Stimulus-dependent Deacylation of Bacterial Lipopolysaccharide by Dendritic Cells

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Abstract

We describe here a previously unrecognized property of dendritic cells (DCs), the ability to deacylate the lipid A moiety of gram-negative bacterial LPSs. Both immature DCs of the XS52 cell line and bone marrow–derived DCs produce acyloxyacyl hydrolase, an enzyme that detoxifies LPS by selectively removing the secondary acyl chains from lipid A. Acyloxyacyl hydrolase expression decreased when DCs were incubated with IL-4, IL-1β, TNFα, and an agonistic CD40 antibody (maturation cocktail), and increased after treatment with LPS, CpG oligodeoxynucleotides, or a gram-positive bacterium (Micococcus luteus). Maturation cocktail treatment also diminished, whereas LPS treatment enhanced or maintained the cells’ ability to kill Escherichia coli, deacylate LPS, and degrade bacterial protein. Enzymatic deacylation of LPS is an intrinsic, regulated mechanism by which DCs may modulate host responses to this potent bacterial agonist.

Key words: lipopolysaccharide • dendritic cell • acyloxyacyl hydrolase • gram-negative bacteria • deacylation

Introduction

Dendritic cells (DCs),* the most potent antigen-presenting cells, initiate and modulate both innate and adaptive immune responses. Immature DCs reside in nonlymphoid tissues, such as the skin, where they are poised to capture and process microbial invaders. After an encounter with bacteria or bacterial components, immature DCs migrate via lymphatic channels to the T cell areas of regional lymph nodes, where they mature, losing their antigen-capturing and -processing ability and becoming specialized for presenting antigens to T cells (1–3). DC maturation can be induced in vitro and in vivo by various stimuli, such as inflammatory cytokines (TNFα and IL-1β), CD40 ligand, and several conserved microbial molecules (LPS, peptidoglycans, bacterial lipoproteins, DNA that contains unmethylated CpG motifs, and viral double-stranded RNA; references 2, 4). Different agonists may drive distinct maturation events and act synergistically to induce the maturation of DCs (5–7).

In addition to inducing adaptive immune responses to microbial antigens, DCs also contribute to innate immunity by ingesting and killing microbes and by secreting mediators that recruit macrophages, natural killer cells, and eosinophils to sites of infection (2, 4). However, little is known about how immature DCs help control bacterial infection and/or prevent harmful host responses to bacteria or bacterial components. In the studies described here, we show that these cells are able to degrade the most potent agonist contained in the gram-negative bacterial cell wall, the lipid A moiety of LPS.

Lipid A, the conserved bioactive center of LPS, has a glucosamine disaccharide backbone. In enterobacterial lipid A, four molecules of 3-hydroxytetradecanoate (3-OH-14:0) attach directly to this backbone. The hydroxyl groups of two or three of the 3-OH-14:0 residues are substituted with secondary acyl chains (laurate and myristate) to form acyloxyacyl groups (Fig. 1 A). Animals have an enzyme, acyloxyacyl hydrolase (AOAH), that removes secondary fatty acyl chains from the lipid A regions of diverse LPSs and greatly reduces the molecules’ ability to elicit toxic responses in vivo (8). Moreover, in vitro studies using human monocytes, endothelial cells, and neutrophils have shown that the partially deacylated LPS produced by AOAH can be an LPS antagonist (9–11). Mice that are unable to produce AOAH due to targeted gene disruption have exaggerated antibody responses to LPS (unpublished results).

Although AOAH had been detected previously only in neutrophils and monocyte macrophages, we found that im-
mature DCs also produce the enzyme. We studied the ability of DCs to alter their expression of AOAH, and their ability to deacylate LPS in ingested *Escherichia coli* in response to host (inflammatory cytokines and CD40 ligand) or bacterial (LPS, *E. coli*, *Micrococcus luteus* [peptidoglycan], and CpG oligonucleotides) stimuli. The results indicate that DCs coordinate AOAH expression and LPS deacylation with many other antibacterial responses, increasing or decreasing their ability to process this important bacterial molecule in response to environmental cues.

