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*J Immunol* 2005; 174:1393-1404; doi: 10.4049/jimmunol.174.3.1393
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Interaction between Phosphatidylserine and the Phosphatidylserine Receptor Inhibits Immune Responses In Vivo

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Phosphatidylserine (PS) on apoptotic cells promotes their uptake and induces anti-inflammatory responses in phagocytes, including TGF-β release. Little is known regarding the effects of PS on adaptive immune responses. We therefore investigated the effects of PS-containing liposomes on immune responses in mice in vivo. PS liposomes specifically inhibited responses to Ags as determined by decreased draining lymph node tissue mass, with reduced numbers of total leukocytes and Ag-specific CD4⁺ T cells. There was also a decrease in formation and size of germinal centers in spleen and lymph nodes, accompanied by decreased levels of Ag-specific IgG in blood. Many of these effects were mimicked by an agonistic Ab-specific for the PS receptor. TGF-β appears to play a critical role in this inhibition, as the inhibitory effects of PS were reversed by in vivo administration of anti-TGF-β Ab.

Surprisingly, little is known regarding the mechanisms by which apoptotic cells inhibit inflammation and adaptive immune responses. Exposure of the anionic phospholipid phosphatidylserine (PS) in the outer leaflet of the plasma membrane is one of the most striking and consistent changes on the surface of apoptotic cells (22–29). It has been known for some time that PS can inhibit macrophage production of proinflammatory cytokines and NO, and that it can block macrophage killing of intracellular parasites (30–36). Data derived from experiments in vitro and in vivo suggest that PS exposure is crucial for the release of TGF-β by phagocytes that accompanies apoptotic cell recognition and uptake (11, 37, 38). Furthermore, PS-containing liposomes can mimic the response to apoptotic cells by generating TGF-β release (11, 38). Blocking PS on apoptotic cells with annexin V (a Ca²⁺-dependent, PS-binding protein) has been shown to eliminate the inhibitory effects of the apoptotic cells on the humoral responses (39).

We hypothesized that PS exposed on apoptotic cells was a key factor in inhibiting the inflammatory response required for survival of Ag-specific T lymphocytes and subsequent activation of the adaptive immune response (40, 41). If this is true, PS should inhibit immune responses in vivo. Using three different immunogens and several different assays to measure immune responses, we determined that PS stereo-specifically inhibited responses of Ag-specific CD4⁺ T and B cells in vivo, and that these effects could be mimicked using an activating Ab directed against the PS receptor. Furthermore, these inhibitory effects could be reversed, at least in part, by the rapid engulfment of apoptotic cells by professional and nonprofessional phagocytes prevents the release of potentially toxic or immunogenic intracellular contents from the dying cells (1–4). Much attention has recently focused on the effects of apoptotic cells on phagocytes engulfing them. Interactions between macrophages and apoptotic cells result in the secretion of anti-inflammatory mediators such as TGF-β, IL-10, and PGE₂, as well as inhibition of the production of proinflammatory mediators (5–11). Similar results have been found with nonleukocytic phagocytes including fibroblasts and epithelial cells (Ref. 12; J. Monks and V. A. Fadok, unpublished observations). In addition, apoptotic cells have also been shown in some cases to inhibit dendritic cell maturation and Ag presentation (13–15).

Apoptotic cells have also been shown to affect inflammation and adaptive immune responses in vivo, although the results are conflicting. For example, apoptotic cells have been shown to be poor adjuvants compared with their necrotic counterparts in the generation of delayed-type hypersensitivity (15). Other evidence suggests that the uptake of apoptotic cells is not an immunologically null event, but is capable of modulating immune responses to self-Ags through induction of T cell-tolerance (16). The tolerizing effects of apoptotic cells have been further demonstrated in studies in which injection of apoptotic cells inhibited rejection of bone marrow allografts (17). By contrast, it has been suggested that apoptotic tumor cells can stimulate antitumor responses in vitro and possibly in vivo (18–21).


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0022-1767/05/S02.00

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1 This work was supported by Grants from the National Institutes of Health GM61031, GM60449, and GM47230.

2 Abbreviations used in this paper: PS, phosphatidylserine; GC, germinal center; KLH, keyhole limpet hemocyanin; FC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-3-glycerophosphocholine; POP-D-S, 1-palmitoyl-2-oleoyl-sn-3-glycerophospho-D-serine; POP-L-S, 1-palmitoyl-2-oleoyl-sn-3-glycerophospho-L-serine; PNA, peanut agglutinin.
part, with anti-TGF-β Ab, implicating TGF-β as an important mediator of the inhibitory response to PS. PS-containing liposomes did not appear to block maturation of bone marrow-derived dendritic cells in response to bacterial endotoxin, TNF-α, or other stimuli as determined by alternation in expression of surface markers or by ability to present Ag to CD4+ lymphocytes. PS-containing liposomes also failed to block the Ag-induced migration of dendritic cells into regional lymph nodes in vivo. Taken together, these findings suggest that PS inhibits the immune response by inhibiting inflammation in tissue.

Materials and Methods

Mice

BALB/cAnN and C57BL/6 male mice were purchased from either The Jackson Laboratory or Harlan Breeders and housed in the Biological Resource Center, National Jewish Medical and Research Center (Denver, CO). Mice were used at age 6–8 wk. DO11.10 CD4+ T cell transgenic mice on the BALB/c background were bred in the Biological Resource Center.

