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Phosphatidylserine Regulates the Maturation of Human Dendritic Cells[1]

Xiao Chen,* Kara Doffek,* Sonia L. Sugg,† and Joel Shilyansky[2]*

Phosphatidylserine (PS), which is exposed on the surface of apoptotic cells, has been implicated in immune regulation. However, the effects of PS on the maturation and function of dendritic cells (DCs), which play a central role in both immune activation and regulation, have not been described. Large unilamellar liposomes containing PS or phosphatidylcholine were used to model the plasma membrane phospholipid composition of apoptotic and live cells, respectively. PS liposomes inhibited the up-regulation of HLA-ABC, HLA-DR, CD80, CD86, CD40, and CD83, as well as the production of IL-12p70 by human DCs in response to LPS. PS did not affect DC viability directly but predisposed DCs to apoptosis in response to LPS. DCs exposed to PS had diminished capacity to stimulate allogeneic T cell proliferation and to activate IFN-γ-producing $CD4^+$ T cells. Exogenous IL-12 restored IFN-γ production by $CD4^+$ T cells. Furthermore, activated CTLs proliferated poorly to cognate Ag presented by DCs exposed to PS. Our findings suggest that PS exposure provides a sufficient signal to inhibit DC maturation and to modulate adaptive immune responses. ***The Journal of Immunology,*** **2004, 173: 2985–2994.**

Dendritic cells (DCs),[3] which are the most potent professional APCs of the immune system, play an important role in the initiation of adaptive immunity (1). DCs acquire, process, and present Ags derived from pathogenic organisms to activate naive T cells, which mount a specific anti-pathogen immune response. DCs also continuously acquire self-Ags from cells that undergo apoptosis as part of normal cell turnover. However, pathological autoimmune conditions occur rarely, suggesting that the ability of DCs to stimulate self-reactive T cells is tightly controlled. The mechanisms permitting DCs to activate vigorous immune responses to most pathogens and to maintain tolerance to tissue-derived "self" Ags are not fully understood (1, 2). There has been a growing body of evidence suggesting that phagocytosis of apoptotic cells under steady state conditions leads to impaired DC maturation and may induce tolerance (3, 4).

The mechanisms by which apoptotic cells inhibit DC maturation or induce tolerizing DCs are not fully understood. CD36, $\alpha_v\beta_5$, $\alpha_v\beta_3$, and other surface molecules have been proposed as possible receptors used by DCs to recognize and phagocytose apoptotic cells (5–8). A recent study using mAbs directed against scavenger receptor CD36 and α_v integrins suggested that these receptors could also modulate DC function (9). Furthermore, *Plasmodium falciparum*-infected erythrocytes have been shown to inhibit human DC maturation, reportedly through CD36 and CD51 (10). Receptors for iC3b, an opsonin present on the surface of apoptotic bodies, have also been shown to mediate engulfment of apoptotic bodies by DCs and to modulate DC maturation (11, 12).

Recognition and removal of apoptotic cells by phagocytes, including DCs, is mediated by changes in the expression of membrane-associated markers on the dying cells. One of the most striking and consistent changes on the cell surface of apoptotic cells is the exposure of phosphatidylserine (PS). PS is an anionic aminophospholipid restricted mostly to the inner leaflet of plasma membrane in live cells (13). However, when cells undergo apoptosis, PS molecules are exposed on the cell surface (14, 15). Exposure of PS has been shown to play a central role in the recognition and phagocytosis of apoptotic cells by macrophages (16–19). Recent studies demonstrate that PS-dependent recognition and ingestion of apoptotic cells by macrophages triggers the release of anti-inflammatory cytokines and inhibits the production of proinflammatory cytokines (20–22). These studies offer a link between the recognition of apoptotic cells and the physiological consequences of their phagocytosis. Whereas the role of PS in the interaction of apoptotic cells with macrophages and the immunological consequence of PS ligation have been broadly delineated, the role of PS in regulating the phagocytosis of apoptotic cells by DCs and the immunological implication of such interactions are not known. Because of the close lineage relationship between macrophages and myeloid DCs, we hypothesized that PS might play a role in the inhibition of myeloid DC maturation. To test this hypothesis, we prepared large unilamellar liposomes containing PS as a simplified model of cell membrane alterations associated with apoptosis. We examined the effect of PS containing liposomes on DC maturation and immunostimulatory capacity. Our results suggest that PS inhibits the ability of DCs to undergo maturation, secrete IL-12, activate T cells, and stimulate IFN-γ-producing $CD4^+$ T cells.

Materials and Methods

Cell separation and generation of DCs

PBMCs were obtained from buffy coats of healthy donors by Ficoll-Paque (Amersham Life Science, Piscataway, NJ) density gradient centrifugation. Purified peripheral blood monocytes were isolated from PBMCs by using $CD14^+$ magnetic beads according to the manufacturer's instructions

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[3] Abbreviations used in this paper: DC, dendritic cell; PS, phosphatidylserine; PC, phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; iDC, immature DC; Rho-PE, rhodamine-conjugated phosphatidylethanolamine; PSR, PS receptor; TNP, trinitrophenol; mDC, mature DC; hu, human; MFI, mean fluorescence intensity.

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(Miltenyi Biotec, Auburn, CA). The purity of $CD14^+$ monocytes by flow cytometry analysis was >98%. To generate DCs, 7.5×10^5 per well of monocytes were cultured in six-well plates (Costar, Cambridge, MA) in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Life Technologies, Carlsbad, CA), recombinant human GM-CSF (800 U/ml), and recombinant human IL-4 (500 U/ml; both from R&D Systems, Minneapolis, MN). Half medium exchange was performed every 2–3 days with fresh cytokine-supplemented medium. After 5–6 days in culture, the DCs were harvested for subsequent experiments.

Preparation of liposomes

Liposomes, large unilamellar vesicles 0.1 μm in diameter, containing either pure phosphatidylcholine (PC; derived from bovine liver; Avanti Polar Lipids, Alabaster, AL) or a 30:70 molar ratio of PS (derived from bovine brain; Avanti Polar Lipids) to PC were prepared using the extrusion method (Lipex Biomembranes, Vancouver, Canada) as previously described (23, 24). Briefly, the individual phospholipids, stored in chloroform, were added to glass tubes and the chloroform was evaporated with argon gas. After adding PBS, the lipid mixture was vortexed for 5 min, subjected to five freeze-thaw cycles, and extruded eight times using a Nucleopore polycarbonate filter (0.1 μm; VWR, San Diego, CA). The final lipid concentration was 5 mM. The concentration of PS liposomes was 30% of the total lipid concentration. Synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids. Pure synthetic POPS and POPC liposomes were prepared according to the procedure described above.

Liposome treatment and DC maturation

Day 5 immature DCs (iDCs) were exposed to liposomes containing 150 μM PS or to liposomes containing 150 μM PC for 18 h at 37°C. Thereafter, DCs were matured with LPS (*Salmonella typhimurium*; Sigma-Aldrich, St. Louis, MO) for 48 h or were left untreated as a control.

