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Lipopolysaccharide deacylation by an endogenous lipase controls innate antibody responses to Gram-negative bacteria

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T cell–independent type 1 agonists such as Gram-negative bacterial lipopolysaccharides can stimulate B lymphocytes to proliferate and produce antibodies by signaling through Toll-like receptors. This phenomenon is well established *in vitro*, yet polyclonal B cell responses after bacterial infection *in vivo* are often weak and short-lived. We show here that B cell proliferation and polyclonal antibody production in response to Gram-negative bacterial infection are modulated by acyloxyacyl hydrolase, a host enzyme that deacylates bacterial lipopolysaccharides. Deacylation of lipopolysaccharide occurred over several days, allowing lipopolysaccharide to act as an innate immune stimulant yet limiting the eventual amount of B cell proliferation and polyclonal antibody production. Control of lipopolysaccharide activation by acyloxyacyl hydrolase indicates that mammals can regulate immune responses to bacterial infection by chemical modification of a Toll-like receptor agonist.

The lipid A moiety of most Gram-negative bacterial lipopolysaccharide (LPS) molecules is a glucosamine disaccharide to which hydroxyacyl chains are attached at positions 2, 3, 2′ and 3′. The hydroxyl moiety of one or more of the hydroxyacyl residues may be further substituted with a secondary acyl chain (typically laurate, myristate or palmitate) in acyloxyacyl linkage. Acyloxyacyl hydrolase (AOAH) selectively removes the secondary acyl chains from LPS[1], leaving the primary hydroxyacyl chains attached to the glucosamine backbone. This reaction results in a partially deacylated LPS product (dLPS) with a relatively stable tetraacyl lipid A structure. The dLPS product is about 0.2–1% as potent as fully acylated LPS, as demonstrated in cell activation assays *in vitro*[2] and in an assay of tissue toxicity *in vivo*[3]. Although those findings suggest possible involvement of AOAH in reducing the stimulatory capacity of LPS during Gram-negative bacterial infection, the conversion of LPS to dLPS by neutrophils, macrophages and dendritic cells may take many hours[4–6], reducing the likelihood that deacylation can prevent inflammatory responses that normally begin within minutes of exposure to LPS. The effect of deacylation of LPS on longer-term immune responses, such as those of B lymphocytes, has not been evaluated.

Antigen-nonspecific stimulation of B cells can increase the plasma concentrations of a wide variety of antibodies, including ones that bind to microbial membranes, activate complement, facilitate phagocytosis of invading microorganisms, deliver antigens to secondary lymphoid organs[7] and react with self molecules[8]. This polyclonal response begins after recognition of conserved microbial molecules by Toll-like receptors (TLRs) expressed on B cells[9]. The known Gram-negative bacterial B cell mitogens (T cell–independent type I antigens) include LPS (TLR4–MD-2 and RP105–MD-1)[9–12], CpG (TLR9)[13], peptidoglycan (TLR2)[14] and bacterial outer membrane porins and lipoproteins (TLR2)[9,15]. B cell responses to these agonists *in vitro* can be extremely robust[16,17], yet the *in vivo* response to immunization with the same agonists, or to infection with bacteria that produce them, is often weak or even absent[17]. As optimal stimulation of mouse B cells *in vitro* requires that LPS be present for over 48 h (ref. 18), we reasoned that direct inactivation of LPS might provide one way by which control of the polyclonal B cell response to bacterial infections is achieved *in vivo*.

To test the hypothesis that AOAH inactivates LPS *in vivo* and thereby limits the extent of the innate immune response, we compared the ability of wild-type (*Aoah*$^{+/+}$) and AOAH-deficient (*Aoah*$^{-/-}$) mice to produce antibodies in response to Gram-negative bacterial infection and LPS challenge. We found that *Aoah*$^{-/-}$ mice had greatly enhanced B cell proliferation and polyclonal antibody production.

