Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-2611

Research Article

Recognition of Live Phosphatidylserine-Labeled Tumor Cells by Dendritic Cells: A Novel Approach to Immunotherapy of Skin Cancer

Michael R. Shurin,[1,2] Alla I. Potapovich,[3] Yulia Y. Tyurina,[3] Irina L. Tourkova,[1] Galina V. Shurin,[1] and Valerian E. Kagan[3]

Departments of [1]Pathology and [2]Immunology, University of Pittsburgh Medical Center, [3]Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

Dendritic cells (DC) loaded with tumor antigens from apoptotic/necrotic tumor cells are commonly used as vaccines for cancer therapy. However, the use of dead tumor cells may cause both tolerance and immunity, making the effect of vaccination unpredictable. To deliver live tumor "cargoes" into DC, we developed a new approach based on the "labeling" of tumors with a phospholipid "eat-me" signal, phosphatidylserine. Expression of phosphatidylserine on live tumor cells mediated their recognition and endocytosis by DC resulting in the presentation of tumor antigens to antigen-specific T cells. In mice, topical application of phosphatidylserine-containing ointment over melanoma induced tumor-specific CTL, local and systemic antitumor immunity, and inhibited tumor growth. Thus, labeling of tumors with phosphatidylserine is a promising strategy for cancer immunotherapy. [Cancer Res 2009;69(6):2487–96]

Introduction

On average, approximately 90 mutant genes can be expressed in a single tumor cell (1, 2), some of which encode tumor antigens (TA). With the identification of more than 1,000 human TA, recent strategies for developing therapeutic cancer vaccines have focused on targeted delivery of TA to dendritic cells (DC), the most effective antigen-presenting cells (APC; ref. 3). Their crucial role in initiating antitumor immunity is due to the processing of TA through the MHC class I and II pathways and presenting antigenic peptides to TA-specific $CD8^+$ and $CD4^+$ T cells. "Packaging" of TA to load DC is pivotal to vaccination strategies as it affects the antigen engagement in processing and presentation pathways that direct T-cell responses (4, 5). Most of ongoing the DC-based clinical trials use either DC pulsed with known TA or DC loaded with killed tumor cells (6). The key advantage of DC loading with tumor cells is that they provide a universal source of TA for broadening T-cell responses against the tumor by inducing MHC class I– and class II–restricted responses without the need for predetermined allele-specific peptides (7). However, a serious drawback is the absence of adequate protocols to kill tumor cells (apoptotically or necrotically) to effectively induce antitumor immune responses rather than tolerance or autoimmunity (8, 9).

Preclinical and clinical studies revealed that apoptotic tumor cells can stimulate antitumor responses *in vitro* and *in vivo* (10–12), and that loading of DC with lysates of necrotic tumor cell materials leads to efficient TA presentation to T cells (13, 14). Antigen loading of DC with apoptotic tumor cells is superior to the use of necrotic cells in terms of antigen presentation to T cells (15, 16). However, uptake of apoptotic cells could inhibit DC maturation and antigen presentation (17, 18), and induce T-cell tolerance (19). Although it has been reported that DC efficiently phagocytized a variety of apoptotic and necrotic tumor cells, only exposure to the latter induced DC maturation and stimulation of both $CD4^+$ and $CD8^+$ T cells (20, 21), other workers concluded that both necrotic and apoptotic tumor cells are an equivalent resource of antigens for DC loading (22, 23). Thus, there is still no agreement on whether apoptotic or necrotic tumor cells should be used for DC-based therapeutic vaccinations (4).

A key step in engulfing dying cells is their recognition by phagocytes, which is facilitated by changes in composition of the cell membrane (24). Normally, cells maintain an asymmetric distribution of phospholipids across the plasma membrane, whereby anionic phospholipid—phosphatidylserine—is strictly confined to the inner cytosolic leaflet. During apoptosis (and possibly necrosis; ref. 25), phosphatidylserine asymmetry collapses, resulting in the egress of phosphatidylserine on the cell surface. Thus, externalized phosphatidylserine acts as an "eat-me" signal for phagocytes, including professional APC (26), and specific receptors defining phosphatidylserine recognition profiles have recently been identified (27, 28). We hypothesized that "labeling" of live tumor cells with signals readily recognizable by DC could be a novel approach to targeted delivery of live tumor "cargoes" into DC. Here, we report that enrichment of the tumor cell surface with phosphatidylserine is a sufficient signal facilitating the uptake of live tumor cells by DC, TA presentation, T-cell activation, and initiation of the local and systemic antitumor immune response in tumor-bearing mice. These findings may lead to development of efficient noninvasive topical therapy for skin cancer, and potentially, other immune-mediated skin diseases.

Materials and Methods

Mice. C57BL/6 male mice (6–8 weeks old), severe combined immunodeficiency (SCID) beige mice (Taconic Farms), and IFN-$\gamma^{-/-}$ mice (H-2^b; Jackson Laboratory) were maintained according to standard guidelines; experimental protocols were approved by Institutional Animal Care and Use Committees.

Cell cultures. Mouse melanoma B16, OVA-expressing B16, T-cell lymphoma EL4, and OVA-transfected EL4 subclone EG7 cells and

Requests for reprints: Valerian E. Kagan, Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA. Phone: 412-624-9479; Fax: 412-624-9361; E-mail: kagan@pitt.edu or Michael R. Shurin, Clinical Immunopathology, Department of Pathology, University of Pittsburgh, Pittsburgh, PA; Phone: 412-647-6140; Fax: 412-648-8158; E-mail: shurinmr@upmc.edu.

doi:10.1158/0008-5472.CAN-08-2611

Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-2611

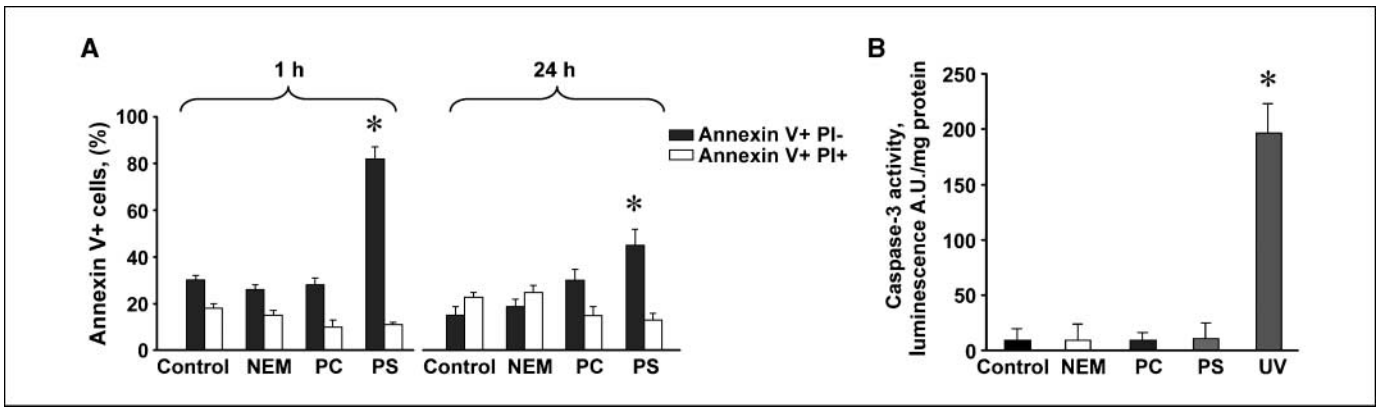

Figure 1. Treatment of B16 cells with NEM and liposomes containing phosphatidylcholine, and phosphatidylserine caused prolonged expression of phosphatidylserine on cell surface (*A*) without up-regulating caspase-3 activity (*B*). *A,* cells were treated with liposomes (see Materials and Methods) and phosphatidylserine externalization in cell membranes was determined with Annexin V (0.5 μg/mL, 1 and 24 h) using FACScan. Cells were lysed and caspase-3 activity was measured. As a positive control, tumor cells were UV irradiated. *Columns,* means; *bars,* SE (n = 3–4); *, $P < 0.05$ vs. control phosphatidylcholine. *PC,* phosphatidylcholine; *PS,* phosphatidylserine; *UV,* UV irradiation.

splenocytes were maintained in a complete RPMI 1640 supplemented with nonessential amino acids, 10% heat-inactivated fetal bovine serum, 2 mmol/L of L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. DC were generated from bone marrow hematopoietic precursors as described earlier (29). For DC maturation, cells were treated with lipopolysaccharide (1 μg/mL) and IFN-γ (10 ng/mL) for 24 h.