**Materials and Methods**

**Materials.** Unless otherwise indicated, reagents were obtained from Sigma-Aldrich. Phosphorothioate-modified CpG (5'-TCCATGACGTTCCTGATGCT-3') and GpC (5'-TCC-ATGACGTTCCTGATGCT-3') oligodeoxynucleotides (ODNs) were obtained from Invitrogen.

**AOAH**<sup>−/−</sup> *Mice.* Targeted disruption of the murine AOAH gene was accomplished in embryonic stem cells from 129S6/SvEvTac mice by inserting a neomycin resistance gene in the first exon of the gene. Memory T cells were cultured in XS medium containing 50 ng/ml LPS on days 3 and 6. To study the impact of living *E. coli* on DC maturation, 10<sup>9</sup> CFU/ml of an overnight culture of *E. coli* O9 was added to XS22 cells (bacteria/cell ratio = 1:5) in XS medium without antibiotics and incubated at 37°C for 1 h. The cells were washed with cRPMI medium without antibiotics, cultured in XS medium that contained 50 μg/ml gentamicin, and were harvested at 24 h. When incubation was continued for 9 d, this procedure was repeated on days 3 and 6, and the cells were harvested on day 9.

**BMDCs and Peritoneal Macrophages.** DCs were generated from bone marrow as described by Inaba et al. (15) with minor modifications. Marrow was flushed out of the femurs and tibias. Red blood cells were lysed using RBC lysis buffer and cells were cultured in 6-well plates at 6 x 10<sup>5</sup> cells per well in 3 ml cRPMI medium supplemented with 10 ng/ml rmGM-CSF. On day 3 of culture, nonadherent cells were removed, and 4 ml of fresh medium containing rmGM-CSF were added. On day 7, nonadherent CD11c<sup>+</sup> cells were purified using anti-CD11c monoclonal antibody N418 coupled to magnetic microbeads (Miltenyi Biotec). Fc receptors were blocked with 0.5 mg/ml of normal mouse IgG (Caltag Laboratories) before the cells were incubated with the beads. Flow cytometric analysis (FACScan<sup>™</sup>, Becton Dickinson) showed that >95% of the sorted cells were CD11c<sup>+</sup>. These purified CD11c<sup>+</sup> cells had DC morphology (veiled and dendritic processes) when viewed using light microscopy. Thioglycollate-elicited peritoneal macrophages were prepared as described previously (16). All cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and air.

**Induction of DC Differentiation.** We followed the method of Yamada and Katz (17). In brief, XS22 cells were cultured in cRPMI medium supplemented with rmGM-CSF and IL-4 (10 ng/ml each) for 6 d; the medium was replaced with fresh medium containing rmGM-CSF and IL-4 on day 3. The cells were cultured for three additional days in the presence of GM-CSF, IL-4, TNF-α (10 ng/ml each, all from R&D Systems), 10 ng/ml IL-1β (BD Biosciences), and 4 μg/ml anti-CD40 (clone 1C40; R&D Systems). To study the impact of LPS on XS52 cell maturation, 10 ng/ml of *E. coli* O9 LPS (purified using hot phenol-water extraction from *E. coli*) was added to the cells in XS medium. Cells were either harvested at 24 h or they were incubated for 9 d; the medium was replaced with fresh XS52 medium containing 10 ng/ml LPS on days 3 and 6. To study the impact of living *E. coli* on DC maturation, 10<sup>9</sup> CFU/ml of an overnight culture of *E. coli* O9 was added to XS52 cells (bacteria/cell ratio = 1:5) in XS medium without antibiotics and incubated at 37°C for 1 h. The cells were washed with cRPMI medium without antibiotics, cultured in XS medium that contained 50 μg/ml gentamicin, and were harvested at 24 h. When incubation was continued for 9 d, this procedure was repeated on days 3 and 6, and the cells were harvested on day 9.