Abs and flow cytometric analysis of DCs

For cell surface marker analysis of DCs, the following mAbs, conjugated with either FITC or PE, were used: CD1a, CD40, CD80, CD86, HLA-ABC, CD14, HLA-DR, and CD83 (all from BD Pharmingen, San Diego, CA). Mouse IgG1-FITC and mouse IgG2a-PE isotype control Abs were purchased from Sigma-Aldrich. Dual-color immunofluorescence staining was performed, and the samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

Detection of TLR-4

TLR-4 expression by DCs was determined by Western blot analysis and flow cytometry. For Western blot analysis, DCs were washed with cold PBS and suspended in lysis buffer (50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitor mixture; Sigma-Aldrich). Total protein was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Lysates were incubated in NuPage LDS loading buffer containing 50 mM DTT at 70°C for 10 min. Proteins (60 μg per lane) were resolved using a NuPage 4–12% Bis-Tris gradient gel (Invitrogen Life Technologies) and were transferred to a polyvinylidene difluoride membrane. The membrane was blocked at 4°C with 5% nonfat milk and was incubated overnight with primary rabbit polyclonal IgG Ab specific for human TLR-4 (Abcam, Cambridge, MA). Staining was detected with goat anti-rabbit IgG conjugated to HRP (Bio-Rad, Hercules, CA). The resulting Ag-Ab complexes were visualized with an ECL detection kit (Amersham Life Science) according to the manufacturer's instructions.

DCs were also analyzed by flow cytometry for surface and intracellular TLR-4 expression. For intracellular staining, DCs were fixed and permeabilized with ice-cold 90% methanol for 15 min. Live and permeabilized cells were then stained with anti-human TLR-4 polyclonal Ab, followed by secondary goat anti-rabbit mAb conjugated to FITC. Samples were analyzed using a FACSCalibur flow cytometer.

Phagocytosis assay

To visualize the binding and phagocytosis of liposomes by DCs, rhodamine-conjugated phosphatidylethanolamine (Rho-PE; Avanti Polar Lipids) was added to PS containing liposomes or PC liposomes as described (25). Rho-PE comprised 1% of the total lipid concentration. DCs were exposed to Rho-PE-labeled liposomes (150 μM) at 37°C for 60 min. In some experiments, unlabeled PS containing liposomes or PC liposomes at a concentration of 500 μM were added to the coculture as competitive inhibitors. The cells were then washed extensively with PBS, and 150 μl of cell suspension was applied onto poly-L-lysine-coated glass slides in a cytocentrifuge (Cytospin 2; Shandon, Pittsburgh, PA). The slides were interrogated using a fluorescent microscope (Carl Zeiss, Thornwood, NY). Cells incubated with Rho-PE-labeled liposomes were also subjected to flow cytometry analysis. In blocking experiments, the DCs were pretreated with purified anti-CD36 blocking Ab (clone 185-1G2; NeoMarkers, Fremont, CA), monoclonal anti-PS receptor (anti-PSR) IgM Ab (clone 217; a kind gift from Dr. P. Henson, National Jewish Medical and Research Center, Denver, CO), or monoclonal anti-trinitrophenol (TNP) IgM Ab (BD Biosciences) for 30 min before the addition of Rho-PE-labeled PS liposomes. We also performed phagocytosis assays at 4°C and in the presence of cytochalasin D (Sigma-Aldrich), an inhibitor of phagocytosis. From here on, liposomes containing a 30:70 molar ratio of PS to PC are referred to as PS liposomes.

Detection of apoptosis

DCs exposed to liposomes were analyzed for evidence of apoptosis. A FITC-conjugated mAb specific for activated caspase-3 (BD Biosciences) was used to detect cells in early apoptosis. Briefly, cells were incubated in Cytofix/Cytoperm solution at a concentration of 2×10^6 cells/ml for 20 min on ice and then were washed and resuspended in 100 μl of Perm/Wash buffer (BD Biosciences). Twenty microliters of FITC-caspase-3 Ab was added and incubated for 30 min at room temperature. Cells were washed and suspended in Perm/Wash buffer and analyzed using a flow cytometer. DCs from the same experiments were also stained with trypan blue and counted to determine necrotic cell death.

T cell proliferation assays

$CD3^+$ T lymphocytes were negatively selected from PBMCs by magnetic bead separation according to the manufacturer's instructions (Miltenyi Biotec). Cell purity was assessed by flow cytometry using PE-conjugated anti-CD3 mAbs (BD Pharmingen) and was shown to be >98%. Purified allogeneic T cells were counted and seeded into 96-well round-bottom microtiter plates (Costar) at 10^5 cells/well. Irradiated (3000 rad) DCs were added to the T cells at varying ratios in triplicate wells. The final volume of each well was 200 μl. T cells alone were used as the background control. After 5 days in culture, 37 MBq of methyl-[^{3}H]thymidine (Amersham Life Science) was added to each well. Eighteen hours later, cells were collected onto GF/C glass fiber filter paper using a MACH III M Harvester (Tomtec, Hamden, CT), and thymidine uptake was quantified using a liquid scintillation counter (Wallac, Gaithersburg, MD). Because the absolute level of [^{3}H]thymidine incorporation was donor dependent, we normalized the data in each experiment and presented it as a percentage of radioactivity incorporated by T cells stimulated with mature DCs (mDCs). For Ag-specific T cell proliferation assays, DCs cultured as described above were pulsed with 25 μg/ml melan-A/MART-1 peptide for 3 h and were used as stimulators. Responder cells were generated by repetitive stimulation of peripheral blood lymphocytes with DCs, which were pulsed with melan-A/MART-1-expressing melanoma cells and treated with a cytokine mixture (TNF-α, IL-1β, IL-6, and PGE_2) to induce maturation. The T cells were $CD8^+$ and specifically lysed melan-A/MART-1 peptide-loaded T2 cells and melanoma cell lines expressing melan-A/MART. Irradiated DCs at varying ratios were incubated with 5×10^4 melan-A/MART-1-specific $CD8^+$ T cells in triplicate wells for 72 h. Proliferation was measured as described above.

Determination of cytokine production by $CD4^+$ T cells

Highly purified $CD4^+$ T cells were isolated from PBMCs using anti-human CD4 Ab-conjugated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). The purity of $CD4^+$ T cells by flow cytometry analysis was always >98%. Purified $CD4^+$ T cells (1×10^5) were cocultured with 1×10^4 irradiated mDCs (3000 rad). Recombinant human IL-12 (R&D Systems) at a concentration of 10 ng/ml was added to some T cell cultures stimulated with DCs exposed to PS liposomes. On day 10, T cells were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin (both from Sigma-Aldrich). After 2.5 h, 10 μg/ml brefeldin A (Sigma-Aldrich) was added and the cells were cultured for an additional 2.5 h. For intracellular cytokine production analysis, cells were harvested, washed, fixed, and permeabilized using the CytoStain kit (BD Pharmingen) according to the manufacturer's instructions. Cells were then stained with 0.5 μg/test of anti-human IL-4-PE (anti-hu-IL-4-PE; BD Pharmingen) and anti-hu-IFN-γ-FITC (BD Pharmingen) and were analyzed by flow cytometry. IFN-γ and IL-4 production by $CD4^+$ T cells was also assessed using the Cytometric Bead Array kit from BD Pharmingen. $CD4^+$ T cells (1×10^5) were incubated with 1×10^4 irradiated DCs. Culture supernatants (100 μl) were collected on days 3, 5, and 7 and were stored at −20°C. The concentration

of IFN-γ and IL-4 in the supernatants was determined according to the manufacturer's instructions using a FACSCalibur flow cytometer and BD CBA software (BD Pharmingen).