Abs and reagents

Abs included allopregocycocin-conjugated and PE-conjugated anti-CD4 (rat IgG2b, clone GK1.5; eBioscience), and CyChromosome anti-CD16/CD32 (rat IgG2b, clone 2.4G2; BD Pharmingen). Anti-keyhole limpet hemocyanin (KLH) (mouse IgG3, C58-1765; BD Pharmingen) was used to determine standard curves for ELISA experiments. Biotinylated KJ1-26 was purchased from Caltag Laboratories. Alexa 488-conjugated streptavidin was from Molecular Probes. FITC-conjugated anti-mouse CD19, FITC-conjugated anti-mouse CD138 (syndecan-1), and PE-conjugated anti-mouse CD11c were purchased from BD Pharmingen. Alexa 488-conjugated F4/80 was purchased from Caltag Laboratories. Mouse monoclonal anti-TGF-β1, -β2, and -β3 was obtained from R&D Systems. Abs used to characterize the mouse dendritic cells by flow cytometry included PE anti-CD40 (rat IgG2a, clone 1C10), PE anti-CD80 (hamster IgG, clone 16-10A1), PE anti-CD86 (rat IgG2b, clone P03.1), PE anti-MHC class II (rat IgG2b, clone M5/114.15.2) all from eBioscience, FITC anti-CD11c (hamster IgG, clone IL3), anti-CD14 (rat IgG1, clone rmC5-3) both from BD Pharmingen, and chicken polyclonal anti-PS receptor (Aves Labs). Isotype controls included PE-rat anti-KLH (rat IgG1, clone G1-2-2 and rat IgG2b, clone G2a-1-1; eBioscience) and chicken preimmune serum (Aves Labs). Secondary Abs included Cy3-goat anti-rat IgG and Cy3-rabbit anti-chicken IgG (Jackson ImmunoResearch). For isolation of CD4+ T cells from spleen and lymph nodes, Abs used included one as previously listed (anti-MHC class II). In addition, anti-CD3 (53-6.7), anti-B220 (RA3-6B2) both from eBioscience, and F4/80 (Caltag Laboratories) were used to coat cells for negative selection.

Bovine brain PS, bovine liver phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-sn-3-glycerophosphorylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-3-glycerophosphatidylserine (POPS) and cholesterol were obtained from Avanti Polar Lipids. One-palmitoyl-2-oleoyl-sn-3-glycerophosphatidylserine (POPS) was synthesized from POPC by phospholipase D-catalyzed headgroup exchange in the presence of n-serine (42, 43). Unilamellar liposomes were prepared as previously described (38). Liposomes contained 80:20 molar ratio of PC to cholesterol, or PS (brain PS, POP-t-S, or POP-t-N) at 30:30:40 PS to PC to cholesterol. In some cases, liposomes contained POP-t-S or POP-t-N at 2:5:8:40 PS to PC to cholesterol.

Synthetic 2PE (awgAlanawavsD, 12-mer) was prepared, purified by reverse-phase chromatography, and analyzed by mass spectrophotometry at the Molecular Resource Center, National Jewish Medical and Research Center. Endotoxin-free KLH was purchased from Calbiochem. CFA was purchased from Sigma-Aldrich.

LPS was purchased from Sigma-Aldrich (Escherichia coli O111:B4) and resuspended in sterile 0.9% NaCl solution. OVA was purchased from Sigma-Aldrich and resuspended in PBS. OVA 223-239 peptide was synthesized by the Molecular Resources Center, National Jewish Medical and Research Center, using florenyl methoxycarbonyl chemistry on a Syntho-Multiplex Peptide Synthesizer. Oligodeoxynucleotides containing CpG motifs (5’-TCCATGACGGTCTCTGATGCT-3’) or, as a control, GpC motifs (5’-TCCATGACGGTCTCTGATGCT-3’) were also synthesized by Molecular Resources Center.

Immunizations

For some experiments, BALB/cAnN mice were injected s.c. in the right rear flank using a 25-gauge needle. Mice were first injected with 100 μl of PBS, PC liposomes (0.5 mg of total lipid), or PS liposomes (0.5 mg of total lipid). PC- and PS-containing liposomes consisted of 60:40 molar ratio PC to cholesterol and 30:40:40 molar ratio PS to PC to cholesterol, respectively. Alternatively, mice were injected with liposomes (0.5 mg of total lipid) containing PC, either POP-t-S or POP-t-N, and cholesterol (30:40 molar ratio). After 1 h, the mice were then injected with 150 μl of emulsion containing 50 μg of OVA or 150 μg of KLH in CFA in the same region. Negative control mice were not immunized.

For other experiments, C57BL/6 mice were injected with PBS, PC liposomes, or PS liposomes as described earlier. After 1 h, the mice were then injected with 150 μl of emulsion containing 50 μg of 2PE and CFA in the same region. Negative control mice were kept in adjacent cages and were not immunized.

Tissue mass determination

Seven days after immunization, mice were euthanized and spleens and lymph nodes were removed carefully and dissected free of surrounding fat. Tissue mass was determined using a Fisher Scientific A-160 electronic balance. Lymph nodes included 2 superficial inguinal, 2 axillary, and 2 lateral axillary nodes (6 nodes/mouse).

Determination of germinal center (GC) development in spleen and lymph nodes

Fourteen days after immunization, mice were euthanized and spleens and superficial inguinal lymph nodes removed. Tissues were embedded in Optimal Cutting Temperature compound using Tissue-Tek Cryomolds (VWR), flash-frozen in 2-methyl butane cooled with dry ice, and stored at −70°C. Sections (7 μm) were cut on a cryostat (International Equipment), mounted on glass slides (Fisher Scientific), and fixed in acetone for 5 min at room temperature.