RESULTS

Antibody responses to bacterial infection

Macrophages from *Aoah*$^{-/-}$ mice are defective in deacylation of both purified LPS and the LPS of phagocytosed *Escherichia coli*[6]. We therefore evaluated serum immunoglobulin concentrations of

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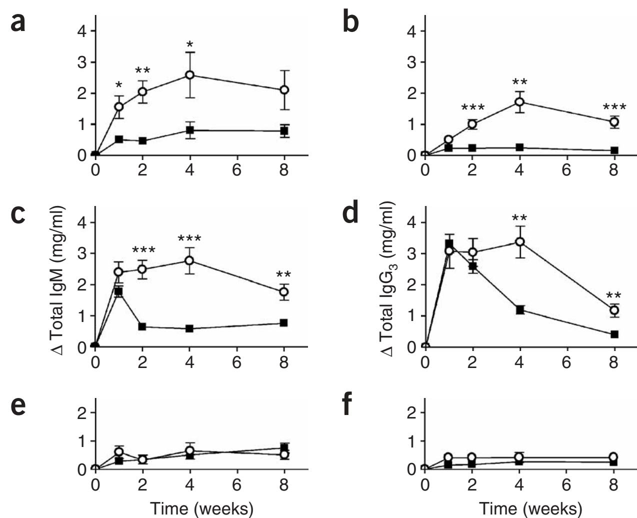

Figure 1 AOAH modulates antibody responses to Gram-negative bacterial infection. B6 mice were injected subcutaneously (**a**,**b**) or intraperitoneally (**c**,**d**) with 1.2×10^7 *N. meningitidis* bacteria or subcutaneously with PBS (**e**,**f**). Data represent the change in total serum IgM and IgG_3 titers after infection at time = 0 for *Aoah*$^{+/+}$ mice (filled squares) and *Aoah*$^{-/-}$ mice (open circles). Mean baseline values: *Aoah*$^{+/+}$, 0.68 mg/ml (IgM) and 0.17 mg/ml (IgG_3); *Aoah*$^{-/-}$, 0.74 mg/ml (IgM) and 0.17 mg/ml (IgG_3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 7$ mice/group. Error bars indicate 1 s.e.m. Representative of two experiments with B6 mice and one experiment with C3H/HeN mice (six to eight mice/group).

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Aoah$^{-/-}$ and *Aoah*$^{+/+}$ C57BL/6 (B6) mice before and after challenge with live *Neisseria meningitidis*. Although the baseline antibody concentrations were not different in the two kinds of mice, challenged *Aoah*$^{-/-}$ mice produced higher titers of immunoglobulin M (IgM) and IgG_3 antibodies than did the B6 control mice (**Fig. 1**). In contrast, serum concentrations of IgG_1 and IgG_2 before and after challenge were unaffected by AOAH deficiency (data not shown). IgM and IgG_3 antibody titers also remained elevated much longer in the *Aoah*$^{-/-}$ mice. Cultures of blood and spleen were sterile at 72 h after infection in both groups ($n = 6$), ruling out the possibility of persistent infection as the stimulus for antibody production.

Aoah$^{-/-}$ mice immunized subcutaneously with purified LPS from *N. meningitidis* also had notable antibody responses, with IgM and IgG_3 titers 5–10 times higher than those in *Aoah*$^{+/+}$ mice (**Fig. 2a,b**). Immunization with LPS or trinitrophenyl-conjugated LPS (TNP-LPS) also induced significantly higher titers of antibody to LPS (anti-LPS) or anti-TNP in *Aoah*$^{-/-}$ mice than in *Aoah*$^{+/+}$ mice ($P < 0.01$; data not shown). Consistent with the broad reactivity of the polyclonal response[17], immunized or infected *Aoah*$^{-/-}$ mice also developed increased serum autoantibodies to several nuclear antigens (**Fig. 2c**).

We also noted AOAH-dependent regulation of IgM and IgG_3 antibody production in two other mouse strains, 129 and C3H/HeN, as well as after subcutaneous injection of B6 mice with either *E. coli* O111 or *Salmonella typhimurium* LPS, which express a lipid A acylation pattern different from that of neisseria LPS[19] (data not shown). Immunization with LPS failed to induce increases in IgM or IgG_3 in mice that were deficient in both AOAH and TLR4, indicating that TLR4-dependent LPS signaling is required for the phenotype of *Aoah*$^{-/-}$ mice (**Fig. 3a**). AOAH-treated *N. meningitidis* LPS (**Fig. 3b**) and *E. coli* O14 dLPS (data not shown) did not stimulate polyclonal antibody production *in vivo*, confirming the ability of AOAH treatment to reduce innate antibody responses to LPS.

