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The Transport and Inactivation Kinetics of Bacterial Lipopolysaccharide Influence Its Immunological Potency In Vivo

Mingfang Lu and Robert S. Munford

The extraordinary potency and pathological relevance of Gram-negative bacterial LPSs have made them very popular experimental agonists, yet little is known about what happens to these stimulatory molecules within animal tissues. We tracked fluorescent and radiolabeled LPSs from a s.c. inoculation site to its draining lymph nodes (DLN), blood, and liver. Although we found FITC-labeled LPS in DLN within minutes of injection, drainage of radiolabeled LPS continued for >6 wk. Within the DLN, most of the LPS was found in the subcapsular sinus or medulla, near or within lymphatic endothelial cells and CD169+ macrophages. Whereas most of the LPS seemed to pass through the DLN without entering B cell follicles, by 24 h after injection a small amount of LPS was found in the paracortex. In wild-type mice, the LPS seemed to pass through the DLN without entering B cell follicles, by 24 h after injection a small amount of LPS was found in the paracortex. In wild-type mice, ≥70% of the injected radiolabeled LPS underwent inactivation by deacylation before it left the footpad; in animals that lacked acyloxyacyl hydrolase, the LPS-deacylating enzyme, prolonged drainage of fully acylated (active) LPS boosted polyclonal IgM and IgG3 Ab titers. LPS egress from a s.c. injection site thus occurred during many weeks and was mainly via lymphatic channels. Its immunological potency, as measured by its ability to stimulate polyclonal Ab production, was greatly influenced by the kinetics of both lymphatic drainage and enzymatic inactivation. The Journal of Immunology, 2011, 187: 3314–3320.

Animals protect themselves from many Gram-negative bacteria by sensing the bacterial cell wall LPS (also called endotoxin), then mounting inflammatory responses that kill the microbes (1). The host response to LPS also typically includes the production of both polyclonal and anti-LPS Abs. Whereas much is known about the fates of LPS molecules that have been injected into the bloodstream (2–5), how LPSs traffic from s.c. tissues to draining lymph nodes (DLN) and distant organs has not been studied. It is important to know how, when, and where LPS is inactivated in tissue sites, because infection usually starts in extravascular tissues and LPS that escapes inactivation there may stimulate cells at other sites within the body. It is also of interest to know how the transport and inactivation of LPS molecules influence an animal’s immunological responses to them.

To follow LPS movement from a peripheral tissue site, we tracked the fate of small doses of LPS injected s.c. into mice. We used both radiolabeled and fluorescent LPS probes to measure LPS movement from an injection site in a footpad or flank to DLN and its appearance in the liver, the major organ for clearing blood-borne LPS.

Inactivation of microbial agonists may also influence host immune responses. We reported previously that acyloxyacyl hydrolase (AOAH), a host enzyme that inactivates LPS by deacylation, limits polyclonal Ab responses to LPS in mice (6). In the present studies we defined the magnitude and time course of LPS deacylation in a s.c. injection site and assessed its impact on the ability of LPS to stimulate downstream B lymphocytes by measuring polyclonal Ab levels in serum.

