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Oxidized Phospholipids Negatively Regulate Dendritic Cell Maturation Induced by TLRs and CD40[1]

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Maturation of dendritic cells (DCs) induced by pathogen-derived signals via TLRs is a crucial step in the initiation of an adaptive immune response and therefore has to be well controlled. In this study, we demonstrate that oxidized phospholipids (ox-PLs), which are generated during infections, apoptosis, and tissue damage, interfere with DC activation, preventing their maturation. ox-PLs blocked TLR-3- and TLR-4-mediated induction of the costimulatory molecules CD40, CD80, CD83, and CD86, the cytokines IL-12 and TNF, as well as lymphocyte stimulatory capacity. CD40 and TLR-2-mediated cytokine production was also inhibited, whereas up-regulation of costimulatory molecules via these receptors was not affected by ox-PLs. Thus, formation of ox-PLs during the course of an inflammatory response may represent a negative-feedback loop preventing excessive and sustained immune reactions through regulating DC maturation. *The Journal of Immunology,* **2005, 175: 501–508.**

Dendritic cells (DCs)[5] are the key regulators of adaptive immunity. In the steady state, DCs reside as sentinels in peripheral tissues as immature APCs and are considered to be tolerogenic (1, 2). However, DCs are capable of sensing changes in their local environment. During infections, DCs are activated by stimulatory signals from invading pathogens, called pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) (3–7). Classical PAMPs are LPS, an integral part of the cell wall of Gram-negative bacteria, which is recognized by TLR-4 (8–10), and lipopeptides from bacterial cell walls and dsRNA, signaling through TLR-2 and TLR-3, respectively (11–14). Peptidoglycan (PGN) is another well-known PAMP, which is sensed by a number of PRRs including Nod proteins (15–17).

Upon stimulation, DCs mature by translocating MHC-peptide complexes to the cell surface, up-regulating costimulatory molecules such as CD40, CD80, and CD86, and are thus becoming fully competent to activate T cells (2, 18–22). In addition, proinflammatory cytokines including IL-12, TNF, and IFN-β are released by DCs during acute infections (3, 23). Host-derived factors associated with tissue damage have been shown to activate or suppress DC function (24, 25).

As a part of the body's defense strategy against invading microorganisms, cells of the innate immune system, mainly neutrophil granulocytes, produce reactive oxygen species capable of killing bacteria (26). Thereby, tissue damage including host lipid peroxidation occurs, resulting in the formation of bioactive oxidized lipids, which are recognized by the immune system (27, 28). Oxidized phospholipids (ox-PLs), which are present in oxidized low-density lipoprotein (29), in apoptotic cells (30, 31), and in inflamed tissue (32), profoundly modulate the course of an inflammatory response by acting on the endothelium, inducing expression of pro- and anti-inflammatory genes (33–36). Moreover, we and others demonstrated that ox-PLs are able to inhibit binding of LPS to CD14 and LPS-binding protein, thereby preventing recognition of LPS by its signaling receptor, TLR-4 (37, 38). However, the impact of ox-PLs on DC function and modulation of the adaptive immune response is not known.

In this paper, we demonstrate that ox-PLs, more precisely lipid oxidation products derived from 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC), represent microenvironmental factors regulating DC activation. We show that OxPAPC efficiently prevents LPS- and polyinosinic:polycytidylic acid (poly(I:C))-induced activation of DC via TLR-4 and -3, respectively, thereby limiting their capacity to stimulate T cells. Moreover, OxPAPC inhibited cytokine production induced by both TLR-2 and CD40L. Thus, oxidative modification of phospholipids leads to the formation of negative regulators of adaptive immune responses through limiting pathogen-induced activation and subsequent maturation of DC.

Materials and Methods

Media and reagents

The cell culture medium RPMI 1640 (Invitrogen Life Technologies) was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Sigma-Aldrich). Recombinant human GM-CSF and IL-4 were kindly provided by Novartis Research Institute (Vienna, Austria). LPS from *Escherichia coli* (serotype 0127-B8), poly(I:C), and PGN from *Staphylococcus aureus* were obtained from Sigma Chemie. Ultrapure LPS (serotype 0111:B4) and Pam3CSK4 were from InvivoGen.

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[5] Abbreviations used in this paper: DC, dendritic cell; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; PGN, peptidoglycan; ox-PL, oxidized phospholipid; (Ox)PAPC, (oxidized) 1-palmitoyl-2-arachidoyl-*sn*-glycero-3-phosphorylcholine; TT, tetanus toxoid; Lyso-PC, lysophosphatidylcholine; COX-2, cyclooxygenase-2.

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Annexin V^{FITC} was provided by Caltag Laboratories. Tetanus toxoid (TT) was purchased from Connaught Laboratories. PAPC and lysophosphatidylcholine (Lyso-PC) were bought at Avanti Polar Lipids. Immobilon-P transfer membranes were products of Millipore.