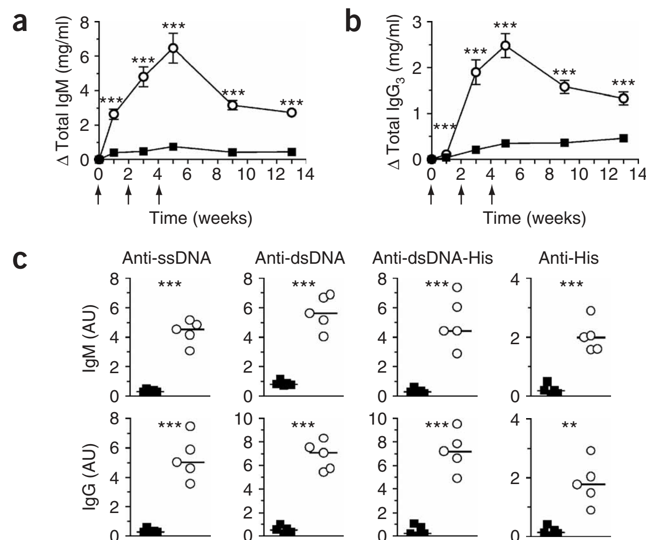

Figure 2 AOAH limits LPS-induced polyclonal antibody production. B6 *Aoah*$^{+/+}$ (filled squares) and *Aoah*$^{-/-}$ (open circles) mice received 10 μg *N. meningitidis* LPS subcutaneously and 5 μg again subcutaneously 2 and 4 weeks later (upward arrows). (**a**,**b**) Change from baseline in total IgM (**a**) and IgG_3 (**b**) titers. Mean baseline values: *Aoah*$^{+/+}$, 0.54 mg/ml (IgM) and 0.10 mg/ml (IgG_3); *Aoah*$^{-/-}$, 0.58 mg/ml (IgM) and 0.14 mg/ml (IgG_3). Error bars are ± s.e.m. ***, $P < 0.001$. Values were pooled from three experiments; $n = 15$–19 mice/group. (**c**) IgM and IgG titers to single-stranded DNA (Anti-ssDNA), double-stranded DNA (Anti-dsDNA), double-stranded DNA–histone complexes (Anti-dsDNA-His) and total histones (Anti-His), determined in sera obtained on day 35. AU, arbitrary units. **, $P < 0.01$; ***, $P < 0.001$. Horizontal lines indicate medians. *Aoah*$^{-/-}$ mice also had increased IgM and IgG_3 anti-nuclear antibody titers after challenge with *N. meningitidis* ($n = 7$; data not shown).

B cell responses *in vitro*

One potential explanation for the increased antibody responses in *Aoah*$^{-/-}$ mice is that *Aoah*$^{-/-}$ B cells are hyper-responsive to LPS. To address that possibility, we compared the ability of splenocytes from *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice to proliferate and produce IgM in response to LPS *in vitro*. Splenocytes from both strains responded similarly to

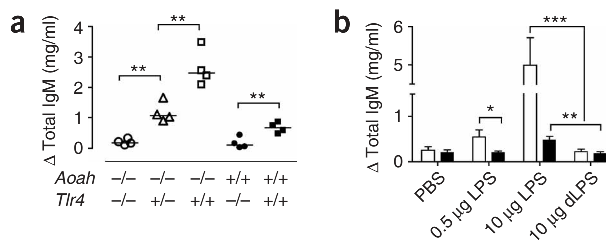

Figure 3 LPS-induced antibody synthesis requires TLR4 and intact LPS. (**a**) Serum IgM titers in mice immunized subcutaneously with 10 μg LPS; serum was obtained 7 d after immunization. Symbols represent individual mice (genotypes, below graph). **, $P < 0.01$. Horizontal bars indicate medians. (**b**) IgM responses of *Aoah*$^{+/+}$ mice (filled bars) and *Aoah*$^{-/-}$ mice (open bars) to immunization with 0.5 or 10 μg LPS, 10 μg AOAH-treated LPS (dLPS) or PBS (control). Mice were immunized on days 0 and 21 and antibody titers were measured on day 28. Error bars are +1 s.e.m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 6$–8 mice/group. Similar results were obtained in three experiments with *N. meningitidis* LPS and dLPS and in one experiment in which *E. coli* 014 LPS and dLPS were used ($n = 7$).

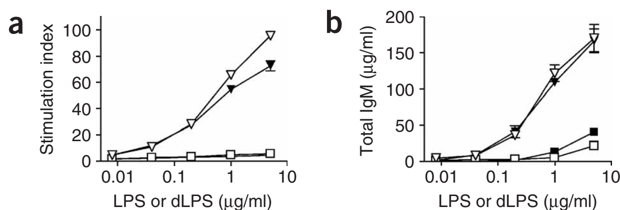

Figure 4 Differential *in vitro* responsiveness of B cells. (**a**) Proliferation assay. Stimulation index of splenocytes (3×10^5) from nonimmunized *Aoah*$^{+/+}$ or *Aoah*$^{-/-}$ B6 mice incubated with various concentrations (horizontal axis) of *N. meningitidis* LPS or dLPS for 24 h before the addition of [^{3}H]thymidine for an additional 16 h. (**b**) Enzyme-linked immunosorbent assay of IgM in cell-free media of splenocytes incubated with LPS or dLPS for 7 d. Triangles, LPS; squares, dLPS; open symbols, *Aoah*$^{-/-}$, filled symbols, *Aoah*$^{+/+}$. $n = 4$ wells/data point. Error bars are ± s.d. Similar results were obtained in four (**a**) or two (**b**) independent experiments.