ELISA

Supernatants from DCs cultured under the conditions described above were collected 48 h after addition of LPS. The concentrations of IL-10, TGF-β, IL-12p40, and IL-12p70 were measured by using human IL-10, TGF-β, IL-12p40, and IL-12p70 OptEIA set ELISA kits (BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis

Student's *t* test was used to compare the experimental groups. Significance was set at $p < 0.05$.

Results

Exposure to PS liposomes inhibited the phenotypic maturation of DCs

To determine whether PS exposure plays a role in modulating DC function, we first examined the effects of PS liposomes on the immunophenotype of DCs undergoing maturation. DCs, generated from $CD14^+$ PBMCs in the presence of GM-CSF and IL-4, exhibited the typical immature phenotype: $CD1a^{high}$, $CD80^{low}$, $CD83^{low}$, $CD86^{low}$, $HLA\text{-}DR^{int}$, and CD14 negative. DCs underwent maturation after treatment with LPS, a TLR-4 ligand, as evidenced by significantly increased expression of CD83, MHC, and costimulatory molecules. Immature DCs were exposed to medium, latex beads (0.1 μm), or PS and PC liposomes for 18 h before LPS was added to induce maturation. DCs exposed to PS failed to significantly increase the expression of HLA-DR, HLA-ABC, CD40, CD80, CD86, and CD83 in response to LPS, resembling iDCs (Fig. 1, *A* and *D*). In contrast, LPS induced significant increase in the expression of surface molecules associated with maturation by DCs exposed to medium alone, latex beads (data not shown), or PC liposomes (Fig. 1, *A* and *C*). Inhibition of the LPS-induced DC maturation by PS was dose dependent (Fig. 1*B*). Whereas PS exposure prevented DC maturation, PS alone, without LPS treatment, had no observable effect on DCs, which retained an immature phenotype (Fig. 1*D*).

To confirm that the inhibitory effect of PS liposomes was mediated by PS and not by a minor contaminant in the natural PS preparation, we prepared pure synthetic POPS and POPC liposomes and tested their effect on DC maturation. Synthetic pure POPS liposomes (150 μM) were equipotent to natural PS-containing liposomes in inhibiting the phenotypic maturation of DCs,

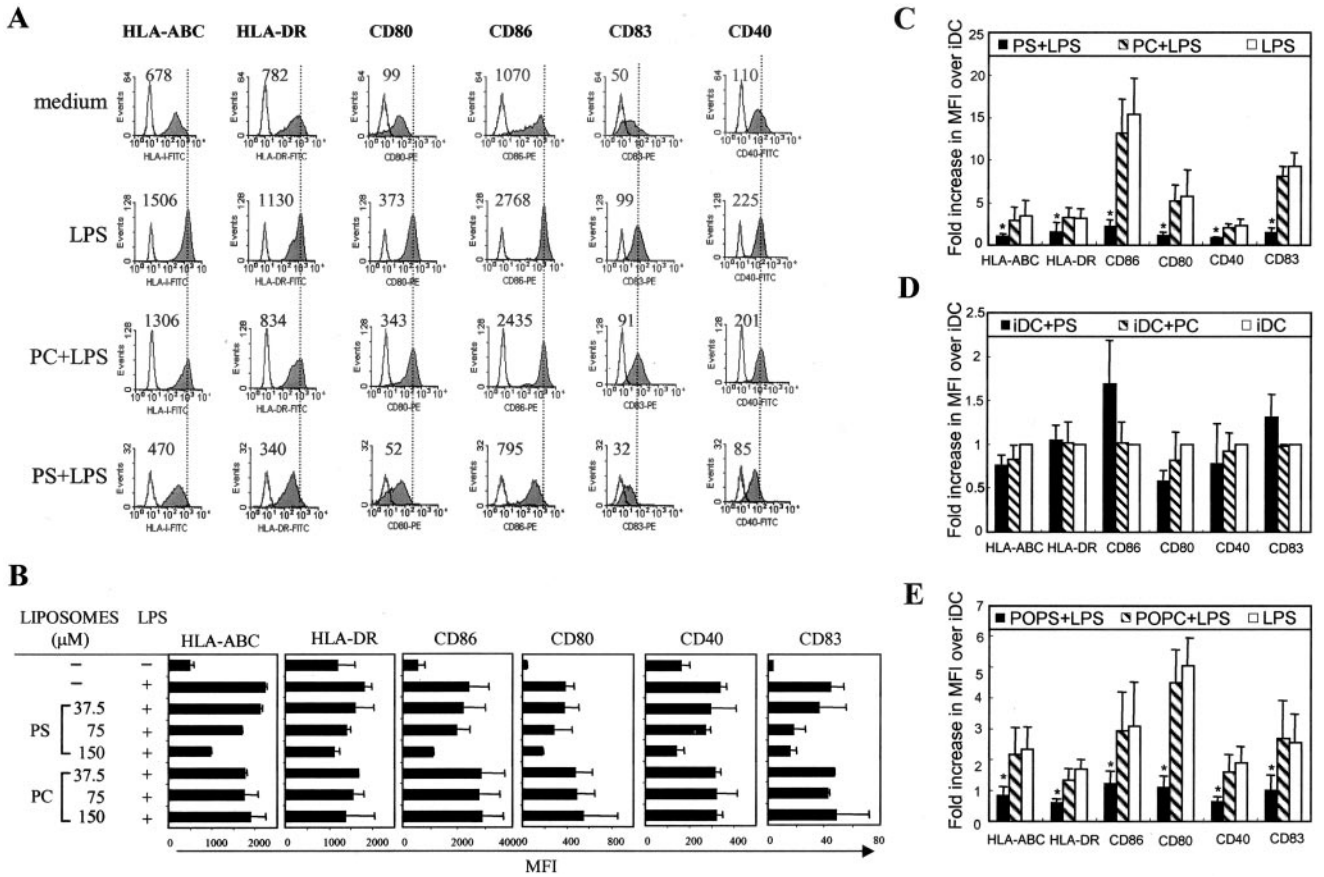

FIGURE 1. PS liposomes inhibit the phenotypic maturation of DCs. *A–C*, Immature DCs were exposed to medium alone, PS liposomes, or PC liposomes for 18 h, and then LPS was added for 48 h to induce maturation. Expression of MHC and costimulatory molecules was determined by flow cytometry. *A*, One representative experiment of at least eight. Open histograms indicate staining with isotype control Abs, and filled histograms represent staining with Abs to the indicated Ags. The mean fluorescence intensity (MFI) corresponding to the expression of surface Ags is indicated in each histogram. *B*, Inhibition of DC maturation by PS was dose dependent. Mean ± SEM from two independent experiments is shown. *C*, Natural PS but not PC liposomes inhibited DC maturation. Mean ± SEM from seven independent experiments is shown. *D*, PS alone did not significantly alter the phenotype of DCs. Immature DCs were exposed to medium alone, PS, or PC liposomes for 66 h. Mean ± SEM from three independent experiments is shown. *E*, Liposomes containing pure synthetic PS inhibited DC maturation. Immature DCs were exposed to medium alone or to liposomes containing pure synthetic POPS or POPC for 18 h. LPS was then added for 48 h to induce maturation. Mean ± SEM from three independent experiments is shown. *C–E*, Expression of surface molecules as a relative value compared with iDCs is depicted. ∗, $p < 0.05$.

