

A Host Lipase Detoxifies Bacterial Lipopolysaccharides in the Liver and Spleen^{*[S]}

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Baomei Shao[‡], Mingfang Lu[‡], Steven C. Katz[§], Alan W. Varley[‡], John Hardwick[‡], Thomas E. Rogers[¶], Noredia Ojogun[‡], Donald C. Rockey[‡], Ronald P. DeMatteo[§], and Robert S. Munford^{‡||1}

From the Departments of [‡]Internal Medicine, ^{||}Microbiology and [¶]Pathology, University of Texas Southwestern Medical School, Dallas, Texas 75390-9113 and the [§]Hepatobiliary Service, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Much of the inflammatory response of the body to blood-borne Gram-negative bacteria occurs in the liver and spleen, the major organs that remove these bacteria and their lipopolysaccharide (LPS, endotoxin) from the bloodstream. We show here that LPS undergoes deacylation in the liver and spleen by acyloxyacyl hydrolase (AOAH), an endogenous lipase that selectively removes the secondary fatty acyl chains that are required for LPS recognition by its mammalian signaling receptor, MD-2-TLR4. We further show that Kupffer cells produce AOAH and are required for hepatic LPS deacylation *in vivo*. AOAH-deficient mice did not deacylate LPS and, whereas their inflammatory responses to low doses of LPS were similar to those of wild type mice for ~3 days after LPS challenge, they subsequently developed pronounced hepatosplenomegaly. Providing recombinant AOAH restored LPS deacylating ability to *Aoah*^{-/-} mice and prevented LPS-induced hepatomegaly. AOAH-mediated deacylation is a previously unappreciated mechanism that prevents prolonged inflammatory reactions to Gram-negative bacteria and LPS in the liver and spleen.

That the liver and spleen play a prominent role in clearing Gram-negative bacteria and their lipopolysaccharide (LPS)² endotoxin from the bloodstream has been known for many years (1, 2). Investigators have also long suspected that these organs may have ways to prevent harmful inflammatory reactions to LPS and other bacterial molecules that enter the bloodstream. Indeed, since the splanchnic bed is a significant source of systemic cytokine production following intravenous LPS challenge (3), hepatic and splenic LPS detoxification may play

an important role in recovery from Gram-negative bacteremia. The potential importance of hepatic endotoxin detoxification is also suggested by evidence that LPS can translocate across the intestinal mucosa, enter the portal blood, and travel to the liver (4, 5), as well as by observations that LPS can stimulate pro-inflammatory responses in several hepatic cell types (4, 6–8). A substantial literature suggests that gut-derived endotoxin may be a co-factor in liver injury induced by other toxins, including alcohol, carbon tetrachloride, and acetaminophen, and that it might contribute to non-alcoholic steatohepatitis (9–13). Other evidence suggests that the limited capacity of the liver to detoxify endotoxin may allow systemic immune activation in patients with human immunodeficiency virus infection (14) or cirrhosis (4).

Numerous blood-borne and cellular mechanisms are known to modulate responses to LPS (15), yet these do not completely inhibit LPS signaling *in vivo*. We show here that partial deacylation of LPS by acyloxyacyl hydrolase (AOAH), an endogenous lipase, is needed to prevent prolonged LPS-induced inflammation in the liver and spleen. We further localize hepatic production of the enzyme to Kupffer cells and show that it deacylates LPS *in vivo*. Providing recombinant AOAH restored hepatic LPS deacylation and prevented LPS-induced hepatomegaly in *Aoah*^{-/-} mice.

EXPERIMENTAL PROCEDURES

LPS and Other Reagents

Salmonella typhimurium PR122 LPS (Rc structure) that contained ~175,000 ³H dpm (in fatty acyl chains) and 10,000 ¹⁴C dpm (in glucosamine backbone) per microgram was prepared as described previously (16) and stored at -80 °C. It was suspended in PBS prior to injection. Prior to radioactivity counting, samples were mixed with 0.4 ml of a solution containing 2% SDS and 5 mM EDTA and added to 3 ml of BudgetSolve (Research Products International, Mt. Prospect, IL). Scintillation counting was performed with external quench correction using a Packard Tricarb 2100 TR scintillation counter (Downers Grove, IL). Non-radioactive LPS was prepared from *Escherichia coli* O14 by phenol-chloroform-petroleum ether extraction. Non-radioactive *S. typhimurium* LPS was purchased from LIST Laboratories (Campbell, CA). The LPS preparations lacked contaminants as assessed by silver-stained SDS-PAGE gel analysis and by their inability to stimulate *Tlr4*^{-/-} peritoneal macrophages to release interleukin-6 *in vitro* (they were at least 500-fold less stimulatory toward *Tlr4*^{-/-} macrophages than toward *Tlr4*^{+/+} cells). Synthetic *N*-palmitoyl-S-(2,3-bis-

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¹ To whom correspondence should be addressed: University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9113. Tel.: 214-648-3480; Fax: 214-648-9478; E-mail: Robert.munford@utsouthwestern.edu.

² The abbreviations used are: LPS, lipopolysaccharide; DC, dendritic cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; PAM3CSK4, *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(R)-Cys-(S)-Serl-(S)-Lys(4)trihydrochloride; AOAH, acyloxyacyl hydrolase; PBS, phosphate-buffered saline; CMV, cytomegalovirus; NFA, non-hydroxylated fatty acids; NK, natural killer; NK-T, natural killer T; rh, recombinant human; Ad, adenovirus.

(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys (4) trihydrochloride (Pam3CSK4) was purchased from InvivoGen (San Diego, CA). Recombinant AOA_H prepared from baby hamster kidney cells (17) transfected with the human AOA_H cDNA (18) was a generous gift from ZymoGenetics, Inc. It was suspended (10 μ g/ml) in PBS that contained 0.2 mg/ml low endotoxin bovine serum albumin prior to injection.

Mice

AOA_H^{-/-} mice were prepared by inserting a neomycin resistance cassette into the first exon of the murine gene, replacing the initial ATG and preventing synthesis of the N-terminal 43 amino acids of the protein (19). AOA_H^{-/-} mouse macrophages do not produce the AOA_H protein (Fig. 1A) and are unable to carry out LPS deacylation (19). The AOA_H^{-/-} construct was backcrossed eight generations into C57Bl/6 (19) and C3H/HeN (20) backgrounds; the AOA_H^{-/-} mice, their AOA_H^{+/+} counterparts, and *Tlr4*^{-/-} C57Bl/10ScN (21) \times AOA_H^{-/-} C57Bl/6 mice were maintained in specific pathogen-free conditions in the University of Texas Southwestern Animal Resources Center and used for experiments when they were 5–12 weeks of age. All protocols were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee.

Time Course Experiments

Wild type and AOA_H-deficient mice were injected via the lateral tail vein with 10 μ g of radiolabeled LPS in 200 μ l of PBS and transferred to metabolic cages (three mice/cage). Urine was collected and measured daily. On days 1, 3, 7, and 14, plasma and tissues were obtained (three mice/time point). Weighed samples of each organ were dispersed by sonication in 600 μ l of lysis buffer (PBS with 0.1% Triton X-100), and aliquots were removed for scintillation counting, ethanol precipitation, and analysis of the LPS-derived [³H]fatty acids as described below.

