addition of 125 and 500 nmol/L native SPARC partially reverted hPMN cytotoxic capacity induced by L-1D cells (Fig. 5*B*), demonstrating that SPARC produced by human melanoma cells modulates PMN antitumor cytotoxic activity.

Isolated hPMNs enter by default to an apoptotic program *in vitro* (28). Coincubation of hPMN with L-1D cells grown as a monolayer had no effect on PMN apoptosis as measured by annexin V staining (Fig. 5*C*). On the contrary, PMN apoptosis was reduced by almost 20% in the presence of L-CMV cells (Fig. 5*C*). These results were confirmed by visual inspection of PMN nuclear morphology; 62.7 ± 8.1% (mean ± SE of six experiments) of control hPMN seemed apoptotic compared with 50.4 ± 5.3% in the presence of L-1D cells (mean ± SE; $P = 0.13$, compared with the control, Wilcoxon rank test) and with 17.2 ± 5.0% in the presence of L-CMV cells (mean ± SE; $P < 0.01$, compared with the control). Moreover, incubation of hPMN with SPARC also induced 44 ± 7% and 46 ± 4% (mean ± SE of three experiments) decreases in the proportion of apoptotic hPMN compared with medium alone as determined by annexin V or propidium iodide staining, respectively (Fig. 5*D* and data not shown). These results indicate that SPARC inhibition of hPMN lytic capacity against melanoma cells might be linked with the inhibition of hPMN apoptosis.

Table 1. *In vivo* rejection of different human melanoma cells coinjected with L-1D cells

	L-1D					L-CMV
	1:0*	1:1	1:0.67	1:0.25	1:0.1	1:1
IIB-MEL-LES	13/14†,‡	0/8	0/8	0/15‡		
L-CMV	13/13‡					
L-βgal	10/10‡	0/5			2/7	
IIB-MEL-J	9/10	2/10				9/10

NOTE: Control cells (5×10^6) were coinjected with different amounts of L-1D cells in the left flank of nude mice, and tumor growth was monitored in all the experiments for 6 months.
*Ratio of coinjected cells (i.e., IIB-MEL-LES: L-1D).
†Number of mice that developed tumors per total number of mice.
‡Cumulative data from two independent experiments.

Discussion

Here, we report a cohesive set of data demonstrating that SPARC plays an essential role in the direct modulation of the antitumor PMN cytotoxic capacity. In addition, knockdown of SPARC expression by human melanoma cells promoted the *in vivo* recruitment of mPMN to the site of injection of tumor cells and the *in vitro* transmigration of hPMN toward melanoma cells.

SPARC expression and polymorphonuclear leukocyte recruitment. We found a clear correlation between the increased *in vivo* recruitment of mPMN at 24 hours and the increased *in vitro* migration of hPMN following knockdown of SPARC expression. This effect seems to be mediated by chemotactic factors produced by the melanoma cells themselves, suggesting that SPARC might modulate a unique signaling pathway that converges into the regulation of leukotrienes, IL-8, and GRO. IL-8 and GRO transcription is stimulated by nuclear factor κB activity (29, 30) but, although the signaling pathways leading to leukotriene production had been explored (31), no link between nuclear factor κB and leukotriene production has been reported. Potential mediators of SPARC signaling are cell surface integrins. In that respect, integrin activation by fibronectin induced IL-8 and GRO expressions in melanoma cells and monocytes (32, 33). In addition, integrins modulate leukotriene production and secretion by neutrophils (34). Because SPARC regulates fibronectin levels in endothelial cells (35) and modulates integrin-mediated melanoma cell adhesion to different matrices,[6] we hypothesize that SPARC might act on an integrin-dependent pathway to regulate IL-8, GRO, and leukotriene production.

