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_J Immunol_ 2007; 178:993-1001; doi: 10.4049/jimmunol.178.2.993

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Oxidized Phospholipids Inhibit Phagocytosis and Impair Outcome in Gram-Negative Sepsis In Vivo

Sylvia Knapp, Ulrich Matt, Norbert Leitinger, and Tom van der Poll

Oxidized phospholipids that are generated during inflammation exert anti-inflammatory properties and prevent death during murine endotoxemia. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) inhibits the interaction of LPS with LPS-binding protein and CD14. In this study, we determined the functional properties of OxPAPC and potential interference with CD14 during abdominal sepsis caused by Escherichia coli. Administration of OxPAPC rendered mice highly susceptible to E. coli peritonitis, as indicated by an accelerated mortality and enhanced bacterial outgrowth and dissemination. CD14−/− mice also displayed increased mortality and bacterial outgrowth and OxPAPC did not further impair host defense in these animals. The mechanisms by which OxPAPC and CD14 deficiency impaired the immune response differed: whereas CD14−/− mice demonstrated a strongly reduced recruitment of phagocytes to the site of the infection, OxPAPC did not influence the influx of inflammatory cells but strongly diminished the phagocytosing capacity of neutrophils and macrophages by a CD14-independent mechanism. Furthermore, OxPAPC potently inhibited uptake of fluorospheres as well as receptor-mediated endocytosis and fluid-phase pinocytosis. These data suggest that oxidized phospholipids such as produced during inflammatory reactions may contribute to mortality during Gram-negative sepsis in vivo via impairment of the phagocytic properties of professional phagocytes. The Journal of Immunology, 2007, 178: 993–1001.

A

cute bacterial peritonitis is a life-threatening infection characterized by the presence of bacteria in the normally germ-free peritoneal cavity. Almost invariably caused by perforation of intestines, the most frequently encountered pathogens are enteric Gram-negative bacteria such as Escherichia coli, which can be found in up to 60% of the cases (1). Mortality rates of peritonitis range between 30 and 50% despite advances in surgery and antimicrobial therapy. A serious complication originating from peritonitis is systemic inflammation and sepsis with mortality rates of up to 80% (2).

The prompt initiation of host defense mechanisms is essential for the host to survive. The innate immune system, that is responsible for mounting an immediate response to invading pathogens, is considered the central element of host defense in peritonitis. LPS is a major constituent of the outer cell wall of Gram-negative bacteria, such as E. coli, and the principal inducer of inflammatory responses to these pathogens. CD14, TLR4, and MD-2 make up the LPS receptor complex involved in the cellular recognition of and signaling by LPS (3–8). LPS-binding protein (LBP)3 greatly augments the transfer of LPS to the CD14–TLR4 complex. Recently, we demonstrated the pivotal role of LBP during murine E. coli peritonitis: the rapid recruitment of polymorphonuclear cells (PMNs) to the site of infection critically depended on the presence of LBP and mice lacking LBP displayed a greatly increased bacterial outgrowth and dissemination that led to early death (9).

PMNs are key phagocytes required for the immediate elimination of bacteria. One of the most powerful weapons generated by activated PMNs is reactive oxygen species that are used for antibacterial defense. However, in parallel with the killing of bacteria, free radicals may damage host molecules, and in particular induce lipid peroxidation. Oxidized phospholipids (OxPL) are generated in vivo at sites of acute and chronic inflammation (10–12). Accumulating evidence suggests that OxPL are not merely by-products of the inflammatory response, but can actively regulate inflammation (13). Most previous studies (13–15) focused on the proinflammatory effects of OxPL, which are thought to play a role in initiating and maintaining chronic inflammation such as atherosclerosis. However, it is increasingly recognized that OxPL at the same time possess potent anti-inflammatory properties, which include the direct antagonism of LPS recognition by cells of the innate immune system. Indeed, OxPL effectively inhibit the interaction of LPS with LBP, CD14, and TLR4 (16). The biological significance of this finding was further underlined by the observation that the exogenous administration of OxPL could prevent mortality of mice exposed to high doses of LPS; in this respect, OxPL reproduced the LPS-resistant phenotype of mice lacking LBP, CD14, or TLR4 (16–20).