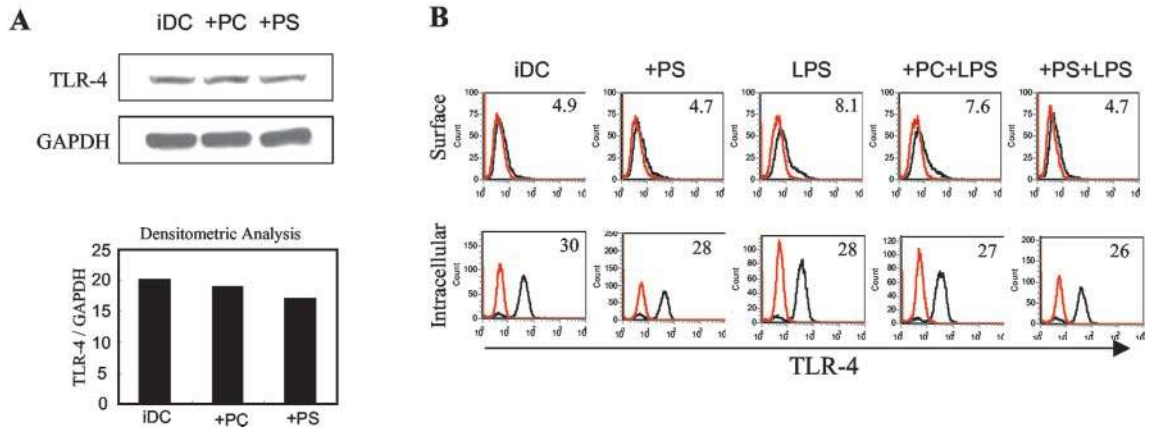

FIGURE 2. Effect of PS on TLR-4 expression by DCs. *A*, DCs were exposed to PS or PC liposomes for 18 h, and cell lysates were subjected to immunoblotting with a polyclonal anti-TLR-4 Ab. The same blot was stripped and reprobed with anti-GAPDH Ab. Relative density of each band is shown. PS did not substantially affect TLR-4 protein expression by DCs. Shown is one representative experiment of two. *B*, Cell surface and intracellular staining of TLR-4 expression by DCs. The MFI of TLR-4 is indicated in each histogram. Shown is one representative experiment of two.

whereas synthetic pure POPC liposomes at the same concentration did not inhibit DC maturation (Fig. 1*E*). The findings show that PS liposomes altered the ability of DCs to respond effectively to inflammatory or "danger" signals, such as LPS, failing to up-regulate the expression of surface molecules that are critical for effective Ag presentation and T cell stimulation.

Effect of PS liposomes on TLR-4 expression by DCs

LPS is recognized by TLR-4, which activates several intracellular signaling cascades in DCs, leading to maturation. We examined the effect of PS exposure on TLR-4 expression using Western blot analysis and flow cytometry. Western blot analysis demonstrated that PS did not alter TLR-4 expression by DCs (Fig. 2*A*). Flow cytometry analysis shown that surface TLR-4 staining of iDCs was very faint, a finding consistent with a previous report (26). However, intracellular staining of iDCs suggested that, in contrast with monocytes, most of the TLR-4 was intracellular. Exposure of iDCs to PS altered neither surface nor intracellular expression of TLR-4 (Fig. 2*B*). Interestingly, treatment with LPS led to increased surface but not total TLR-4 expression, which was blocked by PS exposure (Fig. 2*B*). These data suggest that PS does not interfere with TLR-4 expression but prevents the maturation-associated up-regulation of the receptor on the surface of DCs.

PS liposomes are nontoxic but predispose DCs to apoptosis

We next determined the effect of PS liposomes on DC viability. DCs staining positively for activated caspase-3, a marker of early apoptosis, comprised <2% of all DCs and did not increase after exposure to 150 μM PS for up to 66 h (Fig. 3, *I* and *II*). We also assessed DC viability by trypan blue exclusion and found no differences among DCs exposed to PS, PC, or medium alone (data not shown). These results demonstrated that PS liposomes were not toxic to DCs. We then examined the effect of LPS on DC viability. DCs were exposed to PS liposomes, PC liposomes, or medium alone for 18 h and were matured with LPS for 48 h. DCs exposed to PC or medium alone were resistant to apoptosis, whereas DCs exposed to PS were susceptible to LPS-induced apoptosis (Fig. 3, *III–V*). Decreased resistance to the proapoptotic effects of LPS by DCs exposed to PS could lead to a reduction in their ability to stimulate an immune response.

DCs specifically recognized PS-containing liposomes

We next examined the specificity of DC interaction with PS, which marks cells for phagocytosis. Rho-PE was incorporated into PS or PC liposomes. DCs were exposed to fluorescently labeled liposomes for 60 min, and uptake of dye was examined by flow cytometry and fluorescent microscopy. DCs phagocytosed PS liposomes better than did PC liposomes, as demonstrated by significantly higher fluorescent labeling (Fig. 4, *A* and *B*). The head groups of the PS molecules likely were responsible for the difference in phagocytosis of PC and PS liposomes because the lipid portions were identical in both preparations. DCs failed to incorporate Rho-PE-labeled PS liposomes at 4°C or in the presence of 10 μM cytochalasin D (Fig. 4*C*), which inhibit phagocytosis by interfering with cell metabolism and cytoskeletal rearrangement, respectively. The findings suggested that PS liposomes were phagocytosed as opposed to directly incorporated into, or bound to, cell membranes. Unlabeled liposomes containing PS but not PC

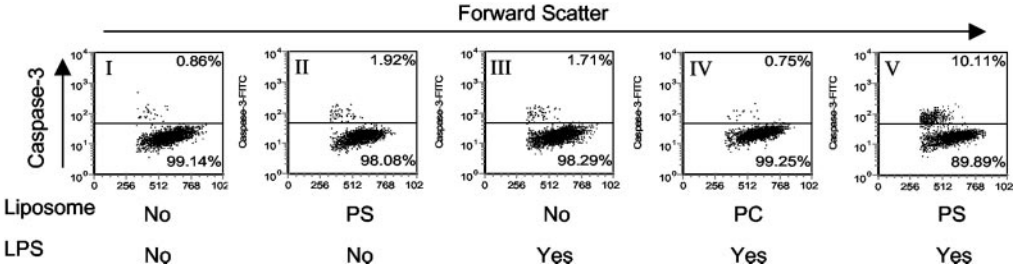

FIGURE 3. PS liposomes do not affect viability directly but predispose DCs to apoptosis in response to LPS. To exclude a cytotoxic effect of liposomes, DCs were exposed to medium alone or to PS liposomes for 66 h (*I* and *II*). Some DCs were treated with LPS for the last 48 h (*III–V*). The cells were stained with FITC-caspase 3 and analyzed for caspase-3 activation by flow cytometry. Shown is one representative experiment of two.