Antibodies

The following murine mAbs were generated in our laboratory: negative control mAb VIAP (calf intestinal alkaline phosphatase specific), DF272 (B7-H1), 1/47 (MHC class II), VIT6b (CD1a), VIM12 (CD11b), VIM13 (CD14), CD33-4D3 (CD33), and 5-216 (ICAM-1/CD54). Hybridomas producing mAb W6/32 (MHC class I) and G28-5 (CD40) were obtained from American Type Culture Collection. The CD14 mAb (MEM18) was kindly provided by An der Grub (Bio Forschungs, Kaumberg, Austria), and the CD19 mAb (HD37) was a gift from G. Moldenhauer (German Cancer Center, Division of Molecular Immunology, University of Heidelberg, Heidelberg, Germany).

The mAbs CD80 DAL-1 (CD80), HB 15 (CD83), and BU63 (CD86) were purchased from Caltag Laboratories. Anti-human CCR7-fluorescein (clone 150503) was obtained from R&D Systems. Polyclonal Abs against phosphorylated form of ERK1/2, p38, and AKT, as well as their unphosphorylated forms, were from Cell Signaling Technology. Polyclonal IκB-α Ab was from Santa Cruz Biotechnology. Peroxidase-conjugated secondary Abs were purchased from Amersham Biosciences.

Lipid oxidation

PAPC was oxidized by exposure of dry lipid to air for 72 h. The extent of oxidation was monitored by positive-ion electrospray mass spectrometry as described previously (32). OxPAPC used for experiments contained <50 pg/ml endotoxin as determined by the *Limulus* amebocyte assay (BioWhittaker).

Cell preparation and stimulation

PBMC were isolated from heparinized whole blood of healthy donors by standard density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech). Subsequently, monocytes and T cells were separated by magnetic sorting using the MACS technique (Miltenyi Biotec) as described (39). Monocytes were enriched by using biotinylated CD14 mAbs VIM13 and MEM18 (purity, >95%). Purified T cells were obtained through negative depletion of CD11b, CD14, CD16, CD19, CD33, and MHC class II-positive cells with the respective mAbs. DCs were generated from $CD14^+$ monocytes cultured in the presence of GM-CSF (50 ng/ml) and IL-4 (100 U/ml) for 6 days. Maturation of DCs was induced by adding 100 ng/ml LPS, 50 μg/ml poly(I:C), 10 μg/ml PGN, 1 μg/ml Pam3CSK4 or trimeric CD40L (kindly provided by Amgen and used as described (40) for 24 h). For lipid pretreatment, DCs were incubated for 20 min at 37°C before addition of maturation stimuli. OxPAPC was used at 60 μg/ml, unless otherwise specified; PAPC was used at 60 μg/ml; and Lyso-PC was used at 20 μg/ml.

T cell proliferation assay

For the MLR, allogenic, purified T cells (1×10^5/well) were incubated in 96-well cell culture plates (Corning Costar) with graded numbers of DC for 6 days. The assay was performed in triplicate. Proliferation of T cells was monitored by measuring [*methyl*-^{3}H]thymidine (ICN Pharmaceuticals) incorporation, added on day 5 of culture. Cells were harvested 18 h later, and incorporated [*methyl*-^{3}H]thymidine was detected on a microplate scintillation counter (PerkinElmer). For the TT MLR, DCs were loaded at day 6 of differentiation with the indicated amount of TT and after 1 h stimulated with LPS or LPS plus OxPAPC for 24 h, and then cocultured with purified T cells of the same donor for 6 days. Again, the assay was performed in triplicate, and proliferation of T cells was monitored by measuring [*methyl*-^{3}H]thymidine incorporation.

Immunofluorescence analysis

For membrane staining, cells (5×10^5) were incubated for 30 min at 4°C with unlabeled mAbs at a concentration of 20 μg/ml. Staining of FcR-bearing cells was performed in the presence of human IgG Abs (20 mg/ml; Beriglobin; Aventis Behring). After washing cells twice with ice-cold PBS containing 1% BSA, binding of the primary mAb was visualized using Oregon Green-conjugated goat anti-mouse Ab from Molecular Probes. Cells were then washed three times with PBS/BSA. Membrane fluorescence was analyzed on a FACSCalibur flow cytometer (BD Biosciences) supported by CellQuest software (BD Biosciences). The exclusion of dead cells was performed by the addition propidium iodide.

Determination of cytokine production

DCs were treated as indicated, and after 24 h, the supernatants were harvested and analyzed by ELISA. Cytokines were measured by sandwich ELISAs using matched-pair Abs. Capture and detection Abs for human IL-10 and IL-12 p70 were obtained from R&D Systems, and for TNF, from BD Pharmingen. Standards consisted of human recombinant material from R&D Systems. Assays were performed in duplicate according to the recommendations of the manufacturers. The lower limit of detection was 20 pg/ml for IL-10, IL-12, and TNF. For T cell polarization assay, MLR supernatants were harvested at the fourth day of coculture and analyzed for IFN-γ by ELISA as described above.

Western blotting

After stimulation, DCs were lysed in Laemmli buffer, and proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride membrane and, after blocking with 5% dry milk/0.1% Tween 20, incubated with primary Abs in the same solution. Bound Abs were detected by anti-IgG conjugated with peroxidase (Amersham Biosciences) and subsequent chemiluminescent detection.

RNA isolation and cDNA preparation

RNA was isolated using TRI reagent (Sigma-Aldrich) according to the protocol of the manufacturers. Nine hundred nanograms of total RNA were reverse transcribed with MuLV-RT using oligo(dT) primers. Design of primers was as follows: The cDNA sequences of the investigated genes were obtained from GenBank. PCR primers were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (41) (code is available at ⟨www.genome.wi.mit.edu/genome_software/other/primer3.html⟩).