FasL and IL-1 are required in the second stage of PMN recruitment that follows knockdown of SPARC expression, suggesting their link to SPARC within a unique signaling pathway. IL-1 production was not detected in melanoma cells, suggesting that it is produced by host cells, most probably by apoptotic PMNs. FasL expression by melanoma cells has been initially associated with tumor escape by virtue of its immune suppressive activity at privileged sites (36). However, cells engineered to express FasL induced a strong PMN-mediated inflammatory response (25). These conflicting results were partially explained by the presence of transforming growth factor β (TGFβ) at immune privileged sites, which seemed to block the proinflammatory activity of FasL (37). Interestingly, SPARC has been shown to induce TGFβ expression and to stimulate the TGFβ signaling pathway (38, 39), suggesting a potential link between SPARC, TGFβ, and FasL on the activation of PMN recruitment and inflammation. Despite some discrepancy (40), SPARC-null mice showed enhanced inflammatory responses compared with wild-type littermates in three different murine models (41, 42), opening the intriguing possibility that SPARC might also regulate inflammatory processes in other scenarios as well.

SPARC expression and activation of polymorphonuclear leukocyte antitumor activity. The *in vivo* data indicate that persistent PMN recruitment is crucial for rejection of SPARC-sup

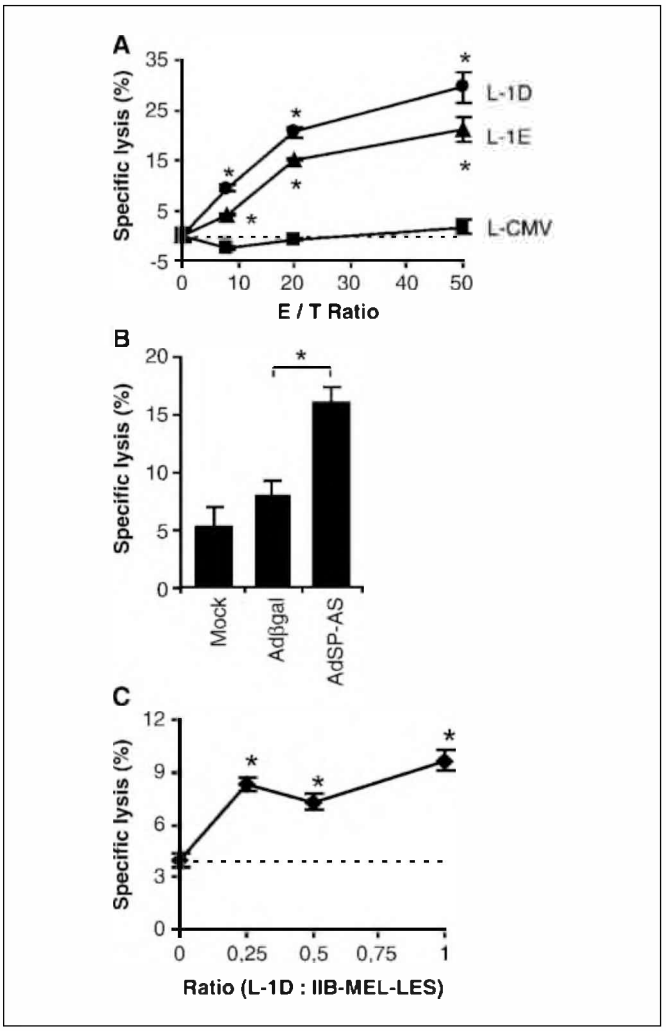

Figure 4. *In vitro* activation of PMN cytotoxic capacity against melanoma cells. *A*, *in vitro* dose-dependent cytolysis of L-1D and L-1E cells by hPMN. ■, L-CMV; ▲, L-1E; ●, L-1D. *Points*, mean; *bars*, SE. Representative of four experiments. *, $P < 0.001$. *B*, *in vitro* lysis of A375N cells by hPMN following transduction of A375N cells with AdSP-AS at 5×10^7 TCID50/mL. PMN cytotoxic activity was assessed 24 hours after cell transduction. *Columns*, mean of six independent experiments; *bars*, SE. *, $P < 0.001$. *C*, *in vitro* cytolytic activity of hPMN against ^{51}Cr-labeled IIB-MEL-LES cells cocultured with different amounts of L-1D cells. *Points*, mean; *bars*, SE. Representative of three experiments. *, $P < 0.001$. *B* to *E*, ratio of PMN to melanoma cells was 25:1.

[6] M.J. Alvarez et al., manuscript in preparation.