**Decaylating Purified LPS.** Double-labeled LPS ([<sup>14</sup>C]-glucosamine backbone and <sup>3</sup>H]-fatty acyl chains; reference 18) was incubated with cell lysates for 16 h at 37°C in AOAH reaction mixture (1 mg/ml of fatty acid–free bovine serum albumin, 5 mM CaCl<sub>2</sub>, 0.05% Triton X-100, and 20 mM Tris-citrate, pH 5). The reaction was terminated by adding 2.5 volumes of 100% ethanol. After centrifugation to precipitate intact LPS and partially deacylated LPS, the <sup>14</sup>C-fatty acids in the supernatant were quantitated by β-scintillation counting (Packard Instrument Co.). Confirmation that the deacylating activity was AOAH-like (releasing only 12:0 and 14:0 from the LPS) was obtained using TLC (19).

**Decaylation of LPS in *E. coli.* *E. coli* LCD25 is unable to produce its own acetate or use acetate as carbon or energy source (20). When LCD25 cells are cultured in minimal medium with sodium 2-[<sup>14</sup>C]acetate (NEN Life Science Products), <sup>14</sup>C is exclusively incorporated into fatty acyl chains. The method of Katz et al. (21) was followed to <sup>14</sup>C-label fatty acyl chains in LCD25, yielding ~20,000 dpm/10<sup>6</sup> CFU. <sup>14</sup>C-Labeled LCD25 bacteria were added to XS52 cells (cell/bacteria ratio = 1:50) in XS medium without antibiotics and incubated for 1 h at 37°C. The cells were washed and incubated for 6 or 24 h in XS medium with 50 μg/ml gentamicin to kill extracellular bacteria. For BMDCs, nonadherent cells were harvested on day 7 of culture, mixed with <sup>14</sup>C-labeled LCD25 (cell/bacteria ratio = 1:50) in cRPMI medium without antibiotics, and incubated at 37°C for 1 h. The CD11c<sup>+</sup> cells were purified by magnetic microbeads and the CD11c<sup>−</sup> cells and unbound bacteria were washed away. The
sorted CD11c+ cells were cultured in cRPMI supplemented with 10 ng/ml mGM-CSF and 50 μg/ml gentamicin for 6 or 24 h. The cells and culture media were pooled to measure LPS deacetylation. At the 0-h time point, samples were harvested immediately after 14C-labeled LCD25 bacteria were added to DCs.

LPS deacetylation was quantitated by calculating the loss of the fatty acyl chains from LPS (21). In brief, the interphase of a Bligh-Dyer extraction (22), which contains LPS and partially deacetylated LPS, was washed three times with chloroform and dried under argon. The interphase was hydrolyzed with 4 M HCl and 4 M NaOH (16). The hydrolyzed interphase was extracted again and the chloroform phase, which contains released fatty acids, was recovered, dried, and resuspended in 100 μl methanol/chloroform 1:1 (vol/vol). The interphase-derived fatty acids were resolved by TLC. First, the primary (3-OH-14:0) and secondary (12:0 and 14:0) fatty acids were separated using TLC system 1 (silica gel G, petroleum ether/diethyl ether/acetic acid 70:30:1). The two bands containing 3-OH-14:0 and the nonhydroxylated fatty acids were visualized by phosphorimager (Molecular Dynamics). The 3-OH-14:0 band was scraped and the radioactivity was quantitated by β-scintillation counting. The nonhydroxylated fatty acids were extracted from the silica gel and resolved on a reverse-phase KC18 plate, using acetic acid/acetonitrile (3:7) as the solvent (TLC, system 2); the plates were developed, dried, and developed again to get a better separation of fatty acids. The bands corresponding to each species of nonhydroxylated fatty acid (12:0, 14:0, and 16:0) were scraped and quantitated. The disintegrations per minute recovered from each band were normalized to the total disintegrations per minute recovered from each sample was assayed in duplicate.

At the end of the maturation experiment, there were more cells in the cytokine-treated wells, and fewer in the LPS-treated wells, than in the untreated wells. The cell mass was estimated by measuring the amount of cell protein in each well.