Materials and Methods

Reagents

Escherichia coli O14 LPS was prepared by the phenol-chloroform-petroleum ether method (7). Neisseria meningitidis LPS, a gift from Michael Apicella (University of Iowa), was purified from a group B (L3,7,9) strain. Re Salmonella typhimurium LPS ([3H/14C]LPS; 14C-labeled fatty acyl chains and 3H-labeled glucosamine backbone) was prepared from S. typhimurium PR122 as described previously (8); 1 μg had ∼150,000 dpm. FITC-LPS or Texas Red (TR)-LPS was prepared as described by Tobias et al. (9). In brief, E. coli O14 LPS (Ra chemotype) was resuspended (2 mg/ml) in 1 M borate (pH 10.5). Radiolabeled LPS (5 μg) was added so that the concentration of the final product could be calculated. Solid FITC (10 mg) was then added to 2.5 ml suspension and incubated for 3 h at 37°C. A 10-fold excess of glycine was added to stop the reaction. The suspension was dialyzed (1000 Da molecular mass cut-off) against PBS at 4°C for 2 wk. The FITC-LPS was then precipitated by adding a 2-fold excess of ethanol. The pellet was washed three times with 70% ethanol and resuspended in PBS. The labeling efficiency was 0.76 FITC/LPS (mol/mol) and 0.15 TR/LPS (mol/mol). The LPS lost 15% of its fatty acyl chains during labeling at alkaline pH, resulting in a FITC-LPS that was ∼10-fold less stimulatory than the LPS used to prepare it. Glycine–FITC was made by mixing glycine with FITC in PBS. The solution was diluted so that its OD494 was the same as that of the FITC-LPS. Trinitrophenol (TNP)-FICOLL was purchased from Biosearch Technologies. TNP-LPS was prepared by the same method used for preparing FITC-LPS. N. meningitidis LPS was incubated with 2,4,6-trinitrobenzenesulfonic acid solution (Sigma-Aldrich) and the TNP-LPS was purified by dialysis and precipitation. The labeling efficiency was 1.13 TNP/LPS (mol/mol).

Abs

Murine monoclonal anti–MD-2/TLR4 Ab, UT12 (10), was prepared as described (11). Abs used for microscopy were B220 (clone RA3-6B2; BD Biosciences), CD169 (clone 3D6.112; AbD Serotech), LYVE1 (polyclonal; Abcam), and CD11c (clone N418; eBioscience). Anti-rat Alexafluor 568 and streptavidin allophtocyanin were from Invitrogen.

The online version of this article contains supplemental material.

Abbreviations used in this article: AOAH, acyloxyacyl hydrolase; DLN, draining lymph node; TNP, trinitrophenol; TR, Texas Red.

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Liposomes containing clodronate or PBS (control) were prepared as described (12).

**Mice**

* Aoah−/− C57BL/6 mice were prepared as described (13). CD11c-DTR (B6.FVB-Tg(Itgax-DTR/EGFP)57LanJ) and Thy−/− (B6.B10ScN-Tly4.6dez/J) JhiJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific-pathogen-free facility and studied using protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center or by the Animal Resources Center of the National Institute of Allergy and Infectious Diseases.

**Studies using fluorescent LPS**

Five micrograms LPS-FITC, 5 μg TR-LPS, or Gly-FITC with the same OD as LPS-FITC were dissolved in 40 μl PBS and injected into a mouse hind footpad or flank using an insulin syringe. The draining popliteal or brachial node was dissected at various time points after injection. Lymph nodes were immersed in Sakura Tissue-Tek OCT Compound (IMEB) and snap-frozen in isopentane chilled in liquid nitrogen. Eight-micrometer sections were cut using a Leica CM1900 cryostat. The sections were fixed and permeabilized in methanol for 10 min at −20°C and blocked with 1 mg/ml BSA, 0.5 mg/ml normal mouse Ig, and 10 μg/ml streptavidin in PBS and then 1 mg/ml BSA and 2 mM biotin in PBS. Sections were stained with primary Abs at 4°C overnight and with the secondary reagents at room temperature for 1 h. After washing, FluorSave reagent aqueous mounting medium (EMD Chemicals) was applied and then coverslips were affixed. Stained sections were examined by using a Leica SP5 X-WLL confocal microscope and analyzed using LAS AF Lite (Leica) software.