Preparation of liposomes. Small unilamellar liposomes containing phosphatidylcholine or phosphatidylserine/phosphatidylcholine (Avanti Polar Lipids) at a 1:1 ratio (4 mmol/L of total lipids) were prepared by sonication (5 × 30 s) in ice-cold PBS (30), and incorporated into the plasma membranes of cells as described by Tanaka and Schroit (31). Briefly, cells were resuspended in PBS (1×10^6 cells/mL), and treated with *N*-ethylmaleimide (NEM, 10 μmol/L, 10 min, 37°C; Sigma) to inhibit aminophospholipid translocase and block inward translocation of phosphatidylserine. Then, cells were treated with liposomes containing phosphatidylcholine (150 nmol/10^6 cells) or phosphatidylserine (as phosphatidylserine/phosphatidylcholine = 1:1 mixture; 150 nmol total phospholipids/10^6 cells) for 30 min at 37°C. Nonincorporated liposomes were removed by washing. Controls included nontreated tumor cells and cells treated with NEM alone. Externalized phosphatidylserine was determined by Annexin V binding (BD PharMingen).

Caspase-3 activity. Caspase-3 activity was measured using Caspase-Glo assay kit (Promega) and expressed as luminescence produced within 1 h of incubation per milligram of protein.

Phagocytosis assay. B16 tumor cells were stained with 1 μmol/L of CellTracker Orange CMTMR. DC were stained with 2.5 μmol/L of CFDA-SE (Vybrant CFDA SE Cell Tracer Kit; Molecular Probes). Cells were cocultured with mature and immature DC in complete medium at tumor cell/DC ratios of 1:1, 2:1, or 4:1 for 24 h at 37°C. Aliquots of DC/tumor cocultures (100 μL) were cytospun (100 × *g*, 3 min; Shandon) and analyzed by fluorescence microscopy (Nikon, Eclipse TE200). Analysis of engulfing of EL4 cells by immature DC used labeling with a red fluorescent dye PKH26 (1 mmol/L, 30 min) and DC with a green fluorescent dye PKH67 (2 mmol/L, 30 min). Then, EL4 cells were treated with NEM and liposomes and cocultured with DC at tumor/DC ratios of 1:1, 2:1, or 4:1 for 4 to 6 h at 37°C. Phagocytosis was assessed by flow cytometry. Phagocytosis was defined as the percentage of DC with double-positive fluorescent signals.

Antigen presentation assay. EL4 and EG7 tumor cells were treated with phospholipid-containing liposomes and cocultured with immature DC at a 1:1 ratio for 24 h at 37°C. After incubation, DC were isolated using CD11c Microbeads (Miltenyi Biotec). A similar procedure was used for isolation of control OVA-pulsed DC (1 mg/mL ovalbumin; Sigma) and nonpulsed DC. Cells were plated in 96-well plates (3×10^4 cells/well/100 μL). The B3Z T-cells (H-$2K^b$) specific for the $OVA_{257-264}$ peptide were then added (5×10^4 cells/100 μL) and cocultured with DC for 24 and 48 h. Control wells contained DC or T cells alone. After incubation, cell-free supernatants were collected and the levels of interleukin 2 (IL-2) produced by activated T cells were measured by ELISA (Endogen).

Animal tumor models. B16 cells were injected s.c. (0.5×10^6 cells/100 μL PBS) in the right flank of syngeneic immunocompetent C57BL/6 mice, SCID mice, or IFN-γ knockout mice. Topical application of ointments was started 1 to 2 days later. Basic ointment formulation consisted of a mixture of oleic acid, phosphatidylcholine or phosphatidylcholine + phosphatidylserine, and NEM at the same concentrations as were used for liposome formulation (molar ratio of oleic acid/DOPS/DOPC = 33:1:7 or a weight ratio = 1.6 g:150 mg:1 g). The ointment was applied twice daily on the skin area covering growing melanomas for 7 to 10 days and tumor size was estimated twice or thrice a week. Experiments included five to seven mice per group.

For analysis of tumor-specific lymphocytes, spleens were aseptically removed and ground; T cells were enriched on the nylon-wool columns and cultured in complete medium (1.0×10^6 cells/mL) with irradiated (20,000 rad) B16 or irrelevant RM1 tumor cells at a 5:1 ratio. Cell-free supernatants were collected 48 to 72 h later and the levels of IL-2 and IFN-γ were assessed by ELISA (Endogen). Determination of tumor-specific CTL *in vivo* was performed by analyzing OVA-specific $CD8^+$ T cells in spleens and lymph nodes in OVA-B16–bearing mice using iTAgTM MHC H-$2K^b$ OVA SA-PE Tetramers (Beckman Coulter). Lymphocytes were isolated 3 weeks after therapy, stimulated with $OVA_{257-264}$ peptide (1 μg/mL) and 10 IU/mL of IL-2 for 5 days, washed, and stained according to the protocols of the manufacturer.

Tumor-infiltrating mononuclear cells were visualized by using immunohistochemistry in Optimal Cutting Temperature (OCT)–embedded tumor samples. Tissue sections (4 μm) were air-dried overnight, immersed in ice-cold acetone for 10 min, and treated with hydrogen peroxide and Superblock (ScyTek Lab). Anti-mouse antibodies CD4 (1/300; BD PharMingen), CD8 (1/100; BD PharMingen), F4/80, and CD11c (1/200; Serotec) were applied overnight at 4°C. After washing, slides were incubated with biotinylated rabbit anti-rat (for CD4, CD8 and F4/80 staining, 1/250; Vector) or goat anti-hamster (for CD11c staining, 1/200; Vector) for 30 min, and treated with avidin-biotin complex (Vector) for 30 min. Amplification procedure using tyramide (1/200; NEN) was applied for detection of $CD8^+$ cells. HRP reaction was developed using amino-9-ethylcarbasole (Scytec) and slides were counterstained with hematoxylin.

1-Palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminocaproyl]-phosphatidylserine penetration through murine skin *in vivo*. 1-Palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminocaproyl] (NBD)–labeled phosphatidylserine (NBD-PS)–containing ointment was topically applied and skin biopsies were collected 0, 1, and 4 h later. Skin sections were prepared from OCT frozen tissues and analyzed on Leica confocal fluorescent microscope. The *Z* step for each scan was set at 0.1 μm and

Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-2611

images were reconstructed using Velocity software after noise reduction and uniform linear adjustments.

Analysis of intratumoral cytokines in live, freely moving animals was performed by a recently developed technique (32). CMA/20 microdialysis probes (CMA Microdialysis) were implanted inside of growing tumors and extracellular interstitial fluid was collected via the micropump-regulated circulation (16 μL/h) of buffer (4% w/v dextran-70 in PBS) in a sampling device. The outer cannula of the probe is made from nanoporous polyethersulfone and has a nominal molecular weight cutoff of 100,000 Da. Samples (~60–70 μL) were collected from animals sitting in specially designed chambers and the levels of 40 cytokines, chemokines, and growth factors were detected by Luminex multiplexed technique.