Estimating LPS Amount and Deacylation in Vivo

In the radiolabeled Rc (short saccharide chain) *S. typhimurium* PR122 LPS used for these experiments, [¹⁴C]glucosamine is incorporated into the most conserved and uniform LPS regions, lipid A and the core oligosaccharide (22). The ¹⁴C content should therefore reflect the number of LPS molecules in a sample. We used two methods to estimate the extent of LPS deacylation in the liver.

Method 1—We measured the ratio of [³H]NFA (non-hydroxylated fatty acids) to [³H]3-OH-14:0 in the LPS recovered from tissue lysates. In the injected LPS, this ratio was 0.5. Since AOA_H removes only the non-hydroxylated secondary acyl chains (myristate, laurate) from the lipid A backbone, AOA_H-mediated hydrolysis decreases the NFA to 3-OH-14:0 ratio. To measure this ratio, tissue lysates were subjected to Bligh-Dyer extraction, LPS was recovered from the interphase (23), and the [³H]fatty acids were cleaved from the LPS by treatment with hot HCl and NaOH before being analyzed by one-dimensional thin-layer chromatography as described previously (19, 23). [¹⁴C]NFA and [¹⁴C]3-OH-14:0 standards were run in adjacent lanes, and the plates were autoradiographed to identify the migration positions of the ³H-labeled fatty acids. Recovery of

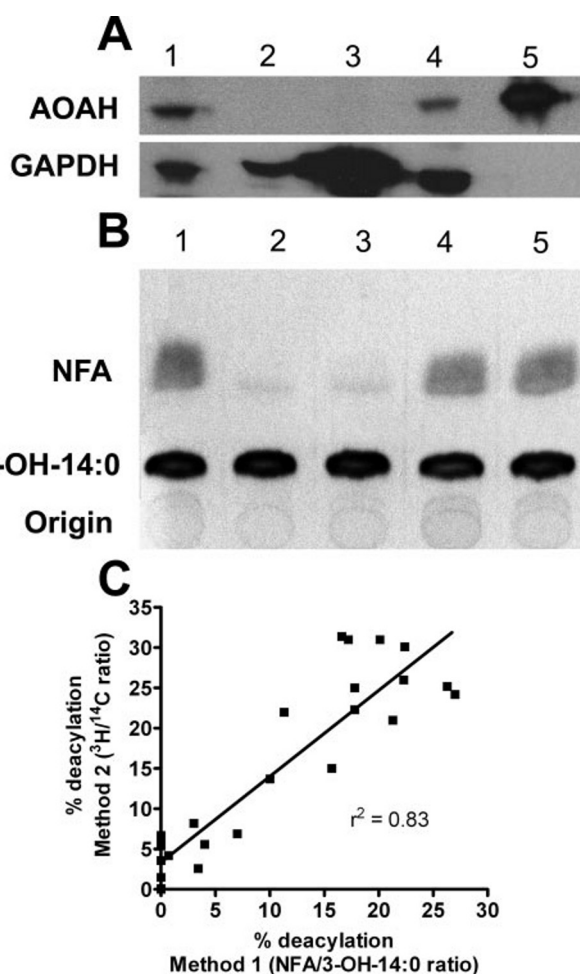


FIGURE 1. A, AOA_H^{-/-} macrophages do not produce AOA_H protein. Western blot analysis was used to identify AOA_H in lysates of C57Bl/6 AOA_H^{+/+} (lane 1) and AOA_H^{-/-} (lane 2) peritoneal macrophages. Controls were a dendritic cell line, 106 (19), that does not produce AOA_H (lane 3), an AOA_H-producing dendritic cell line, XS-52 (19) (lane 4), and 5 ng of recombinant human AOA_H (lane 5). The AOA_H panel shows bands of 70 kDa, the size of the AOA_H precursor (there were no other bands in these lanes), whereas the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) panel (loading control) shows the expected bands at 38 kDa. B, selective removal of secondary acyl chains from LPS in wild type liver. Analysis of LPS recovered from the livers of mice that received radiolabeled LPS 3 days before. The [³H]fatty acids hydrolyzed from the interphase of chloroform-methanol extraction mixtures were analyzed using TLC; fluorography was used to detect the ³H-containing bands. See recovery data in Table 1. Lane 1, LPS, subjected to hydrolysis as a control; lanes 2 and 3, livers from two AOA_H^{+/+} mice; lanes 4 and 5, livers from two AOA_H^{-/-} mice. Note that NFA (12:0 and 14:0) disappeared from the LPS only in the AOA_H^{+/+} livers. C, two methods for estimating LPS deacylation in the liver give similar results. Liver samples were analyzed in parallel from 26 AOA_H^{-/-} and AOA_H^{+/+} mice that had been given [³H][¹⁴C]LPS 1–3 days before study. See "Experimental Procedures" for details.

³H and ¹⁴C radioactivity is detailed in Table 1. A plate that was exposed to film after spraying with En³Hance (PerkinElmer Life Sciences) to bring out the ³H-containing bands is shown in Fig. 1B. Deacylation was estimated as $(1 - [\text{NFA}/3\text{-OH-14:0 ratio in recovered LPS} + 1]/[\text{NFA}/3\text{-OH-14:0 ratio in injected LPS} + 1])$.

Method 2—We measured the ratio of ³H dpm to ¹⁴C dpm in tissue lysates. Releasing [³H]acyl chains from the LPS decreases this ratio, which was 17.5 in the injected LPS. Since the released fatty acids may be retained within the tissue either free or incorporated into various lipids, we added ethanol (200 μ l) to 100 μ l

TABLE 1

Uptake and deacylation of intravenously injected *S. typhimurium* Rc [^3H][^{14}C]LPS by livers of *Aoah* $^{+/+}$ and *Aoah* $^{-/-}$ mice

i.v., intravenous.

	<i>Aoah</i> $^{+/+}$ mice	<i>Aoah</i> $^{-/-}$ mice
Recovery from liver 15 hr after i.v. injection ($n = 6$ mice /group)		
^{14}C dpm (% of injected)	90.1 \pm 12.1	89.6 \pm 10.2
^3H dpm (% of injected)	71.3 \pm 9.6	88.3 \pm 9.5
Recovery from Bligh-Dyer interphase (% of total liver ^3H dpm) ($n = 8$ mice/group)	85.3 \pm 0.4	87.8 \pm 12.0
^3H dpm missing from [^3H][^{14}C]LPS (% of total in injected LPS), 15–16 hrs after i.v. LPS injection ($n = 6$ –12 mice/group)	17.8 \pm 2.8	3.2 \pm 2.4
Cumulative recovery of ^3H dpm from urine ^a during the first 7 days after LPS injection (% of injected dpm). See time course in Fig. 2C. $n = 2$ experiments, 3 mice/box/experiment.	4.6, 5.1	0.9, 1.4

^a The urine ^3H counts (derived from LPS fatty acyl chains) partitioned in the methanol-water phase of a chloroform-methanol extraction mixture and were not recoverable after the samples were allowed to dry (data not shown).

of lysate to precipitate intact LPS. After centrifugation to pellet the ethanol-insoluble precipitate and counting the ^3H dpm in the supernatant, we subtracted the ethanol-soluble ^3H dpm from the total ^3H content of the tissue before calculating the $^3\text{H}/^{14}\text{C}$ ratio. The percentage of deacylation was then estimated as $(1 - [\text{measured ratio}]/[\text{starting ratio}]) \times 100$.

The correlation coefficient (r^2) for the results of the two estimates was 0.83 ($n = 26$ independent liver samples studied using both methods)(Fig. 1C). For most of the experiments, we used Method 2 and chose a time point (15 h after intravenous injection) that reproducibly yielded 15–20% deacylation of 10 μg of LPS in *Aoah* $^{+/+}$ mice.