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LPS stimulation, indicating that *Aoah*$^{-/-}$ cells are not intrinsically hyper-responsive to LPS (**Fig. 4**). In the same experiment we confirmed that treatment of LPS with AOAH significantly reduced its stimulatory capacity *in vitro*, consistent with its conversion to the less potent dLPS form[20] ($P < 0.001$; **Fig. 4**).

LPS deacylation *in vivo*

We reasoned that the increased antibody responses in *Aoah*$^{-/-}$ mice could be caused by the persistence of fully acylated LPS *in vivo*. To study the rate at which LPS undergoes deacylation after subcutaneous injection, we injected 5 μg of double-radiolabeled (^{3}H-labeled fatty acyl chains and ^{14}C-labeled glucosamine backbone) *S. typhimurium* LPS into each hind footpad of *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice and obtained tissues 3, 7, 14 and 41 d later. We measured ^{14}C radioactivity in tissue lysates to determine the amount of LPS in each tissue. We quantified LPS deacylation by measuring the d.p.m. of ^{3}H in the fatty acids recovered from LPS in the same lysates[5,6]. The amount of LPS at the site of injection decreased slowly; 7 d after injection, almost half of the LPS remained in the footpads (**Fig. 5a**). By day 3 after injection, approximately 65% of the LPS in the draining nodes of *Aoah*$^{+/+}$ mice had been converted to dLPS, and 85% was dLPS by day 7 (**Fig. 5b**); we noted a similar deacylation time course for the footpads (data not shown). In *Aoah*$^{-/-}$ mice, in contrast, the fatty acid composition of LPS remained unchanged in the footpads, draining lymph nodes (**Fig. 5b**) and liver for many days after administration. Although deacylation did not influence the rate at which LPS left the inoculation site or accumulated in the draining nodes, the *Aoah*$^{-/-}$ mice developed much larger draining nodes than did *Aoah*$^{+/+}$ mice (**Fig. 5c**), suggesting that the LPS remained stimulatory *in vivo* for several weeks.

B cell stimulation *in vivo*

The enlarged nodes of LPS-immunized *Aoah*$^{-/-}$ mice had significantly increased populations of B220$^+$ cells, including activated B cells (B220$^+$CD69$^+$ and B220$^+$CD86$^+$) and plasmablasts (B220$^+$CD138$^+$; **Fig. 5d**). Lymph nodes from *Aoah*$^{-/-}$ mice also contained more IgM-producing cells per 1×10^6 B cells (by enzyme-linked immunospot assay) on day 7 after immunization than did lymph nodes from *Aoah*$^{+/+}$ mice ($P < 0.02$; $n = 3$ independent experiments), whereas there was no difference between *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice in the fraction of bone marrow cells that produced IgM (data not shown). These data are consistent with the conclusion that by deacylating LPS, AOAH acts to dampen B cell proliferation, B cell activation and antibody secretion *in vivo*.

LPS deacylation can be saturated

If the absence of AOAH-dependent deacylation of LPS enabled LPS to stimulate B cells for longer periods of time *in vivo*, administering multiple doses of LPS to *Aoah*$^{+/+}$ mice might lead to enzyme saturation, less conversion of LPS to dLPS and, therefore, greater antibody production. To test that hypothesis, we injected 10-μg doses of *E. coli* O111 LPS into the footpads of *Aoah*$^{+/+}$ mice every 2–3 d and evaluated serum IgM and IgG_3 titers after the fifth injection. Repeated dosing with LPS resulted in increased concentrations that approximated those of *Aoah*$^{-/-}$ mice given a single injection on day 0 (**Fig. 6**). This result is consistent with inactivation of LPS via the saturable enzyme activity of AOAH.