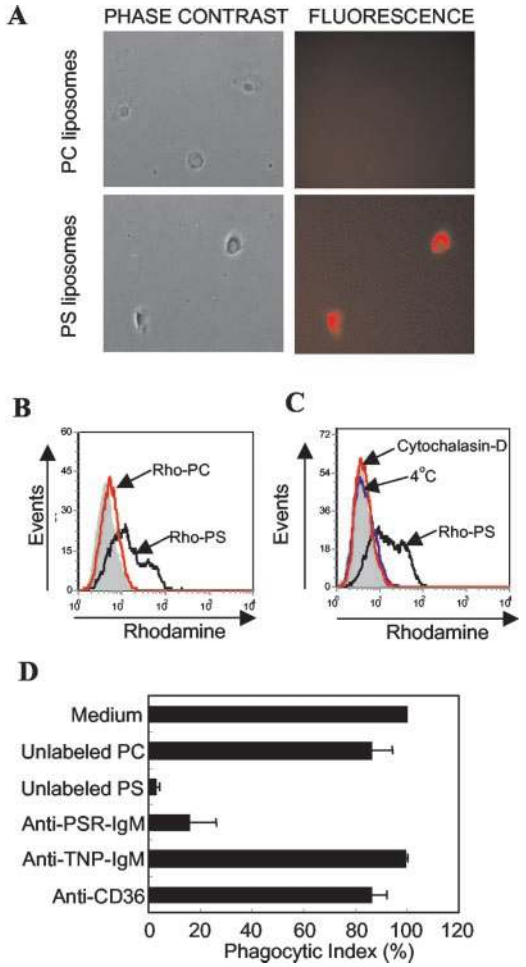

FIGURE 4. DCs recognize and preferentially phagocytose PS liposomes. Day 5 iDCs were exposed to fluorescently labeled PC or PS liposomes for 1 h at 37°C and were washed extensively with PBS. *A*, The cells were applied onto slides and analyzed using fluorescent microscopy. The photomicrographs are representative of the entire cell populations on each slide. *B*, DCs exposed to PS (black line), PC (red line), or medium alone (shaded histogram) were analyzed using flow cytometry. *C*, DCs were treated with cytochalasin D (red line) or were kept at 4°C (blue line) for 30 min before PS liposomes were added. The cells were washed and analyzed using flow cytometry. *D*, DCs were incubated with Rho-PE-labeled PS liposomes in the presence of unlabeled PS or PC liposomes. Alternatively, DCs were treated with blocking anti-CD36 mAb, anti-TNP mAb, or anti-PSR mAb (clone 217) for 30 min before adding PS liposomes. The cells were washed and analyzed using flow cytometry. The phagocytic index is expressed as a percentage of MFI of DCs exposed to Rho-PE-labeled PS liposomes. Mean ± SEM from three independent experiments is shown.

alone also blocked uptake of Rho-PE-labeled liposomes, suggesting that the interaction was saturable and specific (Fig. 4*D*). Finally, a monoclonal IgM Ab directed against PSR, but not anti-TNP monoclonal IgM control Ab or anti-CD36 mAb, blocked the phagocytosis of PS liposomes (Fig. 4*D*). Taken together, the findings demonstrated specific recognition and preferential phagocytosis of PS containing liposomes by DCs via PSR.

DCs exposed to PS stimulated allogeneic T cell proliferation poorly

Because PS inhibited the phenotypic maturation of DCs, we examined its effects on the immunostimulatory capacity of DCs. Immature DCs were exposed to liposomes for 18 h, treated with LPS for 48 h, and used to stimulate allogeneic T cells. As shown in Fig. 5, *A* and *B*, DCs exposed to PS liposomes, but not to PC liposomes or medium alone, had an impaired capacity to stimulate the proliferation of allogeneic T lymphocytes at DC-to-T cell ratios of both 1:100 and 1:500. The diminished immunostimulatory capacity of DCs exposed to PS liposomes may reflect the lower cell surface expression of MHC and costimulatory molecules. Interestingly, when the DC-to-T cell ratio was increased to 1:20 or greater, allogeneic T cell proliferation was restored to the level achieved against control DCs (Fig. 5*A*). The presence of a large number of allogeneic DCs may have overcome the effects of reduced expression of MHC and costimulatory molecules by DCs exposed to PS liposomes. Our findings suggest that PS can impair the activation of T cells even when responding to abundant high-affinity Ags such as alloantigens.

DCs exposed to PS stimulated Ag-specific activated CD8$^+$ T cell proliferation poorly

We next examined whether DCs exposed to PS liposomes would extinguish a secondary Ag-specific response, which is thought to be less dependent on costimulatory molecules. DCs were exposed to liposomes, treated with LPS, and pulsed with a peptide derived from a melanoma-associated Ag, melan-A/MART-1. DCs were then used to stimulate activated melan-A/MART-1-specific CD8$^+$ T cells. When compared with control DCs, DCs exposed to PS liposomes had a significantly diminished capacity to stimulate melan-A/MART-1-specific CD8$^+$ T cell proliferation at DC-to-T cell ratios of 1:10 and 1:50 (Fig. 5*C*). At higher DC-to-T cell ratios, the level of proliferation of melan-A/MART-1-specific CTLs in response to DCs exposed to PS was not different from the levels achieved against control DCs. Diminished proliferation of activated T cells stimulated with DCs exposed to PS liposomes supports the hypothesis that PS can modulate immune responses.

DCs exposed to PS liposomes had impaired ability to induce IFN-γ production

Next, we investigated whether exposure of DCs to PS liposomes affected T cell differentiation. IFN-γ production by T cells is a hallmark of Th1 responses and is required for effective cellular immune responses. IL-4 production is a hallmark of Th2 responses and is required for humoral immune responses. Purified CD4$^+$ T cells were stimulated with DCs that were exposed to PS or PC liposomes and were treated with LPS. As noted above, allogeneic T cell proliferation was not greatly diminished when stimulated at a high DC-to-T cell ratio (1:10). The proportion of CD4$^+$ T cells producing IFN-γ or IL-4 was determined by intracellular cytokine staining. IFN-γ and IL-4 production by CD4$^+$ T cells was determined by cytokine bead array on days 3, 5, and 7 of culture. Compared with control DCs, DCs exposed to PS stimulated a 1.9-fold lower frequency of IFN-γ$^+$, CD4$^+$ T cells ($p = 0.005$; Fig. 6*A*). Correspondingly, secretion of IFN-γ by CD4$^+$ T cells stimulated with DCs exposed to PS was markedly reduced (Fig. 6*C*). Although in some experiments the frequency of IL-4-producing T cells was increased in response to DCs exposed to PS (Fig. 7*A*), the effect was variable, possibly due to the differences between donors. As a result, we did not demonstrate a significant increase in the average frequency of IL-4-producing CD4$^+$ T cells (Fig. 6*B*) or in IL-4 secretion (Fig. 6*D*). The ratios of IFN-γ$^+$ to IL-4$^+$CD4$^+$ T cells induced by DCs exposed to PC and PS were 3.1 ± 0.7 and 1.05 ± 0.8, respectively ($p = 0.002$), further highlighting the effect of PS on DCs and reflecting its modest effect on IL-4 production. These experiments show that DCs exposed to PS liposomes had a significantly diminished capacity to induce IFN-γ-producing CD4$^+$ T cells, even at a high DC-to-T cell ratio, strengthening our