Liver Cells

To isolate specific types of cells from C57B1/6 livers, we used the methods described by Katz *et al.* (24). Briefly, after eluting the blood from the liver *in situ* with 2 ml of collagenase in PBS (5 mg/ml, type IV, Sigma), the liver was cut into small pieces and treated further with collagenase (0.5 mg/ml) for 10 min at 37 °C. We then mechanically disrupted the liver pieces using the flat portion of a plunger from a 3-ml syringe. The cells were passed through 100- μm mesh (BD Biosciences) and then centrifuged at low speed ($50 \times g$ for 3 min, $\times 3$) to pellet hepatocytes. The non-parenchymal cells in the supernatant were pelleted ($500 \times g$ for 15 min) and then isolated on a 40% Optiprep step gradient (Nycomed, Oslo, Norway). In some experiments, the non-parenchymal cells were further fractionated into liver sinusoidal endothelial cells (LSEC, CD45 $^-$), Kupffer cells (CD45 $^+$ CD11c $^-$), and dendritic cells (CD45 $^+$ CD11c $^+$) using immunomagnetic beads (Miltenyi Biotec, Auburn, CA) as described (24, 25). The identity of the cell types was confirmed by flow cytometry and by real-time PCR (SYBR Green method) to measure mRNA for marker proteins (see supplemental Table S1).

Kupffer cells were also isolated from the livers of male Sprague-Dawley rats (450–500 g)(Harlan, Indianapolis, IN) by *in situ* perfusion of the liver with 20 mg/dl Pronase (Roche Applied Science) followed by collagenase (Crescent Chemical, Hauppauge, NY). Dispersed cell suspensions were layered on a discontinuous density gradient of 8.2 and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY). Kupffer cells, present in the lower layer, were further purified by centrifugal elutriation (18 ml/min flow rate) and were grown in 1990R medium containing 20% serum (10% horse serum, 10%

calf serum), which was changed every 24 h. Greater than 90% of the adherent cells took up fluorescent latex beads (carboxylate-modified microspheres, 1.0 μm , Invitrogen). Radiolabeled *S. typhimurium* LPS (100 ng/ml) was added to the Kupffer cells for 24 h to measure their ability to deacylate LPS.

Cell Depletion

Neutrophils (and possibly Gr-1^{hi} monocytes) were depleted by administering anti-Gr-1, an antibody to Ly6G and Ly6C (26). Neutrophil depletion was documented by Wright-Giemsa staining of peripheral blood smears and by flow cytometry of liver non-parenchymal cells (neutrophils were CD11b $^+$ Ly6G $^+$). To deplete macrophages, we gave an intravenous infusion of 200 μl of clodronate liposomes (prepared using clodronate provided by Roche Applied Science) (27) 2 or 3 days prior to administering LPS. PBS liposomes were used as the control. Kupffer cell depletion was documented as a decrease in the number of F4/80 $^+$ cells in frozen sections of liver obtained 15 h after LPS or PBS infusion. To minimize the impact of photobleaching, digital photographs were taken (four different fields/liver section, $\times 200$ magnification), and cells were counted from these images.

Flow Cytometry

The antibodies used were rat anti-mouse Ly-6G (clone 1A8), rat anti-mouse F4/80 (clone A3-1), rat anti-mouse CD11b (M1/70), hamster anti-mouse CD11c (HL3), rat anti-mouse CD3 (17A2), mouse anti-mouse NK1.1 (PK136), rat anti-mouse CD45R/B220 (RA3-6B2), hamster anti-mouse $\alpha\beta$ T cell receptor (H57–597), hamster anti-mouse $\gamma\delta$ T cell receptor (GL3), rat anti-mouse CD4 (RM4–5), and rat anti-mouse CD8 (53-6.7). The isotype controls were rat IgG2a, rat IgG2b, and rat IgG2a. Kupffer cells were CD11c $^-$ F4/80^{hi}, dendritic cells were CD11b $^+$ CD11c $^+$, B cells were B220 $^+$ CD3 $^-$, NK cells were CD3 $^-$ NK1.1 $^+$, and NK-T cells were CD3 $^+$ NK1.1 $^+$. NK-T cells were also quantitated, with similar results, using tetramers (mCD1d/PBS 57) obtained from the NIH Tetramer Core (Centers for Disease Control and Prevention, Atlanta, GA). All antibodies were from Pharmingen except rat anti-mouse F4/80, which was from Caltag Laboratories. Flow cytometry was performed using a FACSCalibur machine (BD Biosciences) and analyzed using FlowJo v4.6.2. software.

Recombinant Adenoviral Vector

An adenoviral vector that produces recombinant human AOA from the early CMV promoter (Ad-CMV-rhAOAH) was prepared and used as described (28). Ad-CMV-luciferase was the control.

Assays

AOAH activity was assayed as described previously (16). Western blotting was performed (29) using a mouse anti-murine AOA IgG monoclonal antibody (2F3-2A4) that recognizes the large subunit of the enzyme. The membrane was also blotted with a cross-reactive rabbit antibody to glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, UK). Serum amyloid A was measured in mouse plasma using an enzyme-linked immunosorbent assay kit obtained from BIOSOURCE (Camarillo, CA). Interleukin-6 was measured using enzyme-linked immunosorbent assay reagents obtained from Pharmin-gen. Triglycerides were assayed using a Sigma kit (343-25P) with a glycerol standard. Alanine-2-oxoglutarate aminotransferase was measured using reagents from ThermoElectron Corp. (Louisville, CO).

RESULTS

To increase the likelihood that intravenously injected LPS would be taken up rapidly by the liver, we used a rough (short saccharide chain) LPS. As expected (28, 30, 31), the *S. typhimurium* R_c LPS disappeared from the circulation within a few minutes of injection (data not shown). Fifteen hours later, almost all of the injected LPS could be recovered from the liver (Table 1). The recovery of ¹⁴C radioactivity (a marker for the carbohydrate backbone of LPS) from the liver did not decrease significantly over the 7–14-day study period in either *Aoah*^{-/-} or *Aoah*^{+/+} mice (Fig. 2A). Although the ³H dpm recovered from the livers of *Aoah*^{-/-} mice was also stable over time, in *Aoah*^{+/+} mice, the ³H radioactivity in the liver decreased as the [³H]fatty acyl chains were released from the LPS backbone. Approximately 18% of the ³H radioactivity in the liver-associated LPS had been removed by 15 h after injection in wild type C57Bl/6 mice (Table 1). Since AOA only cleaves two of the six fatty acyl chains from LPS (maximal deacylation is ~33%), approximately half of the LPS molecules in the liver had thus been deacylated at this time point. Deacylation was complete by day 3 (Fig. 2B). Analysis of the fatty acid composition of the LPS recovered from the liver showed that there had been selective deacylation of the non-hydroxylated acyl chains (myristoyl, lauryl) from the backbone, consistent with the known activity of AOA toward LPS (Fig. 1B). The recovery of ³H dpm from the urine peaked as deacylation occurred in the wild type mice (Fig. 2C). Although only 2.8 ± 1.4 and 5.3 ± 1.5% of the injected dose of LPS was taken up by the spleens of *Aoah*^{+/+} and *Aoah*^{-/-} mice, respectively, the time course and extent of LPS deacylation in the spleen closely paralleled that in the liver (data not shown).