Phospholipid analysis. For evaluation of phosphatidylserine distribution after topical therapy of melanoma-bearing mice, ointment contained 20% of fluorescently labeled phosphatidylserine (NBD-PS; Avanti Polar Lipids). B16 tumors grew in mice for 7 days followed by application of NBD-PS ointment over the subcutaneous melanoma twice daily for 3 consecutive days. After tumor incision and triple enzyme digestion (collagenase/DNase/hualuronidase), tumor cells were washed and cytospun. For labeling nuclear DNA, cells were preincubated with 1 μg/mL of Hoechst (Molecular Probes) at 30°C for 30 min. Blue and green fluorescent staining was analyzed by confocal microscopy.

Lipids were extracted using Folch's procedure (33) and separated by two-dimensional high-performance TLC on silica G plates (5 × 5 cm, Whatman; ref. 34). Phospholipids were visualized by exposure to iodine vapors. Spots were scraped and lipid phosphorus was determined by a micro method (35).

Statistical analysis. Standard Student's t test and one-way or two-way ANOVA were performed to evaluate the significance of differences between experimental groups. The differences were considered statistically significant at $P < 0.05$. Data are means ± SE.

Results

"Phosphatidylserine-labeled" live tumor cells are actively recognized and engulfed by DC *in vitro*. We used phosphatidylserine-containing liposomes to enrich the surface of live B16 melanoma cells with phosphatidylserine. Because phosphatidylserine is known to be effectively internalized by aminophospholipid translocase (36), we treated cells with NEM, a nonspecific inhibitor of translocase activity. An assay with fluorescently labeled Annexin V revealed that up to 70% of B16 cells expressed phosphatidylserine on their surface ($P < 0.001$; Fig. 1*A*). The level of Annexin V+ cells decreased gradually to 30% after 24 h ($P < 0.05$). No activation of caspase-3, a biomarker of apoptosis, occurred in B16 cells treated with NEM and liposomes ($P > 0.1$; Fig. 1*B*). Simultaneous staining with propidium iodide revealed no induction of necrotic cell death. Furthermore, trypan blue exclusion confirmed the unchanged

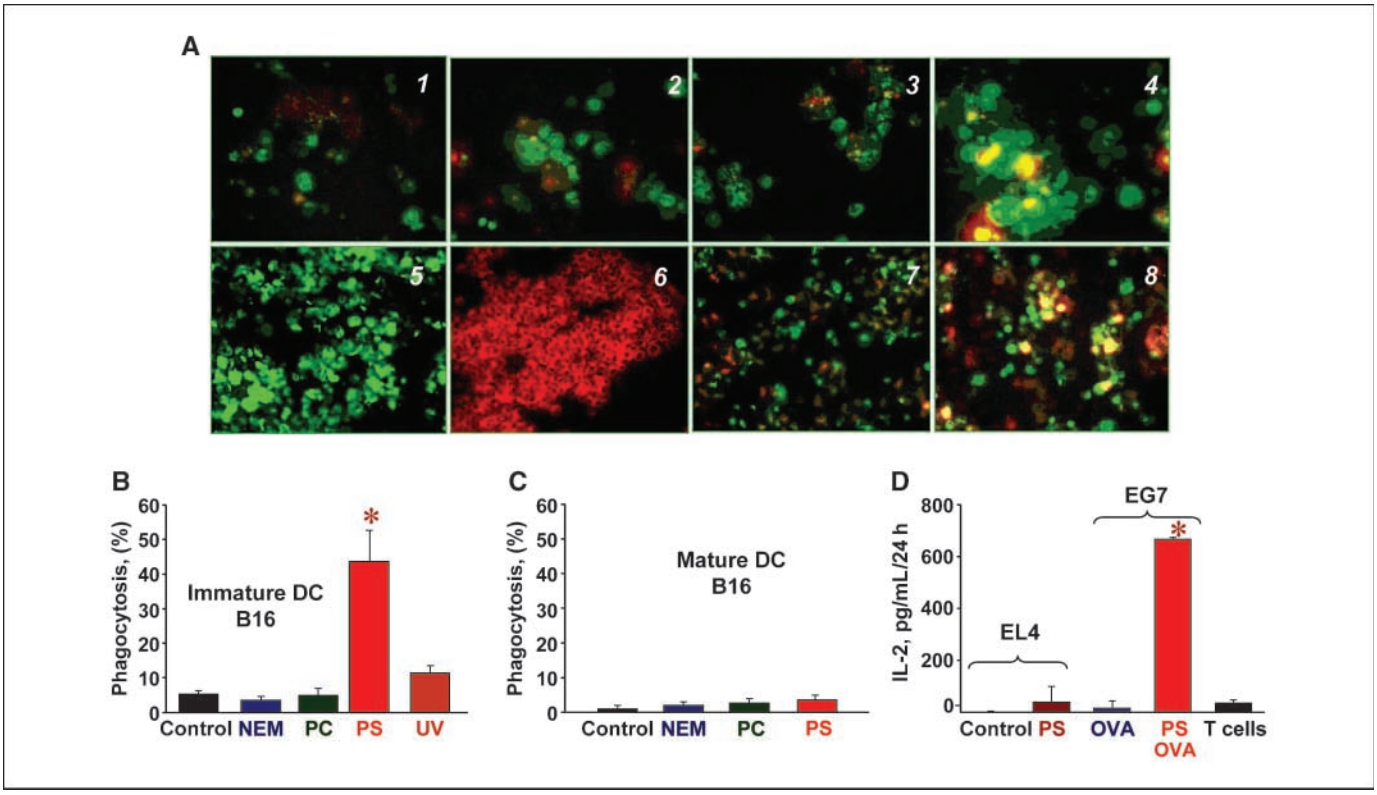

Figure 2. *A*, immature, but not mature, DC actively engulfed live tumor cells pretreated with phosphatidylserine-containing liposomes (*4* and *8*), but not phosphatidylcholine-liposomes (*3* and *7*); no engulfment was observed for tumor cells pretreated with NEM alone (*2*) or non-pretreated tumor cells (*1*). Bone marrow–derived DC were labeled with CMTMR cell Tracker Orange (1 μmol/L, 30 min; *6*) and coincubated with tumor cells labeled with CFDA CE green (2 μmol/L, 30 min; *5*) for 8 to 10 h (1:1–1:2 tumor/DC ratio). DC engulfing tumor cells were counted 10 h later as a percentage of the total number of DC in each sample in 10 high-power fields. Single and double-positive cells were analyzed by confocal microscopy. Representative images are shown ($n = 10$). *1-4,* B16 cells; *7-8,* EL4 cells. Only immature (*B*) but not mature (*C*) DC were able to engulf phosphatidylserine-labeled tumor cells. Uptake of UV-exposed B16 cells is shown for comparison (*B*). *D,* engulfment of phosphatidylserine-enriched live OVA-transfected EL4 cells (*EG7*) by DC was associated with presentation of $OVA_{257–264}$ peptide to antigen-specific $CD8^+$ B3Z T-cell clones. DC were coincubated overnight with nontreated EL4 cells, EL4 cells pretreated with phosphatidylserine-containing liposomes (*PS*), nontreated EG7 cells (*OVA*), and EG7 cells pretreated with phosphatidylserine-enriched liposomes (*PS-OVA*). Then, CD11c+ DC were isolated from tumor cells by magnetic microbeads. To detect the presence of $H\text{-}2K^b/OVA_{257–264}$ complexes on DC, B3Z cells specific for $OVA_{257–264}$ peptide were used. DC (3×10^4cells/well) were mixed with T-cell hybridoma (5×10^4 cells/well) for 18 h, and IL-2 production by activated T cells was assessed by ELISA. Negative controls: T cells, EL4, and EG7 cells pretreated with NEM and phosphatidylcholine-liposomes and coincubated with medium alone. Positive controls: DC pulsed with dialyzed OVA, which repeatedly provided 300 to 400 pg/mL of IL-2 production. *Columns,* means; *bars,* SE ($n = 4–6$); *, $P < 0.05$ vs. control, NEM, and phosphatidylcholine. *PC,* phosphatidylcholine; *PS,* phosphatidylserine.