The biological role of OxPL during Gram-negative infection with viable bacteria has not been studied so far. We therefore decided to investigate the effects of OxPL during E. coli-induced abdominal sepsis in vivo and the role of CD14 herein.
Materials and Methods

Mice

Pathogen-free 9- to 11-wk-old male C57BL/6 wild-type (WT) mice were purchased from Harlan Sprague Dawley (Horst). CD14 gene-deficient (CD14−/−) mice were obtained from The Jackson Laboratory (21) and backcrossed to C57BL/6 background six times. Age- and sex-matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Phospholipids

1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholine (PAPC) and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma-Aldrich. DMPC was used as negative control due to the fact that DMPC lacks unsaturated fatty acids and thus cannot be oxidized. Oxidized PAPC (OxPAPC) was generated by air oxidation as described recently and stored in chloroform at −70°C (16). The extent of PAPC oxidation was routinely confirmed by electrospray ionization-mass spectrometry (22), and only preparations showing a reproducible pattern of lipid oxidation products were used in the study. These preparations were routinely tested in in vitro assays for biological activity as well as LPS content using the Limulus assay. Directly before use, OxPAPC were dripped in glass tubes under the stream of N2 and resuspended in NaCl by vortexing.

Induction of peritonitis

Peritonitis was induced as described previously (9, 23, 24). In brief, E. coli O18:K1 was cultured in Luria-Bertani medium (Difco) at 37°C, harvested at mid-log phase, and washed twice before inoculation. Mice were injected i.p. with 1–2 × 10⁶ CFU E. coli in 200 μl of sterile saline. The inoculum was plated on blood agar plates to determine viable counts. OxPAPC (12.5 mg/kg) or control lipids (DMPC; 12.5 mg/kg in 200 μl of NaCl) or the same volume of carrier were injected i.p. immediately before bacterial inoculation.

Monitoring of mortality and enumeration of bacteria

In survival studies, 8–12 mice per treatment group were inoculated with E. coli. Because mortality occurs primarily between 24 and 48 h after infection in this model, mortality was assessed every 2 h in this period; thereafter, mortality was monitored every 6 h. In separate studies, mice were sacrificed 4 or 20 h after infection; at these time points, mice were anesthetized by inhalation of isoflurane and peritoneal lavage was performed with 5 ml of sterile isotonic saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes, washed twice, and cells were seeded in tissue culture plates to get rid of adherent macrophages. Nonadherent cells were counted after 2 h and consisted of >95% PMNs. Phagocytosis assay was performed as described above. The phagocytosis index of each sample was calculated: (mean fluorescence × percent positive cells at 37°C) minus (mean fluorescence × percent positive cells at 4°C). The same procedure was followed when using RAW 264.7 cells that were expanded from peritoneal lavage of C57BL/6 mice (CD14−/−). Peroxidase activity of RAW 264.7 cells that were cultured for 24 h with or without PAPC was measured as described.

Cell counts and differentials

Cell counts, determined on each peritoneal lavage sample stained with Giemsa, were counted in a hemocytometer (Türck counting chamber). The cells were then diluted to a final concentration of 1 × 10⁶ cells/ml and differential cell counts were performed on cytospin preparations stained with Giemsa.

Cytokine/chemokine assays

Cytokines and chemokines (TNF-α, IL-6, IL-10, and MCP-1) were measured using the cytometric bead array multiplex assay (BD Biosciences) according to the manufacturer’s instructions. The detection limits were 5 pg/ml. Keratinocyte-derived chemokine (KC), MIP-2, and IL-1β concentrations were determined using commercially available ELISA (R&D Systems).

Bacterial killing assay

Bacterial killing was determined according to a protocol published recently (25). In brief, RAW 264.7 cells (American Type Culture Collection) were mobilized using 5 mg/ml lidocaine in PBS, washed, and plated in 24-well plates at a density of 2 × 10⁵ cells/well. Cells were allowed to adhere for 2 h at 37°C and washed thoroughly with serum-free RPMI 1640. E. coli O18:K1 were added at a multiplicity of infection of 10 and spun onto cells at 2000 rpm for 5 min, after which plates were placed at 37°C for 10 min. Each well was then washed five times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake at 10 min, triplicate of wells were lysed with sterile H₂O and designated as t = 0. Prewarmed serum-free RPMI 1640 with or without 50 μg/ml OxPAPC was added to remaining wells and plates were placed at 37°C for 5, 10, 30, 60, or 90 min, after which cells were again washed five times with ice-cold PBS and lysed as described above. Cells were then allowed to adhere in 24-well plates in 5 ml of sterile isotonic saline using an 18-gauge needle. Lavage fluid and bacterial counts were enumerated after 16 h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t = 0 (percent killing = 100 − (no. of CFU at time ×/no. of CFU at time 0) × 100).