Both the limited extent of deacylation (loss of no more than one-third of the LPS-associated ³H dpm) and the analysis of the fatty acyl composition of the recovered LPS (selective loss of non-hydroxylated fatty acids) were consistent with the occurrence of partial deacylation due to acyloxyacyl hydrolysis. In

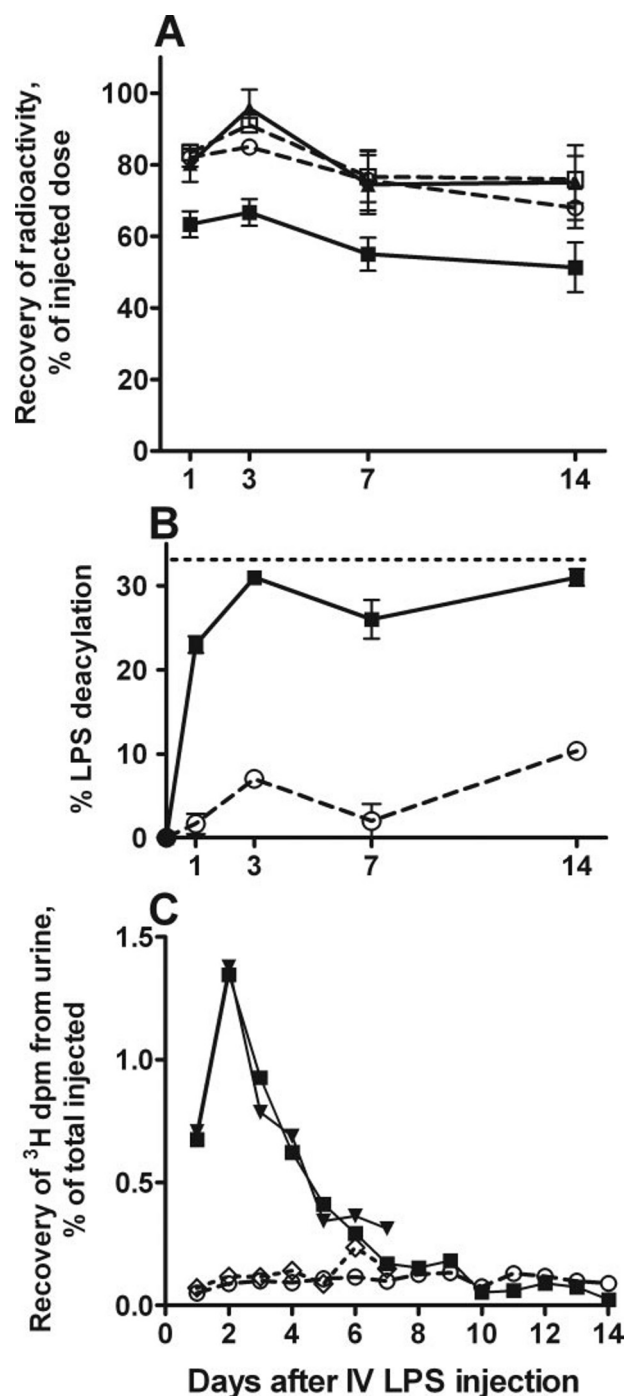


FIGURE 2. LPS uptake and deacylation by the liver. A, time course of ¹⁴C and ³H recovery in the livers of wild type and AOA-deficient mice. Closed boxes = ³H dpm, *Aoah*^{+/+}; closed triangles = ¹⁴C dpm, *Aoah*^{+/+}; open circles = ³H dpm, *Aoah*^{-/-}; and open triangles = ¹⁴C dpm, *Aoah*^{-/-}. LPS deacylation (B) was calculated using Method 2. Closed symbols = *Aoah*^{+/+}, and open circles = *Aoah*^{-/-}. *n* = 3 mice/group at each time point. This experiment was repeated over a 7-day period with similar results. The dotted horizontal line indicates maximal deacylation (release of 33% of the total ³H dpm from the ¹⁴C-containing backbone). C, time course of appearance of ³H dpm in the urine. Results from the two time course experiments are shown. Closed symbols indicate wild type mice, and open symbols indicate AOA-deficient mice. Note that the excretion of ³H radioactivity (C) coincided in time with LPS deacylation in the liver (B). IV, intravenous.

addition, LPS underwent very little deacylation in *Aoah*^{-/-} mice (Fig. 2B). It is likely that slow, non-AOA-mediated deacylation also occurred *in vivo* since the ratio of ³H dpm to

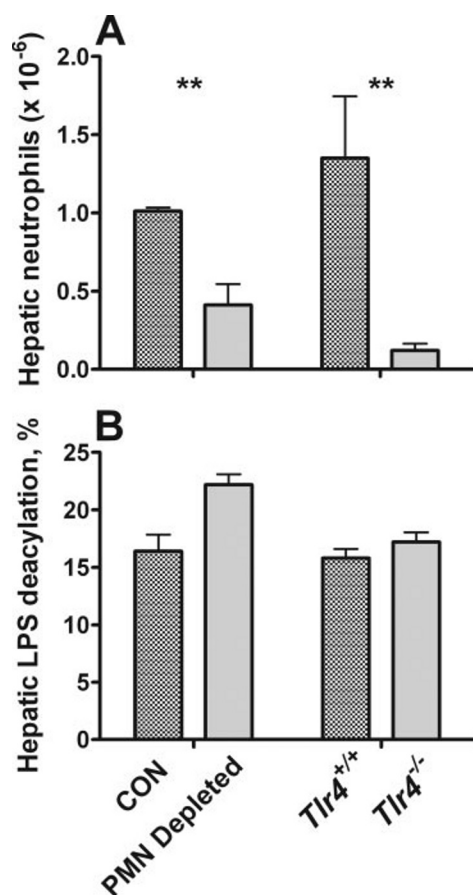


FIGURE 3. Hepatic LPS deacylation does not require recruited neutrophils. Two experiments are shown: PBS-treated (CON) versus Gr-1 pretreated (PMN-depleted) mice (left) and *Tlr4*^{+/+} versus *Tlr4*^{-/-} mice (right). Each experiment was repeated with similar results. **A**, hepatic neutrophils. The bars show the means (\pm 1 S.D.) of the CD11b⁺Ly6G⁺ cells in the non-parenchymal liver cell fraction, per gram of liver wet weight, in mice that had received 10 μ g of *E. coli* 014 LPS intravenously 15 h earlier. ** = $p < 0.01$. $n = 4$ mice/group. **B**, hepatic LPS deacylation. Deacylation was measured 15 h after intravenous injection of 10 μ g of radiolabeled LPS. Hepatic LPS deacylation did not correlate with hepatic neutrophil abundance in either experiment. PMN, polymorphonuclear cells (neutrophils).

¹⁴C dpm declined gradually over the 14-day period in the livers of both wild type and *Aoah*^{-/-} mice (data not shown).