Sections were blocked for nonspecific staining with PBS containing 5% FCS for 10 min at room temperature. Sections were then incubated with biotin-peanut agglutinin (PNA; Vector Laboratories) at a final concentration of 5 μg/ml for 30 min at room temperature. Sections were then washed with PBS and biotin-PNA was detected using Vectastain ABC kit and DAB substrate kit (Vector Laboratories) according to the manufacturer’s directions. Sections were counterstained using a standard H&E stain. Number and size of GCs were determined for each spleen section using a phase-contrast microscope and IP Labs software. For each mouse, at least two sections were randomly chosen that were from areas of the spleen at least 100-μm apart.

Ag stimulation of spleen and lymph node cells

Ten days after KLH immunization, mice were euthanized and spleens and lymph nodes removed. Tissues were harvested and cells isolated as described earlier. Spleen and lymph node cells were then plated in triplicate in 200 μl of complete medium (RPMI 1640 containing 10% FCS (Atlanta Biologicals), 1% penicillin/streptomycin–glutamine, 1 mM sodium pyruvate, 0.2% sodium bicarbonate, 0.1 mM essential and nonessential amino acids, 14.4 M 2-ME, and 2.92 mg/ml 1-glutamine per well in 96-well plates at a concentration of 1 × 106 and 5 × 105 cells per well, respectively. KLH was added to each well at a final concentration of 100 μg/ml. Cells were then incubated at 37°C and 5% CO2 for 3 days.

Determination of serum IgG level

Immunization of BALB/cAnN male mice with liposomes and KLH/CFA were conducted as previously described. Nine days after immunization, the mice were euthanized and whole blood collected by cardiac puncture. The whole blood was allowed to clot at room temperature for 1 h, centrifuged at 14,000 rpm for 15 min, and serum removed. The serum was then diluted with ELISA buffer (PBS with 3% BSA and 0.02% sodium azide) from 1/200 to 1/3200. Ninety-six-well ELISA plates (BD Falcon) were coated as previously described (44) with KLH protein in ELISA buffer (PBS). The dilutions of serum were added to the plates (50 μl/well) and allowed to incubate for 2 h at room temperature. The wells were washed with PBS five times and then peroxidase anti-mouse IgG (Jackson ImmunoResearch) was added at a final dilution of 1/100 and incubated at room temperature for 2 h. After five washes with PBS, the amount of secondary Ab bound to the plate was detected with peroxidase substrate solution (KPL). Color development was assessed using a microplate autoreader (EL309) by BioTek Instruments. Data were analyzed using a log/log curve fit option from Delta Soft 3 software for Macintosh (BioMetallcs).

Adaptive transfer of DO11.10 transgenic T cells

For experiments in which adoptive transfer of DO11.10 transgenic T cells was performed, each BALB/c male or female mouse was injected i.v. (tail
Dendritic cell cultures

Murine dendritic cells were cultured from bone marrow using methods previously described with slight modifications (45). Briefly, femurs, tibiae, and pelvic bones were removed from 8- to 12-wk-old male BALB/c or C57BL/6 mice. Bone marrow was flushed with HBSS using a syringe and a 25-gauge needle. The extracted cells were then drawn up twice into a 25-gauge needle. The extracted cells were then plated in RPMI 1640 containing 10% FCS (Atlanta Biologicals), 1% penicillin/streptomycin/l-glutamine, 1 mM sodium pyruvate, 0.2% sodium bicarbonate, 0.1 mM essential and nonessential amino acids, 14.4 M 2-ME, and 2.92 mg/ml l-glutamine.

Spleens and lymph nodes were removed from adult DO11.10 transgenic males. The tissues were homogenized in RPMI 1640 and passed through 40-μm cell strainers. These cell suspensions were then washed once with RPMI 1640, resuspended in RPMI 1640 with 5% FCS, and placed on ice. CD4+ T cells were enriched by negative selection using magnetic bead separation. Purity was assessed by flow cytometry; the population was enriched to 97% CD4+, 98% T cells (data not shown). Briefly, ~10^6 T cells were resuspended in 1 ml of RPMI 1640 with 5% FCS and incubated with 5 μg per 10^6 cells of anti-CD16/CD32 for 10 min on ice to block FcγR. Abs targeting other cell types (anti-MHC class II, anti-B220, anti-CD8, and F4/80; all rat IgG) were then added at 5 μg per 10^6 cells for 30 min on ice. Cells were then washed twice with RPMI 1640 and resuspended in PBS with 1% BSA and 5 mM EDTA. MACS microbeads coated with sheep anti-rat IgG and anti-NK.1.1 were then used to enrich CD4+ T cells according to manufacturer’s recommendations (Miltenyi Biotec).

Stimulation of dendritic cells

Primary murine dendritic cells were cultured in 100-mm petri dishes or six-well plates as earlier described. Day 8 murine dendritic cells were stimulated with varying doses of liposomes containing 60:40 PC to cholesterol or 30:30:40 PS to cholesterol (prepared as described earlier). The dose of liposomes that was found to be most effective with minimal nonspecific effects was 100 μM total lipid. Thus, this concentration was used for all experiments. Liposome stimulation was conducted at 37°C for 1 h. Prior to stimulation, the cells were washed with RPMI 1640 containing 10% FCS to remove tissue debris. The cells were then plated in RPMI 1640 supplemented with 2% FCS, and appropriate amount of murine GM-CSF as determined previously.