Phagocytosis assays

Phagocytosis was evaluated in essence as described before (26, 27). Peritoneal lavage was performed in WT and CD14−/− mice (n = 8/strain) using 5 ml of sterile saline. Lavage fluid was collected in sterile tubes and put on ice. Peritoneal macrophages (PM) were washed, counted, and resuspended in RPMI 1640 at a final concentration of 1 × 10⁶ cells/ml. Cells were then allowed to adhere in 12-well microtiter plates (Greiner) overnight. Adherent monolayer cells were washed thoroughly with HBSS and incubated with FITC-labeled heat-killed E. coli (O18:K1, 1 × 10⁶ CFU/ml) in the presence or absence of 50 μg/ml OxPAPC at 37°C or 4°C for 30 min. Immediately thereafter, cells were put on ice, washed in PBS, suspended in Quenching solution (ORPEGEN Pharma), and analyzed using a FACS Calibur (BD Biosciences). To obtain primary PMNs, WT mice (n = 8) were injected i.p. with 4% proteose peptone (Difco). The next day mice were anesthetized by inhalation of isoflurane (Abbott Laboratories) and peritoneal lavage was performed with 5 ml of sterile saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes, washed twice, and cells were seeded in tissue culture plates to get rid of adherent macrophages. Nonadherent cells were counted after 2 h and consisted of >95% PMNs. Phagocytosis assay was performed as described above. The phagocytosis index of each sample was calculated: (mean fluorescence × percent positive cells at 37°C) minus (mean fluorescence × percent positive cells at 4°C). The same procedure was followed when using RAW 264.7 cells that were expanded from peritoneal lavage of C57BL/6 mice (CD14−/−). Peroxidase activity of RAW 264.7 cells that were cultured for 24 h with or without PAPC was measured as described.

Endocytosis/pinocytosis assays

Cellular uptake of HRP (Sigma-Aldrich), FITC-dextran (Molecular Probes), Lucifer yellow (LY; Molecular Probes), or mannosylated BSA-FITC (Sigma-Aldrich) was determined as reported earlier (28). RAW 264.7 cells were cultured in 12-well plates at a concentration of 0.5 × 10⁵ cells/well and allowed to adhere overnight in RPMI 1640 supplemented with 10% FCS. The next day, cells were washed thoroughly in serum-free RPMI 1640 and FITC-dextran (1 mg/ml), LY (1 mg/ml), or mannosylated BSA-FITC (10 μg/ml) was added and cells were incubated at 37°C for indicated times. In some experiments, excess mannan (from Saccharomyces cerevisiae; Sigma-Aldrich) was added at a concentration of 3 mg/ml. At indicated time points, cells were put on ice, washed thoroughly with cold PBS, and analyzed by FACS analysis. Cells pulsed at 4°C were used to determine background uptake. The uptake index of each sample was calculated: (mean fluorescence × percent positive cells at 37°C) minus (mean fluorescence × percent positive cells at 4°C). To determine uptake of HRP, cells were cultured as described above. HRP was added at indicated concentrations and incubated for 3 h at 37°C. Next, cells were washed four times and lysed with 0.05% Triton X-100 in 10 mM Tris buffer (pH 7.4) for 30 min. The enzyme activity of the lysate was measured using o-phenylenediamine and H₂O₂ as substrate.

Statistical analysis

Differences between groups were calculated using the Mann-Whitney U test or one-way ANOVA. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SEM. A p < 0.05 was considered statistically significant.
Results

OxPL impair survival during E. coli peritonitis in vivo

OxPL have been shown to improve survival during murine endotoxemia due to their capacity to reduce the bioavailability of LPS, thereby attenuating overwhelming systemic inflammation (16). We consequently were interested in studying the effect of OxPL, such as OxPAPC, on the course of septic peritonitis induced by viable Gram-negative bacteria, i.e., microorganisms that express LPS. Mice were inoculated i.p. with E. coli along with OxPAPC or vehicle and observed for 5 days. As depicted in Fig. 1A, OxPAPC-treated mice started to succumb as early as 20 h after infection while all control mice remained alive until t = 38 h. In total, 92% (11 of 12) of OxPAPC-treated mice died vs only 42% (5 of 12) of the control animals (p = 0.004). Thus, the administration of OxPAPC rendered mice more susceptible to E. coli peritonitis.