We performed several experiments to identify the cells that produce AOAH and carry out LPS deacylation in the liver. Following an intravenous injection of LPS, neutrophils accumulate in the liver; there were 5×10^5 to 25×10^5 neutrophils/g of liver 15 h after intravenous LPS injection, whereas the livers of PBS-challenged mice had fewer than 1×10^5 neutrophils/g. Since neutrophils produce AOAH and deacylate LPS (32, 33) and others have found that Gram-negative bacteria can be phagocytosed by neutrophils prior to ingestion by Kupffer cells (34), we tested the hypothesis that infiltrating neutrophils deacylate LPS in the liver. First, we depleted neutrophils prior to administering radiolabeled LPS and found that reducing the recruited neutrophil population by $\sim 60\%$ did not diminish LPS deacylation (Fig. 3A). In a second approach, we measured the deacylating ability of LPS-hyporesponsive *Tlr4*^{-/-} mice, which do not mount pro-inflammatory responses to LPS and thus do not recruit inflammatory cells, including neutrophils and monocytes, to the liver. Although there was no increase in neutrophil

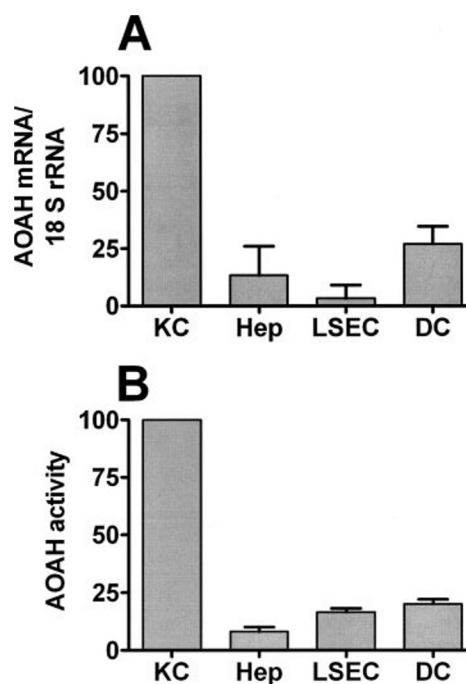


FIGURE 4. AOAH mRNA and enzymatic activity in liver cell types. **A**, AOAH mRNA (relative to 18S rRNA) in KC, hepatocytes (Hep), LSEC, and DC. The data are normalized to let the Kupffer cell value = 100. **B**, AOAH activity ([³H]fatty acids released from LPS/mg of cell protein/h) for each of the cell types. The results are expressed relative to the activity in Kupffer cells. The results are means of 2 or 3 independent cell preparations, each tested in duplicate. Bars indicate the maximum value (range).

or monocyte abundance in the liver after intravenous LPS injection, LPS deacylation occurred in the *Tlr4*^{-/-} livers to the same extent as in *Tlr4*^{+/+} controls (Fig. 3B). These results thus suggest that LPS deacylation in the normal liver is not augmented significantly by recruited cells.

It therefore seemed likely that one or more intrinsic hepatic cell types deacylate LPS. To identify the AOAH-producing cell(s), we isolated different cell types from the livers of wild type C57Bl/6 mice, confirmed their identity by quantitating mRNA levels of marker genes (see supplemental Table S1), and tested them for AOAH mRNA and enzymatic activity. As shown in Fig. 4, Kupffer cells (KCs) produced ~ 4 -fold more AOAH mRNA (relative to 18S rRNA) than did the other cell types. Dendritic cells also produced AOAH mRNA, but they are much less abundant than are KCs (in normal livers, we found $3.6 \pm 1.2 \times 10^6$ KCs and $0.51 \pm 0.14 \times 10^6$ DCs/g ($n = 6$, mean \pm 1 S.E.)), so their contribution to overall AOAH production in the liver is probably much less than that of KCs. AOAH enzymatic activity was not confined to these cells; in particular, LSEC, which produced very little AOAH mRNA, had significant enzymatic activity in each of the three preparations studied. A possible explanation for this observation is uptake of AOAH by LSEC from the extracellular fluid, either *in vivo* or during cell isolation in the presence of fetal bovine serum, which contains trace amounts of AOAH. Hepatocytes also may have low amounts of AOAH mRNA and enzymatic activity, although one of the three preparations studied had none.

To confirm that live Kupffer cells can deacylate LPS, we studied Kupffer cells prepared by elutriation from normal rat liver

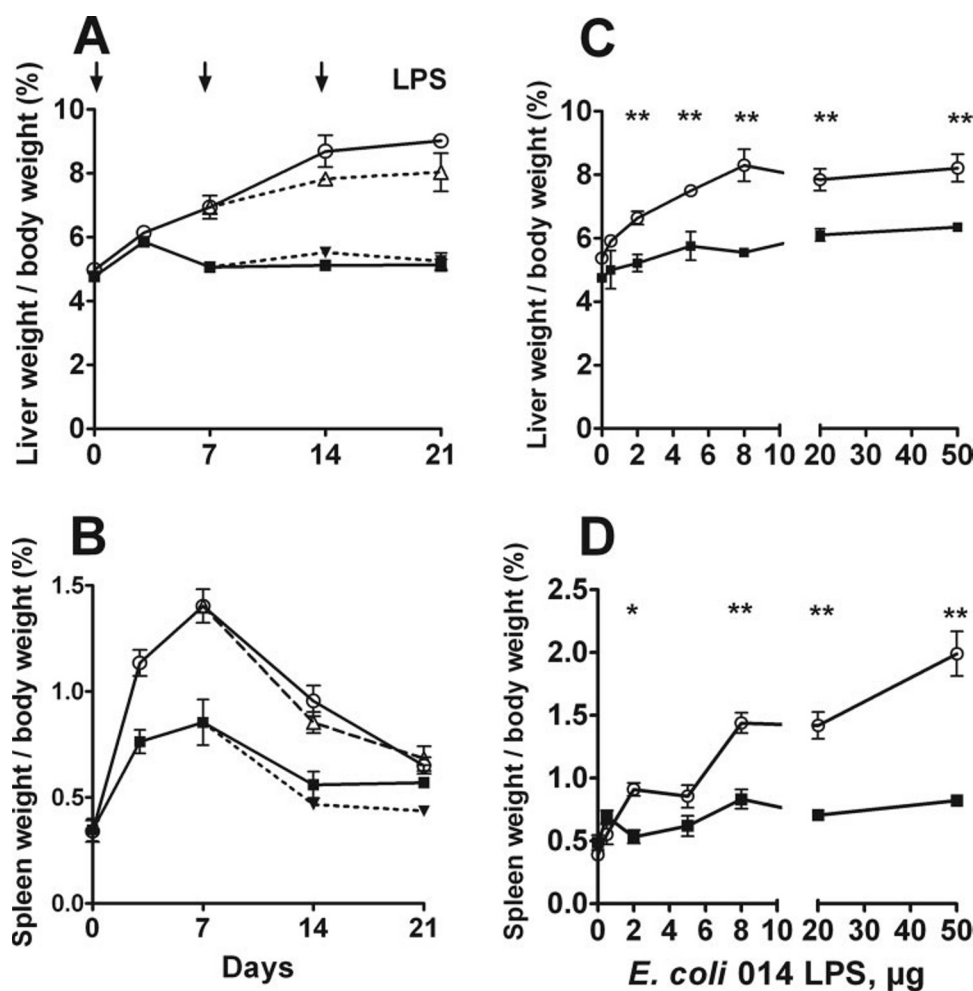


FIGURE 5. LPS induces hepatosplenomegaly. Intravenous (i.v.) injection of 10 µg of *E. coli* O14 LPS (dashed lines) induced significant enlargement of the liver (A) and spleen (B), especially in *Aoah*^{-/-} mice (open symbols). Injecting 20 µg of *E. coli* O14 LPS on day 7 induced further hepatic enlargement, whereas a third injection of 20 µg of *S. typhimurium* LPS on day 14 did not (solid lines). (Different LPS preparations were used to minimize antibody-mediated LPS neutralization.) Solid symbols = *Aoah*^{+/+} mice. Dose-response relationships are shown for the liver (C) and spleen (D). Seven days after intravenous LPS challenge, hepatomegaly was inducible with as little as 2 µg of *E. coli* O14 LPS. $n = 3$ or 4 mice/group. *, $p < 0.05$ (*Aoah*^{+/+} versus *Aoah*^{-/-}), **, $p < 0.01$. For *Aoah*^{+/+} mice, liver and spleen weights (as the percentage of body weight) exceeded those of the PBS-injected controls ($p < 0.05$, 2-way analysis of variance) at doses of 8 µg and above (C and D). Closed circles = *Aoah*^{+/+}, open circles = *Aoah*^{-/-}.

and carried briefly in culture. Deacylation of radiolabeled LPS occurred in a dose- and time-dependent fashion. As described above for murine Kupffer cells, lysates of these cells selectively released the non-hydroxylated LPS fatty acids in an AOA-like pattern (data not shown).

