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

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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 LPS 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, >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 polyvalent 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: 000–000.

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 fate 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. Rc Salmonella typhi LPS ([3H/14C]LPS; ’H-labeled fatty acyl chains and 14C-labeled glucosamine backbone) was prepared from S. typhi/um PR122 as described previously (8); 1 μg had ~150,000 dpm [3H] and 10,000 dpm [14C]. 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 0.1 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 OD280 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 Serotec), LYVE1 (polyvalent; Abcam), and CD11c (clone N418; eBioscience). Anti-rat Alexafluor 568 and streptavidin allopbyocyanin were from Invitrogen.
Lipsosomes containing clodronate or PBS (control) were prepared as described (12).

Mice

Aoah<sup>−/−</sup> C57BL/6 mice were prepared as described (13). CD11c-eDTR (B6.FVB-Tg[Itgax-DTR/EGFP]57Lanj) and Tlr4<sup>−/−</sup> (B6.129S7-Tlr4<sup>−/−</sup>/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 mouse normal 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 after brachial node dissection were dissected and analyzed for colocalization of green and red fluorescence. As a control, 40 μl 0.02% suspension of 1.0 μm yellow-green or red fluorescent microspheres (Invitrogen) were injected into the same sites in other mice and their location in the brachial node was determined 2 d later. Microspheres were known to be carried from skin to DLN by monocyte-derived dendritic cells (14).

Quantitation of radiolabeled LPS

To follow the location and deacetylation of LPS over time, we inoculated the footpads of mice with 10 μg [1<sup>4</sup>C] LPS (8) (see above) and sacrificed them by cervical dislocation at 3, 7, 14, and 41 d. The feet were solubilized by immersion in Solvable (Packard Instruments, Meriden, CT). DLN and livers were homogenized in PBS. To estimate LPS deacetylation, the ratio of 3H to 14C was used (12).

Inoculation site removal experiment

AOAH wild-type and knockout mice were injected with 10 μg N. menigitidis LPS s.c. at a shaved site on the back. Red microspheres were included in the LPS suspension to mark the injection location. On day 1 or day 4 after injection, a skin patch that included the injection site was excised. For control mice, similar sizes of skin patches were excised from the opposite (noninjection) sites. Mice were bled before LPS injection and 7, 14, and 21 d thereafter. Serum total IgM and IgG3 levels were measured using ELISA.

ELISA

Standard ELISA methods were used. To assay Ab concentrations, microtiter wells were coated with goat anti-mouse polyclonal IgGs (IgG, IgM, and IgA) from Sigma-Aldrich. The detection Abs were HRP-conjugated goat anti-mouse IgM (Sigma-Aldrich) and anti-mouse IgG3 (SouthernBiotech). HRP substrate (BD Pharmland) was used. Plates were read on an MRX microplate reader (Dynex Technologies, Chantilly, VA). IgM and IgG standards were provided by E. Viettta (University of Texas Southwestern Medical Center, Dallas, TX). In all cases, differences between post- and preimmunization are reported (6).

Cell depletion experiments

To deplete neutrophils, anti-Gr-1 ascites was injected i.p. to mice on day −1 before LPS injection and again on day 1 after LPS injection. About 65 and 88% of neutrophils were depleted in the footpad and LN respectively, as assessed by myeloperoxidase assay of solubilized tissue (Supplemental Fig. 1A, 1B). To deplete dendritic cells in CD11c-eDTR mice, 100 ng diphtheria toxin was injected i.p. on day −1 before LPS injection on day 0. To deplete resident macrophages, clodronate liposomes (50 μl) (12) were injected into footpads on day −5; control animals received PBS-liposomes. The depletion of DCs or macrophages was documented by a 92% decrease in CD11c<sup>+</sup> cells in spleens measured by flow cytometry (LSRFortessa; BD Biosciences) or by the disappearance of subcapsular and medullary CD169<sup>+</sup> cells in DLN by immunomicroscopy, respectively (Supplemental Fig. 1C, 1D–G).

Results

LPS moves rapidly from a s.c. injection site to its DLN, yet drainage continues for many weeks

Previous studies on lymphatic drainage from s.c. injection sites to DLN showed that molecules of higher molecular weight, including LPS aggregates, appeared in the subcapsular sinuses 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; coinjection 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<sup>+</sup> lymphatic endothelium (Fig. 1A, 1B, 1D). FITC-LPS was also located in, or on the surfaces of, CD169<sup>+</sup> 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 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<sup>+</sup> 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 is 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 DNs 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 DNs. 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 fullyacylated 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 mice that underwent skin excision on day 1 had IgM and IgG3 responses that were similar to those of Aoah+/+ mice and significantly lower than those observed in control Aoah−/− mice (s.c. LPS with mock excision) (Fig. 4). Aoah−/− mice that underwent excision of the injection site on day 4 had slightly lower Ab responses than did control Aoah−/− mice. The robust Ab responses observed in Aoah−/− mice thus require a continuous source of LPS at the inoculation site from days 1 to ∼4. Although LPS deacylation at the inoculation site occurs over several days in wild-type mice, it is sufficient to limit their B cell responses. In contrast, AOAH does not act quickly enough to be able to diminish LPS-induced cytokine production, which occurs within minutes to hours after LPS injection (11).