The amplified cDNA regions were chosen to span one or more large introns in the genomic sequence, thus avoiding coamplification of genomic DNA under our amplification protocol. The testing of primer specificity included melting point analyses, agarose gel electrophoresis of the PCR products, and subsequent DNA sequencing. Primer sequences used are as follows: cyclooxygenase-2 (COX-2), forward, 5′-CCGCAAACGCTTTATGCTGAA-3′, and reverse, 5′-TGGCCGAGGCTTTTCTACCA-3′; IFN-β, forward, 5′-TGCATTACCTGAAGGCCAAGG-3′, and reverse, 5′-GCAATTGTCCAGTCCCAGAGG-3′.

Real-time RT-PCR

Quantitative RT-PCR was performed using a LightCycler (Roche Molecular Biochemicals) using SYBR Green I detection. Protocol for amplification and quantification was described (42).

EMSA

Nuclear extracts from DC were prepared as described (43). Oligonucleotides resembling the consensus binding site for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were purchased from Santa Cruz Biotechnology. The double-stranded oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. After labeling, 5 μg of nuclear extract was incubated with 120,000 cpm labeled probe in the presence of 3 μg of poly(dI:dC) at room temperature for 30 min. This mixture was separated on a 6% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8.5. Control experiments were performed as described (44). For specific competition, 5 pmol of unlabeled NF-κB oligonucleotide was included, and for nonspecific competition, 5 pmol of double-stranded AP-1 oligonucleotides was used.

Results

ox-PLs inhibit LPS-induced up-regulation of costimulatory molecules in DCs

To test the impact of ox-PLs on DC maturation, we used OxPAPC, a well-characterized mixture of phospholipid oxidation products (32). Immature DCs were treated with LPS (100 ng/ml) with or without addition of OxPAPC (60 μg/ml). Those cells treated with LPS acquired a characteristic morphologic phenotype, and when analyzed by flow cytometry, displayed characteristic markers of mature DCs. Pretreatment of DCs with OxPAPC blocked the up-regulation of surface markers induced by LPS, including costimulatory molecules CD40, CD80, and CD86, cell adhesion molecules like CD54 (ICAM-1) and MHC class I and MHC class II, all of

which are necessary for the induction of an adaptive immune response. Furthermore, LPS-induced up-regulation of the maturation marker CD83, and of the inhibitory B7-H1 (45), as well as induction of CCR7, which is critically involved in lymph node homing of DCs (46), all were inhibited by OxPAPC (Fig. 1*A*). In contrast, treatment of DCs with OxPAPC did not alter expression of the typical differentiation marker CD1a (Fig. 1, *A* and *B*). Inhibition required intermediate oxidation products of PAPC, because neither unoxidized PAPC nor Lyso-PC interfered with LPS-induced DC maturation (Fig. 1*C*). Although LPS potently induced up-regulation of tested surface markers, OxPAPC itself significantly up-regulated only CD86 and MHC class II ($p < 0.05$, paired Student *t* test; Fig. 1*B*).

To examine whether OxPAPC would exert cytotoxic effects or induce apoptosis in DC, we performed control experiments using propidium iodide or Annexin V^{FITC} staining, respectively. Neither OxPAPC nor OxPAPC in combination with LPS significantly induced cell death or increased the percentage of apoptotic cells (data not shown).

LPS-induced cytokine production in DCs is inhibited by OxPAPC

Activated DCs produce a set of cytokines, which contributes to modulation of the T cell response (47–50). OxPAPC pretreatment of DC blocked LPS-induced production of both the active IL-12p70 heterodimer and the p40 subunit (Fig. 2, *A* and *B*), as well as expression of the pro- and anti-inflammatory cytokines TNF and IL-10 (*C* and *D*, respectively). Although OxPAPC also inhibited LPS-induced expression of COX-2 and IFN-β (Fig. 2, *E* and *F*), OxPAPC did not induce expression of IL-10, and neither OxPAPC nor LPS induced expression of the immunosuppressive protein TGF-β (not shown), indicating that neither IL-10 nor TGF-β was involved in the inhibitory effects of OxPAPC.

We have shown previously that OxPAPC interferes with binding of LPS to CD14 and LPS-binding protein, thereby inhibiting recognition of LPS by its signaling receptor, TLR-4 (38), a mechanism that may also account for the inhibitory effect of OxPAPC on LPS-activated DCs. However, in DCs, we observed striking differences in inhibitory potency of OxPAPC toward LPS-induced cytokine expression vs LPS-induced up-regulation of costimulatory molecules. At a concentration of 5 μg/ml, OxPAPC significantly inhibited LPS-induced expression of both IL-12 and TNF, whereas LPS-induced up-regulation of CD83 was not affected. In contrast, concentrations of $\geq$30 μg/ml OxPAPC were necessary to significantly inhibit LPS-induced CD83 up-regulation (Fig. 3). To exclude impurities in our LPS, which have been shown to signal via TLR-2 (51), we also performed the experiments using purified LPS, where we obtained similar results (Fig. 3*B*). These results indicate that at least two different mechanisms account for the inhibitory effects of OxPAPC on LPS-activated DCs.