To quantitate the Kupffer cell contribution to hepatic LPS deacylation *in vivo*, we studied LPS deacylation in *Aoah*^{+/+} mice that had received either clodronate liposomes or PBS liposomes 2 days previously. Intravenously injected clodronate (dichloromethylene-bisphosphonate) liposomes selectively deplete Kupffer cells from the liver and monocytes from the circulation (35, 36). Examination using immunostaining for F4/80⁺ cells on snap-frozen tissue sections confirmed the expected pattern of depletion; although 32.3 ± 5.4 F4/80⁺ cells were found per field in livers of PBS-treated mice, none was seen after clodronate treatment ($n = 4$ mice/group). Clodronate treatment decreased the hepatic uptake of intravenous LPS by 57% ($27.6 \pm 4.5\%$ of the injected LPS after clodronate

treatment versus $63.7 \pm 7.8\%$ in mice that received PBS liposomes, $n = 6$, $p < 0.003$) and, in addition, the deacylation of liver-associated LPS decreased by greater than 90% ($0.8 \pm 1.6\%$ release of LPS-derived ³H dpm in mice that had received clodronate versus $14.8 \pm 3.7\%$ in PBS liposome controls, $n = 6$ mice/group, $p < 0.01$). Macrophage depletion also reduced LPS deacylation in the livers of *Tlr4*^{-/-} mice; 15 h after intravenous injection, $12.4 \pm 3.9\%$ of the ³H dpm had been released from LPS in *Tlr4*^{-/-} mice that received PBS liposomes versus $1.4 \pm 2.2\%$ in clodronate-treated mice ($n = 3$, $p < 0.04$). These data suggest strongly that Kupffer cells are required for LPS deacylation in the liver, either directly (by internalizing and deacylating LPS) or indirectly (by producing and releasing AOA that acts on or within other liver cell types).

We did not detect significant differences between *Aoah*^{+/+} and *Aoah*^{-/-} C57Bl/6 mice in the levels of interleukin-6 in plasma obtained 6 h, 24 h, 5 days, or 7 days after intravenous LPS challenge. The concentrations of serum amyloid A protein (an acute phase reactant) in the plasma were also similar in the two strains on post-inoculation days 1, 3, and 7. Moreover, the abundance of the different non-parenchymal cells in the liver at 15 h after intravenous injection did not differ significantly between *Aoah*^{-/-} and

Aoah^{+/+} mice (data not shown). On the other hand, we noticed that *Aoah*^{-/-} mice developed significant hepatosplenomegaly during the week following intravenous LPS injection (Fig. 5, A and B). By day 7, the livers of *Aoah*^{-/-} mice were 30–40% heavier than those of *Aoah*^{+/+} mice. LPS-induced hepatomegaly persisted for at least 3 weeks after a single intravenous injection of 10 µg of LPS. When the mice received a second intravenous injection of 20 µg of LPS on day 7, the livers of the *Aoah*^{-/-} animals became even larger (Fig. 5A). We found that a significant increase in liver weight could be detected in *Aoah*^{-/-} mice following a single intravenous dose of 2 µg of LPS and that the response was maximal at a dose of 8 µg (Fig. 5C). An impressive (~3-fold) increase in *Aoah*^{-/-} spleen weight also occurred after single doses of 8 µg or greater (Fig. 5D). At the higher LPS doses, *Aoah*^{+/+} mice also developed significant hepatosplenomegaly relative to the PBS-injected controls (Fig. 5, C and D), suggesting that the greater increases seen in *Aoah*^{-/-} mice are exaggerations of a normal response.