Bacterial Protein Degradation. To label bacterial proteins, LCD25 cells were cultured in minimal medium plus 1 mM sodium acetate with 0.04 μM 3H-arginine (NEN Life Science Products) and 10 μM of nonradiolabeled t-arginine, yielding ~3,500 3H dpm/106 bacterial CFUs. 3H-Arginine–labeled LCD25 were added to XS52 cells (cell/bacteria ratio = 1:50). Incubation, washing, and harvesting were performed as described in the LPS deacetylation protocol described in previous paragraphs. The 0-h time point samples were harvested immediately after the 3H-arginine–labeled LCD25 bacteria were added to DCs. 0.5 ml of each sample was mixed with an equal volume of 20% TCA, incubated at 4°C for 20 min, and centrifuged. The radioactivities in the supernatant and pellet were counted. The fraction of the counts that was TCA-insoluble at 0 h was considered to represent 100%, and the values at later time points were converted to percentages by comparing them to the t = 0-h value. Each experimental condition was assayed in duplicate.

Flow Cytometry. XS52 cells were washed with PBS and harvested with PBS containing 2 mM EDTA. BMDCs were purified by magnetic cell sorting. After blocking Fe binding with 0.5 mg/ml of normal mouse IgG, cells were incubated with anti-CD14-PE (mCS-3), anti-CD40-PE (3.23), anti-CD86-PE (GL1), anti-CD11c-FITC (HL3), and corresponding isotype controls. These antibodies were all purchased from BD Biosciences and used at 5 μg/ml. The samples were analyzed by FACScan®, gating on cells that excluded propidium iodide. Data were analyzed using CELLQuest™ (Becton Dickinson) software.

Phagocytosis. 106 DCs were washed with PBS and suspended in 0.2 ml cRPMI medium. 0.05 ml of 106 bacteria/ml Bodipy–E. coli (Molecular Probes) was added to the cells, which were incubated at 37°C in the dark for 1 h before they were chilled on ice to stop phagocytosis. To quench extracellular fluorescence, 0.25 ml of 0.2% trypan blue in PBS was added before analysis by flow cytometry. Control cells were either pretreated with 10 μM cytochalasin D for 0.5 h before adding Bodipy–E. coli.

Bactericidal Activity. LCD25 bacteria, labeled with 3H-arginine as described in a previous paragraph (Bacterial Protein Degradation), were added to XS52 cells in X medium without antibiotics. Bacteria were added at a cell/bacteria ratio of 1:50. After incubation at 37°C for 0.5 h, the cells were washed and incubated for 0.5 or 2 h in X medium without antibiotics. Cells and media were harvested together and cells were lysed by adding Triton X-100 (final concentration = 0.2%). Samples were serially diluted in PBS, and 100 μl of several dilutions were spread on LB agar plates. After overnight incubation at 37°C, the colonies were counted and the total number of CFUs recovered from each well was calculated. The total number of bacteria associated with cells after the wash was determined by measuring the cell-associated 3H dpm at this time and dividing by the 3H dpm/CFU.

mRNA Analysis. Total RNA was isolated from untreated or treated XS52 cells (RNAqueous Kit; Ambion). A region of the AOAH cDNA was amplified using primers Seq_mAOAH-ex12F (5’- CCAACTCCTGTGGAACGTGATTTT-3’) and Seq_mAOAH-ex12R (5’-TCTCAACAGTGGTAAATGGATTTT-3’). The probe (TagMan® MGB, FAM™ dye-labeled) 5’-ACGAGTGAATTTGGAAG-3’ and primers were designed and synthesized by Applied Biosystems. Plasmid PMF612, which contains murine AOAH cDNA, was used as a reference molecule for the standard curve calculation. TagMan® rodent GAPDH control reagents were used to measure GAPDH gene expression. All real-time PCR reactions were performed in a 25-μl mixture with TaqMan® one-step RT-PCR Master Mix Reagents Kit on a sequence detection system (model ABI PRISM® 7700).

Results

Immature (XS52) DCs and BMDCs Express AOAH. We first measured LPS-deacetylating activity in lysates of XS52 cells, immature BMDCs, a mature DC line, XS106, and several murine macrophage lines. Both XS52 cells and BMDCs had LPS-deacetylating activity similar to that of murine peritoneal macrophages and greater than that of the other cell lines tested (Fig 1 B). Mature DCs (XS106) expressed much lower activity than immature DCs (XS52).