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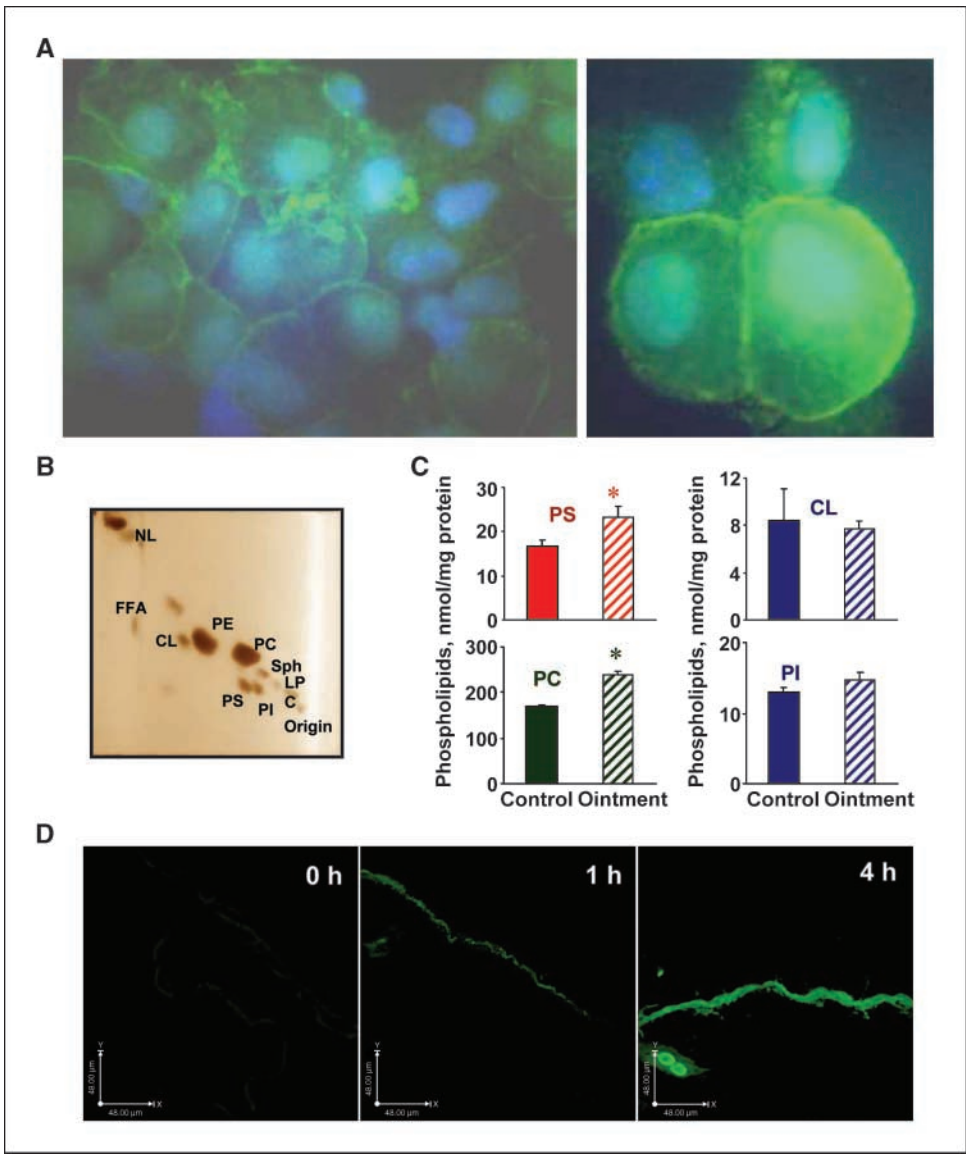

Figure 3. Visualization of green NBD-PS on tumor cells after topical application of NBD-PS–containing ointment on skin over growing B16 melanoma in mice (*A*) and analysis of phospholipid composition of mouse B16 cells (*B* and *C*). Tumor-bearing mice received either topical application of phosphatidylserine ointment over growing subcutaneous melanoma for 3 d (*Ointment*) or no treatment (*Control*). Tumors were removed, enzymatically digested, and cells were cytospun for confocal microscopy (*A*) or lipid analysis (*B* and *C*). *A,* for imaging phosphatidylserine in tumor cells *in vivo*, 20% of phosphatidylserine in ointment was replaced with fluorescent NBD-PS. Typical images of NBD-PS–positive (*green*) tumor cells isolated from ointment-treated mice. No fluorescence was seen in control animals. *B,* typical two-dimensional high-performance TLC of total lipids from *in vivo* growing B16 cells. *C,* quantitative analysis of phospholipids in melanoma cells. Both phosphatidylserine and phosphatidylcholine were markedly increased in tumor cells treated with phosphatidylserine-containing ointment ($n = 3$). *, $P < 0.01$ vs. control; *NL,* neutral lipids; *FFA,* free fatty acids; *CL,* cardiolipin; *PE,* phosphatidylethanolamine; *PC,* phosphatidylcholine; *PS,* phosphatidylserine; *PI,* phosphatidylinositol; *Sph,* sphingomyelin; *LPC,* lysophosphatidylcholine. *D,* analysis of murine skin permeability for phosphatidylserine from topically applied ointment *in vivo*. Ointment containing fluorescently labeled NBD-PS was applied on exposed areas of skin and biopsies were collected 0, 1, and 4 h later. Skin sections (10 μm) were analyzed by confocal microscopy.

longevity of tumor cells after liposome/NEM treatments. The rate of tumor cell proliferation assessed by ^{3}H-thymidine incorporation was not changed after the treatments (data not shown). Similar results were also obtained with EL4 cells (data not shown). Thus, we were able to enrich melanoma and lymphoma cells' surface with phosphatidylserine without triggering apoptotic or necrotic death pathways. Modified live tumor cells with increased levels of phosphatidylserine might provide an eat-me signal for phagocytic cells.

We next tested whether live phosphatidylserine-labeled tumor cells could be recognized by DC. B16 and EL4 tumor cells were labeled with a green fluorescent dye (Fig. 2*A5*) and DC were labeled with an orange/red dye (Fig. 2*A6*), and cells were mixed at different ratios for 4 to 24 h followed by the analysis of single-colored and double-colored cells by confocal microscopy. Figure 2 shows immature DC mixed for 10 h with nontreated B16 cells (Fig. 2*A*), B16 cells pretreated with NEM alone (Fig. 2*A2*), or B16 cells pretreated with phosphatidylcholine or phosphatidylserine-containing liposomes (Fig. 2*A3* and *A4*, respectively). Immature DC were able to phagocytose B16 cells expressing phosphatidylserine (*yellow cells,* Fig. 2*A4*). Similar results were obtained with EL4 cells; Fig. 2*A8* shows DC and phosphatidylserine-liposome–treated EL4 cells, whereas Fig. 2*A7* shows a representative negative control with phosphatidylcholine-liposome–treated tumor cells. Analysis of cells at higher magnifications confirmed the presence of tumor cell materials in DC.