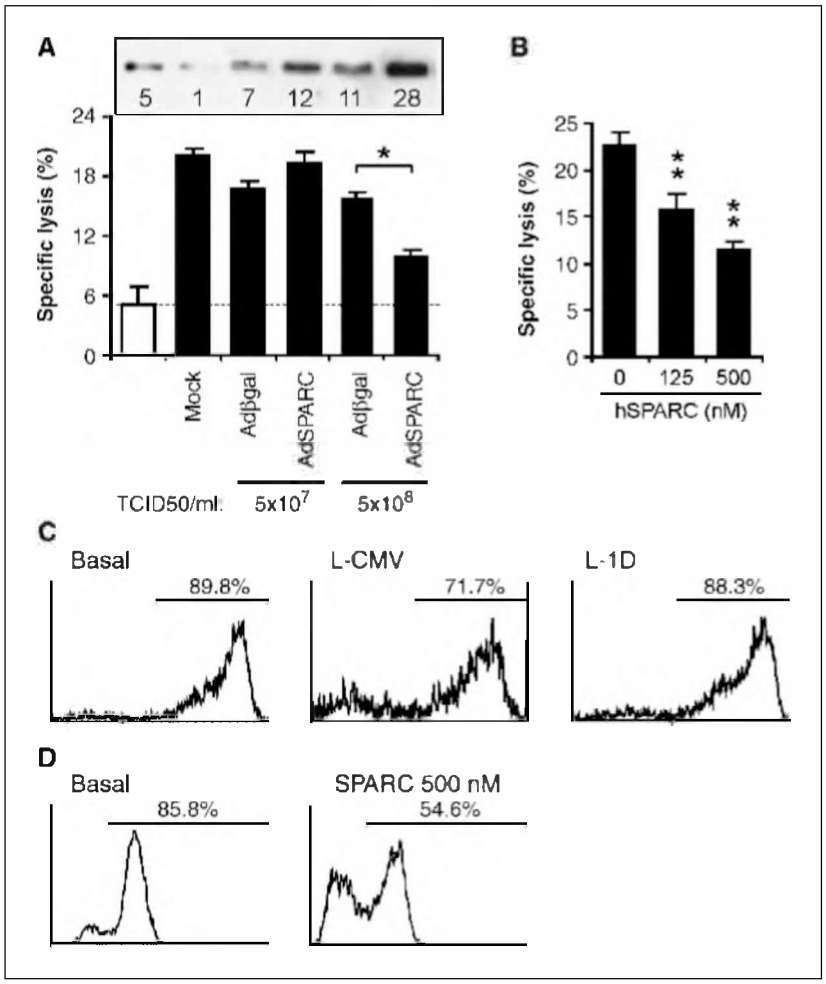

Figure 5. SPARC inhibits PMN cytotoxic activity against L-1D cells. *A*, SPARC reexpression in L-1D cells inhibits hPMN cytotoxicity against melanoma cells. Cytotoxic activity of hPMN was assessed 24 hours after adenovirus-mediated gene transfer. *Columns*, mean of four independent experiments (*white columns*, L-CMV cells; *black columns*, L-1D cells); *bars*, SE. *, $P < 0.001$. *Inset*, SPARC immunoblot of 1 μg of total protein produced by L-CMV and L-1D cells 48 hours after adenovirus-mediated gene transfer. Numbers indicate the relative absorbance of each band. *B*, purified human SPARC inhibits hPMN-mediated cytotoxicity of L-1D cells *in vitro*. *Columns*, mean of four independent experiments; *bars*, SE. **, $P < 0.0001$. *C*, control L-CMV but not SP-AS L-1D cells inhibit the default apoptosis program of hPMN *in vitro*. Annexin V staining of hPMN preincubated 16 hours with L-CMV or L-1D cells. Only metabolically active cells, identified by propidium iodide exclusion, were analyzed by flow cytometry. Proportion of apoptotic cells in percentage. *D*, SPARC protein inhibits the default apoptosis program of hPMN *in vitro*. Apoptosis was evaluated by annexin V staining as depicted in *C*.