XS52 Cells and BMDCs Deacetylate the LPS in Whole Bacteria in an AOAH-like Manner. In the assay described in the previous paragraph, we used purified LPS as the substrate and measured its deacetylation by cell lysates in the presence of detergent. Next, we studied the ability of DCs to deacetylate LPS in its natural setting, the outer membrane of gram-negative bacteria. Because the fatty acids cleaved from LPS, and other bacterial lipids can be degraded by host cells and/
or incorporated into cellular lipids, we measured the disappearance of individual fatty acids from the LPS backbone. We found that both XS52 cells and BMDCs deacylated the LPS in whole bacteria in an AOAH-like manner (Fig. 2, A and B). 3-OH-14:0, the primary fatty acyl chain of lipid A, was not removed from the LPS backbone, whereas the nonhydroxylated (secondary) fatty acids (12:0 and 14:0) were cleaved over time. Confirmation that AOAH is the deacylating enzyme came from a comparison of BMDCs from AOAH wild-type (+/+/+) and AOAH null (−/−/−) 129 mice. BMDCs from AOAH−/− mice deacylated significantly less LPS than their wild-type counterparts (Fig. 2, B and C); the apparent removal of 20–30% of the 12:0 by AOAH−/− BMDCs is unexplained because they were unable to deacylate purified LPS (unpublished data), and peritoneal macrophages from AOAH−/− mice did not deacylate the LPS in E. coli (Fig. 2, D and E).

**Figure 1.** LPS deacylation by cell lysates. (A) Structure of the lipid A moiety of E. coli LPS. AOAH (arrows) cleaves the secondary fatty acyl chains (laurate, 12:0; myristate, 14:0) attached in ester (acyloxyacyl) linkage to the glucosamine-linked 3-hydroxymyristoyl residues. (B) LPS deacylation by cell lysates. Lysates of cultured cells were assayed for their ability to deacylate purified 3H/14C-LPS as described in Materials and Methods. Each bar shows the mean of three or more independent experiments. Error bars represent 1 SEM. Asterisks, significantly different from XS52 cells: **, P < 0.01; ***, P < 0.001 (Student’s t test).

**Figure 2.** Deacylation of LPS in E. coli. (A) Deacylation of LPS in E. coli by XS52 cells. (B and C) Deacylation of LPS in E. coli by BMDCs from AOAH+/+ (B) and AOAH−/− (C) mice. (D and E) Deacylation of LPS in E. coli by thioglycollate-elicited peritoneal macrophages from AOAH+/+ (D) and AOAH−/− (E) mice. The data in each panel are representative of two or more experiments with similar results. FA, fatty acid.

**Regulation of AOAH Activity in XS52 Cells.** In vitro, DCs can be induced to mature by inflammatory cytokines, by CD40 ligand, or by microbes or microbial molecules such as LPS and peptidoglycan. Following the protocol of Yamada and Katz (17), we treated XS52 cells with 10 ng/ml IL-4 for 6 d and for an additional 3 d with a maturation cocktail that included IL-4, TNFα, IL-1β, and an agonistic CD40 antibody. To assess the maturation state of the cells, we measured cell surface markers by using flow cytometry. Increased surface expression of CD40, a costimulatory molecule, was used to reflect maturation (17). The LPS binding receptor, CD14, which is constitutively expressed on the XS52 cell surface, was shown to be down-regulated on XS52 cells treated with IL-4 and maturation cocktail. We also measured the phagocytic activity of untreated and treated cells. Immature DCs, which are highly phagocytic, lose this capability when they mature (5, 23).

After treatment with IL-4 for 6 d and the cytokine cocktail for three more days, CD40 expression on XS52 cells had increased a little (Fig. 3 A), whereas CD14 expression (Fig. 3 A) and phagocytic activity (Fig. 3 B) had decreased. These changes were accompanied by a sixfold decrease in AOAH activity (Fig. 3 C).