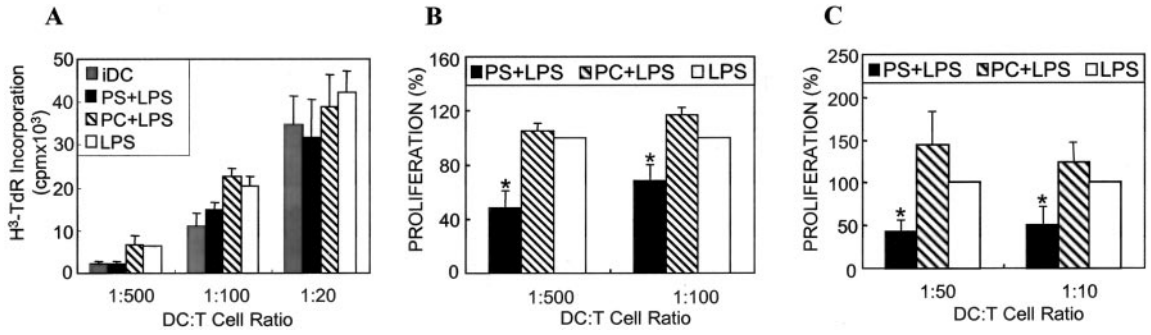

FIGURE 5. DCs exposed to PS liposomes had diminished capacity to stimulate T cell proliferation. DCs were exposed to medium alone or PS or PC liposomes, treated with LPS for 48 h, and used to stimulate T cells. Two hundred, 1000, or 5000 DCs were added to triplicate wells containing 10^5 purified allogeneic T lymphocytes in complete medium. After 5 days, [^{3}H]thymidine was added and the cells were analyzed 18 h later. *A*, Shown is a representative experiment. Results represent the mean of triplicate cultures and SDs are indicated by bars. *B*, Mean ± SEM from three independent experiments is shown. The results are expressed as a relative value compared with the proliferation of allogeneic T cells stimulated with mDCs. *, $p < 0.05$. *C*, DCs were exposed to medium alone or to PS or PC liposomes, treated with LPS for 48 h, and pulsed with melan-A/MART-1 peptides for 3 h. After washing, varying numbers of DCs (1000 or 5000) were added in triplicate wells containing 5×10^4 melan-A/MART-1 peptide-specific T cells in complete medium. After 3 days, [^{3}H]thymidine was added and the cells were analyzed 18 h later. The results are expressed as a relative value compared with the proliferation of CTLs stimulated with mDCs. Mean ± SEM from three independent experiments is shown. *, $p < 0.05$.

hypothesis that PS modulates the immune response by modifying DC function.

PS suppressed IL-12p70 secretion by DCs

IL-12 plays a central role in driving the development of Th1 immune responses and IFN-γ production (27–29). Because PS liposomes significantly inhibited the ability of DCs to induce IFN-γ-producing T cells, we examined IL-12 secretion by DCs. We also examined the effect of PS on the production of the anti-inflammatory cytokines TGF-β and IL-10 by DCs, because macrophages were shown to produce TGF-β in response to PS and DCs were shown to produce IL-10 in response to apoptotic cells (9, 20, 22, 30). IL-12p40-, IL-12p70-, IL-10-, and TGF-β-specific ELISAs were used to assay supernatants from DCs exposed to liposomes or medium alone and stimulated with LPS for 48 h to induce maturation. As shown in Fig. 8*A*, DCs treated with PS liposomes produced significantly less IL-12p70 in response to LPS, compared with DCs exposed to PC liposomes or medium alone ($p < 0.01$). There was not a statistically significant difference for IL-12p70 production between DCs exposed to medium and PC liposomes ($p = 0.09$). IL-12p40 secretion was also diminished, but to a lesser extent (data not shown). Interestingly, we did not observe significantly enhanced production of IL-10 or TGF-β (Fig. 8, *B* and *C*). Instead, IL-10 production, which is stimulated by LPS, was inhibited by PS exposure.

Exogenous IL-12 restored IFN-γ production by CD4$^+$ T cells stimulated with DCs exposed to PS liposomes

Because PS dramatically inhibited IL-12p70 secretion, we examined the effect of restoring IL-12 on the CD4$^+$ T cell response. The addition of exogenous IL-12 (10 ng/ml) restored IFN-γ production by T cells stimulated with DCs exposed to PS liposomes (Fig. 7, *A* and *B*). The findings suggest that DCs exposed to PS liposomes fail to secrete sufficient IL-12p70 and, as a result, have a diminished ability to stimulate IFN-γ-producing CD4$^+$ T cells.

Discussion

An emerging paradigm suggests that under steady state conditions, apoptotic cells modulate DC function, inducing immune tolerance to self-Ags and preventing autoimmunity (3, 4, 9). Early apoptosis is characterized by loss of membrane asymmetry and exposure of PS on the cell surface (13). PS recently has been shown to play a central role in the recognition of apoptotic cells by macrophages and may be a signal to secrete anti-inflammatory cytokines (17, 20). However, the effects of PS on the maturation and function of human monocyte-derived DCs, whose lineage is closely related to macrophages, have not been well described. We found that PS, which is recognized specifically by DCs, triggers phagocytosis and plays a key role in modulating DC maturation and function.

To study the effects of PS on DCs, we used large unilamellar liposomes containing PS or PC as a simplified model of cell membranes, avoiding the heterogeneity of signals likely provided by cells induced to undergo apoptosis. We found that DCs specifically recognized and preferentially phagocytosed PS liposomes (Fig. 4, *A* and *B*). Inhibiting cellular metabolism or cytoskeletal rearrangement prevented phagocytosis and eliminated incorporation of PS liposomes (Fig. 4*C*). Excess unlabeled PS, but not PC liposomes, blocked the incorporation of fluorescently labeled PS (Fig. 4*D*). Phagocytosis was likely triggered through PSR, because anti-PSR Ab, which blocks PS binding to the PSR but not mAb to CD36, a scavenger receptor type B, prevented the phagocytosis of PS liposomes (Fig. 4*D*). In the current study, PS alone was sufficient to trigger phagocytosis and to modulate DC function. This is in contrast with macrophages, where PS alone was reportedly insufficient to promote phagocytosis of erythrocytes or to modulate cytokine production (21). A second tethering interaction with the phagocyte was necessary to trigger either event in macrophages. Our divergent results in DCs may stem from the difference in DC and macrophage function. Alternatively, it is possible that the requirements for phagocytosis of liposomes and erythrocytes are different or that the density of PS on liposomes is greater than on erythrocytes loaded with PS. Although PSR appears to be essential for DC phagocytosis of PS liposomes, we did not rule out a potential role for other receptors, such as $\alpha_v\beta_3$ integrins, that could interact with PS (5, 6, 9). Our findings, for the first time, demonstrate that PS is specifically recognized via PSR expressed on the surface of DCs and triggers phagocytosis.