OxPL facilitate bacterial growth and dissemination

To obtain insight in the mechanism underlying the accelerated and higher mortality of mice that received OxPAPC, we repeated this experiment and sacrificed mice 4 or 20 h after infection to enumerate bacterial counts in peritoneal lavage fluid (PLF; the primary site of infection), blood, and liver (to evaluate to which extent the infection became systemic). Already 4 h after induction of peritonitis, bacterial counts in OxPAPC-treated mice were up to 1 log higher than in controls, but the differences between groups did not reach statistical significance (control vs OxPAPC): PLF, 2.0 ± 0.6 vs 3.6 ± 0.3; blood, 1.8 ± 0.8 vs 2.9 ± 9.6; liver, 8.8 ± 6.0 vs 27.8 ± 12.5). Thereafter, E. coli grew exponentially in all body compartments but much faster in OxPAPC-treated animals and significantly increased CFU counts were recovered from PLF, blood, and liver from these mice 20 h after infection (Fig. 1, B–D). Therefore, the i.p. administration of OxPAPC is associated with an increased bacterial outgrowth and dissemination during E. coli peritonitis.

OxPL transform WT mice into a CD14-deficient phenotype

Because earlier findings pointed toward the fact that OxPL inhibit the interaction of LPS with LBP and CD14 (16) and because LPS

FIGURE 1. OxPAPC increases mortality and facilitates bacterial outgrowth and dissemination. Mice received either OxPAPC (12.5 mg/kg i.p.; open symbols) or carrier (saline; filled symbols) and were i.p. infected with 1.2 × 10^4 CFU E. coli. A, Survival data are representative of two independent experiments of n = 12/group; p value indicates the difference between survivals by log rank test. B, In separate experiments, mice were treated as above and PLF, blood, and liver CFU were determined 20 h after infection. Data are mean ± SEM of n = 8 mice/group. Results are representative of two independent experiments. *, p < 0.05 vs control mice.

FIGURE 2. OxPAPC does not further increase the enhanced mortality of CD14^-/- mice. WT and CD14^-/- mice were i.p. infected with 2 × 10^4 CFU E. coli in addition to administration of 12.5 mg/kg control lipids (DMPC; A) or OxPAPC (B). All four groups of mice were infected simultaneously and survival curves were separated for reasons of clarity (all CD14^-/- and/or OxPAPC-treated mice succumbed at the same pace with p < 0.05 vs WT mice that received DMPC). Survival was monitored over 1 wk, n = 8/group; p value indicates the difference between survivals by log rank test. Experiments were repeated and mice were infected i.p. with 1.2 × 10^4 CFU E. coli 20 h before bacterial counts were enumerated in PLF (C), blood (D), and liver (E). *, p < 0.05 vs WT DMPC mice.
CD14 during Gram-negative peritonitis and lay emphasis on CD14 significance. Together, these findings underline the importance of OxPL as a potential target molecule that might explain the detrimental effects induced by OxPL in vivo.

**OxPL do not influence early cytokine/chemokine responses**

Having shown that OxPL impaired outcome, we then asked whether the effects of OxPAPC described above could be explained by a functional CD14 blockade in the presence of these phospholipids. For this purpose, CD14−/− and WT mice were treated with OxPAPC or control lipids (DMPC) and infected i.p. with *E. coli*. Survival studies disclosed that CD14−/− mice were highly susceptible to *E. coli* peritonitis and succumbed quickly (Fig. 2A). The administration of OxPAPC transformed WT mice into a CD14−/− phenotype, whereas no additional effect on lethality was observed in CD14−/− animals treated with OxPAPC (Fig. 2B). Of note, to exclude potential nonspecific effects of lipids, we used control lipids (DMPC) instead of carrier (NaCl) in this set of experiments. As expected, DMPC-treated mice behaved exactly like mice that received carrier in earlier experiments, i.e., they showed an improved survival when compared with OxPAPC-treated animals. Mortality rates were slightly higher in this experiment (compared with Fig. 1A), due to the, in retrospect, higher number of bacteria mice were infected with. We then repeated this experiment and enumerated bacterial counts 20 h after infection. CD14−/− mice treated with control lipids (DMPC) displayed significantly increased bacterial counts in PLF, liver, and blood when compared with DMPC-treated WT mice (Fig. 2, C–E). OxPAPC treatment enhanced bacterial outgrowth in WT mice, confirming the experiments shown in Fig. 1 (again illustrating the fact that DMPC treatment results in identical results as carrier treatment). Importantly, OxPAPC did not further increase bacterial loads in CD14−/− mice (Fig. 2, C–E). In addition, although the numbers of *E. coli* CFU tended to be higher in blood and livers of OxPAPC-treated CD14−/− mice than in OxPAPC-treated WT mice (Fig. 2, D and E), the differences between groups did not reach statistical significance. Together, these findings underline the importance of CD14 during Gram-negative peritonitis and lay emphasis on CD14 as a potential target molecule that might explain the detrimental effects induced by OxPL in vivo.