To distinguish between cell-free and cell-mediated transport, LPS-FITC and LPS-TR were injected s.c. at separate locations on the back. Both injection sites drained to the same brachial node. If the two LPS preparations were carried by cells to DLN, they should not colocalize within the node. Four, 24, and 48 h later, brachial nodes were dissected and analyzed for colocalization of green and red fluorescence. As a control, 40 μl of 0.02% LPS aggregates, appeared in the subcapsular sinus within 10–12 min after injection, but were largely excluded from the lymph node cortex (parenchyma). In contrast, Randolph et al. (14) found that fluorescent microspheres, which are carried from skin to DLN by mainly dendritic cells, appeared 24 h or more after injection in the DLN paracortex; conjoint of LPS delayed microsphere transport by preventing the differentiation of monocytes into dendritic cells (16). We found evidence for both cell-free and cell-mediated transport of LPS from a s.c. site to DLN. Confirming the results of Gretz et al. (15), we found FITC-LPS in the ipsilateral popliteal node within 3–10 min after injecting it into a hind footpad, strongly suggesting that the first LPS molecules that arrived in the DLN were not transported by cells (Fig. 1A–D). Some of the FITC-LPS colocalized with LYVE1 lymphatic endothelium (Fig. 1A, 1B, 1D). FITC-LPS was also located in, or on the surfaces of, CD169+ macrophages in the subcapsular sinus, yet it did not extend into the B cell zone (Fig. 1A–G). In sections obtained as long as 5 d after injection, most of the FITC appeared within the subcapsular sinus and medulla (as in Fig. 1A), and the amount of detectable FITC gradually diminished over time.

To distinguish cell-free from cell-mediated transport from the injection site to DLN, we injected FITC-LPS and TR-LPS into adjacent intradermal sites on a flank. Because LPS moves more slowly to DLN when injected into sites on the back when than when injected into a footpad, we excised the draining brachial node 4 h after LPS injection. The distribution of the two fluorescent labels overlapped in the subcapsular sinus and medulla, where many cells had taken up both labels (Fig. 1H, 1J). Although it seems likely that the two LPS preparations traveled cell-free to the DLN, where they could then be taken up by the same cells in the subcapsular sinus and medulla, it is also possible that the LPS was carried by cells and passed from cell to cell after arriving in the DLN; the slow rate with which LPS is released from macrophages in vitro argues against the latter interpretation (17, 18). One or 2 d after injection, in contrast, there were small, discrete collections of FITC-LPS and TR-LPS in the paracortex of the brachial node (Fig. 1K–M). Fluorescent microspheres had a similar paracortical location 2 d after they were injected into adjacent s.c. sites (Supplemental Fig. 2). This LPS was probably carried to the paracortex by cells (19); in keeping with Randolph et al. (14), we found some of it within, or in close proximity to, CD11c+ cells (Fig. 1M). When we injected glycine-FITC into a footpad as a control, we did not find FITC in the DLN.

We conclude that LPS can traffic from a s.c. site to DLN both cell-free and carried by cells. Most of the injected LPS passed through the DLN via the subcapsular sinus and medulla, beginning within minutes of injection and continuing for weeks; a smaller fraction of the injected LPS made its way to the paracortex, where contact with B and T cells would be expected (20).

We used radiolabeled LPS to quantitate LPS movement from the footpad to the DLN over time. The amount of 14C is a reliable
marker for the number of LPS molecules because $^{14}C$ in the glucosamine backbone of lipid A and this part of the molecule does not undergo catabolism in vivo. The $^{14}C$ dpm remaining in the footpad declined for at least 6 wk following injection, as did the amount of $^{14}C$ in the popliteal and inguinal nodes (Fig. 2A). At each time point from 3 to 41 d, the nodes contained 3–8% of the $^{14}C$ dpm recovered from the footpad. At no point after day 3 was accumulation of $^{14}C$ radioactivity evident in the node. LPS deacylation did not alter this pattern (Aoah$^{+/+}$ versus Aoah$^{-/-}$ mice) (Fig. 2A).

LPS slowly traffics to the liver

We also measured the amount of $[^{14}C]LPS$ in plasma and liver at different time points after footpad injection (Fig. 2B). At no point was >1% of the injected dose found in the plasma. Whereas we found in previous studies that >80% of an i.v. dose of the same radiolabeled LPS appeared in the liver within 5 min (12), accumulation of s.c.-injected LPS by the liver was much more gradual, so that by 14 d after injection the liver contained ~20% of the injected dose [at this time point, ~40% of the inoculum was still in the footpad (Fig. 2A) and ~10% had been excreted in the urine or feces (data not shown)].