Next, we quantified the percentage of DC engulfing B16 melanoma cells versus the total number of DC by counting 10 high-power fields on each slide (Fig. 2*B*). As the endocytic potential of DC is developmentally regulated, mature DC—known to display low phagocytic activity—served as a negative control (Fig. 2*C*). Immature, i.e., endocytically active, but not mature DC, were able to ingest phosphatidylserine-labeled live tumor cells: up to 50% of DC contained engulfed tumor materials ($P < 0.01$; Fig. 2*B*). We found that B16 cells were phagocytosed by immature DC at an early time point after UV irradiation (4 h; Fig. 2*B*). This is in line with the known facts that UV irradiation induces early apoptosis of tumor cells resulting in phagocytosis of dying tumor cells by APC (37). However, phagocytosis of UV-irradiated apoptotic B16 cells was less effective than phagocytosis of tumor

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cells after treatment with phosphatidylserine-containing liposomes (Fig. 2*B*). This can be due to a markedly higher level of phosphatidylserine present on the surface after incubation with phosphatidylserine-containing liposomes versus amounts of phosphatidylserine externalized as a result of UV irradiation. Similar results were obtained with EL4 cells cocultured with immature DC. Up to 60% of DC were double-positive after 4 h of coincubation with phosphatidylserine-enriched, but not phosphatidylcholine-enriched tumor cells, with a maximum endocytosis observed at 1:1 to 1:3 DC/EL4 ratio (data not shown). Altogether, results show that DC could engulf live tumor cells with externalized phosphatidylserine on their surface *in vitro*.

We further studied whether DC could process and present TAs from ingested live tumor cells. We used EG7 cells, OVA-expressing

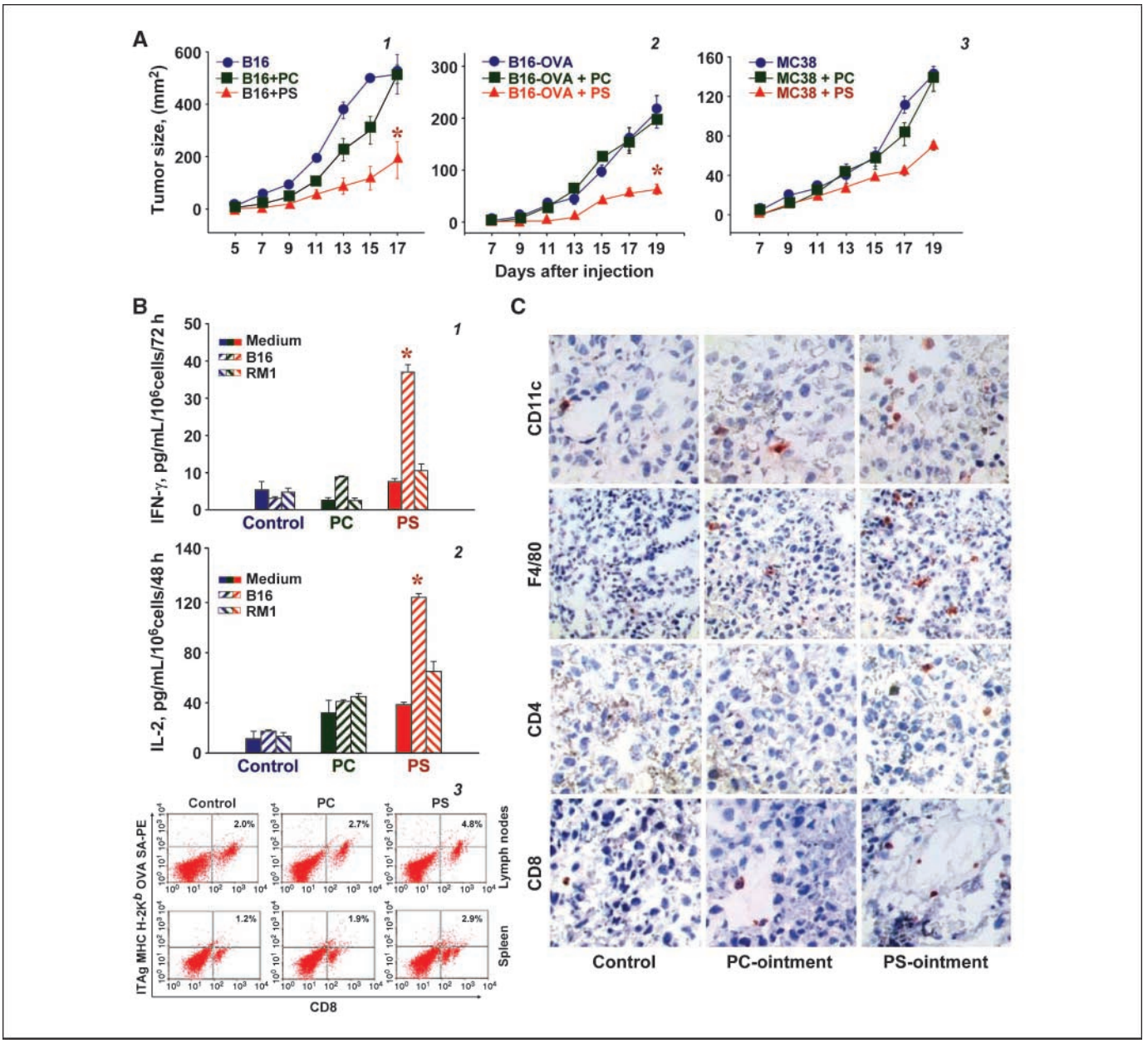

Figure 4. Topical application of phosphatidylserine ointment caused significant inhibition of tumor growth in mice (*A*) associated with generation of tumor-specific T cells (*B*) and tumor infiltration by DC, macrophages, and CD8 T cells (*C*). Wild-type B16 melanoma (*1*), OVA-expressing B16 melanoma (*2*), or colon adenocarcinoma MC38 (*3*) cells were inoculated s.c. and topical phospholipid ointment–based therapy was initiated 24 h later. Ointments were applied for 2 wk and tumor size was determined every other day with a caliper (*A*). *Points,* means; *bars,* SE ($n = 3$); *, $P < 0.01$ vs. phosphatidylcholine ointment and saline therapy.
B, T cells were isolated from the spleens (*1*, *2*, and *3*) and lymph nodes (*3*) obtained from wild-type (*1* and *2*) and OVA-expressing (*3*) B16-bearing mice treated with phospholipid-containing ointments. In wild-type B16 model (*1* and *2*), cells were stimulated with medium or irradiated B16 or irrelevant RM1 cells and IFN-γ and IL-2 levels in cell-free supernatants were assessed by ELISA. *Columns,* means; *bars,* SE ($n = 3$); *, $P < 0.001$ vs. control and phosphatidylcholine (one-way ANOVA). In the B16-OVA model (*3*), nylon wool–enriched T cells from tumor-bearing mice were isolated on day 21 after tumor injection and topical therapy and stimulated for 5 d with $OVA_{257–264}$ peptide SIINFEKL (1 μg/mL) and IL-2 (10 IU/mL). Cells were analyzed for the presence of $CD8^+$ T cells specific for OVA peptide. Percentage of $CD8^+$ MHC class I tetramer+ cells are shown from a representative experiment ($n = 2$). *C,* tumors were harvested 1 wk after the application of ointments over B16 growing melanoma. Immunohistochemical analysis was performed using CD11c Ab for DC, F4/80 Ab for macrophages, and CD4, CD8 Ab for T cells. Representative results are shown ($n = 3$). *PC,* phosphatidylcholine; *PS,* phosphatidylserine.