In the same experiments, we asked if exposing the cells to LPS or gram-negative bacteria would alter their ability to phagocytose or to express AOAH. After XS52 cells had been incubated with LPS or whole gram-negative bacteria for 9 d, their surface expression of CD40 increased, whereas CD14 expression, phagocytic activity, and AOAH activity were maintained or slightly increased (Fig. 3, A–C).

Prolonged incubation in the presence of LPS or E. coli was associated with some loss of XS52 cell viability. When
we treated XS52 with LPS or *E. coli* for 24 h, with no loss of cell numbers, the results were similar: maintenance of CD14 expression, phagocytic ability, and AOAH activity were accompanied by increased expression of CD40 (unpublished data).

To investigate the basis for the changes in AOAH activity, we measured AOAH mRNA abundance in XS52 cells using real-time PCR. As shown in Fig. 3 D, AOAH mRNA abundance decreased eightfold after treatment with the maturation cocktail and increased twofold after treatment with LPS.

Maturation of XS52 cells, when induced by proinflammatory cytokines and an agonistic CD40 antibody, was thus associated with decreases in CD14 expression, phagocytic activity, and AOAH activity, whereas CD40 expression was enhanced. Although treatment with LPS or gram-negative bacteria was also followed by increased expression of the costimulatory molecule, CD40, the cells maintained their ability to recognize LPS, internalize bacteria, and deacetylate LPS.

**Regulation of AOAH Activity in BMDCs.** We treated BMDCs with IL-4 and the same maturation cocktail for 2 d. CD40 and CD86 expression increased (Fig. 4 A), whereas phagocytic activity decreased (Fig. 4 B). Immature BMDCs expressed very little CD14, and treatment with maturation cocktail did not change its expression (unpublished data). Cytokine-induced maturation decreased AOAH activity by sixfold (Fig. 4 C).

When BMDCs were treated with 10 ng/ml LPS for 2 d, they expressed more surface CD40 and CD86 (Fig. 4 A) and slightly more CD14 (reference 24; unpublished data), whereas their phagocytic activity decreased (Fig. 4 B). AOAH activity increased by threefold (Fig. 4 C). Up-regulation of costimulatory molecules has also been described previously in Salmonella-infected BMDCs in vitro (25) and in response to LPS in vivo (26). Thus, the maturation cocktail and LPS both augment cell surface CD40 and CD86 expression in BMDCs and decrease their phagocytic activity. The maturation cocktail decreases BMDC AOAH activity whereas LPS increases it.

**Other Bacterial Agonists Also Increase AOAH Expression in BMDCs.** Next, we wanted to know if the augmentation of AOAH activity is LPS-specific because LPS is an AOAH

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**Figure 3.** Stimulus-induced changes in XS52 cells. (A) Cell surface expression of CD14 and CD40 on untreated XS52 cells and on cells treated with cytokines (IL-4 and maturation cocktail), 10 ng/ml LPS, or 10⁶ CFU/ml *E. coli* for 9 d (Materials and Methods). Black line, specific antibody; gray line, control antibody of the same isotype. (B) Flow cytometric analysis of phagocytosis of Bodipy-labeled *E. coli*. Dotted line, cells only; black line, cells with Bodipy-*E. coli*; gray line, after pretreatment with cytochalasin D to inhibit phagocytosis. (C) LPS-deacylating activity in cell lysates. Each experimental condition was assayed in duplicate; the results are combined from nine independent experiments. (D) AOAH mRNA abundance as assessed by real-time PCR measurements of AOAH and GAPDH mRNA. The data are combined from two independent experiments. (C and D) Bars represent means ± 1 SD.
substrate. LPS is thought to activate DCs by interacting with the toll-like receptor (TLR) 4–MD-2 signaling complex. To find out if cell activation via other TLRs can increase or decrease AOAH expression, we incubated BM-DCs with bacterial CpGs (TLR9 agonist; references 27, 28) and *M. luteus* (gram-positive bacterium; agonist for TLR2 [29], possibly also other TLRs). When we tested the working suspension of each preparation using Limulus amebocyte lysate (Cape Cod Inc.), the endotoxin levels were <0.03 EU/ml. As shown in Fig. 4 C, CpG and *M. luteus* both increased AOAH activity whereas GpC did not. These results indicate that BMDC AOAH can be regulated via signals downstream of TLRs other than TLR4.