Flow cytometric detection of dendritic cell surface markers

Detection of surface markers on day 7 human dendritic cells was performed by suspending 5 × 10^6 cells in 200 μl staining media (RPMI 1640 supplemented with 2% FCS) in each well of a 96-well plate. To each well was added 2.5 μg of one of the previously described Abs against CD11c, CD14, CD80, CD83, CD86, MHC class II L CCR7, or PS receptor for 30 min on ice. For unconjugated primary Abs (anti-CD14 and anti-PS receptor), the cells were washed twice with HBSS, resuspended at 5 × 10^6 cells in 200 μl of staining media per well, and Cy3-conjugated secondary Abs then added at 1/200 final concentration. After a 30-min incubation on ice, the cells were washed twice with HBSS, resuspended in flow media (PBS supplemented with 2% FCS), and transferred to flow tubes. Flow cytometry was performed on a FACSscan or FACSCaliber cytometer and data were analyzed with either FACLyss software or CellQuest software (BD Biosciences).

For detection of cell surface markers on mouse bone marrow-derived dendritic cells, the staining conditions were identical with those previously described for the human dendritic cell experiments, with the exception that two-color flow cytometry was conducted. Thus, FITC anti-CD11c Ab (used to distinguish immature dendritic cells from undifferentiated precursors) was added simultaneously with either PE-conjugated Abs (anti-CD40, anti-CD80, anti-CD86, or anti-MHC class II) or unconjugated primary Abs (anti-CD14 or anti-PS receptor, mAb 21B7G8E9) followed by Cy3-conjugated secondary Abs. Detection of two-color fluorescence and analysis was conducted as described for human dendritic cells.

Ag presentation assays in vitro

Presentation of either OVA protein or OVA323-339 peptide was conducted as follows: murine bone marrow-derived immature dendritic cells were cultured in six-well plates at a density of 3 × 10^6 cells in 4 ml of complete medium/well. On day 8, some of the immature dendritic cells were stimulated with 100 μM liposomes containing either PC to cholesterol or PS to cholesterol (prepared as already described) for 30 min at 37°C. The dendritic cells were then pulsed with either OVA protein or OVA323-339 peptide for 3 h at 37°C, which allowed sufficient time to ingest the soluble OVA protein. LPS was then added to some of the wells at a final concentration of 100 ng/ml and the cells allowed to mature overnight (18–24 h). After overnight incubation, the dendritic cells were washed twice with HBSS and fixed in 0.75% paraformaldehyde for 30 min on ice.

T lymphocytes

DO11.10 T cell hybridoma cells were cultured in RPMI 1640 containing 10% FCS (Atlanta Biologicals), 1% penicillin/streptomycin/l-glutamine, 1 mM sodium pyruvate, 0.2% sodium bicarbonate, 0.1 mM essential and nonessential amino acids, 14.4 M 2-ME, and 2.92 mg/ml l-glutamine.

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The fixed dendritic cells were then washed twice with HBSS and resuspended in RPMI 1640 with 5% FCS. Either DO11.10 hybridoma T cells or CD4+ T cells isolated from the spleen and lymph nodes of DO11.10 transgenic mice as described were washed twice with HBSS and resuspended in RPMI 1640 with 5% FCS. T cells (5 x 10^6) and fixed dendritic cells (5 x 10^6) were combined in 200 μl per well of a 96-well plate and incubated at 37°C for 24 h. The cells were then pelleted by centrifugation and supernatants collected. The supernatants were analyzed for the presence of IL-2 by ELISA as previously described.

**Determination of TNF-α secretion by dendritic cells**

For determination of TNF-α, murine dendritic cells were cultured in six-well plates as previously described. On day 8 the cells were stimulated with either PC or PS liposomes followed by LPS as previously described. After 48 h, the supernatants were collected, centrifuged at 14,000 rpm to remove cells and debris, and stored at -20°C. The mouse TNF-α ELISA was purchased from ELISA Tech; the lower limit of detection was 7.8 pg/ml.

**Statistical analysis**

Univariate analysis was conducted on data sets for each experiment to test normality. General linear modeling with contrast between two treatments was used to compare means for normally distributed data. Analysis was conducted with SAS software (SAS Institute). For adoptive transfer experiments and in vitro experiments with dendritic cell, data was analyzed by ANOVA and Tukey-Kramer test, using JMP software (SAS Institute).

**Results**

*Injection of PS liposomes reduced lymph node enlargement in response to immunization*

Enlargement of secondary lymphoid tissues after immunization results from influx of lymph as well as recruitment and proliferation of cells responding to immunogens (46). Therefore, measurement of the mass of the spleen and draining lymph nodes in mice responding to immunization was used as an initial, simple assay for immune response. BALB/c mice were immunized with OVA emulsified in CFA (OVA/CFA) and C57BL/6 mice with the immunogen synthetic peptide 2W (AWGALANWAVDS, recognized in context of I<sup>A</sup>) emulsified in CFA (2W/CFA) (47–49). Immunization of BALB/c mice with OVA/CFA resulted in a large increase in both spleen and draining lymph node mass (Fig. 1). Mice in which PS-containing liposomes were first injected into the same area with OVA/CFA, as described. Draining lymph nodes harvested 7 days later showed reduced numbers of total leukocytes as well as reduced numbers of OVA-specific CD4+ T cells. The effect of the l-steroisomer of PS was evident even when used at 2% total lipid (indicated as “low” concentration). Using a limited number of mice, we also assessed the effects of PS-containing liposomes on the adjuvant effects of IFA or LPS on the response to OVA by DO11.10 cells adoptively transferred into BALB/c mice. Lymph nodes were harvested at 3 days and 7 days after injection. Similar repressive effects on percentage of DO11.10 CD4+ T cells were noted (Fig. 2B).