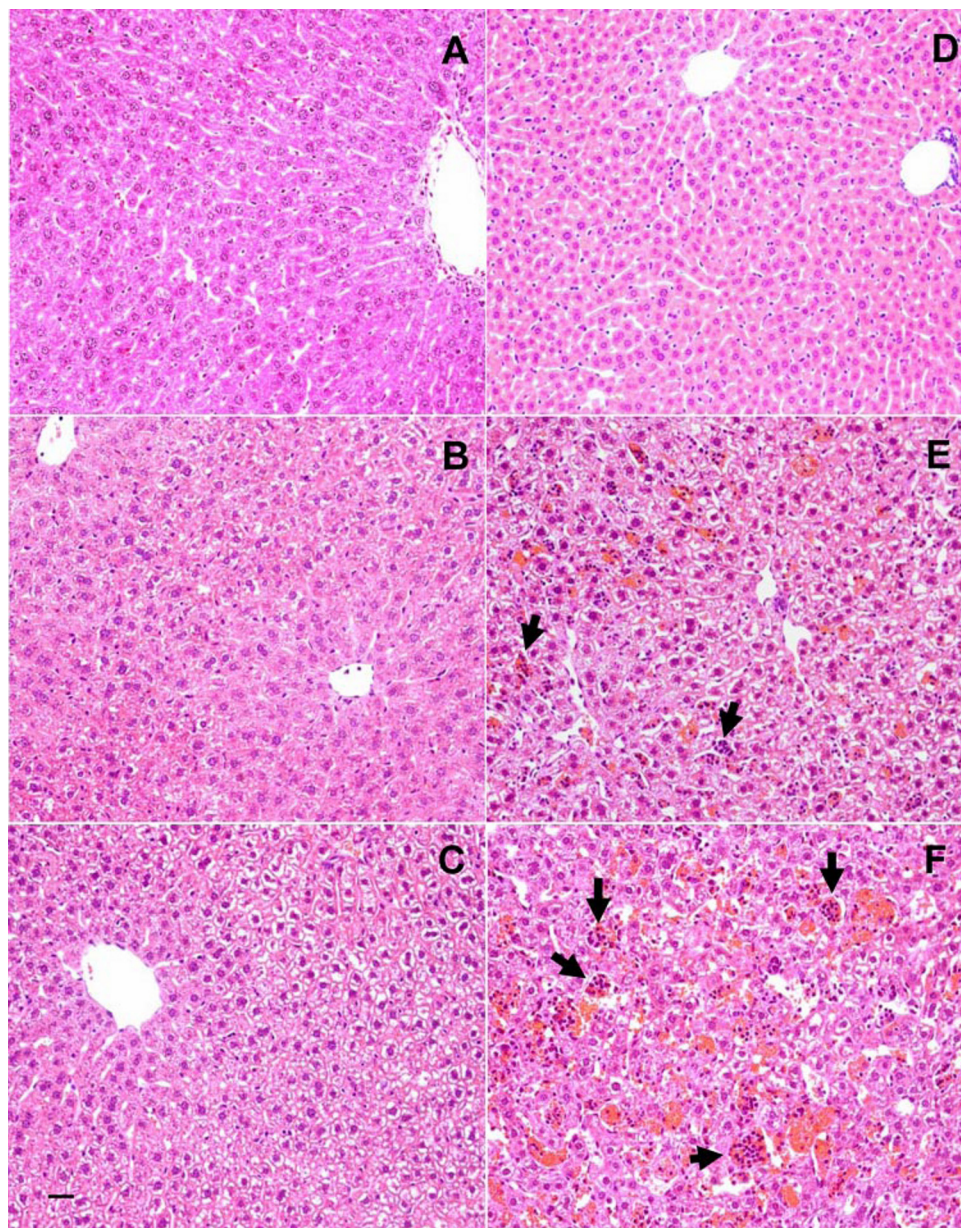


FIGURE 6. LPS induces leukocyte accumulation in *Aoah*^{-/-} liver. Hematoxylin-eosin-stained sections of paraformaldehyde-fixed liver are shown. A–C, *Aoah*^{+/+} C57Bl/6 liver. D–F, *Aoah*^{-/-} C57Bl/6 liver. A and D, normal, liver not perfused; B and E, 7 days after 10 μ g of intravenous LPS, liver perfused prior to fixation; C and F, on day 21 after receiving LPS on days 0 (10 μ g), 7 (20 μ g), and 14 (20 μ g); liver not perfused. Note the increased numbers of intrasinusoidal leukocytes in E and F (arrows). All images $\times 200$. Bar in C = 20 μ m.

LPS injection also induced significantly greater hepatomegaly in *Aoah*^{-/-} than in *Aoah*^{+/+} C3H/HeN mice, excluding the possibility that this phenotype is peculiar to the C57Bl/6 background. Hepatomegaly did not develop in mice that were given LPS subcutaneously to induce robust polyclonal antibody responses (20). That the phenotype is LPS- and *Tlr4*-dependent was confirmed by finding that AOA-treated (partially deacylated) LPS (10 μ g intravenously) did not induce hepatosplenomegaly in *Aoah*^{-/-} mice, that LPS did not induce hepatomegaly in *Aoah*^{-/-} *Tlr4*^{-/-} (double knock-out) mice, and that two weekly intraperitoneal injections of PAM3CSK4 (7.5 mg/kg), a TLR1/2 ligand (37), also did not induce hepatomegaly (data not shown). A single injection of 5×10^7 colony-forming units of *E. coli* O14 induced hepatomegaly within 7 days (liver weight/

body weight for *Aoah*^{+/+} mice was $5.6 \pm 0.2\%$, versus $8.0 \pm 0.5\%$ for *Aoah*^{-/-} mice; $p < 0.02$, $n = 3/\text{group}$).

We performed several studies to characterize the prolonged LPS-induced responses in *Aoah*^{-/-} mice. First, we found that the triglyceride concentrations in *Aoah*^{-/-} and *Aoah*^{+/+} livers were not significantly different 7 days after intravenous LPS injection and that alanine-2-oxoglutarate aminotransferase levels were not elevated in *Aoah*^{-/-} serum (data not shown). These results suggested that hepatocyte (parenchymal cell) integrity was minimally affected. On the other hand, hematoxylin-eosin-stained sections of livers from LPS-challenged *Aoah*^{-/-} mice showed blood-filled sinusoids with many leukocytes (Fig. 6); these findings were confirmed using scanning electron microscopy (not shown). Analysis of hepatic non-parenchymal cells using flow cytometry revealed increases in the numbers of several leukocyte cell types within *Aoah*^{-/-} livers 7 days after LPS injection; the greatest increases above *Aoah*^{+/+} levels (>6 -fold) were found for neutrophils, B cells, and (CD4⁺CD8⁻) T cells (data not shown). An inability to deacylate LPS is thus associated with prolonged LPS-induced inflammation within the sinusoids of the liver.

The increase in spleen weight in *Aoah*^{-/-} mice peaked at 7 days after intravenous LPS injection and was accompanied by the presence of 6-fold more macrophages (F4/80⁺CD11b⁺) than were found in *Aoah*^{+/+} mice. There were also more neutrophils in *Aoah*^{-/-} spleens, yet the abundance of the other cell types studied (B cells, T cells, NK cells, NK-T cells, dendritic cells) did not differ between *Aoah*^{-/-} and *Aoah*^{+/+} spleens over the time period studied (data not shown). There were no consistent differences in the microscopic appearance of the splenic architecture in *Aoah*^{+/+} and *Aoah*^{-/-} mice (hematoxylin-eosin staining).

If endogenous AOA normally carries out LPS deacylation, providing AOA to *Aoah*^{-/-} animals should restore LPS deacylating ability *in vivo* and prevent LPS-induced hepatomegaly. We tested this hypothesis in two ways. First, *Aoah*^{-/-} mice were given either an AOA-producing or a luciferase-producing adenoviral vector (10^9 plaque-forming units, via tail

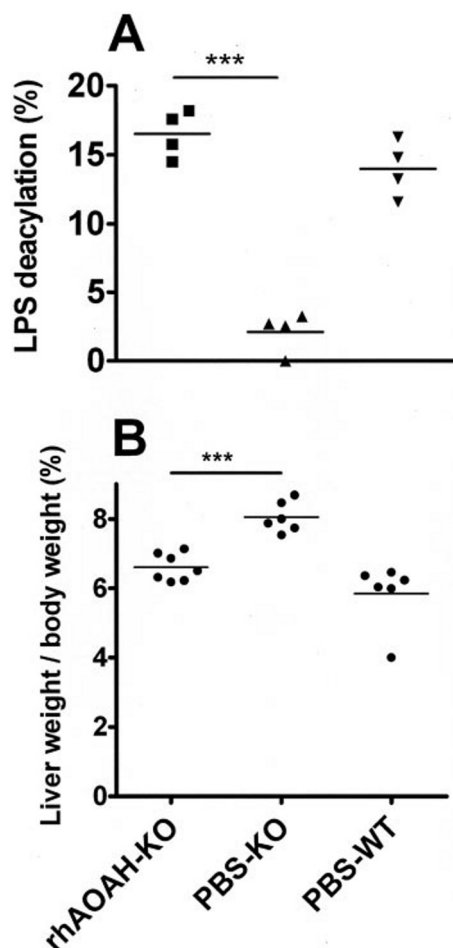


FIGURE 7. Recombinant AOA restores LPS deacylation and prevents LPS-induced hepatomegaly. A, recombinant AOA restores LPS deacylating ability to *Aoah*^{-/-} mouse liver. One μ g of rhAOAH was injected intravenously into *Aoah*^{-/-} mice; other *Aoah*^{-/-} mice and *Aoah*^{+/+} controls received vehicle (PBS with 0.2 mg/ml bovine serum albumin). Two hours later, all mice received 5 μ g of radiolabeled LPS. Deacylation was measured in the liver 4 h later. B, recombinant AOA prevents LPS-induced hepatomegaly in *Aoah*^{-/-} mice. Mice received 1 μ g of rhAOAH or vehicle as in A 2 h prior to an intravenous injection of 8 μ g of *E. coli* O14 LPS. Liver weight/body weight ratios were measured 7 days later. *** = $p < 0.001$. Each symbol represents one mouse.

vein) 3 days prior to intravenous administration of 5 μ g of radiolabeled LPS. The extent of deacylation was measured 1 h later. In *Aoah*^{-/-} mice that had received Ad.CMV-AOAH, 25.4 \pm 2.6% of the [³H]fatty acids had been released from the LPS in the liver 1 h after intravenous injection, whereas only 0.9 \pm 0.3% had been released in the livers of mice that received Ad.CMV-luciferase. This experiment was repeated at a 4-h time point with similar results. Producing recombinant human AOA from an adenoviral vector thus enables rapid LPS deacylation in the livers and spleens of *Aoah*^{-/-} mice. We also found that injecting 1 μ g of recombinant human AOA (rhAOAH) intravenously 2 h prior to injecting radiolabeled LPS enabled the livers of *Aoah*^{-/-} mice to deacylate LPS at the wild type rate (Fig. 7A).