OxPAPC blocks intracellular signaling events initiated by LPS and prevents activation of NF-κB

Signaling mechanisms that play a role in DC maturation involve the p38 and ERK1/2 MAPK pathway as well as activation of NF-κB and a concerted action of those was shown to be important for the induction of CD83, CD86, CD40, and ICAM-1 (52). To analyze the influence of OxPAPC on activation of signaling pathways by LPS, we determined the intracellular levels of the phosphorylated forms of p38 and ERK1/2 as well as phosphorylation of AKT. As depicted in Fig. 4, LPS-induced phosphorylation of p38, ERK1/2, and AKT was blocked by the addition of OxPAPC. OxPAPC itself induced phosphorylation of ERK1/2 MAPK after 15 min, which was no longer detectable after 30 min, whereas it had no effect on phosphorylation of p38 and AKT. To test the influence of OxPAPC on LPS-induced NF-κB activation, we determined IκB-α levels after 15 and 30 min of LPS treatment with or without addition of OxPAPC. Addition of OxPAPC prevented LPS-induced degradation of IκB-α (Fig. 4*D*), but did not interfere with CD40L-induced IκB-α degradation (*E*). OxPAPC itself did not induce degradation of IκB-α (not shown). Moreover, OxPAPC inhibited in a concentration-dependent manner LPS-induced NF-κB activation and translocation analyzed by an EMSA (Fig. 5). Pretreatment of DCs with 60 μg/ml OxPAPC completely inhibited LPS-induced NF-κB activation, whereas 10 μg/ml OxPAPC, a

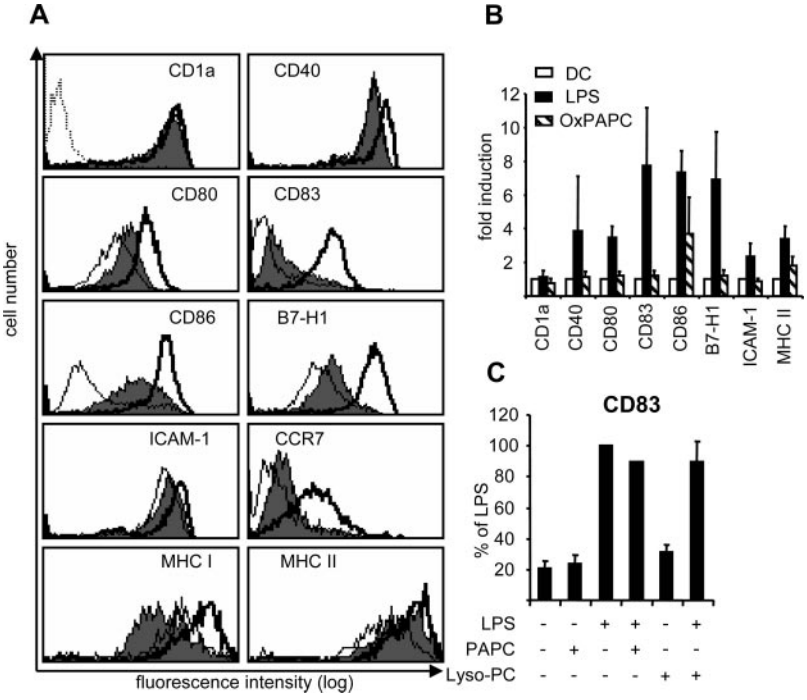

FIGURE 1. OxPAPC, but not unoxidized PAPC nor Lyso-PC, inhibits expression of maturation markers induced by LPS. *A*, Human peripheral blood monocytes were cultured for 6 days in GM-CSF and IL-4 to receive immature monocyte-derived DCs (open histograms, thin line), and were then stimulated with 100 ng/ml LPS either with (gray histograms) or without OxPAPC pretreatment (open histograms, thick line) (OxPAPC at 60 μg/ml 20 min before addition of LPS). After 24 h, the cells were harvested and the surface expression level of the indicated markers was measured by flow cytometry. The dotted line in the CD1a histogram represents VIAP staining (isotype control). Results are representative of at least five independent experiments. *B*, Induction of maturation markers by LPS (100 ng/ml) and OxPAPC alone (mean of five independent experiments), shown as fold induction of immature DCs. *C*, DCs were stimulated with LPS with or without PAPC or Lyso-PC pretreatment as well as PAPC and Lyso-PC alone, and induction of CD83 was measured by flow cytometry. Mean values of mean fluorescence intensity from three independent experiments were calculated as percentage of control LPS responses.