**OxPAPC impairs the phagocytic capacity of PM and PMN**

Having shown that OxPAPC affects CD14-independent pathways of the innate immune response, we next aimed to investigate the

### TABLE I. OxPAPC does not impair the early cytokine/chemokine response

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<tr>
<th></th>
<th>PLF</th>
<th>Plasma</th>
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<tr>
<td></td>
<td>Co</td>
<td>OxPAPC</td>
</tr>
<tr>
<td></td>
<td>Co</td>
<td>OxPAPC</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td></td>
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<tr>
<td>IL-6</td>
<td>28.4 ± 6.7</td>
<td>256.6 ± 77.7*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8.8 ± 0.5</td>
<td>143.4 ± 20.6*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>136 ± 51</td>
<td>358 ± 38*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>152.6 ± 29.1</td>
<td>548.9 ± 137.8*</td>
</tr>
<tr>
<td>KC</td>
<td>25.5 ± 5</td>
<td>273.5 ± 104*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>125.6 ± 0.6</td>
<td>149.9 ± 19.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>89.4 ± 5.2</td>
<td>98.1 ± 4.1</td>
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</table>

WT mice (*n = 8*) per group and time point) were inoculated i.p. with 1.2 × 10⁶ CFU *E. coli* and 12.5 mg/kg OxPAPC or carrier (NaCl, control mice). PLF and plasma was obtained after 4 h and cytokines/chemokine concentrations were assayed as described in Materials and Methods. Data are mean ± SEM. ND, Not detectable.

* *p < 0.05 vs NaCl-treated control mice.

OxPL does not influence early cytokine/chemokine responses

Having shown that OxPL impaired outcome, we then asked which factors might be involved in the outgrowth and spread of *E. coli*. Responses accountable for an appropriate innate immune response during peritonitis include the local production of proinflammatory cytokines and chemokines at the site of the infection (27, 30, 31). Because OxPL can inhibit LPS bioavailability (16), we considered it possible that OxPAPC would impair the early cytokine/chemokine response to *E. coli*, thereby enhancing susceptibility to abdominal sepsis. To address this issue, we measured IL-6, TNF-α, IL-1β, MCP-1, KC, MIP-2, and IL-10 in PLF and plasma. As shown in Table I, OxPL did not impair the early induction of proinflammatory cytokines after i.p. infection with *E. coli*. Peritoneal IL-6, TNF-α, MCP-1, and KC concentrations were even higher in OxPL-treated mice at *t* = 4 h, which indicates that a suppressed early immune response cannot explain differences in outcome and bacterial elimination (Table I). Because PMN influx to the peritoneal cavity is a critical component of the innate immune response during peritonitis, we next determined leukocyte counts in PLF. However, 4 h after induction of peritonitis, we did not find any indication for an impaired leukocyte influx (Table II). On the contrary, OxPAPC-treated mice had the tendency to attract more leukocytes than control mice, likely as a result of the higher bacterial load in these mice. Thus, the number of PMN at the site of infection does not explain differences in bacterial clearance and outcome. When examining the local peritoneal and systemic cytokine/chemokine response in WT and CD14−/− mice at *t* = 20 h, we identified an enormously increased release of IL-6, TNF-α, and MCP-1 in PLF and plasma of CD14−/− animals after 20 h (Fig. 3), whereas MIP-2 and KC levels were similar in all groups (data not shown). These values by far exceeded the levels measured in WT mice treated with OxPAPC. Of interest, CD14−/− control mice (i.e., DMPC treated) had the highest cytokine/chemokine concentrations, whereas CD14−/− animals that received OxPAPC showed less pronounced elevations (Fig. 3). Therefore, OxPAPC modestly reduced the cytokine response in CD14−/− but not in WT mice in vivo. In contrast, CD14−/− animals displayed a severely impaired ability to attract PMNs and macrophages to the peritoneal cavity, irrespective of OxPAPC treatment (Table III). Even 20 h after the induction of peritonitis when vast amounts of bacteria were encountered in all mice, the number of PMNs and macrophages was significantly reduced in the absence of CD14. Thus, although OxPAPC did not impair the attraction of inflammatory cells to the site of infection, CD14 crucially contributes to this important host defense mechanism.