cells. However, melanoma cells expressing SPARC did also promote the initial recruitment of PMNs that were unable to inhibit tumor growth. This, in turn, was followed by a decrease in PMN recruitment. Based on this evidence, we propose that SPARC levels are critical in directing PMN activation. Moreover, the lytic activity of PMNs obtained from all the donors was regulated by SPARC, indicating that SPARC is a key molecule in this process. If SPARC expression is suppressed, the intimate contact between PMNs and tumor cells promotes their antitumor cytotoxic activation. However, if high SPARC levels repress their activation, recruited PMN could favor tumor establishment. In agreement with this hypothesis, a dual PMN functionality is currently being suggested by the literature. PMN potentiates tumor progression of squamous epithelial carcinoma through degradation of extracellular matrix, activation of fibroblasts, and enhanced angiogenesis (43). Moreover, mouse melanoma cells require host PMN for rapid growth after injection in nude mice (44). On the other hand, PMNs do play an effector role in tumor rejection on activation with cytokines, chemokines, or FasL expression (14). The molecular basis for this dual role of PMN is still unclear as it is difficult to reconcile the dual role of IL-8 and GRO as chemotactic factors for PMN and autocrine growth factors for melanoma growth. Recently, it was shown that IL-8 production by melanoma cells stimulated the *in vivo* growth of a metastatic melanoma cell line in the presence of a strong but inactive PMN infiltrate (45). In the same study, IL-8 induced a PMN-mediated rejection of another nonmetastatic melanoma cell line, suggesting that a metastatic "status" dictates whether IL-8 will promote tumor progression or regression (45). Instead, we propose that PMN activation is not related to the metastatic status but to the presence or not of a PMN "trigger signal" in malignant cells. In the absence of this signal, PMN recruited by chemotactic signals will eventually promote tumor progression (21). Thus, tumor cell rejection by PMN is not merely dependent on their recruitment but there are further downstream signals necessary to activate them locally. We propose that SPARC might modulate a general mechanism that governs both PMN recruitment and activation. An intriguing possibility is that SPARC might affect PMN capacity to attack tumor cells by modulating their apoptotic default pathway. It can be hypothesized that the inhibition of PMN apoptosis *in vivo* might avoid recruitment of further waves of PMN and then favor tumor establishment.

The fact that exogenously added SPARC reverted the *in vitro* lytic capacity of PMN but was unable to revert the *in vivo* bystander effect even when SPARC-producing cells accounted for 90% of total injected cells might indicate a role for additional yet unidentified proteins *in vivo* that might be involved in tumor rejection and are under SPARC regulation. Alternatively, endogenous SPARC might induce an intracellular change that exogenous SPARC is unable to revert. SPARC is widely accepted as a secreted protein that seems to exert its effect from outside the cell. However, very recent evidence indicates that SPARC might be intracellularly incorporated by certain cell types and translocated

to the nuclei (46). Moreover, we observed SPARC expression in nuclei of human melanoma samples through immunohistochemical studies using a SPARC specific antibody.[7] Finally, both alternatives might coexist *in vivo*.

Role of SPARC produced by malignant cells in tumor progression. SPARC expression was associated with the neoplastic progression of different types of human cancer. Interestingly, SPARC is expressed by malignant cells, fibroblasts, and tumor endothelium in melanomas and gliomas (47, 48), whereas it is mainly expressed by tumor fibroblasts and tumor endothelium in most adenocarcinomas (9, 10). An intriguing question is whether SPARC produced by the malignant cells has a different role compared with SPARC produced by tumor stroma cells. In this regard, SPARC expression by the malignant cells stimulated the invasive capacity of melanoma and glioma cells (4, 6, 49), whereas exogenously added SPARC obtained from different sources promoted migration of prostate and breast cancer cells toward a bone extract, indicating its possible role in the tropism of adenocarcinoma cells for metastatic sites (5). Recent attempts to elucidate the role of host-derived SPARC using SPARC null mice led to controversial results (42, 50). Whereas Brekken et al. (50) showed enhanced tumor growth in SPARC null mice, Sangaletti et al. (42) showed impaired tumor growth in SPARC null mice. Whereas these differences might depend on the tumor model, both studies suggested a link between tumor formation and host-dependent extracellular matrix assembly, which in our studies was impossible to assess due to the prompt recruitment of PMN and rejection of malignant cells. The present study, in which the modulated variable (SPARC production by the tumor cell) is indeed the one that changes during some tumorigenic processes (47, 48), validates and strengthens the role of SPARC as a direct inhibitor of the antitumor cytotoxic capacity of PMN and suggests its potential use as a target for cancer therapy.

[7] M.J. Alvarez, data not shown.

Acknowledgments

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