DC apoptosis may play a central role in immune regulation in vivo (3). We examined the effect of PS on DC viability. Exposure to PS liposomes alone did not induce apoptosis, but increased the susceptibility of DCs to apoptosis 48–72 h after treatment with LPS (Fig. 3). We speculate that PS may prevent the activation of DC survival pathways induced by LPS, permitting proapoptotic pathways to predominate (31–33). Induction of apoptosis may lead

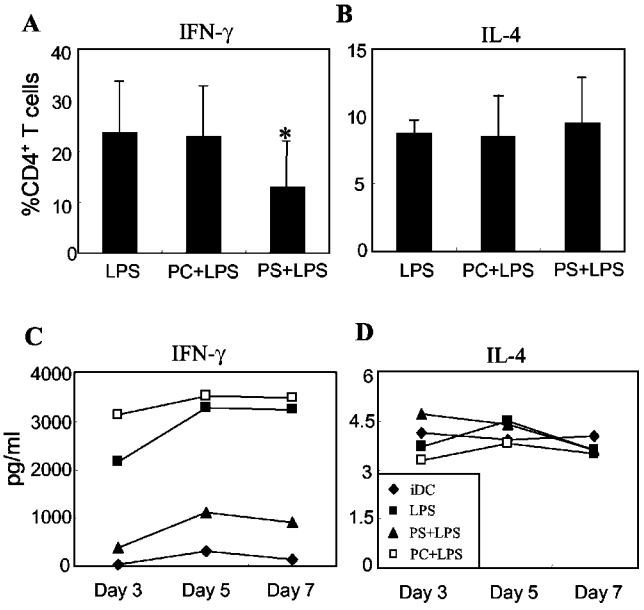

FIGURE 6. DCs exposed to PS liposomes had reduced capacity to stimulate IFN-γ production by $CD4^+$ T cells. DCs were exposed to PS or PC liposomes and were treated with LPS for 48 h and used to stimulate purified $CD4^+$ T cells (1×10^5). DC-to-T cell ratio was 1:10. *A* and *B*, On day 10, quiescent T cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were then stained with 0.5 μg/test of anti-hu-IL-4-PE and anti-hu-IFN-γ-FITC to detect intracellular cytokine production. Mean ± SEM from three independent experiments is shown. *, $p < 0.01$. *C* and *D*, Supernatants were collected on days 3, 5, and 7 of culture, and IFN-γ and IL-4 content were analyzed using cytometric bead arrays. Shown is one representative experiment of two with similar results.

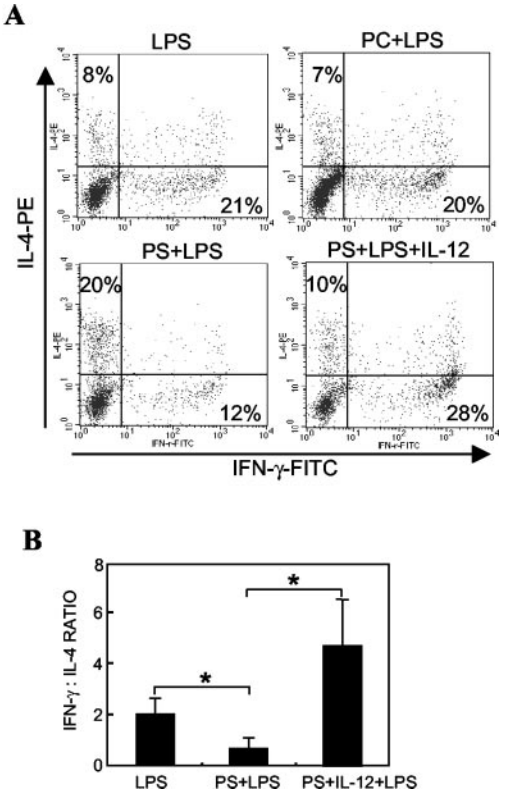

FIGURE 7. Exogenous IL-12 restored IFN-γ production by $CD4^+$ T cells stimulated with DCs exposed to PS liposomes. *A*, DCs were exposed to PS or PC liposomes and were treated with LPS for 48 h. A total of 1×10^4 DCs were used to stimulate 1×10^5 purified $CD4^+$ T cells. IL-12 (10 ng/ml) was added to some T cells stimulated with DCs exposed to PS liposomes. On day 10, T cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were then stained with 0.5 μg/test of anti-hu-IL-4-PE and anti-hu-IFN-γ-FITC to detect intracellular cytokine production. One representative experiment of three with similar results is shown. *B*, The ratio of IFN-γ^+ to IL-4^+ $CD4^+$ T cells is depicted. Mean ± SEM from three independent experiments is shown. *, $p < 0.01$.

to decreased number of DCs migrating to draining lymph nodes and diminished T cell activation. Alternatively, DCs undergoing apoptosis may amplify a tolerogenic response as they carry Ags to secondary lymph nodes where they can be phagocytosed by resident DCs, preventing their maturation (3, 34). By predisposing DCs to apoptosis in response to proinflammatory signals, PS may suppress the activation of adaptive immunity.

Previous studies have demonstrated that exposure to PS or apoptotic cells modulated the activation of macrophages and microglial cells by LPS (13, 20, 22, 25, 35–37). We examined the effect of PS on the expression of surface molecules associated with DC maturation. Compared with control groups, DCs exposed to PS liposomes expressed significantly lower levels of HLA-ABC, HLA-DR, CD80, CD86, CD40, and CD83 in response to LPS. Our findings show that although PS alone did not alter the phenotype of DCs (Fig. 1*D*), it inhibited the LPS-triggered maturation of DCs in a dose-dependent fashion (Fig. 1*B*). Lack of DC maturation was not the result of reduced DC viability, because in our studies PS liposomes were not directly toxic to DCs. Phagocytosis alone was not responsible for inhibiting maturation, because DCs that took up latex beads with the same size as liposomes acquired a mature phenotype in response to LPS (data not shown). PS also did not directly interfere with LPS receptor expression. Both total and cell surface expression of TLR-4 remained unaffected after exposure to PS alone. Furthermore, apoptosis of DCs exposed to PS occurred only after treatment with LPS, suggesting that TLR-4 remained functional. PS exposure also inhibited DC maturation in response to other maturation signals such as anti-CD40 agonist Ab (our unpublished results), suggesting that the inhibitory effects of PS were not the result of direct interference with LPS binding, but of modulation of signaling associated with maturation. Indeed, we found that, similar to its effect on MHC and costimulatory molecules, PS inhibited the increase of cell surface TLR-4 expression in response to LPS. Surface TLR-4 expression was low, however,

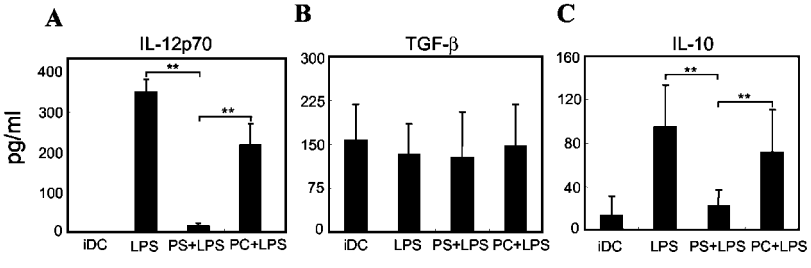

FIGURE 8. DCs exposed to PS liposomes had diminished capacity to produce IL-12p70. Day 5 iDCs were exposed to PS or PC liposomes and treated with LPS for 48 h, and culture supernatants were collected. The concentration of cytokines was measured by using human IL-12p70 (*A*), TGF-β (*B*), and IL-10 (*C*) OptEIA set ELISA kits. Mean ± SEM from five independent experiments is shown. **, $p < 0.01$.

and it has not been established whether such an increase in surface TLR-4 expression is required for DC maturation.