**After Exposure to Maturation Cocktail or LPS, XS52 Cells Alter Their Ability to Deaclylate LPS in Whole Bacteria.** We explored how different stimuli affect the ability of XS52 cells to deaclylate the LPS in whole bacteria. XS52 cells treated with IL-4 for 6 d and maturation cocktail for three additional days were compared with XS52 cells treated with LPS and with control cells that had been maintained without stimulation. The ability of the cells to deaclylate LPS in *E. coli* was abolished in the maturation cocktail–treated group (Fig. 5 B) and was increased by LPS treatment (Fig. 5 C). In parallel experiments, we studied the ability of the cells to degrade bacterial protein. Protein degradation was fourfold lower in maturation cocktail–treated XS52 cells than in untreated cells, whereas LPS treatment maintained the protein degradation rate (Fig. 5 D). Under the conditions studied here, maturation thus altered the cells’ ability to degrade bacterial LPS and protein in a similar fashion.

**Differentiation Also Influences the Bactericidal Activity of DCs.** IL-4– and maturation cocktail–treated XS52 cells were less able (by 50%) to kill *E. coli*, whereas LPS treatment maintained or slightly increased killing (Fig. 6).

**Discussion**

Decylation of the lipid A moiety of LPS was first described (30) in *Dictyostelium discoideum*, a slime mold that digests internalized bacteria as a foodstuff. The discovery that human neutrophils can also carry out LPS decylation was reported in 1983 (19), and subsequent work identified an LPS-deacylating enzyme, AOAH, in myeloid lineage cells from numerous animals. Mouse, rabbit, and human enzymes have over 70% amino acid sequence identity/similarity (31), whereas *D. discoideum* and mouse AOAH genes encode proteins that have ~30% overall amino acid sequence similarity, with identity in four of the five sequence motifs that place the enzyme in the GDSL lipase family (reference 32; unpublished data). Although the enzyme has thus been highly conserved during evolution, the role(s) that it plays in modulating immune responses to LPS are not well understood.

The results of the present experiments provide strong evidence that AOAH is the major, if not the only, mammalian enzyme that decylates the LPS contained in phagocytosed bacteria. We also identified a previously unsuspected role in LPS decylation for DCs, key cells in the innate immune response to invading bacteria.

We first found that lysates of immature DCs, whether derived from skin (XS52 cells) or bone marrow, had...
AOAH activity that was equivalent to the activity found in peritoneal macrophages and considerably greater than that in several macrophage cell lines. Second, both XS52 cells and BMDCs deacylated, in an AOAH-like manner, the LPS contained in the E. coli that they ingested; this ability was greatly diminished in BMDC and macrophages from AOAH-deficient mice, indicating that this enzyme is largely, if not entirely, responsible for LPS deacylation in these cells. The absence of enzymes that remove any of the four glucosamine-linked hydroxylated fatty acids from LPS suggests that animals may have other mechanisms for digesting, and/or disposing of, partially deacylated LPS (33).

The high levels of AOAH activity found in immature DCs and in macrophages raise the possibility that these cells play important roles in regulating the body’s responses to bioactive LPS. In this regard, it is intriguing that AOAH-deficient mice have exaggerated antibody responses to LPS (unpublished results); because the ability of extracellular AOAH to deacylate LPS is quite limited (34) and B cells do not produce the enzyme, partial deacylation of LPS by phagocytes may be required to limit B cell responses to gram-negative bacterial LPS in vivo.