The stereo-specificity of the response to PS suggested the involvement of a receptor specific for PS. One such receptor was described recently (50). An Ab specific for this protein has been shown to mimic the effects of apoptotic cells and of PS-containing liposomes. Therefore 200 μg of highly purified Ab or its isotype control were injected s.c. over the tailhead, followed by OVA/CFA, as described. Draining lymph nodes harvested 7 days later showed reduced numbers of total leukocytes as well as reduced numbers of DO11.10 CD4+ T cells, suggesting that the PS receptor was mediating the stereo-specific responses to PS (data not shown).

*Injection of PS liposomes or anti-PS receptor Ab inhibited production of effector cytokines from DO11.10 T cells following adoptive transfer*

We next determined the cytokine response of lymph node cells from the adoptive transfer experiments. The number of cells added...
to the wells was adjusted so that the number of DO11.10 T cells per well was equivalent among the various treatment groups. The cells were incubated in serum-free medium for 24 h in the presence (termed “Ag restimulation” in Fig. 3) or absence (“spontaneous”) of OVA. Supernatants were collected and analyzed for IL-2, IFN-γ, IL-4, IL-10, and TGF-β production by ELISA. As is shown in Fig. 3, A and B, spontaneous levels of IL-2 and IFN-γ were decreased in cells from mice pretreated with POP-L-S, but not POP-D-S. Furthermore, the levels of these cytokines remained significantly lower in lymph node cells from POP-L-S-treated mice, even if the cells were restimulated with Ag. The repression of these two effector cytokines did not appear to be mediated by either IL-4 or IL-10. In the case of the former, low levels were produced by cells from mice immunized with OVA/CFA, but the levels were significantly decreased by POP-L-S (Fig. 3C). IL-10 was not detectable by ELISA in this system. We did find, however, that TGF-β levels were elevated in the samples from POP-L-S but not POP-D-S treated mice (Fig. 3D), suggesting its potential role as an inhibitory cytokine in this system.

FIGURE 2. Injection with l. stereoisomer but not d stereoisomer of PS reduces accumulation of leukocytes and Ag-specific CD4+ T cells responding to immunization, and anti-PS receptor Ab mimics this effect. BALB/c mice were injected i.v. with DO11.10 T cells derived from the spleen and lymph nodes of transgenic mice. After 24 h, the mice were injected s.c. over the tailhead with liposomes (0.5 mg of total lipid; see Materials and Methods for exact composition) containing either 30% or 2% POP-L-S or POP-n-S, followed by s.c. injection of OVA/CFA. Seven days later, the lymph nodes were harvested and total leukocytes were determined (A). The percentage of CD4+ DO11.10 T cells was determined by flow cytometry, and the total number of these T cells was determined by multiplying the percentage by total number of leukocytes (B); n = 20 mice per group. A statistically significant difference (*) from OVA/CFA alone (p < 0.05) is indicated. BALB/c mice were injected with 100 μg of anti-PS receptor mAb 217G8E9 before injection with Ag and adjuvant (C); n = 5 mice per group. A statistically significant difference (**) from OVA/CFA alone (p < 0.05) is indicated.

FIGURE 3. Injection with PS or anti-PS receptor Ab inhibits effector cytokine production by Ag-specific CD4+ T cells in response to Ag. BALB/c mice were treated as described in Fig. 2. Lymph node cells were cultured as described in Materials and Methods. The following cytokine levels were determined by ELISA: IL-2 (A), IFN-γ (B), IL-4 (C), TGF-β (D); p < 0.05.
Injection of PS liposomes reduced GC formation in spleen and lymph nodes responding to immunization

Because of our results and the key role CD4⁺ T cells play in adaptive immune responses, it was important to determine whether PS liposomes also had effects on the humoral immune response. An important component of adaptive immune response is the formation of GCs in secondary lymphoid tissues (51). GC formation was of particular interest in our system, because GCs failed to