Most LPS molecules are deacylated at the injection site

In wild-type mice, almost all of the LPS that remained in the footpad had been deacylated by 1 wk following injection (Fig. 3A). The amount of fully acylated LPS in the footpad thus became quite low at a time when more than half of the injected LPS was still in the footpad (Fig. 2A). LPS recovered from DLNs and the liver had been even further deacylated (Fig. 3B, 3C). Although LPS may be deacylated by Kupffer cells, which express AOAH (12), it seems likely that most of the LPS recovered from the liver had undergone deacylation within the footpad or in the DLN. Very little deacylation occurred in Aoah$^{-/-}$ mice (6) (Fig. 3A–C), so fully acylated LPS continued to drain to the DLN for many weeks (Fig. 3D).

Prolonged movement of fully acylated LPS from a peripheral site to DLN promotes exaggerated Ab responses

If LPS deacylation by AOAH takes more than a week to reach completion, how could such a slow reaction limit LPS-induced polyclonal Ab production by B cells? We hypothesized that fully acylated LPS must reach the DLN over a prolonged period of time to be able to produce maximal Ab responses. If this hypothesis is correct, the exaggerated Ab responses seen in Aoah$^{-/-}$ animals...
should be prevented by excising the injection site, thus removing the supply of fully acylated LPS. To test this idea, we injected LPS into a s.c. site on the back on day 0. We then excised the injection site skin from Aoah−/− mice on day 1 or day 4 after injection and measured serum Ab levels 7, 14, and 21 d after injection. We then excised the injection site skin from Aoah−/− mice (triangles, left axis label). Open symbols indicate Aoah+/-; closed symbols indicate Aoah+/+. n = 3–6 mice/time point. Data were combined from two independent experiments with similar results. Error bar, 1 SEM.

FIGURE 2. A, LPS traffics from footpad injection site to DLN. Recovery of 14C dpm from LPS injected into footpads (Foot) and from their draining popliteal and inguinal lymph nodes. Symbols show the means ± SE of three measurements per time point. Decay curves were generated using GraphPad Prism software (two-phase exponential decay). The results from a second experiment (larger symbols) are plotted alongside the best-fit curves from the first experiment (smaller symbols), which has been previously reported (6). Closed symbols and solid lines indicate Aoah+/-; open symbols and interrupted lines indicate Aoah−/−. Blue indicates feet; red indicates DLN. B, LPS traffics to the liver. 14C radioactivity (dpm, as a percentage of the injected dose) recovered from the plasma and liver following footpad injection of 10 μg [3H/14C]LPS is shown. Fewer than 1% of the injected 14C counts were recovered from the plasma at any time point (round symbols, right axis label), whereas 14C gradually accumulated in the livers of both Aoah+/- and Aoah−/− mice (triangles, left axis label). Open symbols indicate Aoah−/−; closed symbols indicate Aoah+/-; n = 3–6 mice/time point. Data were combined from two independent experiments with similar results. Error bar, 1 SEM.

FIGURE 3. LPS undergoes deacylation at the injection site. A–C, LPS deacylation time course in the footpad, DLN, and liver. Circles indicate Aoah−/−; boxes indicate Aoah+/-; Maximum deacylation is 33% (AOAH removes two of the six fatty acyl chains from the LPS backbone). In Aoah+/- mice, almost all of the LPS in the footpad had been deacylated by day 7 after injection. D, Amount of fully acylated LPS remaining in the feet of Aoah−/− mice (○) and Aoah+/- mice (●), expressed as a percentage of the LPS inoculum. The figure was plotted using data from Figs. 2A and 3A. Error bars, 1 SEM. In mice that lack AOAH, fully acylated LPS continued draining from the footpad long after almost all of the LPS had been deacylated in wild-type animals.