It is tempting to speculate that the effect of PS on DC maturation is mediated by the PSR. PSR ligation has been previously shown to inhibit the activation of macrophages by LPS (22, 35), and in the current study it was required for DCs to recognize and take up PS liposomes. However, we have not excluded the possibility that an alternative receptor mediated the immunomodulatory effects of PS. We could not use the 217 mAb to block the immune effects of PS because it is a PSR agonist (35). The role and function of PSR in DCs continue to be the subjects of active investigation in our laboratory.

Previous reports have suggested that iDCs can induce immune unresponsiveness in vitro and in vivo, possibly due to reduced Ag presentation and T cell costimulation (38–41). Inhibition of DC maturation by exposure to PS is a potential mechanism for inducing immune unresponsiveness or tolerance. We found that DCs exposed to PS had impaired ability to activate allogeneic T cells (Fig. 5, *A* and *B*) and to stimulate IFN-γ production (Fig. 6, *A* and *C*). Increasing the ratio of DCs to responders restored the proliferation of allogeneic T cells; however, IFN-γ production by allogeneic $CD4^+$ T cells was still impaired. Increased susceptibility to apoptosis by DCs exposed to PS after LPS treatment could not explain the inability to stimulate IFN-γ-producing T cells in vitro, because IL-4-producing T cells were still induced. Furthermore, exogenous IL-12, which does not affect DC viability, restored IFN-γ production by T cells. The findings suggest that PS did not simply inhibit the ability to activate T cells, but selectively modulated the ability to induce IFN-γ-producing T cells.

We also demonstrated that activated CTLs proliferated poorly in response to cognate Ag presented by DCs exposed to PS. Stimulation of activated $CD8^+$ T cells is thought to be independent of CD80 and CD86 costimulation or IL-12 production (42, 43). Impaired proliferation by CTLs could be the result of reduced expression of MHC and Ag presentation or of direct inhibition by DCs exposed to PS. We did not directly examine the effect of PS on the expression of costimulatory molecules such as 4-1-BB ligand that are thought to enhance the proliferation of activated CTLs (44–47). Taken together, these findings suggest that DCs exposed to PS would fail to initiate and may extinguish cellular immune responses.

Cytokines produced by DCs play an important role in defining the T cell response. The immunostimulatory form of IL-12, IL-12p70 heterodimer consisting of p35 and p40 subunits, is required to induce Th1 responses (27–29). We found significantly reduced secretion of IL-12p70 by DCs exposed to PS, providing a mechanism for the diminished capacity to stimulate IFN-γ production by T cells. The addition of exogenous IL-12 restored IFN-γ production in response to DCs exposed to PS. The findings suggest that diminished capacity to stimulate IFN-γ-producing T cells by DCs exposed to PS resulted from diminished IL-12p70 secretion. IL-12p40 secretion was also diminished, but to a lesser extent. IL-12p40 is usually produced in great excess and can form homodimers, which may serve as natural antagonists of biologically active IL-12 and have been shown to induce tolerance and to prevent autoimmunity in murine models (48). Although the effect in humans is controversial, unopposed IL-12p40 has been thought to induce Th2 differentiation or anergy (49).

We also examined the effect of PS on secretion of IL-10 and TGF-β, which could modulate DC function and have been associated with resolution of inflammation (50–52). Macrophages exposed to PS or apoptotic cells produce TGF-β, which in turn inhibits TNF-α secretion in response to LPS (22, 35). Notably, we did not observe an increase in TGF-β secretion by DCs exposed to PS. These findings suggest that despite the close lineage relationship between macrophages and DCs, the responses to PS by DCs and macrophages appear substantially different, possibly because they play distinct immunological roles. The mechanisms responsible for modulating DC maturation by apoptotic cells are still not clear. Urban et al. (9) reported that human DCs exposed to apoptotic cells and treated with LPS secreted IL-10, but the inhibition of DC maturation was not IL-10 dependent. Takahashi and Kobayashi (53) did not find an increase in either IL-10 or TGF-β secretion by murine DCs exposed to apoptotic cells. Stuart et al. (30) and Morelli et al. (12) also examined the effect of apoptotic cells on murine DC function. They found that inhibition of DC maturation was not dependent on either IL-10 or TGF-β. The discrepancy between studies may arise from differences in the source of DCs, species examined, and type of serum or medium used. In addition, apoptotic cell preparations are heterogeneous and may include live, necrotic, early apoptotic, and late apoptotic cells. Such cells may express an array of surface molecules and secrete a myriad of cytokines that could affect DC maturation. We found that PS did not stimulate IL-10 secretion by DCs; instead, production of IL-10 in response to LPS was inhibited by PS. These findings are consistent with a recent report suggesting that PS inhibits the secretion of proinflammatory cytokines by microglial cells, without altering IL-10 or TGF-β production (37). Although PS did not induce IL-10 or TGF-β production by DCs in our in vitro model, TGF-β produced by tissue macrophages in response to PS and IL-10 produced by regulatory T cells may play important roles in modulating DC maturation in vivo (41, 50, 52, 54–57).

PS, externalized on the surface of apoptotic cells, tumor cells, and cells infected with some intracellular pathogens, represents a possible mechanism for tolerance to self-Ags and for immune evasion (10, 22, 57–64). Our findings suggest that PS exposure directly inhibits DC maturation and modulates their ability to activate T cell responses. PS is specifically recognized by DCs and triggers phagocytosis via the PSR. Our findings suggest that PS modulates the adaptive immune response at three different levels: 1) PS inhibits survival, Ag presentation, and costimulation by DCs, leading to ineffective T cell proliferation; 2) PS inhibits IL-12p70 secretion by DCs, diminishing the ability to stimulate IFN-γ-producing T cells characteristic of Th1 responses; and 3) DCs exposed to PS have reduced ability to stimulate the proliferation of activated $CD8^+$ T cells, even in the presence of abundant Ags. PS may synergize with additional signals, such as CD200, IL-10, and TGF-β, expressed by apoptotic, inflammatory, or stromal cells (20, 22, 50, 51, 56, 65, 66). Further understanding of the mechanisms by which PS modulates DC function may lead to the development of effective immunotherapies for autoimmune diseases and for cancer.

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