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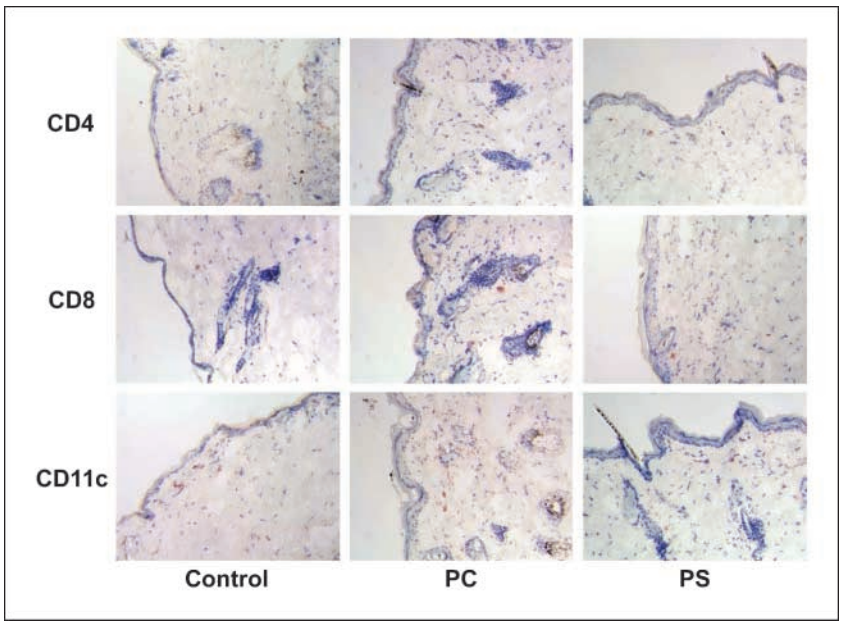

Figure 5. Immunohistochemical analysis of normal skin treated with topical ointments containing phosphatidylcholine and phosphatidylserine. Two sides of the shaved back in a mouse were treated with phosphatidylcholine-containing and phosphatidylserine-containing ointments by daily topical application (~50 μL) for 7 d. Immunohistochemical staining of tissue was performed as described in Materials and Methods. Staining with secondary antibodies without primary antibodies served as a control; *red-brown cells,* positive staining.

EL4 cells, pretreated them with NEM/phosphatidylserine-containing liposomes and then coincubated these cells with DC. To detect the presence of H-2K^b/OVA$_{257–264}$ complexes on DC, B3Z cells, a CD8$^+$ T-cell hybridoma specific for the OVA$_{257–264}$ peptide, were used as responders with the IL-2 production reflecting the level of T-cell activation (Fig. 2*D*). IL-2 expression was up-regulated 20-fold to 25-fold in T cells stimulated by DC cocultured with phosphatidylserine-labeled (but not with nonphosphatidylserine-enriched) OVA-positive tumor cells. Thus, DC were able to present a specific antigenic peptide from a model TA OVA in the context of a MHC class I complex when live tumor cells, used as a source of TA, expressed phosphatidylserine on their surface.

Topical therapy with phosphatidylserine ointment induced local and systemic antitumor immune responses and inhibited tumor growth in murine melanoma models *in vivo*. Next, we examined phosphatidylserine as a modifier of tumor cells *in vivo* with the ultimate goal of inducing antitumor immune response in a tumor-bearing host. Initially, we tested whether the application of phosphatidylserine-containing ointment onto skin over growing melanomas resulted in phosphatidylserine penetration through skin and incorporation into the tumor cell membrane. Phosphatidylserine ointment containing green fluorescent NBD-PS was applied daily for 3 consecutive days over growing B16 melanomas. Green NBD-PS was readily detectable on the surface of 45 ± 7% (n = 3) of all tumor cells isolated from ointment-treated, but not control, mice (Fig. 3*A*). Considering that only 20% of phosphatidylserine in ointment was labeled with NBD, these data suggest that topical skin application of phosphatidylserine-containing ointment was associated with phospholipid penetration through skin and incorporation in surrounding tumor cells. This conclusion was confirmed by direct measurement of phospholipid levels in tumor cells isolated from phosphatidylserine ointment–treated or nontreated B16-bearing mice. Lipids were extracted and separated by two-dimensional high-performance TLC (Fig. 3*B*). Up to 30% increased levels of phosphatidylserine and phosphatidylcholine content in melanoma cells isolated from ointment-treated, but not control (i.e., nontreated), mice were observed (P < 0.01; Fig. 3*C*). In addition, we found that phosphatidylserine entered the upper layer of the skin (epidermis) within 1 hour and was seen below the dermis in adipose tissue in 4 hours (Fig. 3*D*). These results confirm that phosphatidylserine from topical ointment application is able to penetrate through mouse skin and incorporate into cell membranes of intradermal and subcutaneous tumor cells *in vivo*.

To determine whether ointment-derived phosphatidylserine affects tumor growth *in vivo*, B16 melanoma-bearing mice were treated with phosphatidylserine ointment or control phosphatidylcholine ointment for 2 weeks and tumor size was determined before and during therapy (Fig. 4*A1*). Statistical analysis of data revealed significant growth inhibition after treatment with phosphatidylserine-based, but not phosphatidylcholine-based ointment (P < 0.01, two-way ANOVA). Average tumor size was 192 ± 26 mm^2 in mice treated with phosphatidylserine-containing ointment, whereas tumors reached 500 to 530 mm^2 in mice treated with saline or phosphatidylcholine-containing ointment.

Inhibition of tumor growth by phosphatidylserine ointment–based therapy was associated with the generation of B16-specific T cells as evidenced by measurements of cytokine production by splenic T-lymphocytes restimulated with tumor cells after topical therapy (Fig. 4*B1* and *B2*). There was a 3-fold to 7-fold increase in IFN-γ and IL-2 production in T cells obtained from B16-bearing mice treated with phosphatidylserine ointment and restimulated with B16 cells, but not irrelevant RM1 tumor cells (P < 0.001 versus control, phosphatidylcholine, medium, and RM1 groups; ANOVA). Thus, application of phosphatidylserine ointment in melanoma-bearing mice caused the generation of tumor-specific IFN-γ–producing and IL-2–producing T cells *in vivo*. Furthermore, as shown in Fig. 4*A2*, topical therapy of OVA-expressing B16 tumors with phosphatidylserine ointment resulted in the generation of OVA-specific CTL at a significantly higher level than in control saline or phosphatidylcholine-treated mice. This was also associated with a significant inhibition of tumor growth in phosphatidylserine-treated animals (P < 0.01, two-way ANOVA). This suggests that phosphatidylserine-based therapy induced TA-specific immune response *in vivo* which is associated with significant inhibition of tumor growth.

Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-2611

Given that the presence of intralesional mononuclear infiltrate correlates with the intensity of the immune response in a variety of cancers (38), we next evaluated tumor-infiltrating leukocytes in B16-bearing mice 1 week after completion of therapy. Application of phosphatidylserine ointment, but not phosphatidylcholine ointment, caused a statistically significant increase in tumor-infiltrating DC, macrophages, and $CD8^+$ T cells ($P < 0.05$, ANOVA; Fig. 4*C*). Although the number of $CD4^+$ tumor-infiltrating T cells were high in the same areas of tumor, no statistical analysis of these cells was possible due to the overall low number of cells in all tested specimens. Thus, immunohistochemical data also suggest that topical phosphatidylserine ointment therapy was associated with the development of antitumor immune response in tumor-bearing mice.

In addition, we showed that when murine colon carcinoma MC38 cells (of noncutaneous origin) were injected s.c. in syngeneic animals and mice treated with saline, phosphatidylcholine-based or phosphatidylserine-based ointments, phosphatidylserine-containing ointment significantly inhibited tumor growth in comparison to both control groups ($P < 0.01$, ANOVA). However, growth inhibition of MC38 tumor was lower than in B16-bearing mice (Fig. 4*A3*).

Using freshly isolated splenocytes as a control for nonmalignant cells, we found that treatment with phosphatidylserine-containing liposomes did not induce their engulfment by immature DC *in vitro*, suggesting that nonmalignant cells are not readily phagocytized by DC (Fig. 4*B3*). To test whether phosphatidylserine ointment affects nonmalignant cells *in vivo* and induces inflammatory responses in normal skin, the skins of tumor-free mice were treated with phosphatidylcholine-containing or phosphatidylserine-containing ointments and subjected to histologic analyses of leukocyte infiltration. No signs of increased infiltration of normal skin by T cells and DC was seen after ointment therapy (Fig. 5*FB*). H&E staining also did not reveal increased infiltration by mononuclear cells, suggesting that the tested ointments did not induce significant inflammatory responses in exposed skin areas.