AOAH does not modulate Ab responses to a non-LPS TLR4 agonist or to a TI-II Ag

It is important to be sure that phenomena attributed to AOAH are indeed due to its ability to deacylate LPS. Accordingly, we have previously injected mice with UT12, an agonistic mAb to MD-2/TLR4 that, similar to LPS, induces murine B cell proliferation in vitro and in vivo and is a potent activator of TLR4-dependent intracellular signaling (10). We found virtually identical IgM and IgG3 responses in Aoah+/- and Aoah−/− mice (21), suggesting strongly that the enzyme’s action on LPS accounts for its ability to modulate Ab responses in vivo. In this study, we asked whether Aoah−/− mice have exaggerated responses to a TI-II Ag, Ficoll-TNP. Unlike LPS and UT12, Ficoll-TNP did not induce polyclonal Ab responses, so we measured anti-TNP responses. Ficoll-TNP elicited similar anti-TNP IgM and IgG3 responses in Aoah+/- and Aoah−/− mice, whereas anti-TNP responses were much higher in LPS-TNP-immunized Aoah−/− mice than in Aoah+/- mice (Supplemental Fig. 3). These results confirm that AOAH modulates Ab responses to its substrate, LPS, but not to a different TI Ag.

Multiple cell types contribute to LPS deacylation in vivo

AOAH is produced by monocytes/macrophages, neutrophils, and dendritic cells. To identify the cells that contribute to deacylation in the footpad, we depleted macrophages by giving clodronate-liposomes, neutrophils by pretreatment with anti–Gr-1 mAb, and dendritic cells by injecting diphtheria toxin into mice engineered to produce the diphtheria toxin receptor downstream of the CD11c promoter (Supplemental Fig. 1). We found that each of these cell types partially contributes to LPS deacylation in the footpad (Fig.
5A); depleting macrophages had the greatest effect. In contrast, depleting each cell type did not increase the amount of LPS that remained in feet or alter the amount of LPS that was recovered from DLN 3 d after injection (Fig. 5B, 5C). These phagocytes thus may not play a critical role in transporting LPS, or perhaps other cells can compensate for the absence of a single cell type.

To characterize further the role played by macrophages in LPS inactivation, we studied the Ab responses of wild-type and Aoah−/− mice after depleting macrophages using clodronate-liposomes. If footpad macrophages deacylate LPS, depleting them should allow a larger fraction of the LPS injected to remain acylated, drain to regional nodes, and elicit Ab production. Alternatively, LPS-responsive macrophages might produce cytokines or other mediators that would stimulate B cells and indirectly augment Ab production. When we injected footpads of Aoah+/+ mice with clodronate-liposomes 5 d before injecting LPS, we found elevated total IgM and IgG3 levels 7 and 14 d later (Fig. 5D, 5E), in keeping with a prominent role for macrophages in LPS inactivation. Clodronate-liposome pretreatment did not alter LPS-induced Ab production in Aoah−/− mice. Because subcapsular CD169+ macrophages in the DLN were depleted by clodronate (Supplemental Fig. 1D–G), this finding is also evidence that these cells do not play an essential role in LPS-induced polyclonal Ab production, despite their close association with LPS in the sinus (Fig. 1E–G) and their ability to transfer lymph-borne virus or immune complexes to B cells and promote B cell activation (22, 23).

**TLR4 influences LPS trafficking and deacylation in vivo**

In previous studies we found that TLR4-deficient macrophages, which are unable to mount an inflammatory response to LPS, take up extracellular LPS and deacetylate it at the same rates observed for wild-type macrophages (17). When we injected LPS into the footpads of Tlr4−/− mice, the amount of LPS remaining in the footpad 2–3 d after injection was significantly less than that observed in wild-type mice, whereas the amount recovered from the DLN was significantly higher (Fig. 6A). The local inflammatory response thus may slow the rate at which the LPS leaves an s.c. site. In other experiments we found that TLR4-deficient mice had deacylated ~50% less of the LPS in their feet on day 3 after

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**FIGURE 4.** Prolonged LPS drainage increases polyclonal Ab responses in Aoah−/− mice. Mice received 10 μg LPS s.c. at a site on the right flank on day 0. The overlying skin and s.c. fat in the Aoah−/− mice was excised on day 1 (∆) or day 4 (○). Titers of IgM (A) and IgG3 (B) were measured before and 7, 14, and 21 d after LPS inoculation. Controls included Aoah−/− (○) and Aoah+/+ (●) mice that received sham excision (from the left flank) on day 1 after LPS inoculation. In Aoah−/− mice, removing the inoculation site 1 d after injection reduced Ab titers almost to levels seen in Aoah+/+ mice. Data were combined from two experiments, each with n = 4 mice/group. Error bar, 1 SEM. Significantly different from Aoah−/− response: *p < 0.05, **p < 0.01, ***p < 0.001. ∆, Change from preimmune sera.