**OxPAPC impairs the phagocytic capacity of PM and PMN**

Having shown that OxPAPC affects CD14-independent pathways of the innate immune response, we next aimed to investigate the

### TABLE II. OxPAPC does not attenuate early cell recruitment

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<tr>
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<th>PMN</th>
<th>PM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>4 h (cells × 10⁷/ml)</td>
</tr>
<tr>
<td>NaCl</td>
<td>37.2 ± 11.4</td>
<td>23.0 ± 6.6</td>
</tr>
<tr>
<td>OxPAPC</td>
<td>108.0 ± 37.9</td>
<td>57.0 ± 22.8</td>
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WT mice (*n = 8*) were infected i.p. with 1.2 × 10⁶ CFU *E. coli* and 12.5 mg/kg OxPAPC or carrier (NaCl) and PLF was obtained after 4 and 20 h. Total cell counts were determined and differentials were done on cytospin preparations stained with Giemsa. Data are mean ± SEM.

* *p < 0.05 vs NaCl-treated control mice.*
Figure 3. Cytokine/chemokine response in WT and CD14−/− mice treated with OxPL. WT (■) and CD14−/− (□) mice were treated with 12.5 mg/kg control lipids (DMPC) or OxPAPC and infected with 1.2 × 10⁶ CFU E. coli i.p. PLF and plasma TNF-α, IL-6, and MCP-1 concentrations were measured after 20 h. Mean ± SEM. * p < 0.05 vs indicated group.

OxPAPC impairs endocytosis by macrophages

To further understand the impact of OxPL on phagocytosis, we then asked whether our finding of impaired phagocytosis in the presence of OxPAPC is specific for pathogens such as E. coli or reflects a more general phenomenon. Because we performed all phagocytosis assays (Fig. 4) under serum-free conditions using nonopsonized bacteria, the possibility that OxPAPC interferes with Fc receptor or complement-mediated phagocytosis is unlikely. However, scavenger receptors (SR)-A have been identified as important receptors that specifically contribute to Fc receptor-independent phagocytosis of E. coli (32). We therefore investigated the potential contribution of SR-A and found, in accordance with earlier reports (32), that blocking SR-A with anti-SR-A Abs significantly reduced uptake of E. coli by macrophages (Fig. 5A). The addition of OxPAPC alone showed exactly the same inhibitory effect as anti-SR-A alone and no additive effect was observed when the combination of both reagents was tested (Fig. 5A). We then tried to more closely imitate the in vivo situation seen during peritonitis, when PM encounter LPS or bacteria that activate them before they phagocytose whole bacteria, and preincubated macrophages with LPS before the addition of E. coli. As expected, phagocytosis of E. coli was tremendously increased when macrophages were preactivated by LPS (Fig. 5B; compare y-axes of A and B). In addition, when anti-SR-A or OxPAPC were added to these activated macrophages, the inhibitory action of OxPAPC clearly exceeded that of anti-SR-A (Fig. 5B). Together these findings indicate that OxPAPC either interferes with phagocytosis receptors other than SR-A or that OxPAPC uses additional (possibly activation-dependent) pathways. To assess SR-A-independent phagocytosis and the role of OxPAPC herein, we next investigated the uptake of polystyrene microspheres and identified that OxPAPC dose-dependently inhibited the uptake of these microspheres (Fig. 5C). Importantly, control lipids (DMPC) did not show this effect.