**FIGURE 4.** Injection with PS liposomes reduces GC formation and levels of specific IgG in response to immunization. C57/BL6 mice were injected s.c. with either PBS (positive control), PC liposomes, or PS liposomes, as described for Fig. 1. After 1 h, the mice were immunized in the same area with 2W/CFA. After 14 days, spleens and draining lymph nodes were removed. Cryosections were then cut, fixed, stained, and examined by light microscopy. A, Images demonstrating presence of large GCs in both spleen and lymph nodes of mice in positive control and PC groups are represented. In contrast, mice in PS and negative control groups showed a paucity of GC formation in spleen and lymph nodes. Quantitative analysis (2 spleen sections/mouse) showed lower number (B) and average size (C) of GCs in spleen sections of mice injected with PS as compared with positive or PC controls. Six mice were analyzed per group and results indicate mean ± SEM for each group. A statistically significant difference (*) between the PS group and both positive and PC control groups (p < 0.05) is shown. BALB/cAnN mice were injected s.c. with either PBS (positive control), PC liposomes, or PS liposomes, as described for Fig. 1. After 1 h, the mice were immunized in the same area with KLH/CFA. After 10 days, serum was collected and analyzed for IgG levels by ELISA. D, Results demonstrate that lower levels of IgG were detected in mice in which PS liposomes were injected before KLH immunization. E, Similarly, lower levels of IgG were detected in mice that received injections with liposomes containing L-stereoisomers of PS, but not D-stereoisomers, before KLH immunization. Some 7–10 mice were analyzed per group and results indicate mean ± SEM for each group. A statistically significant difference (*) between the PS group and both positive and PC control groups is shown, as well as a statistically significant difference (**) between the POP-L-S and POP-D-S control group (p < 0.05).
form in response to Ags to which tolerance has first been induced, even when given with adjuvants (52), and the ability of PS liposomes to inhibit effector cytokine production following Ag restimulation suggests the possibility that PS-containing liposomes may be a tolerizing agent. So, experiments were conducted using C57BL/6 mice injected with PC- or PS-containing liposomes followed by immunization with 2W/CFA. Because GC formation has been detected most clearly 10–15 days after immunization (53), spleens and lymph nodes were removed on day 14 and stained for GCs (PNA-positive cells) using immunohistochemistry. Representative images demonstrate the significant increase in GC number and size in spleens and lymph nodes of immunized mice compared with nonimmunized mice (Fig. 4). Mice injected with PS-containing liposomes had a noticeable paucity of GC formation in spleen and lymph node compared with positive and PC controls.

To quantify the effects of PS on GC formation, spleen sections were scored by light microscopy for number and size of GCs. Injection of PS-containing liposomes had the effect of reducing both the number and size of GCs in immunized mice, whereas PC-containing liposomes did not (Fig. 4, A–C). The variable nature of the lymph node sectioning made quantification of GC formation in this tissue impractical. However, qualitative analysis of lymph nodes sections revealed similar effects as found in the spleen.

Injection of PS liposomes reduced serum levels of Ag-specific Abs produced in response to immunization

The results showing that PS-containing liposomes reduced formation of GCs support the notion that exposure to PS results in ineffective T cell-B cell interactions. The effects of PS liposomes on the levels of serum IgG generated in response to Ag were examined in a system using BALB/c mice immunized with the strong immunogen KLH, which has been shown to elicit a strong Ab response (54, 55). Mice in which PS liposomes were injected had lower levels of serum IgG specific for KLH compared with positive and PC controls (Fig. 4D).

To investigate whether PS was being recognized in a stereo-specific manner, liposomes containing either POP-L-5 or POP-D-5 were injected before KLH/CFA immunization. Similar to the results from the DO11.10 adoptive transfer experiments described in this study, only the L-stereoisomer, not the D-stereoisomer, reduced serum levels of Ag-specific IgG when compared with PC-containing liposomes or positive controls (Fig. 4E).

PS failed to inhibit maturation of bone marrow-derived dendritic cells following stimulation with LPS or CpG motif-containing DNA

Because we found that PS inhibited immune responses in vivo, it was important to determine whether these liposomes could directly inhibit dendritic cell maturation in response to inflammatory stimuli including bacterial endotoxin and CpG motif-containing DNA. For this purpose, we used bone marrow-derived dendritic cells cultured in the presence of GM-CSF. To test for maturation, we determined the change in expression of surface markers including CD40, CD80, CD86, and MHC class II by flow cytometry. We also examined the ability of these dendritic cells to present Ag (OVA) in vitro to DO11.10 T cells from transgenic mice and DO11.10 T cell hybridomas. As shown in Fig. 5, mouse dendritic cells responded to CpG treatment by up-regulation of maturation markers; the control oligonucleotide GpC failed to induce maturation (data not shown). Murine dendritic cells stimulated with PS liposomes did not exhibit lower expression of maturation markers compared with controls in response to CpG motif-containing DNA. Murine dendritic cells stimulated with PS liposomes showed lower levels of CD40, CD80, and CD86 in response to LPS as compared with LPS alone or PC controls, but the differences were not statistically significant (p > 0.05). Culturing the bone marrow cells in the presence of IL-4 in addition to GM-CSF failed to reverse this responsiveness. We also observed that secretion of TNF-α after stimulation with LPS was not reduced by first stimulating the dendritic cells with either PS-containing liposome or anti-PS receptor Ab (data not shown). Given these results and the observation that these cells fail to express PS receptor, as determined by their failure...
to bind to anti-PS receptor Ab (mAb 217G8E9; data not shown), we suggest that this receptor is required for the anti-inflammatory.

Ag presentation assays were set up using dendritic cells stimulated as described pulsed with OVA protein. The responder T cells used in these assays were either DO11.10 hybridoma CD4⁺ T cells or CD4⁺ T cells purified from spleens of DO11.10 transgenic mice. Supernatant was collected after overnight coculture of dendritic cells and CD4⁺ T cells and levels of IL-2 measured by ELISA. A, *Left*, 5 × 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *Right*, 5 × 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells. PS had a slight inhibitory effect on the ability of dendritic cells to process and present OVA to primary CD4⁺ T cells, but no effect was seen using hybridoma CD4⁺ T cells. The number of dendritic cells and T cells used were 5 × 10⁴ and 5 × 10⁵, respectively. B, *Upper left*, 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *upper right*, 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *lower left*, 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells; *lower right*, 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells. PS was found to have no effect on ability of LPS to induce mature dendritic cells capable of presenting Ag to either type of T cell. All results are from one experiment performed in duplicate and expressed as mean ± SEM.