LPS-induced adjuvant activity

In contrast to the prominent differences noted for polyclonal antibody production in *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice, the ability of LPS to enhance IgM and IgG_1 antibodies to two protein antigens, ovalbumin and BSA, was similar in the presence and absence of AOAH (**Fig. 7**). This result was unexpected, as published studies using bacterial lipid A acylation mutants have reported that removal of both secondary acyl chains

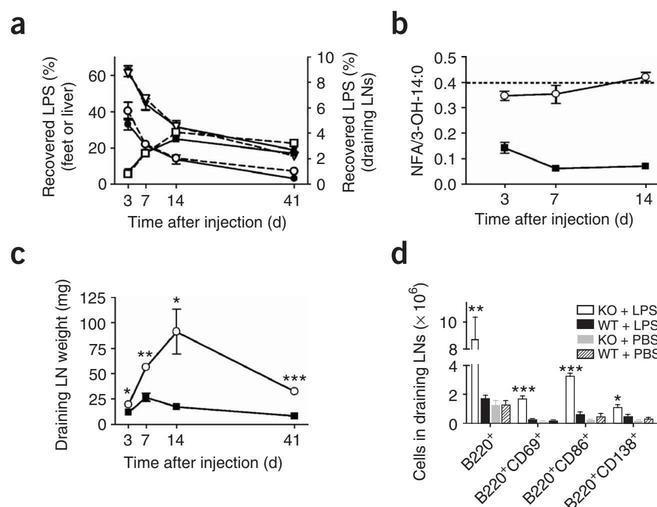

Figure 5 AOAH reduces LPS-induced B lymphocyte proliferation *in vivo*. (**a**) *In vivo* distribution of LPS after footpad inoculation. Data represent the fraction (%) of the injected ^{14}C d.p.m. recovered from the feet (triangles), draining lymph nodes (circles) and livers (squares) of *Aoah*$^{+/+}$ mice (filled symbols) and *Aoah*$^{-/-}$ mice (open symbols). Data for feet and liver (left axis) and lymph nodes (right axis) have different scales for their vertical axes. Less than 2% of the ^{14}C d.p.m. appeared in the spleen. (**b**) LPS deacylation in draining lymph node, estimated from the ratio of d.p.m. of ^{3}H-labeled nonhydroxylated fatty acids to d.p.m. of ^{3}H-labeled 3-OH-14:0 in the LPS isolated from each sample (NFA/3-OH-14:0). The ratio for the injected LPS was 0.4 (horizontal dashed line). *Aoah*$^{+/+}$, filled squares; *Aoah*$^{-/-}$, open circles. (**c**) Lymph node (LN) enlargement. Data represent the average of the combined weights of the inguinal and popliteal nodes from each mouse at each time point. For **a**–**c**, $n = 3$ mice at each time point. The experiment was repeated with similar results. (**d**) At 7 d after inoculation, the draining lymph nodes from *Aoah*$^{-/-}$ mice have increased numbers of B cells, including activated B cells (B220$^+$CD69$^+$ or B220$^+$CD86$^+$) and plasmablasts (B220$^+$CD138$^+$). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, *Aoah*$^{-/-}$ (KO) versus *Aoah*$^{+/+}$ (WT). Data represent means of three independent experiments; error bars are +1 s.e.m.

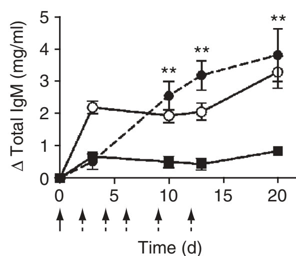

Figure 6 Multiple doses of LPS can mimic AOAH deficiency. *Aoah*$^{+/+}$ mice were immunized in one footpad either with one 10-μg dose of *E. coli* O111 LPS on day 0 (solid upward arrow) or with repeated doses (dashed upward arrows). A group of *Aoah*$^{-/-}$ mice received a single dose on day 0. Total IgM antibody was measured. Open circles, *Aoah*$^{-/-}$; filled squares, *Aoah*$^{+/+}$, single dose; filled circles, *Aoah*$^{+/+}$, multiple doses. $n = 5$ mice/group. **, $P < 0.01$, *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ multiple doses versus *Aoah*$^{+/+}$ single dose. Error bars are ± 1 s.e.m.