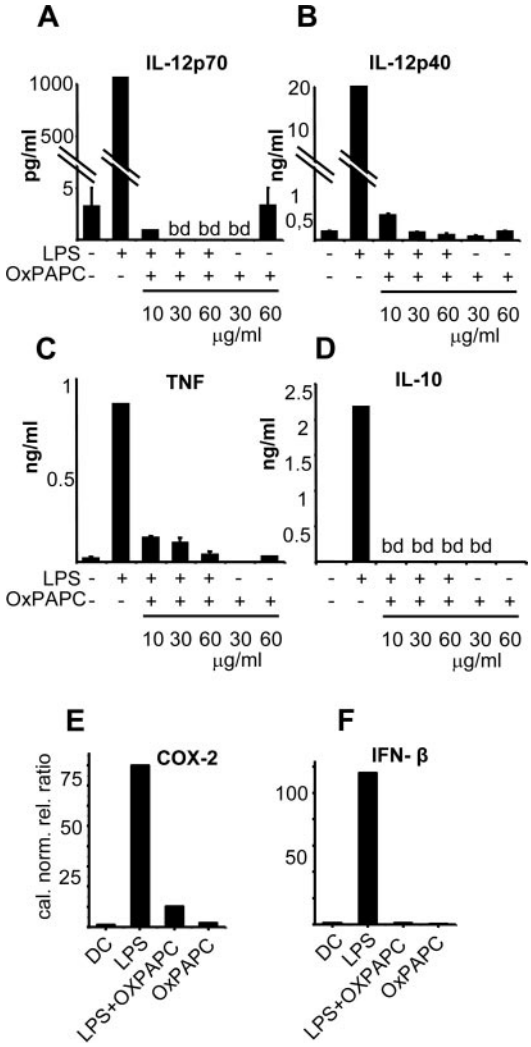

FIGURE 2. OxPAPC inhibits LPS-induced cytokine and proinflammatory mediator production. *A–D*, DCs were cultured with LPS (100 ng/ml) with or without pretreatment with 10, 30, or 60 μg/ml OxPAPC, or 30 or 60 μg/ml OxPAPC alone for 24 h, and the supernatant was analyzed for the indicated cytokines by ELISA. *E* and *F*, DCs were cultured with LPS (100 ng/ml) with or without pretreatment with 60 μg/ml OxPAPC or 60 μg/ml OxPAPC alone for 1 h, and RNA was isolated and COX-2 and IFN-β mRNA expression was determined by RT-PCR.

concentration sufficient to block LPS-induced cytokine production (Figs. 2 and 3), did not interfere with activation of NF-κB.

OxPAPC exerts different effects on DC maturation induced by poly(I:C), CD40L, Pam3CSK4, and PGN

Next, we investigated whether the inhibitory effects of OxPAPC were confined to LPS-induced, TLR-4-mediated maturation of DCs. We found that OxPAPC strongly inhibited phenotypic maturation induced by the TLR-3 ligand poly(I:C) (Fig. 6*A*). However, OxPAPC did not affect DC maturation induced by CD40L and the TLR-2-ligand Pam3CSK4, as evidenced by a lack of inhibition of up-regulation of costimulatory molecules (Fig. 6, *B* and *C*). When using PGN derived from *S. aureus* to induce maturation of DCs, we observed only a slight inhibition by OxPAPC (Fig. 6*D*). Furthermore, OxPAPC blocked poly(I:C)- and Pam3CSK4-induced expression of IL-12 p40 and TNF (Fig. 7, *A* and *B*, *E* and *F*), however did not interfere with PGN-induced TNF production, whereas it still inhibited PGN-induced IL-12 production (Fig. 7, *G* and *H*). It is of note that OxPAPC also inhibited CD40L-mediated

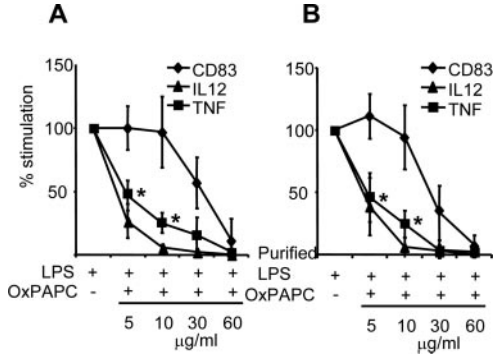

FIGURE 3. Different concentrations of OxPAPC are required for inhibition of LPS-induced costimulatory molecules and cytokines. DCs were stimulated with 100 ng/ml LPS (*A*) or purified LPS (*B*) and pretreated with the indicated amounts of OxPAPC. Inhibition of LPS-induced control responses (LPS = 100%) $\pm$ SD by increasing concentrations of OxPAPC were calculated from at least three independent experiments for CD83. $*$, $p < 0.05$ for all indicated concentrations of OxPAPC.

production of IL-12 and TNF (Fig. 7, *C* and *D*), whereas CD40L-induced up-regulation of costimulatory molecules and activation of NF-κB was not affected by OxPAPC (Figs. 5 and 6).

OxPAPC pretreatment suppresses TLR-induced T cell-stimulatory capacity of DCs

Functionally, mature DCs are characterized by their ability to initiate strong lymphocyte responses (2). To test the stimulatory properties of DCs activated with LPS with or without the addition of OxPAPC, we performed allogenic MLR. LPS-matured DCs gained strong stimulatory capacity, which was reduced by the addition of OxPAPC to the level of immature DCs (Fig. 8*A*), consistent with the inhibition of costimulatory molecules and production of cytokines (Figs. 1 and 2). DCs treated with OxPAPC alone