Next, we tested whether phosphatidylserine ointment–based topical therapy modulated the cytokine network in a tumor microenvironment. Intratumoral cytokines were assessed in specimens harvested for 3 days from B16 tumors during phosphatidylcholine- and phosphatidylserine ointment–based therapy (Fig. 6). Although many cytokines were undetectable inside of a growing melanoma, the application of phosphatidylserine ointment was associated with a decrease of intratumoral IL-4 and tumor necrosis factor-α and an increase in intratumoral IL-1α and MIP-1α ($P < 0.05$, ANOVA); this suggests that topical therapy altered the intratumoral cytokine network in melanoma. These results confirm that the cytokine network of many common tumors may be rich in inflammatory cytokines but generally lacks cytokines involved in specific and sustained immune responses (39).

To further test the involvement of the immune system in antitumor activity of phosphatidylserine-containing ointment, we used immunocompetent mice, which received two subcutaneous injections of B16 cells on both right and left flanks, but phosphatidylserine ointment was applied over a growing melanoma on the left side only (i.e., only one tumor was treated). Results

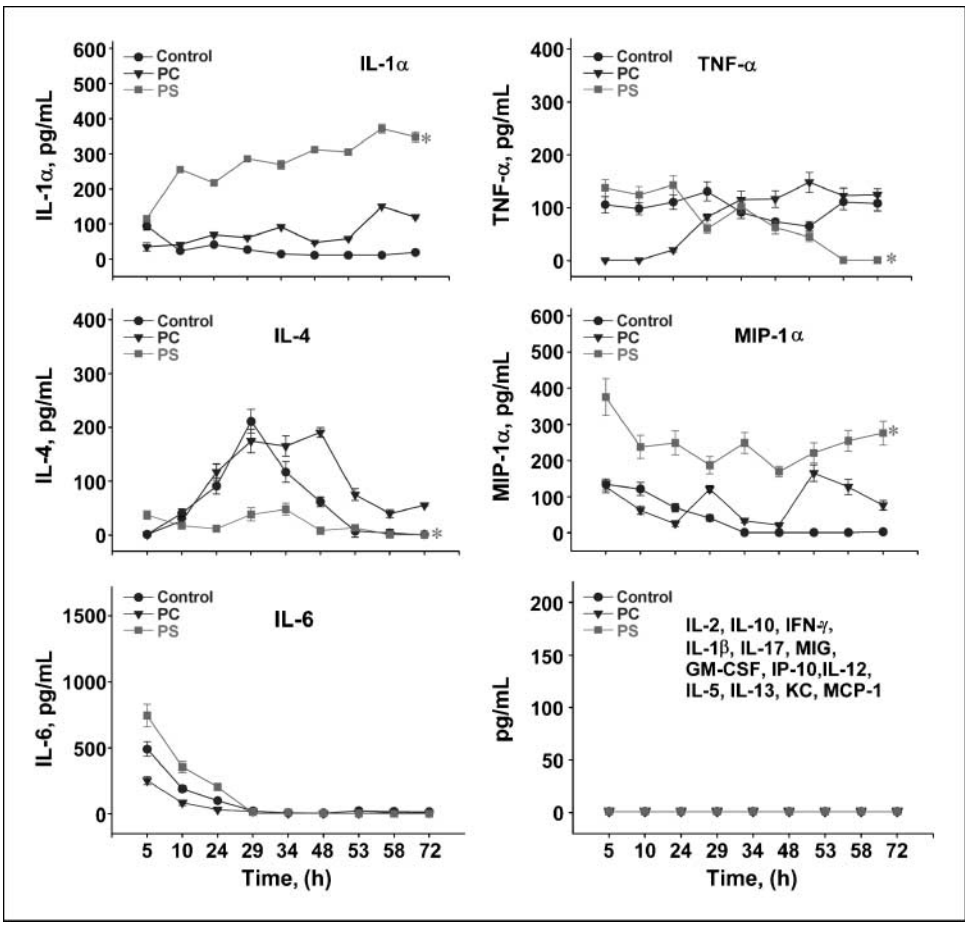

Figure 6. Alteration of intratumoral cytokines in B16-bearing mice treated with phospholipid-containing ointments. Mice were inoculated with B16 cells and 7 d later were treated with topical phosphatidylserine-containing or phosphatidylcholine-containing ointments or saline (*control*). CMA/20 microdialysis probes (10 mm in length) were implanted and fixed inside of growing melanomas 24 h prior to the initiation of topical therapy. *Points,* mean; *bars,* SE ($n = 3$); *, $P < 0.05$ vs. phosphatidylcholine group. *PC,* phosphatidylcholine; *PS,* phosphatidylserine.

Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-2611

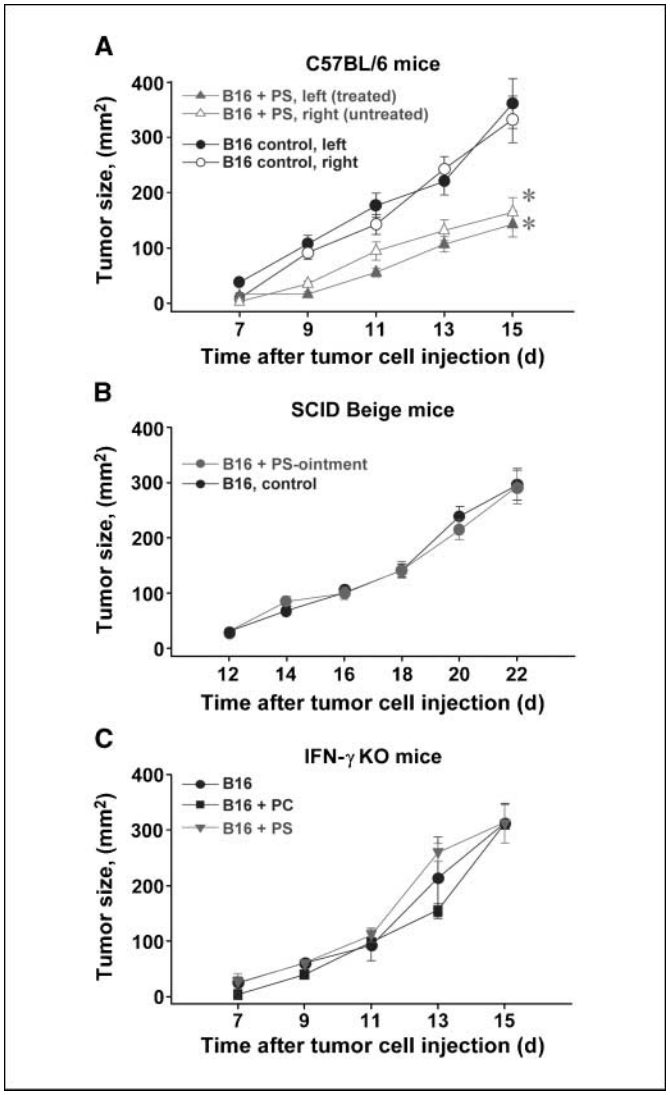

Figure 7. Evaluation of antitumor potential of phospholipid-containing ointments in immunodeficient melanoma-bearing mice. Immunocompetent C57BL/6 mice (*A*) and immunocompromised SCID (*B*) and IFN-$\gamma^{-/-}$ (*C*) mice received s.c. injections of B16 cells and were treated with the phosphatidylserine-containing ointment (*PS*) or saline (*control*) twice a day for 7 to 10 d. IFN-γ knockout mice (*IFN-γ KO*) were also treated with phosphatidylcholine-containing ointment as an additional control. Immunocompetent mice (*A*) received tumor cell injections on both left and right flanks. Tumors on the left side were treated with ointment. Tumor size was measured twice or thrice a week and expressed as tumor area in square millimeters. *Points,* mean; *bars,* SE ($n = 3$); *, $P < 0.01$ vs. controls.

revealed that tumor growth was significantly ($P < 0.01$, Student's t test, $n = 3$; Fig. 7*A*) inhibited on both sides (143 ± 23 and 165 ± 26 mm^2 versus nontreated controls 361 ± 45 and 332 ± 42 mm^2 2 weeks after tumor cell inoculation), although treated tumors grew slightly slower than their nontreated counterparts. Thus, topical application of phosphatidylserine-containing ointment is associated with the induction of systemic immune response to TA. We also carried out a pilot study in which mice received a topical application of phosphatidylserine ointment on the tumor-free left side, whereas tumor cells were injected on the right side. Tumor growth was compared with mice receiving no therapy. The results of this experiment revealed no significant differences in tumor growth between the two groups ($P > 0.05$, Student's t test, $n = 3$), suggesting that the application of phosphatidylserine ointment over a growing tumor is a prerequisite for the induction of effective antitumor immune responses.