FIGURE 6. Stimulation of murine dendritic cells with PS liposomes does not reduce their capacity to process and present Ag to T cells. Murine dendritic cells cultured as described in *Materials and Methods* were stimulated with either PC or PS liposomes for 1 h, pulsed with OVA protein for 3 h, and then stimulated with LPS overnight. Immature dendritic cells and LPS-matured dendritic cells were included as negative and positive controls, respectively. The responder T cells used in these assays were either DO11.10 hybridoma CD4⁺ T cells or CD4⁺ T cells purified from spleens of DO11.10 transgenic mice. Supernatant was collected after overnight coculture of dendritic cells and CD4⁺ T cells and levels of IL-2 measured by ELISA. A, *Left*, 5 × 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *Right*, 5 × 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells. PS had a slight inhibitory effect on the ability of dendritic cells to process and present OVA to primary CD4⁺ T cells, but no effect was seen using hybridoma CD4⁺ T cells. The number of dendritic cells and T cells used were 5 × 10⁴ and 5 × 10⁵, respectively. B, *Upper left*, 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *upper right*, 10⁴ dendritic cells added to 5 × 10⁵ primary CD4⁺ T cells; *lower left*, 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells; *lower right*, 10⁴ dendritic cells added to 5 × 10⁵ hybridoma CD4⁺ T cells. PS was found to have no effect on ability of LPS to induce mature dendritic cells capable of presenting Ag to either type of T cell. All results are from one experiment performed in duplicate and expressed as mean ± SEM.
presentation to either DO11.10 hybridoma cells or CD4\(^+\) transgenic DO11.10 T cells (Fig. 6A). This experiment was repeated using two concentrations of dendritic cells and no inhibitory effect was seen using dendritic cells stimulated with PS liposomes (Fig. 6B). Thus, the results suggest stimulation of immature dendritic cells with PS liposomes does not inhibit their ability to process and present Ag to T cells.

**Ab against TGF-\(\beta\) reverses the inhibitory effects of PS-containing liposomes**

As shown in Fig. 3, the production of IL-2, IL-4, and IFN-\(\gamma\) by draining lymph node cells was reduced in those mice treated with PS before stimulation in vivo with OVA and CFA. We detected no IL-10, but significant increases in TGF-\(\beta\), implying a potential role for this cytokine in mediating the inhibitory effects. We therefore tested the effects of a mouse mAb against TGF-\(\beta\) injected s.c. at the time of liposome injection and again at the time of OVA/CFA injection. The draining lymph nodes were harvested 7 days later, and the number of total leukocytes determined for each mouse. In addition, the lymph node cells were stained to identify CD4\(^+\) DO11.10 transgenic T lymphocytes, CD19\(^+\) B lymphocytes, CD138\(^+\) plasma cells, F4/80\(^+\) macrophages, and CD11c\(^{hi}\) dendritic cells. We found again that treatment with PS liposomes significantly reduced the total number of leukocytes as well as the number of DO11.10 transgenic T cells (Fig. 7). PS liposomes also significantly reduced the number of B cells and the number of plasma cells. Although the total number of macrophages was reduced significantly, there was no significant decrease in total numbers of dendritic cells. As is shown in Fig. 7, anti-TGF-\(\beta\) Ab partially or completely reversed the inhibitory effects of PS liposomes, restoring the total number of leukocytes, as well as the numbers of transgenic T cells, B cells, plasma cells, and macrophages. The isotype control had no effect (data not shown). These data support a critical role for TGF-\(\beta\) in the inhibitory effects of PS liposomes on immune responses.

**PS liposomes do not block Ag/adjuvant-induced migration of dendritic cells to regional lymph nodes in vivo**

One potential explanation for the inhibitory effects of PS liposomes on the immune response could be that they blocked the migration of Ag/adjuvant-activated dendritic cells to draining lymph nodes. This seemed less likely, given that the total number of dendritic cells in the draining lymph nodes was not affected (Fig. 7), but it was important to address this question directly. Mice were therefore injected s.c. with CFSE and fluorescent OVA to determine whether liposomes containing PS had any effect on dendritic cells migration from the s.c. tissue into the lymph nodes. As is shown in Fig. 8, dendritic cells from mice treated with PS liposomes migrated to the inguinal lymph nodes as well as those treated with PC liposomes.

**Discussion**

A small body of literature exists that support the hypothesis that the phospholipid PS represses inflammation in vitro and in vivo (11, 30–36, 50). Inflammation (i.e., activation of the innate immune system) is believed to be a necessary precursor for the development of an effective adaptive immune response. Given the previous work demonstrating the potentially inhibitory effects of
apoptotic cells on immune responses and given that apoptotic cells expose PS externally, we set out to investigate whether this phospholipid alone, delivered in liposomes, could repress an immune response following immunization with well-characterized Ags including chicken OVA, KLH, and the synthetic peptide 2W. We also sought to determine whether inhibition of an immune response to OVA could be mediated by direct stimulation of the PS receptor, using the anti-PS receptor Ab (mAb 217G8E9). The data show that PS-containing liposomes were sufficient to inhibit adaptive immune responses. Particularly, PS stereo-specifically inhibited immune responses to immunization as determined by draining lymph node tissue mass, accumulation of adoptively transferred Ag-specific CD4^+ T cells in the draining lymph nodes, formation of GCs in spleen and lymph nodes, cytokine production by CD4^+ T cells responding to Ag, and production of Ag-specific Ab. Thus, it appears that prior exposure to PS in the peripheral tissues modulates an Ag-specific immune response by altering the phenotype of CD4^+ T cells responding to Ag present in those tissues.