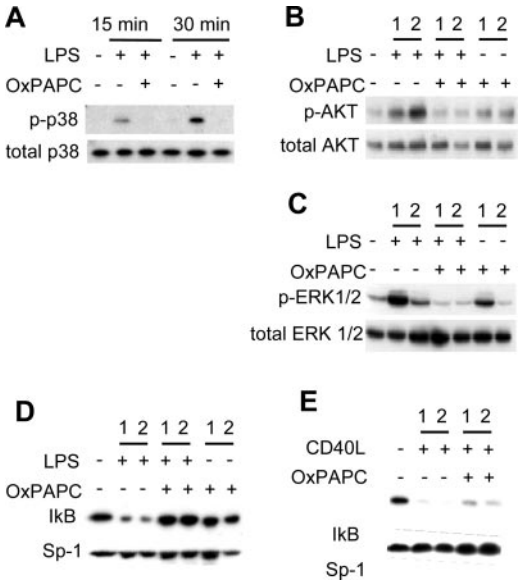

FIGURE 4. LPS-induced signaling events are blocked by OxPAPC. *A–C*, DCs were stimulated for 15 min (*1*) and 30 min (*2*) with LPS (100 ng/ml) with or without 20-min pretreatment with OxPAPC 60 μg/ml, harvested, and analyzed by Western blot for phosphorylated p38 MAPK (*A*), phosphorylated AKT (*B*), and phosphorylated ERK1/2 (*C*). *D* and *E*, DCs were stimulated for 15 min (*1*) and 30 min (*2*) with LPS (100 ng/ml) or CD40L with or without 20-min pretreatment with OxPAPC and probed with Abs directed against IκB-α.

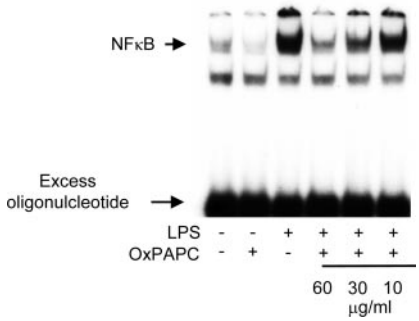

FIGURE 5. OxPAPC prevents LPS-induced activation of NF-κB. DCs were treated with LPS (100 ng/ml) with or without pretreatment with the indicated amount of OxPAPC. After 70 min, total nucleoprotein was extracted. ^{32}P-Labeled oligonucleotides containing a NF-κB consensus sequence were incubated at room temperature with 5 μg of nuclear extracts, followed by non-denaturating gel electrophoresis. Similar results were obtained in two independent experiments.

did not significantly differ from immature DCs in their ability to induce T cell proliferation (not shown). In addition to proliferation, we analyzed T cell cytokine production in the MLR. LPS-induced expression of the Th1 cytokine IFN-γ was inhibited by OxPAPC (Fig. 8*B*). To test whether T cell activation in response to a recall Ag was influenced by OxPAPC, DCs were loaded with graded concentrations of TT for 1 h, matured with LPS with or without the addition of OxPAPC, and incubated with autologous T cells. OxPAPC blocked proliferation of T cells in response to TT-pulsed DCs matured with LPS (Fig. 8*C*). These data show that OxPAPC is able to abrogate the increased capacity of LPS-matured DCs to induce proliferation and cytokine production of Ag-specific T cells.

Furthermore, OxPAPC also prevented poly(I:C)-induced T cell-stimulatory capacity (Fig. 8*D*); however, when CD40L, Pam3CSK4, or PGN were used as maturation stimuli, OxPAPC only slightly reduced the ability of those DCs to initiate lymphocyte proliferation (Fig. 8, *E–G*).

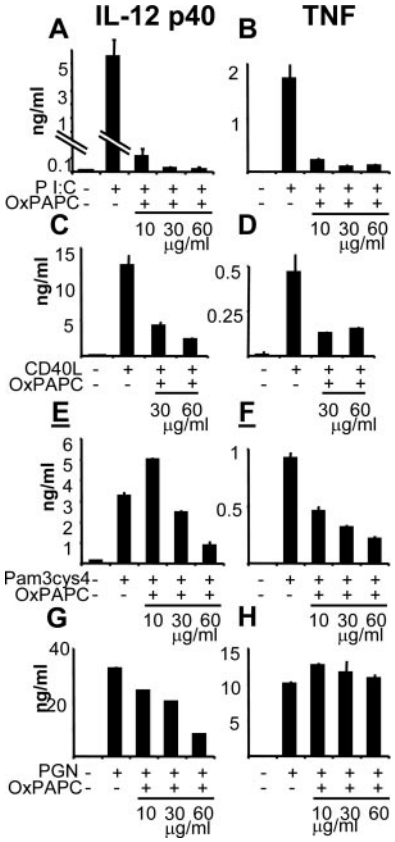

FIGURE 7. OxPAPC prevents poly(I:C) and CD40L-mediated cytokine production and inhibits PGN-induced IL-12. DCs were treated with poly(I:C) (*A* and *B*), CD40L (*C* and *D*), Pam3CSK4 (*E* and *F*), and PGN (*G* and *H*) with or without pretreatment with the indicated amount of OxPAPC. Twenty-four hours later, the supernatants were harvested and analyzed for IL-12 p40 (*A*, *C*, *E*, and *G*) and TNF (*B*, *D*, *F*, and *H*) by ELISA. Data are representative of three independent experiments.