Considering the fact that macrophages are quite unique in their capacity to not only phagocytose but also ingest particles via fluid-phase pinocytosis and macropinocytosis, the latter also involving actin remodeling (33), we next examined whether these properties were also influenced by OxPAPC. For this purpose, we first analyzed the uptake of peroxidase (HRP) by macrophages and found reduced internalization when OxPAPC was added (Fig. 6A). To study effects on a single cell level, we then chose to test the uptake of FITC-dextran, which is internalized via macropinocytosis, representing an uptake mechanism that is quite uniquely found in...
macrophages, dendritic cells, and epithelial cells (33). For this purpose, macrophages were incubated with FITC-dextran and OxPAPC clearly diminished the macropinocytotic uptake of FITC-dextran (Fig. 6B). Because HRP and dextran uptake has been shown to partly depend on mannose receptor (28), we then investigated the uptake of a well-defined, pure mannose receptor ligand, namely, manniosylated BSA-FITC (28). The addition of OxPAPC reduced receptor-mediated endocytosis of manniosylated BSA when compared with DMPC (Fig. 6C) or carrier (Fig. 6D). Moreover, addition of excess mannan abolished the uptake of manniosylated BSA.
BSA, thus confirming the specificity of this mechanism (Fig. 6C). We finally studied whether OxPAPC also interferes with receptor-independent, fluid-phase endocytosis. LY has been described as a receptor-independent ligand that is taken up by cells via pinocytosis (28, 34). We found that even small amounts of OxPAPC diminished the uptake of LY (Fig. 6, E and F) as compared with carrier (●). A representative histogram at t = 120 min is depicted in F (thin line, 4°C control; thick line, 37°C carrier; filled gray, 37°C OxPAPC). Mean ± SEM. * p < 0.05 vs carrier.

Discussion

Gram-negative peritonitis is a life-threatening condition frequently associated with systemic dissemination of bacteria and septic shock. Host defense in peritonitis is an established domain of the innate immune system as the rapid response to invading pathogens is essential for the host to survive. OxPL are endogenous mediators of inflammation and products of oxidative stress that have been shown to also exert anti-inflammatory effects (11, 16, 35). Inhibition of LPS-LBP and LPS-CD14 interactions by OxPAPC protected mice from overwhelming inflammation during endotoxia (16). In the present study, we examined the functional in vivo role of OxPAPC and its potential interaction with CD14 during murine E. coli peritonitis. Our key finding was that OxPAPC, in sharp contrast to its protective effect during LPS-induced shock, rendered mice highly susceptible to abdominal sepsis induced by viable E. coli by a mechanism that is CD14 independent. Although both administration of OxPAPC and CD14 deficiency resulted in increased mortality and bacterial growth after i.p. infection with E. coli, the mechanisms accounting for these findings differed: OxPAPC interfered with crucial functional properties of recruited phagocytes reducing their phagocytic capacity, whereas CD14 deficiency resulted in an attenuated recruitment of phagocytes to the site of the infection. The present data reveal for the first time that OxPL may impair host defense against Gram-negative infection.

Host defense in peritonitis is a delicate balance between proinflammatory pathways aimed at the rapid elimination of bacteria and anti-inflammatory pathways intended to prevent systemic inflammation (36). Any imbalance in pro- or anti-inflammatory mediators might prove harmful. We initially considered it conceivable that OxPAPC, due to their anti-inflammatory properties described in models of acute inflammation (16), might represent an endogenous mediator that assists preventing overwhelming inflammation.
and, therefore, would reduce mortality in mice suffering from severe peritonitis. Our findings, however, could not confirm this hypothesis but rather disclosed an important, although detrimental, role for OxPAPC in host defense against *E. coli* in vivo. In the presence of OxPAPC, a higher proportion of mice succumbed and this was accompanied by an increased bacterial load in all organs tested. The important attraction of phagocytes to the peritoneal cavity was not compromised by OxPAPC. It has been reported earlier (11, 14, 37) that OxPL themselves induce the production of chemokines such as IL-8, KC, and MCP-1 in vitro as well as in vivo. In our hands, the addition of OxPAPC during *E. coli* peritonitis in vivo was accompanied by increased peritoneal MCP-1 and KC concentrations, which may have contributed to the elevated number of PMNs and monocytes/macrophages in peritoneal fluid early (t = 4 h) after induction of peritonitis.