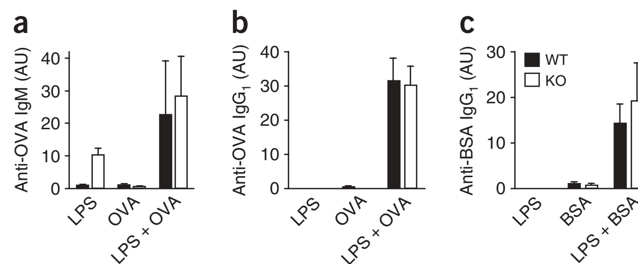

Figure 7 AOAH does not diminish LPS adjuvanticity. (**a**,**b**) Titers of IgM (**a**) and IgG_1 (**b**) in response to ovalbumin (OVA) after immunization of B6 *Aoah*$^{+/+}$ mice (filled bars) or *Aoah*$^{-/-}$ mice (open bars; $n = 6$) with 5 μg LPS or 40 μg ovalbumin or both. The injections were repeated on day 14 and serum was obtained on day 21. In **a**, IgM antibodies to OVA were induced during the polyclonal response to LPS ($P < 0.01$). (**c**) IgG_1 titers in response to BSA on day 35 in mice ($n = 7$) that received 5 μg LPS, 50 μg BSA or both on day 0 and were 'boosted' with 50 μg BSA on day 28. Error bars are +1 s.e.m.

reduces LPS adjuvant activity[21]. LPS adjuvanticity is thought to involve LPS-induced production of costimulatory molecules and cytokines by 'accessory' cells, however, and we found that LPS-stimulated *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ marrow-derived dendritic cells had equivalent cell surface expression of costimulatory molecules (CD40 and CD86) and secretion of cytokines (interleukins 2 and 6 and tumor necrosis factor) over a 24-hour period *in vitro* (data not shown). Before LPS is inactivated by deacylation *in vivo*, it thus may induce mediators that promote T cell–dependent antibody responses to protein antigens.

DISCUSSION

Mechanisms proposed for reducing the stimulatory potency of bacterial LPS in tissues have included natural antibodies, neutrophil-derived cationic peptides (such as CAP37 and bactericidal permeability-increasing protein), lactoferrin and alkaline phosphatase[22]. In addition, it has been assumed that animals exposed to LPS become tolerized, because subsequent exposure to LPS does not stimulate a robust response similar to that seen in the primary exposure[23] and there are several negative regulators of TLR4 signaling[24]. Our data now indicate that an additional mechanism for inactivating LPS *in vivo* involves the host lipase AOAH.

LPS inactivation through deacylation is the first defined *in vivo* function to our knowledge for AOAH, an unusual heterodimer in which a 50-kilodalton subunit, a GDSL-motif lipase[25], is disulfide-linked to an approximately 14-kilodalton member of the sphingolipid activator protein (saposin) family[26]. An AOAH-like sequence has been found in the genomes of all vertebrates sequenced so far, except fish. In the most thoroughly studied animals, mice and humans, the enzyme is produced mainly by monocyte-macrophages, dendritic cells, neutrophils and renal cortical epithelial cells[4,6,27]. AOAH mRNA abundance and enzyme activity increase in response to LPS stimulation both *in vitro* and *in vivo*[6,28]. Although it is possible that B cells internalize and deacylate LPS *in vivo*[29], mouse B cells produced very little if any AOAH (data not shown). Moreover, the time course of deacylation in the footpad and draining lymph nodes suggests that most of a subcutaneous LPS inoculum undergoes deacylation at the inoculation site; over the entire time course, as much as 80% of the total LPS injected into the footpads was deacylated before it reached the draining lymph nodes. It seems likely that deacylation is accomplished by dendritic cells, macrophages and/or neutrophils, although it is possible that the enzyme acts in these cells or extracellularly[30] or both.

In the absence of AOAH, total IgM and IgG_3 responses to *N. meningitidis* infection were enhanced and prolonged. *N. meningitidis* produces several TLR agonists that can stimulate B cell proliferation *in vitro*, including LPS, peptidoglycan, CpG and outer membrane porins[31]. Although our findings suggest that LPS is the most potent of these ligands, it is also possible that LPS acts synergistically to enhance the potency of one or more of the other agonists[31,32]. The existence of an effective enzymatic mechanism for inactivating LPS raises the possibility that similar mechanisms (DNAses, proteases and muramidases) limit TLR-dependent responses to other microbial ligands.

The LPS concentrations needed to stimulate B cell proliferation *in vitro* are greater than those required to stimulate cytokine production by macrophages and dendritic cells. It is thus possible that as LPS is deacylated *in vivo*, the LPS concentration in draining lymph nodes falls below the threshold needed to stimulate polyclonal B cell activation, whereas LPS concentrations sufficient to activate dendritic cells and macrophages persist much longer. This phenomenon could account for the ability of AOAH to reduce T cell–independent antibody production (which requires direct stimulation of B cells by LPS) but not T cell–dependent antibody synthesis (in which LPS induces expression of costimulatory molecules by macrophages and dendritic cells). In addition, because LPS deacylation occurs over several days *in vivo*, there may be sufficient time for LPS to induce dendritic cells and macrophages to produce the mediators that promote T cell–dependent antibody responses to protein antigens.