Discussion

During infection, the innate immune response involves activation and maturation of DC that is induced by microbial components, which are recognized by TLRs. The bridging function between the innate and adaptive immune system, translating an unspecific activation signal into an Ag-specific immune response conceivably has to be well controlled (3, 7). In this study, we demonstrate that

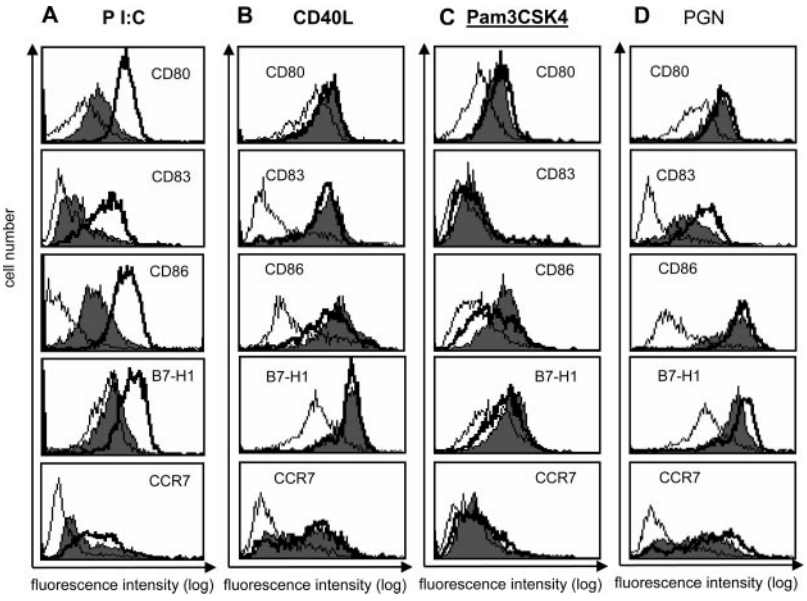

FIGURE 6. OxPAPC modulates surface marker induction by various inducers of DC maturation. DCs (open histograms, thin line) were stimulated with either poly(I:C) (*A*), CD40L (*B*), Pam3CSK4 (*C*), or PGN (*D*) (open histograms, thick line) with or without OxPAPC pretreatment (OxPAPC pretreatment, gray histograms) (OxPAPC at 60 μg/ml/20 min before addition of maturation stimuli). After 24 h, the cells were harvested, and the surface expression level of the indicated markers was measured by flow cytometry. Data are representative of three independent experiments.

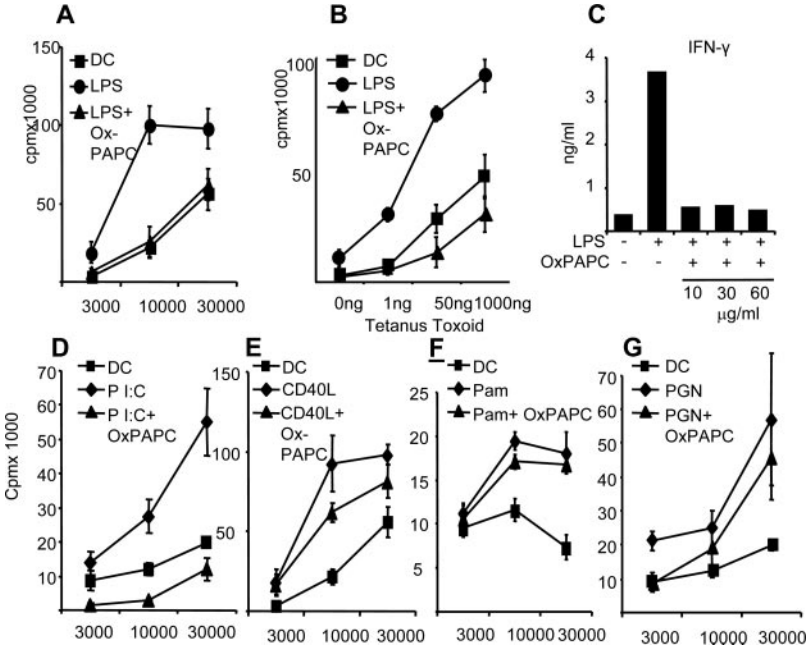

FIGURE 8. OxPAPC reduces LPS-induced T cell-stimulatory capacity. *A*, Purified T cells were stimulated with graded numbers of allogenic immature or LPS-treated (with or without pretreatment with 60 μg/ml Ox-PAPC) DCs, and proliferation of T cells was monitored on day 5 of culture by adding [*methyl*-^{3}H]thymidine followed by measuring [*methyl*-^{3}H]thymidine incorporation 18 h later. *B*, Supernatant of an MLR where DCs treated as indicated were used as stimulators was taken and analyzed for IFN-γ by ELISA. *C*, Purified T cells were stimulated with autologous immature or LPS-treated (with or without pretreatment with 60 μg/ml Ox-PAPC) DCs that have been loaded with the indicated amount of TT, and proliferation of T cells again was monitored on day 5 of culture by adding [*methyl*-^{3}H]thymidine followed by measuring [*methyl*-^{3}H]thymidine incorporation 18 h later. *D–G*, Purified T cells were stimulated with graded numbers of allogenic immature or poly(I:C)-, CD40L-, Pam3CSK4-, or PGN-treated (with or without pretreatment with 60 μg/ml OxPAPC) DCs, and proliferation of T cells was monitored on day 5 of culture by adding [*methyl*-^{3}H]thymidine followed by measuring [*methyl*-^{3}H]thymidine incorporation 18 h later.