The third significant finding from these experiments is that DCs can regulate their ability to deacylate LPS according to external cues. In response to a mixture of inflammatory cytokines and an agonistic CD40 antibody, DCs down-regulated their AOAH activity, whereas LPS treatment increased it. The enzymatic activity measured in cell lysates changed in concert with the ability of the cells to deacylate the LPS in phagocytosed bacteria. The regulation of AOAH expression was due, at least in part, to differential expression or degradation of AOAH mRNA. The finding that LPS treatment can increase AOAH mRNA abundance in murine macrophages (10–20-fold) and in vivo in mouse lung and liver (3–6-fold) was described by Cody et al. (35); whereas none of the stimuli used in their paper decreased AOAH mRNA or enzymatic activity in macrophages, we found that treatment with IL-4 and maturation cocktail greatly reduced AOAH in DCs. Thus, it appears that both up- and down-regulation of this low abundance enzyme can occur in phagocytes. The results of microarray analyses of AOAH mRNA abundance in human peripheral blood leukocytes (36) support this conclusion.

Fourth, we found that both XS52 cells and BMDCs also regulate their ability to internalize and kill E. coli. Although XS52 cells responded to IL-4 and maturation cocktail treatment decreased XS52 cells’ ability to kill E. coli by ~50% (*, P < 0.05, Student’s t test), whereas LPS maintained bactericidal activity or increased it by ~20% (#, P = 0.1). Data shown are mean ± 1 SD from three independent experiments.
These results are consistent with the currently accepted paradigm in which immature DCs can internalize and process bacterial antigens (37), whereas mature DCs lose these capabilities as they gain in antigen-presenting ability (2, 4, 38). Together with the results of others (39–41), our findings suggest that immature DCs contribute not only to the processing of bacterial antigens but also to the host’s innate armamentarium for killing invading bacteria and disabling their stimulatory molecules.

When BMDCs were treated with LPS, in contrast, their phagocytic activity decreased as AOAH activity increased. This finding suggests that phagocytosis (as well as endocytosis [42]) and AOAH expression may be regulated independently in BMDCs. Differential regulation downstream of the LPS signal has also been suggested by the reduced ability of LPS to induce cytokines in MyD88-deficient DCs whereas LPS induction of costimulatory molecule expression was intact (43, 44). Similarly, inhibition of p38 SAPK prevented LPS-induced up-regulation of CD80, CD83, and CD86 in monocyte-derived DCs, but did not affect changes in macrophagocytosis or HLA-DR and CD40 expression (45). Furthermore, Rescigno et al. (46) found that LPS induced NF-κB translocation and that inhibition of NF-κB with a serine protease inhibitor prevents D1 (a murine DC line) maturation (CD86 and class II expression), but does not interfere with the ability of LPS to prevent DC apoptosis. In contrast, Sallusto et al. (47) found that human monocyte-derived DCs respond to LPS as well as TNF and IL-1 with a coordinated series of changes that include down-regulation of macrophagocytosis and Fc receptors, disappearance of the class II compartment, and up-regulation of adhesion and costimulatory molecules.

Finally, it is noteworthy that AOAH activity increased as DCs recognized microbial agonists that activate them via several different TLRs. Thus, maintaining or increasing the ability to deacylate LPS seems to be a DC response to sensing diverse microbial molecules, including those in a gram-positive bacterium (M. luteus), which does not contain LPS. Expression of TLR4 is required for LPS, but not gram-negative bacteria, to induce the maturation of BMDCs (39).

In all phagocytes studied in vitro to date, LPS deacylation has occurred over many hours (21, 48). Thus, it is unlikely that AOAH influences the ability of the phagocytosing cell to respond to the LPS in a bacterial cell wall. Because the long-term fate of LPS within phagocytes is unknown, it is possible that LPS deacylation might either diminish a phagocyte’s late responses to LPS, or even reduce the responses of other cells that encounter LPS (either on the surface of the phagocyte (49, 50), or after the LPS has been released into the phagocyte’s environment; reference 51). It is also intriguing that animals should deacylate LPS so selectively: when AOAH acts on ingested E. coli, all of the hydroxylated fatty acyl chains remain attached to the glucosamine backbone of lipid A. Previous authors have raised the possibility that the partially deacylated (thus, tetra-acyl) LPS produced by AOAH could act as an LPS antagonist in vivo (9, 10, 52). The discovery of stimulus-regulated deacylation of LPS by DCs provides a new impetus for investigating the biological significance of LPS degradation by animals and the role(s) that DCs play in antibacterial host defense.

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