

Lipid Extraction and Analysis by Mass Spectrometry. Total lipids were extracted from thymocytes by chloroform/methanol as described previously (1). In brief, viable or apoptotic cell pellets ($\sim 10^9$) were treated with 1 ml of methanol containing 0.3 mM BHT (Sigma-Aldrich) and 3 mM PMSF (Sigma-Aldrich), and 2 ml of methanol/4 ml of chloroform were added. After vortexing, the mixtures were kept on ice for 1 h, 6 ml of distilled H_2O was added, and the mixtures were centrifuged at 1,800 g for 10 min. The chloroform phase was collected and dried under nitro, and DMPC internal standard was added to the samples. For each sample, three separate extractions were performed. Data were pooled from two experiments. To test recoveries for this extraction method, known quantities of the individual lipids to be analyzed were separately extracted and 80–90% recovery was confirmed.

The levels of oxidized phospholipids were analyzed by liquid chromatography multiple reaction monitoring (LC/MRM) as described previously (2). In brief, dried lipid extracts containing internal standard were redissolved in 200 μ l MeOH/ H_2O (98:2) and loaded onto a PrepSep-C18 extraction column previously activated with 3 ml MeOH/ H_2O (98:2). The columns were eluted with 2 ml of MeOH/ H_2O (98:2) and 10 ml of MeOH. The pooled eluate was analyzed by quantitative LC/MRM analysis. For this analysis, samples were injected onto a C8 column (betasil 250 \times 2 mm, 5 μ m; Keystone Scientific) equilibrated in 80% MeOH containing 1 mM ammonium acetate. The column was eluted at 0.4 ml/min with a linear gradient to 100% MeOH containing 1 mM ammonium acetate over 45 min. The column effluent was split, and $\sim 20\%$ of the sample was directed to the Ionspray mass spectrometer source and the rest was collected for additional analyses including inorganic phosphate measurement. Argon containing 10% nitrogen was used as collision gas (instrumental CGT setting of 110) with an orifice voltage of 100. The ion currents for the transition of each preselected parent ion (m/z 782 [PAPC], 496.2 [lysoPC], 594.3 [POVPC], 610.3 [PGPC], 678.5 [DMPC], and 828.5 [PEIPC]) to the corresponding most abundant MS/MS fragment ion (in all cases this was the ion for phosphocholine ([OP{OH}₂OCH₂CH₂N⁺{CH₃}]₃) at m/z 184.1) were recorded. Retention times for each phospholipid were determined using synthetic phospholipids (PAPC, lysoPC, POVPC, PGPC, and DMPC) or bioactive oxidized phospholipids isolated from Ox-PAPC (PEIPC). Instrument-supplied software was used for the extraction of MRM traces from each dataset. The amount of each phospholipid was calculated from the peak areas using calibration curves constructed with a fixed amount of DMPC and varying amounts of the phospholipids.

Chemiluminescent Immunoassay. Copper oxidized LDL (OxLDL), MDA-LDL, and POVPC-LDL were prepared as described previously (1). Phosphorylcholine-conjugated keyhole limpet hemocyanine (PC-KLH) and KLH were obtained from Biosearch Technologies Inc. In this assay, 5 μ g/ml of antigen in dilution buffer (50 mmol/L PBS, pH 7.5, containing 0.27 mmol/L EDTA, 0.02% NaN₃) was added to wells of a 96-well white, round-bottomed MicroFlur microtitration plate (Dynex Technologies) and incubated overnight at 4°C. Plates were washed three times with washing buffer (PBS containing 0.27 mmol/L EDTA, 0.02% NaN₃) using an automated plate washer. Sera from NIH/Swiss-Webster or C57BL/6 mice were diluted at 1:400 and 1:250, respectively, in dilution buffer containing 2% BSA and incubated in each well for 1 h at room temperature. After three washes, bound antibodies were detected by 1 h incubation of 50 μ L/well of an alkaline phosphatase-labeled goat anti-mouse IgM or IgG (μ -chain or γ -chain specific, respectively; Sigma-Aldrich) diluted in 2% BSA/TBS according to the supplier's specification. After three washes, 25 μ l of a 50% solution of Lumi-Phos 530 (Lumigen Inc.) were incubated with each well for 1–2 h at room temperature in the dark. Luminescence was determined using a Dynatech luminometer (Dynex Technologies). Antibody binding was measured as relative light units (RLUs) measured over 100 ms. All measurements of antibody binding to each antigen were done in a single assay, and each plasma sample was assayed in triplicate. The intra-assay coefficient of variation for these assays was 6–10%.

References

1. Hökkö, S., D.A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J.A. Berliner, P. Friedman, E.A. Dennis, L.K. Curtiss, W. Palinski, and J.L. Witztum. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J. Clin. Invest.* 103:117–128.
2. Subbanagounder, G., J.W. Wong, H. Lee, K.F. Faull, E. Miller, J.L. Witztum, and J.A. Berliner. 2002. Epoxyisoprostane and epoxycyclopentenone phospholipids regulate monocyte chemotactic protein-1 and interleukin-8 synthesis. Formation of these oxidized phospholipids in response to interleukin-1 β . *J. Biol. Chem.* 277:7271–7281.