phospholipid oxidation products, which are generated during inflammatory processes and apoptosis, represent counterregulators of DC activation induced by PRRs (TLR-2, TLR-3, TLR-4) and CD40-CD40L interactions

We observed that OxPAPC (60 μg/ml) inhibits LPS-induced up-regulation of costimulatory molecules CD40, CD80, and CD86 (Figs. 1 and 6), all of which are necessary for initiation of T cell responses (2). Moreover, LPS-induced surface expression of both MHC classes I and II was significantly inhibited by OxPAPC. As a consequence, T cell-stimulatory capacity of DCs that had been treated with LPS and OxPAPC was drastically reduced (Fig. 8). OxPAPC also strongly inhibited CCR7 surface expression in LPS-treated DC (Fig. 1). It has been shown that down-regulation of CCR7 results in failure of DC to emigrate from the inflamed tissue to the lymph node (53). Thus, inhibition of CCR7 surface expression by OxPAPC may limit emigration of DCs from damaged tissue to draining lymph nodes.

Furthermore, OxPAPC inhibited LPS-induced activation and translocation of NF-κB, as well as MAPK activation. The inhibitory capacity of OxPAPC on LPS-induced signaling cascades, on up-regulation of DC activation markers (CD83, CD86), as well as on cytokine production (IL-12) suggest that OxPAPC acts primarily through blocking of LPS binding to its receptor TLR-4, a mechanism recently described by us (38). However, at OxPAPC concentrations $\leq$10 μg/ml, OxPAPC is now no longer capable of blocking LPS-induced signaling (i.e., NF-κB activation) as well as up-regulation of the DC activation marker CD83 (Figs. 5 and 3, respectively). These results suggest that activation of DC by LPS via TLR-4 does occur at low concentrations of OxPAPC. Nevertheless, LPS-induced expression of cytokines IL-12 and TNF is still inhibited in DC by OxPAPC concentrations as low as 5 μg/ml (Fig. 3). Moreover, at these low concentrations, the ability of LPS-treated DCs to polarize T cells in an MLR toward IFN-γ-producing Th-1 cells is abrogated. These results demonstrate that, in addition to its ability to interfere with LPS/TLR-4 interaction, OxPAPC modulates responsiveness of DC for LPS by targeting the Th-1-driving capacity.

We further show that OxPAPC inhibits DC maturation induced by poly(I:C), which signals through TLR-3. Similarly to the effects on LPS-induced DC maturation, addition of OxPAPC prevented induction of costimulatory molecules, production of cytokines, and increase in T cell-stimulatory capacity in DCs activated by poly(I:C) (Figs. 6 and 7). However, effects of OxPAPC on maturation of DCs induced via TLR-2 or CD40 were less pronounced. OxPAPC inhibited neither up-regulation of costimulatory molecules nor activation of NF-κB induced by CD40L, whereas OxPAPC potently inhibited CD40L-induced TNF and IL-12. Also, in case of Pam3CSK4-induced maturation, OxPAPC did not interfere with up-regulation of costimulatory molecules, but potently inhibited TNF and IL-12 production. In contrast, when PGN was used to activate DCs, we observed a very moderate reduction in the expression of costimulatory molecules, unaltered levels of TNF, but a strong inhibition of IL-12 by OxPAPC (Figs. 6 and 7). These data identify OxPAPC as a strong negative regulator of IL-12 production in DCs by all of the maturation stimuli used in this study. The mechanism by which OxPAPC inhibits IL-12 production is currently investigated.

OxPAPC has been shown to induce several signaling pathways causing an inflammatory response in endothelial cells (54). When DCs were treated with OxPAPC, we did not observe typical signs of activation such as induction of the activation marker CD83 or cytokine production. Most importantly, the T cell-stimulatory function of immature DCs was not affected by OxPAPC. Thus, in our experiments, OxPAPC alone did not have major direct impact on the functional behavior of DCs. The only effects seen were activation of ERK1/2 MAPK and increased surface expression of CD86 and MHC class II upon OxPAPC treatment of DCs. Other lipid oxidization products such as Lyso-PC or oxidized low-density lipoprotein were described to induce maturation and cytokine production as well as increased T cell-stimulatory capacity in DCs (55, 56), suggesting that OxPAPC acts on DCs via different, so-far-unknown mechanisms.

It has been shown that anti-inflammatory cytokines such as IL-10 or TGF-β contribute to down-modulation of the immune response (57, 58). However, we demonstrate that OxPAPC does not increase the expression of IL-10 or TGF-β, and LPS-induced IL-10 expression was strongly inhibited by OxPAPC (Fig. 2), excluding the possibility that these cytokines mediate the inhibitory effect of OxPAPC.

Taken together, these data suggest a regulatory role of ox-PLs on DC activation during inflammation. In tissues with increased oxidative stress, the formation of OxPAPC may limit generation of IL-12-producing DCs, thereby diminishing their capacity to initiate Th-1 responses. Ultimately, in the case of Gram-negative and viral infections, accumulation of OxPAPC at sites of increased tissue damage may limit maturation of DCs, thereby contributing to shut off adaptive immune responses.

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Disclosures

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