OxPAPC can inhibit the interaction of LPS with CD14 (16). Because CD14 is a major component of the LPS-signaling complex on innate immune cells and an important player in host defense against Gram-negative bacterial infections (3, 38–42), we were interested in studying whether our observations of increased bacterial outgrowth and mortality in OxPAPC-treated mice were linked to an inhibitory action of OxPL on the interaction of LPS with CD14. Using CD14 gene-deficient mice, we first were able to demonstrate an important role of CD14 in the innate immune response during *E. coli* peritonitis in vivo. Analogous to our earlier studies, where we investigated the role of LBP in this infection model and in line with earlier reports in *Salmonella* peritonitis, an inadequate onset of inflammation in CD14−/− mice led to early systemic dissemination and increased bacterial outgrowth (9, 43). Although bacterial dissemination and survival rates were identical in CD14−/− and OxPAPC-treated mice, the underlying mechanisms differed. OxPAPC administration had no influence on the number of PMNs attracted to the peritoneal cavity but recruited PMNs imposed covered by bacteria. This inspired us to investigate the functional properties of PMNs and macrophages and led us to discover an impaired phagocytosis by professional phagocytes in the presence of OxPAPC. Indeed, RAW cells as well as primary PMNs and PM were less capable of effectively phagocytosing *E. coli* in the presence of OxPAPC. Although CD14 was attributed to phagocytosis of Gram-negative bacteria by an earlier report (44), we could not disclose a role for this receptor. In line, our laboratory previously found no effect of a blocking anti-CD14 Ab i.v. administered to human subjects on phagocytosis by blood monocytes and PMNs (42). However, CD14 is known to participate in the elimination of apoptotic cells and OxPAPC, due to its capacity to interfere with CD14, is certainly a candidate molecule that might interact with this process (45). In fact, one report (46) showed an impaired phagocytosis of apoptotic cells in the presence of minimally modified low-density lipoprotein that bind to CD14 and concurrently enhance the uptake of oxidized low-density lipoprotein. Nevertheless, the potential impact of OxPL on phagocytosis of bacteria has not been investigated thus far and we hereby report for the first time that OxPAPC interfere with the elimination of bacteria in vivo and that this mechanism does not depend on CD14.

Another receptor that crucially contributes to opsonin-independent phagocytosis of *E. coli* is SR-A (32). SR-A recognizes a wide range of polyanions including LPS from Gram-negative bacteria. Although not known so far, the possibility exists that OxPAPC interferes with the binding of polyanions to SR-A, thereby preventing the phagocytosis of Gram-negative bacteria. However, our data from activated macrophages clearly demonstrate that OxPAPC inhibits phagocytosis to a greater degree than blocking Ab against SR-A. Addition of OxPAPC to anti-SR-A further reduced the uptake of *E. coli*, thus indicating different inhibitory pathways. Of note, although the class B SR CD36 has been shown to bind OxPAPC (47), CD36 is not involved in the phagocytosis of *E. coli* (48), which precluded us from investigating CD36 as a target receptor that might explain the effects of OxPAPC observed in this study.

Our data indicate that the inhibitory action of OxPAPC is not restricted to phagocytosis of *E. coli*. Considering the multitude of endocytotic pathways elicited by macrophages, we found OxPAPC to not only impair phagocytosis of bacteria but also polystyrene particles as well as receptor-mediated and fluid-phase endocytosis. These findings definitely underline the broad, and potentially harmful, impact of oxidation products generated during bacterial infections or chronic inflammation in vivo. Although the pathophysiological impact of these findings remains to be established, we propose a detrimental role for OxPL during bacterial infections. Beside infections, OxPL are found predominantly during chronic inflammation such as atherosclerosis. The observations described in this study of impaired endocytosis in the presence of OxPL could explain the very recent finding of diverse bacterial products within a single plaque specimen from patients with coronary heart disease (49). The possibility exists that OxPL, present in atherosclerotic plaques, prevent the effective elimination of bacteria that are encountered during asymptomatic phases of bacteremia, such as after dental procedures or translocation from the intestines, thus leading to the accumulation of intracellular bacterial products that in turn might contribute to ongoing inflammation.

It should be noted that although OxPAPC and CD14 deficiency negatively influenced the outcome of *E. coli* peritonitis by different mechanisms, OxPAPC did not further impair host defense in CD14−/− mice. Importantly, however, OxPAPC and CD14 deficiency both impacted on PMNs and macrophages, and although OxPAPC profoundly diminished the capacity of CD14−/− PMNs and macrophages to phagocytose *E. coli* in vitro, apparently this immune suppressing effect did not further impact on the outcome of CD14−/− mice in vivo due to the fact that these animals had very few PMNs and macrophages in their peritoneal cavity and as a consequence already had a severely hampered cellular immune response.

In conclusion, we demonstrate that OxPAPC reduce host defense against abdominal sepsis caused by *E. coli* most likely by inhibiting the phagocytosing capacity of cells involved in innate immunity by a CD14-independent mechanism. Although OxPL might be able to prevent overwhelming inflammation in settings of sterile inflammatory disorders, our results suggest that OxPL generated at sites of inflammation impair the innate immune response to bacterial infections.

**Acknowledgments**

We thank I. Kop and J. Daalhuisen for expert technical assistance.

**Disclosures**

The authors have no financial conflict of interest.

**References**