Furthermore, the antitumor potential of phosphatidylserine-containing ointment was tested in lymphocyte-deficient SCID mice. Figure 7*B* shows that topical application of the phosphatidylserine ointment was unsuccessful in treating tumor-bearing immunodeficient mice ($P > 0.1$), suggesting the key role of immune effectors in antitumor activity. Finally, similar experiments were conducted using IFN-γ knockout mice. Both control and phosphatidylserine-containing ointment-treated mice survived for less than 10 days after tumor cell inoculation; no significant differences in tumor growth between nontreated and treated mice were observed (Fig. 7*C*). Short-term survival of these mice was likely due to the fast spreading of melanoma cells and the formation of multiple metastases.

Altogether, these results show that phosphatidylserine ointment was ineffective in immunodeficient SCID mice and IFN-γ knockout mice bearing B16 melanoma, but induced local and systemic antitumor effects in immunocompetent animals.

Discussion

Nationally, there are more new cases of skin cancer each year compared with the combined incidence of cancers of breast, prostate, lung, and colon cancers with more than 1.5 million skin cancers diagnosed yearly in the United States. One in five Americans will develop skin cancer in the course of their lifetime. These and other epidemiologic findings strongly justify the development of novel, especially noninvasive therapeutic approaches for skin malignancies.

Tumor cells undergoing cell death have been established as useful sources of TA in immunizations, in particular, in approaches based on DC (40, 41). However, the question of whether necrotic or apoptotic tumor cells are most beneficial for the loading of DC *ex vivo* still remains unanswered. It is even less clear whether triggering the apoptosis or necrosis of tumor cells is an appropriate way for augmenting antitumor immunity *in vivo*. We reasoned that targeting and recognition of live tumor cells by APC as a source of TA both *in vitro* and *in vivo* may represent a different and novel approach.

Here, using the B16 murine melanoma model, we provide first "proof-of-principle" results demonstrating that topical application of phosphatidylserine-containing ointment over skin tumor results in marked inhibition of tumor growth. An inhibitory effect was associated with infiltration of the tumor bed by APC and T lymphocytes, induction of tumor-specific CTL, and the development of systemic antitumor activity in mice. Molecular mechanisms underlying the effect of phosphatidylserine were revealed in *in vitro* studies in which we showed that phosphatidylserine-labeled live melanoma cells were recognized and engulfed by DC, followed by the TA processing and presentation to TA-specific CD8$^+$ T cells. Phosphatidylserine plays a key role in this phenomenon because incorporation of another phospholipid, phosphatidylcholine, did not mediate tumor cell recognition by DC. We found no signs of increased infiltration of normal skin by T cells and DC after ointment therapy. In addition, we did not reveal increased infiltration by mononuclear cells, suggesting that the tested ointments did not induce significant inflammatory responses in exposed skin areas. The reasons for these differences between incorporation of phosphatidylserine by nonmalignant and

malignant cells and associated immune reaction *in vivo* are not quite clear. Because tumor cells rely mostly on glycolysis rather than on mitochondrial respiration (42), they are less effective than normal cells in generating ATP that is required for the internalization of exogenously added phosphatidylserine. It is also possible that integration of phosphatidylserine into plasma membrane of tumor cells from liposomes may be lessened by the specifics of their membrane lipid composition (43). We found that phosphatidylserine-containing ointment significantly inhibited tumor growth. However, growth inhibition of MC38 colon carcinoma was less than in B16-bearing mice. Because B16 tumor is more aggressive than MC38 and grows faster in subcutaneous models, these results cannot be explained by the rate of tumor growth *in vivo*. Furthermore, CTL response was markedly lower in the case of MC38 cells compared with B16 cells. This suggests that phosphatidylserine ointment induced a stronger antitumor immune response in the B16 model than in the MC38 model. Thus, it is possible that application of ointment caused targeted destruction of normal melanocytes and supported the induction of antimelanoma immunity, as has been described in a different experimental system (44). However, it is unclear whether tumor cells or host melanocytes were the primary targets of phosphatidylserine ointment–triggered functional T cell responses against antigens that are shared by melanoma cells and normal host melanocytes. Furthermore, growth inhibition of OVA-expressing B16 tumors was more effective than inhibition of wild-type B16 tumors and was associated with the appearance of OVA-specific CTL. Finally, the application of phosphatidylserine ointment to normal skin was not associated with detectable inflammation and mononuclear cell infiltration. Overall, these results suggest that both mechanisms, i.e., induction of "classic" direct antitumor immunity, as well as the involvement of at least minimal destruction of normal melanocytes, should be considered as two pathways through which phosphatidylserine-based ointment inhibits the growth of tumor in melanoma model in mice.

Recent work has identified several receptors involved in the recognition of phosphatidylserine by professional phagocytes (27, 28, 45, 46). This explains the complexity and controversies of phosphatidylserine-mediated signaling in DC. Girolomoni and colleagues reported that phosphatidylserine was capable of up-regulating the induction of contact hypersensitivity in mice by stimulating the APC function of epidermal DC (47). This effect could be due to the ability of phosphatidylserine to enhance binding of peptides to MHC molecules (48). In contrast, Chen and colleagues (49) reported that phosphatidylserine may inhibit DC maturation and T-cell stimulatory potential. The effects of phosphatidylserine-containing liposomes on DC might be quite different from those of phosphatidylserine-expressing cells with additional signaling molecules present. Our *in vitro* data showing that DC were able to efficiently phagocytose, and present a model TA, imply that phosphatidylserine expressed on the surface of live tumor cells caused no inhibition of DC function. Furthermore, our *in vivo* data on increased levels of APC, including DC and macrophages, as well as effector $CD8^+$ T cells in tumors treated with the phosphatidylserine-containing ointment, suggest that phosphatidylserine was able to induce or up-regulate the development of TA-specific immune responses.

Although proven effective *in vivo*, optimization of the phosphatidylserine-containing ointment composition and the frequency and schedule of its application require further studies and are in progress in our laboratories. For instance, unique oxidized molecular species of phosphatidylserine may be specifically recognized by CD36 scavenger receptor (50). This indicates that use of oxidized forms of phosphatidylserine, possibly with immunostimulatory cytokines, chemokines, or TLR ligands (e.g., CD40L, IL-12, IL-15, CXCL14, and CpG) might represent further imminent improvements of our proposed strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/8/2008; revised 12/17/2008; accepted 12/22/2008; published OnlineFirst 3/10/09.

Grant support: NIH CA84270 (M.R. Shurin), U19 AIO68021 (V.E. Kagan), NIOSH OH008282 (V.E. Kagan), Skin Cancer Foundation (G.V. Shurin), and Human Frontier Science Program (V.E. Kagan).

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Recognition of Live Phosphatidylserine-Labeled Tumor Cells by Dendritic Cells: A Novel Approach to Immunotherapy of Skin Cancer

Michael R. Shurin, Alla I. Potapovich, Yulia Y. Tyurina, et al.

Cancer Res 2009;69:2487-2496. Published OnlineFirst March 10, 2009.