Vertebrates restrain their antibody responses to specific antigens in several ways, including antigen elimination or sequestration[33], epitope masking[34] and feedback inhibition via FcγIIB receptors[35,36]. Our results indicate that T cell–independent polyclonal antibody responses to microbial molecules are also controlled *in vivo*. In the absence of AOAH, LPS-induced polyclonal antibody production is not effectively suppressed by antibody feedback (epitope masking). Our findings do not suggest how restraining the polyclonal antibody response might confer evolutionary advantage, although the time course we noted for LPS deacylation coincides with the transition from innate to acquired immunity[37,38]. In any case, limiting B cell activation and antibody, especially autoantibody, production in response to T cell–independent type 1 antigens poses a special situation, because too-rapid disposal or inactivation of TLR ligands might prevent animals from mobilizing many elements of the innate immune response[39]. Our findings suggest that, at least in those animals for whom LPS is a B cell mitogen, an

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enzyme that takes a few days to deacylate LPS could be an important solution to this problem.

Finally, mice unable to deacylate LPS experienced swollen lymph nodes for several weeks, raising the possibility that abnormalities in AOAH expression might contribute to persistent inflammation in other settings. Much observational and experimental evidence points to possible links between chronic LPS exposure and inflammatory diseases such as atherosclerosis[40], asthma[41,42] and alcoholic steatohepatitis[43,44]. Our results suggest possible involvement of LPS deacylation in the pathogenesis of these and other chronic inflammatory conditions.

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METHODS

LPS and deacylated LPS. *N. meningitidis* LPS was purified from a group B (L3,7,9) strain. For the preparation of dLPS, 400 μg of nonradiolabeled LPS were mixed with trace amounts of LPS containing ^{14}C-labeled fatty acids[45] in reaction buffer (30 mM sodium acetate, pH 5.5, 0.1% Triton X-100, 0.2 mg/ml of fatty acid–depleted BSA (Sigma) and 0.9% NaCl). The LPS was incubated at 37 °C with 1 μg of recombinant human AOAH until about 30% of the ^{14}C-labeled fatty acids were released. 'Mock-treated' LPS was incubated without AOAH. The extent of deacylation was confirmed by thin-layer chromatography and autoradiography of the ^{14}C-labeled fatty acids that remained attached to the LPS[6]. The dLPS and mock-treated LPS were extracted once as described[46] to remove traces of AOAH and BSA. Double-labeled Rc *S. typhimurium* LPS (^{3}H-labeled fatty acyl chains and ^{14}C-labeled glucosamine backbone) was prepared as described[47]; 1 μg had about 150,000 d.p.m. ^{3}H and about 10,000 d.p.m. ^{14}C.

Mutant mice. Targeted disruption of mouse *Aoah* was accomplished by replacement of most of the first *Aoah* exon with a neomycin-resistance gene[6]. The mutation was 'bred' to both the B6 and C3H/HeN genetic backgrounds; *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice from the eighth backcross were kept in a specific pathogen–free barrier facility. To obtain mice that lacked both AOAH and TLR4, B6 *Aoah*$^{-/-}$ mice were bred with C57BL/10ScN mice (provided by J. Thomas, The University of Texas Southwestern Medical Center, Dallas, Texas), which have a disabling deletion in *Tlr4*. *Tlr4* genotyping was done by amplification of DNA from tail tissue using primers that have been described[48]; *Aoah* was detected by assay of AOAH activity in urine and by DNA amplification with the primers 5′-GGAAGGGACTGGCTGCTATTGGGC GAA-3′ and 5′-TCTTCAGCAATATCACGGGTAGCCAACGCT-3′, which produce a 431–base pair product that spans the downstream neomycin-resistance gene–*Aoah* exon 1 insertion junction (R.L. Kitchens, The University of Texas Southwestern Medical Center, Dallas, Texas). All mouse protocols were approved by the Animal Research Care and Utilization Committee of The University of Texas Southwestern Medical Center (Dallas, Texas).