**FIGURE 5.** A, Multiple cell types contribute to LPS deacylation in the footpad. Wild-type PBS-treated (control), macrophage-depleted, neutrophil-depleted, or dendritic cell (DC)-depleted mice were injected with [3H/14C]LPS on day 0 and their feet were harvested for study on day 3. Results are expressed relative to the amount of LPS measured in the wild-type mice that were pretreated with PBS (n = 6–10 mice/group). **, ***, p < 0.01. B and C, Radiolabeled LPS was recovered in the ipsilateral foot (B) and DLN (C) 3 d after LPS injection into footpads of mice that had undergone depletion of macrophages, neutrophils, or DCs. Recovery relative to PBS controls (100%) is shown. No significant differences from the PBS-injected controls were seen (n = 6–10). Error bar, 1 SEM. D and E, Macrophage depletion increases LPS-induced IgM (D) and IgG3 (E) in wild-type mice. Clodronate-liposomes were injected into the footpads of Aoah+/+ and Aoah−/− mice 5 d prior to injecting LPS. Both IgM and IgG3 titers were higher in macrophage-depleted Aoah+/+ mice (▲) than in Aoah+/+ controls (PBS liposomes before LPS, ●). Clodronate pretreatment did not alter responses in Aoah−/− mice (∆, clodronate; ○, PBS). Data were combined from two experiments, each with n = 5/group. Error bar, 1 SEM. **p < 0.01; ***p < 0.001, significantly different from PBS liposome control. ∆, Change from the preimmune value.
inoculation than had wild-type mice (Fig. 6B), raising the possibility that TLR4 signaling may enhance AOAH expression in resident phagocytes and/or promote recruitment of AOAH-expressing cells such as neutrophils and monocytes. When we tested this hypothesis, we found that injecting LPS into one footpad of wild-type mice increased AOAH enzyme activity (Fig. 6C) and enhanced the deacylation of [3H/14C]LPS that was given into the same footpad 2 d later (Fig. 6D).

Discussion

This study of LPS trafficking in vivo yielded three noteworthy findings. First, we found that LPS moves from a local injection site principally via lymphatics. Endotoxemia, or the presence of LPS in the circulating blood, is a much feared complication of many Gram-negative bacterial diseases. LPS in an extravascular tissue might enter the blood directly, via venous capillaries, or indirectly, by draining first through lymphatic channels to the thoracic duct. Our results indicate that lymphatics provide an important conduit from an inflamed s.c. site to the circulating blood, just as lymphatic drainage is a major route of LPS clearance from infected peritoneal fluid (24). The slow course of lymphatic drainage not only provides greater time for LPS inactivation by deacylation prior to entering the blood, but the LPS may also be exposed to inhibitory factors in lymph (25, 26). It is possible that some LPS moved directly from the footpad into the blood, or that it was internalized by footpad phagocytes that then left the injection site and entered the bloodstream, but these seemed to be minor modes of LPS egress from the injection site. Although almost 5% of the LPS injectate was found in the liver on day 1 after injection, the LPS recovered from the livers of wild-type mice had lost more than half of its secondary acyl chains, consistent with AOAH-mediated deacylation/inactivation either prior to, or after (12), hepatic uptake of LPS from the circulation.

Second, most of the injected LPS passed through the DLN without entering the paracortex or B follicles. Radiolabeled LPS deacylation in footpad 3 d after injection (p < 0.05, **p < 0.01, ***p < 0.001). Error bar, 1 SEM.