One potential mechanism by which PS could exert its modulating effects is by delivering a inhibitory or tolerizing signal directly to dendritic cells. However, a series of in vitro experiments using bone marrow-derived dendritic cells showed that addition of PS-containing liposomes failed to significantly inhibit phenotypical responses (e.g., macrophages, fibroblasts, epithelial cells, endothelial cells) by down-regulation of inflammation; however a more detailed analysis of the effects of PS on dendritic cell behavior in vivo is required and is underway. As shown in Fig. 8, however, treatment with PS failed to block the migration of dendritic cells activated by Ag and adjuvant in vivo to regional lymph nodes, confirming the fact that these liposomes were not toxic to dendritic cells contained within the skin.

The stereo-specific effects of PS demonstrated in this study suggest the involvement of a specific receptor. The PS receptor recently cloned in our laboratory is a strong candidate in that it has been shown to recognize PS in a stereo-specific manner and is crucial for the TGF-β released from phagocytes upon interaction with apoptotic cells (37, 50). Moreover, the PS receptor is expressed on the surface of a variety of cells found in peripheral tissue including fibroblasts, epithelial cells, endothelial cells, and macrophages. Interestingly, it was not present on the bone marrow-derived dendritic cells used in our in vitro studies. Using adoptive transfer of DO11.10 cells, we found that injection of the anti-PS receptor Ab mAb 217G8E9 at the site of subsequent Ag/adjuvant administration significantly repressed accumulation of leukocyte and Ag-specific T cells, as well as reduced effector cytokine production. Therefore, it is conceivable that PS-PS receptor interactions during apoptotic cell clearance in the peripheral tissues contributes to anti-inflammatory effects in these tissues as well as tolerizing events occurring in secondary lymphoid tissues. In fact, Li et al. (60) recently reported that mice deficient in PS receptor died at birth; histologic analysis of multiple organs showed pronounced inflammation and free apoptotic bodies, supporting the notion that this receptor is important in apoptotic cell clearance and the lack of inflammation associated with it. Furthermore, Kunisaki et al. (61) have also developed a PS receptor-deficient mouse that showed defective fetal liver erythropoiesis and abnormal T cell development; regrettably, these animals also died shortly after birth.

Because TGF-β1 levels were increased in lymph node cultures from mice pretreated with PS liposomes or anti-PS receptor Ab, we wondered whether this cytokine played a role in repression of the response to OVA. By blocking TGF-β with a mouse mAb in vivo, we were able to demonstrate a critical role for this anti-inflammatory cytokine in the suppressive effects of PS on immune response. Mice treated with PS and anti-TGF-β showed partial or complete restoration of total leukocyte numbers in the draining lymph nodes, as well as Ag-specific T lymphocytes, B lymphocytes, plasma cells, and macrophages. Interestingly, there were no significant differences in the number of dendritic cells obtained from draining lymph nodes from animals treated with PS liposomes compared with those treated with control liposomes (Figs. 7 and 8). Anti-TGF-β Ab also had no significant effect on dendritic cell numbers. These findings imply that dendritic cell migration to and/or proliferation in draining lymph nodes was not affected by PS liposomes.

TGF-β1 has emerged as an important regulator of immune responses (reviewed in Refs. 62, 63). Its effects on T lymphocytes, like its effects on other cells, is context-dependent; i.e., it can provide activating or inhibitory signals depending on the mix of cells present and the state of differentiation of the cells when exposed to TGF-β1. Thus, TGF-β1 has been suggested to repress Th1 responses, while enhancing Th2 responses (62). It does appear that TGF-β1 is an important mediator of tolerance induction, particularly when present during primary stimulation of naive T lymphocytes; the mechanisms by which it does so are still being elucidated. Furthermore, subsets of regulatory T cells are known to produce TGF-β1, which is required for suppression of autoimmune responses in several model mouse models (reviewed in Ref. 63). Exposure of CD4^+ T lymphocytes to TGF-β during TCR activation induces their development into CD4^+CD25^+ T regulatory cells, which are anergic and which then suppress proliferation of Ag-specific responder T cells. Our data suggest that PS alone, in the absence of other repressive signals undoubtedly expressed on apoptotic cells, can induce the production of TGF-β, which contributes to repression of Ag-specific immune responses. We are currently in the process of determining whether the DO11.10 cells injected into the recipient mice can become regulatory T cells. The mechanisms by which this PS-induced inhibition is mediated are
likely to be complicated, given that PS receptor is or can be expressed on all cells, and that all cells expressing PS receptor can be stimulated to produce TGF-β by PS presented on lymphotoxins, symmetrytized RBCs, or apoptotic cells.

In conclusion, we believe that the interaction of PS receptor with its ligand PS play a critical role in tissue homeostasis, an important part of which is the regulation of inflammation. Our data are most consistent with the hypothesis that PS represses inflammation in part of which is the regulation of inflammation. Our data are most consistent with the hypothesis that PS represses inflammation in part.

Acknowledgments

We thank the staff of the Biological Resources Center, National Jewish Medical and Research Center, particularly Ellen Kruk, Dawn Bohrer-Kunter, and Michelle Mocilac, for their help in tissue harvest. William Kossack in the Biostatistics Department, National Jewish Medical and Research Center, provided crucial assistance with data analysis, and Lyndsay Guthrie provided technical assistance during sample processing.

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