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. 3).
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. 5A), 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 subsplenic 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 (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 deacylate 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 decayed almost 50% less of the LPS in their feet on day 3 after 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. Δ, Change from preimmune sera.

**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 (◇). Titters 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. Δ, 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 [14C]LPS on day 0 and their feet were harvested for study on day 3. Results are expressed relative to the amount of deacylation measured in the wild-type mice that were pretreated with PBS (n = 6–10 mice/group). **p < 0.001. 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. **p < 0.01; ***p < 0.001, significantly different from PBS liposome control. Δ, 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 $[^{3}H/^{14}C]$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 did not accumulate in the DLN. FITC-LPS that entered the DLN was found principally within the subcapsular sinus, where it was associated with macrophages (CD169$^+$) and lymphatic endothelial cells (LYVE1$^+$), and also in the medulla. With time, discrete foci of FITC-LPS and TR-LPS appeared in the paracortex, closely resembling the pattern observed for s.c.-injected fluorescent beads, suggesting that some of the LPS is carried to the DLN by cells. This was more prominent at later time points (1–2 d after injection). Although a minor fraction of the injected LPS was found in the paracortex, this may be sufficient to bring LPS into direct contact with B cells (20), a prerequisite for B cell stimulation by LPS in mice (M.F. Lu, unpublished results).

Third, our results document the important role that AOAH plays in modulating the bioactivity of LPS in vivo. AOAH is a lipase, found principally in myeloid cells, that removes two of the six fatty acids that are required for LPS to be sensed by its receptor on animal cells, the MD-2/TLR4 complex. In three different experimental settings, which involved B cells, Kupffer cells, and peritoneal macrophages, mice that lacked AOAH were unable to restore homeostasis after they were exposed to small amounts of LPS in vivo (6, 11, 12). In each instance, the response to LPS exposure was exaggerated and prolonged in the absence of AOAH. In this study, we found that $>70\%$ of the injected LPS was deacylated by AOAH before it left the injection site in Aoah$^{-/-}$ mice. There was strong evidence that phagocytes took part in deacylating LPS, but we were unable to distinguish extracellular from intracellular deacylation. AOAH has an acid pH optimum and resides within intracellular granules, yet it may be secreted and taken up by other cells in a mannose-6-phosphate–dependent fashion (27). Additionally, Gioannini et al. (28) found that soluble CD14 and 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.

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, CD14, and MD-2/TLR4 (30–32). It thus seems unlikely that this degree of deacylation would alter the ability of FITC-LPS to interact normally with cells.

To our knowledge, these are the first studies to track the fates of LPS molecules in tissues other than the bloodstream. We did not expect to find the very slow disappearance kinetics from the injection site, the prominent role played by lymphatics in removing LPS from the s.c. tissue, the passage of a large fraction of the LPS directly from the footpad into the blood, or that it was internalized by resident phagocytes that then left the injection site and entered 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.

**FIGURE 6.** TLR4 influences LPS trafficking and deacylation in vivo. A, Recovery of $[^{14}C]$LPS from footpad and DLN 3 d after footpad injection into Tlr4$^{-/-}$ and wild-type mice. Data from day 2 after injection were similar. B, LPS deacylation in footpad 3 d after injection ($n = 10–12$ mice/group). Error bar, 1 SEM. C and D, Pretreatment with LPS enhances LPS deacylation in the footpad. Aoah$^{-/-}$ mice were injected with PBS ($n = 3$) or 4 $\mu$g nonradioactive LPS ($n = 3$) in both footpads on day −3. On day 0, $[^{3}H/^{14}C]$LPS was injected to the right footpads of all mice. Two days later, the left feet were harvested to measure AOAH activity in tissue lysates (C) and the right feet were used to measure deacylation of the injected $[^{14}C]$LPS (D). Maximal deacylation = $\sim 33\%$. Error bar, 1 SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. 

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the lymph. The immunological potency of LPS in vivo, measured as well as by lymph node anatomy.

Disclosures

The authors have no financial conflicts of interest.

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