We attempted to prevent LPS-induced hepatomegaly in *Aoah*^{-/-} mice by using Ad.CMV-rhAOAH to overexpress AOA in the liver, but the control vector (Ad.CMV-luciferase) itself stimulated greater hepatomegaly by day 7 than did LPS

alone (data not shown), in keeping with the well known inflammatory properties of adenoviral vectors (38). On the other hand, giving 1 μ g of rhAOAH intravenously 2 h prior to intravenous challenge with 8 μ g of LPS significantly reduced hepatomegaly in *Aoah*^{-/-} mice (Fig. 7B).

DISCUSSION

Previous attempts to identify a hepatic mechanism for inactivating endotoxin have been unsuccessful (4, 10, 39). In retrospect, two factors may have contributed to this difficulty: the relatively slow rate with which enzymatic deacylation of LPS proceeds *in vivo* and the fact that its impact on the liver becomes apparent several days after the initial inflammatory response has subsided.

Previous studies of LPS inactivation by degradative enzymes found that dephosphorylation of LPS could be demonstrated *in vitro* and that administering alkaline phosphatase rescued rodents from endotoxin challenge *in vivo* (40–42). Whether dephosphorylation by native enzymes is sufficient to detoxify LPS *in vivo* is not known. Others have also studied LPS deacylation. Freudenberg and Galanos (43) found that rat liver catabolizes smooth (*Salmonella abortus equi*) LPS by removing some of the fatty acids from lipid A. Three days after intravenous injection, they were able to recover 35% of the radioactivity that was originally present in the liver. They extracted and partially repurified the remaining molecules and found that they were still active in several bioassays (43), suggesting that hepatic catabolism failed to detoxify the recovered LPS. In retrospect, their data indicate that the recovered molecules retained most of their secondary acyl chains. Although we cannot entirely account for the differences between these results and our own, it is possible that much of the smooth (long polysaccharide chain) LPS used in their study was delivered directly to hepatocytes after binding to circulating lipoproteins, whereas our rough (short saccharide) LPS was taken up by Kupffer cells (31, 44). Intact Gram-negative bacteria are also principally taken up from the bloodstream by Kupffer cells (44, 45), which, as shown here, contribute greatly to LPS deacylation in the liver.

Others have also described the catabolism of LPS by Kupffer cells. van Bossuyt *et al.* (46) found that rat Kupffer cells can degrade LPS *in vitro* without detoxifying it. Fox *et al.* (47) noted that isolated rat Kupffer cells modify ¹²⁵I-labeled LPS in a way that changes its buoyant density; they also found evidence that Kupffer cells modify LPS by enriching its lipid content (48). As has been found in most other studies of LPS degradation by liver cells, the recovered LPS retained bioactivity in various assays (43). There is also a report that rat hepatocytes can take up LPS *in vitro* and release 3-hydroxymyristate into the culture medium (49). We are unable to account for the differences between these results and those presented here, which indicate that AOA carries out LPS deacylation in murine liver, that it is produced principally in Kupffer cells, and that it inactivates LPS *in vivo*.

Three further points deserve emphasis. First, our results confirm the important role that acyloxyacyl-linked secondary acyl chains play in LPS recognition by animals. Each of the LPS preparations used in our studies was less active in *Aoah*^{+/+}

than in *Aoah*^{-/-} mice, suggesting that, at least *in vivo*, tetraacyl LPS molecules are significantly less active toward murine cells than are hexaacylated ones. This was unexpected since there is strong evidence that murine MD-2-TLR4 recognizes tetraacyl lipid A analogs (50, 51). It is possible that further degradative reactions contributed to the loss of activity *in vivo* so that deacylated LPS also underwent loss of (for instance) one or both lipid A-linked phosphates. Alternatively, the presence of the polysaccharide chain might decrease recognition of tetraacyl lipid A by murine MD-2-TLR4 (52, 53).

Second, we found that LPS deacylation *in vivo* occurs slowly over time, reaching completion only after 2–3 days. In keeping with the slow deacylation rate, AOA expression had little impact on several early pro-inflammatory responses to LPS. On the other hand, persistently acylated LPS remained stimulatory in the liver and spleen. The findings in these organs thus resemble those in the draining lymph nodes of *Aoah*^{-/-} mice, which respond to persistently acylated LPS by remaining enlarged for prolonged periods of time (20). In confirmation of Cody *et al.* (54), who studied AOA mRNA expression *in vivo*, we found that administering low doses of LPS increases AOA activity 3-fold in the livers of wild type mice over a 12-h period (data not shown). It is thus possible that subsequent doses of LPS would be deacylated more rapidly than was the single bolus injection studied here. Indeed, Cody *et al.* (54) also found that macrophage AOA expression could be induced *in vitro* by tumor necrosis factor and interferon- γ , was not inhibited by interleukin-10 or dexamethasone, and occurred in endotoxin-tolerant cells. Increases in AOA activity may thus play a role in reducing responses to recurring exposures to LPS or Gram-negative bacteria *in vivo*.

Third, Kupffer cells, the principal LPS-responsive cells within the liver, are also the major AOA-producing cells. Previous studies have found that LPS moves from Kupffer cells to hepatocytes over time (44); both of these cell types are LPS-responsive *in vitro*, as are sinusoidal endothelial cells and stellate cells (55–57). The observation that deacylation is required to inactivate LPS raises the possibility that, in the absence of AOA, LPS that has been internalized by Kupffer cells can be released and then stimulate one or more downstream cell types. Further studies are required to reveal how persistently acylated LPS induces prolonged inflammation in the liver and spleen. Since AOA is also a phospholipase and acyl transferase, at least *in vitro* (58), it is possible that the absence of these activities also influences the pathologies observed in LPS-treated *Aoah*^{-/-} mice.

The MD-2-TLR4 mechanism for sensing Gram-negative bacteria recognizes hexaacyl lipid A molecules that have four primary and two secondary acyl chains (59, 60). This general lipid A configuration is found in the LPSs of almost all of the aerobic or facultatively anaerobic Gram-negative bacteria that can inhabit the gastrointestinal tract (60). It may not be coincidental that these LPSs are optimal substrates for AOA, which has been very highly conserved during animal evolution (60); since these LPSs are more likely than others to enter the portal blood, inactivating them may be the major function of hepatic AOA. As noted in the Introduction, gut-derived endotoxin

may be a co-factor in several liver diseases and might also contribute to systemic immune activation in patients with human immunodeficiency virus infection or cirrhosis. Our findings thus raise the possibility that AOA, by inactivating LPS within the liver and spleen, is an important endogenous control mechanism. If this is true, increasing hepatic AOA activity might be beneficial in several clinical settings.

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