Immunization. *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice 4–10 weeks of age (five to seven mice per group) were injected by the intraperitoneal or subcutaneous route with group B meningococci in the logarithmic growth phase (1.2×10^7 colony-forming units in 200 μl saline). In other experiments, mice were injected subcutaneously on the dorsum of the neck with 10 μg of either *N. meningitidis* or *E. coli* O111 LPS; 5 μg was injected by the same route 2 and 4 weeks later. In some experiments, the draining lymph nodes (axillary and anterior cervical) were removed for analysis.

For assay of the adjuvant activity of LPS, groups of mice were injected subcutaneously with 5 μg LPS or 50 μg ovalbumin (grade V; Sigma) or with 5 μg LPS mixed with 50 μg ovalbumin. Then, 2 weeks later, mice received a second injection and blood was obtained 7 d after the boost. In a separate experiment, 50 μg endotoxin-free BSA (Sigma) was injected instead of ovalbumin; a second dose of BSA was given on day 28 and blood was obtained 7 d later.

Enzyme-linked immunosorbent assay. Standard methods were used for enzyme-linked immunosorbent assays[49]. For assay of antibody concentrations, microtiter wells were coated with goat anti-mouse polyvalent immunoglobulins (IgG, IgM and IgA) from Sigma. For measurement of antibodies to LPS or TNP-LPS, wells were coated with 10 μg/ml of *N. meningitidis* LPS or 25 μg/ml of TNP-BSA (Southern Biotechnology Associates). For measurement of antibodies to BSA or ovalbumin, wells were coated with 50 μg/ml of either antigen. Detection antibodies were horseradish peroxidase–conjugated goat anti-mouse IgM or IgG (Sigma) and horseradish peroxidase–conjugated goat anti-mouse IgG_3 or IgG_1 (Southern Biotechnology Associates). Horseradish peroxidase substrate (BD-PharMingen) was used. Plates were 'read' on a MRX Microplate Reader (Dynex Technologies Incorporation). IgM and IgG standards were provided by E. Vitetta (The University of Texas Southwestern Medical Center, Dallas, Texas). In all cases, differences between after and before immunization are reported.

Measurement of anti–single-stranded DNA, anti–double-stranded DNA, anti-histone and anti–histone-DNA were done as described[50]; double-stranded calf thymus DNA was obtained from Sigma and total histone was from Boehringer Mannheim. Samples from experimental mice were compared with an antiserum prepared from mice hyperimmunized with LPS as a standard (arbitrary units).

Quantification of radiolabeled LPS *in vivo*. To monitor the *in vivo* location and deacylation of LPS over time, we inoculated the footpads of mice with Rc *S. typhimurium* PR122 LPS[47] (discussed above). Mice were killed at 3, 7, 14 and 41 d. Feet were solubilized by immersion in Solvable (Packard Instruments). Aliquots of the other tissues were lysed (0.1% Triton-X-100 in PBS) and aliquots were suspended in Budget-Solve (Research Products International) for β-scintillation counting (Packard Instrument). Tissues were subjected to Bligh-Dyer extraction, LPS was recovered from the chloroform-methanol interface and fatty acyl chains were hydrolyzed as described[5,6]. The nonhydroxylated fatty acids (myristate and laurate) and 3-OH-14:0 (3-hydroxymyristate) were separated by thin-layer chromatography, and bands corresponding to ^{14}C-labeled fatty acid standards (run in adjacent lanes and detected by autoradiography) were scraped and then suspended in Budget-Solve for β-scintillation counting. The percent deacylation (loss of secondary acyl chains) was calculated as described[6]. The amount of radioactivity remaining in the nodes at day 41 was insufficient to allow accurate quantification of the extent of deacylation.

Splenocyte mitogenicity assay. Splenocyte proliferation was measured as described[20]. The stimulation index is the ratio of the [^{3}H]thymidine incorporated into stimulated versus unstimulated (control) cells.

Analysis of cell populations in lymph nodes. Popliteal and inguinal nodes from individual mice were combined. Single-cell suspensions were washed with staining medium (Hank's balanced salt solution, 2% BSA and 10 mM HEPES, pH 7.4). For blockade of Fc receptors, cells were incubated on ice for 45 min with 0.5 mg/ml of normal mouse IgG (Caltag Labs). Anti–CD45R and B220–peridinine chlorophyll protein (RA3-6B2), anti-CD69–phycoerythrin (HI.2F3), anti-CD86–phycoerythrin (GLI), and anti-CD138 (Syndecan-1)–phycoerythrin (281-2), all from BD-PharMingen, were diluted 1:100 before use. Samples were processed on a FACScalibur (Becton Dickinson), and the data were collected and evaluated with Cell Quest (Becton Dickinson) software.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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