To our knowledge, these are the first studies to track the fates of LPS molecules quantitatively in vivo, we injected LPS s.c. into a footpad or a site on the back. Although this approach was meant to mimic the LPS released by bacteria into an infected tissue site, it required relatively high doses of LPS and used purified LPS instead of intact bacteria. Using rough (Ra or Rc) LPS or lipo-oligosaccharide (N. meningitidis) offered several advantages over smooth (long polysaccharide-containing) LPS preparations: each of the LPS preparations used in this study has a relatively uniform structure, with six fatty acyl chains attached to the lipid A moiety of most molecules, and each potently activates MD-2/TLR4 (29). A disadvantage is the tendency of rough LPS preparations to form aggregates or micelles that, while possibly resembling the size of bacterial outer membrane fragments, are clearly artificial. LPS is not soluble in methanol, yet it is possible that cell-free FITC-LPS was washed away during tissue fixation for microscopy. Although our FITC-LPS was partially deacylated during the labeling process, there is strong evidence that partially deacylated LPS structures bind to LPS-binding protein can bind LPS in a structure that makes it accessible to deacylation by AOAH. Although there is evidence that LPS can be released after being processed by cultured macrophages (18), the absence of a defined mechanism for this phenomenon makes it seem less likely than extracellular deacylation within the footpad by secreted AOAH.

To be able to follow LPS molecules quantitatively in vivo, we injected LPS s.c. into a footpad or a site on the back. Although this approach was meant to mimic the LPS released by bacteria into an infected tissue site, it required relatively high doses of LPS and used purified LPS instead of intact bacteria. Using rough (Ra or Rc) LPS or lipo-oligosaccharide (N. meningitidis) offered several advantages over smooth (long polysaccharide-containing) LPS preparations: each of the LPS preparations used in this study has a relatively uniform structure, with six fatty acyl chains attached to the lipid A moiety of most molecules, and each potently activates MD-2/TLR4 (29). A disadvantage is the tendency of rough LPS preparations to form aggregates or micelles that, while possibly resembling the size of bacterial outer membrane fragments, are clearly artificial. LPS is not soluble in methanol, yet it is possible that cell-free FITC-LPS was washed away during tissue fixation for microscopy. Although our FITC-LPS was partially deacylated during the labeling process, there is strong evidence that partially deacylated LPS structures bind to LPS-binding protein can bind LPS in a manner that makes it accessible to deacylation by AOAH. Although there is evidence that LPS can be released after being processed by cultured macrophages (18), the absence of a defined mechanism for this phenomenon makes it seem less likely than extracellular deacylation within the footpad by secreted AOAH.
the lymph. The immunological potency of LPS in vivo, measured here as B cell activation to produce polyclonal Abs, was greatly influenced by the kinetics of drainage and enzymatic inactivation as well as by lymph node anatomy.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. Neutrophil, DC and macrophage depletion. A, B. Neutrophils were depleted by injection of anti-Gr-1 ascites i.p. one day before and one day after LPS injection. Myeloperoxidase activity was measured in feet and DLN on day 3. n = 6. C. Diphtheria toxin was injected i.p. to CD11c-DTR transgenic mice one day before LPS injection. CD11c+ cell numbers in spleens were measured by FACS. n = 6. D-G. Macrophages were depleted by injecting clodronate-liposomes into mouse footpads. Popliteal nodes from PBS-liposome injected mice have CD169+ macrophages (C). Bright field image of the LN is shown in D. Five days after clodronate liposome injection, the CD169+ macrophages in the DLN had disappeared (E and F). Dotted lines show the margins of the DLN.

Supplemental Figure 2  Microspheres traffic from a subcutaneous injection site to DLN. Green and red microbeads were injected into adjacent subcutaneous sites on one flank. Forty eight hrs later, green (A) and red (B) beads were found in the paracortex of DLN, where they did not co-localize (C). B cells are blue. Dotted lines show the margins of the LN.

Supplemental Figure 3  The exaggerated antibody response in AOAH null mice is limited to the AOAH substrate, LPS. 40 μg Ficoll-TNP or 5μg LPS-TNP were injected s.c. on days 0 and 21, the mice were bled on days 7, 14 and 28, and their anti-TNP responses were measured by ELISA. n = 7-8/group. **, p < 0.01; ***, p < 0.001. Delta is the change from the pre-immune value. Error bar = 1 SEM.