ACYLOXYACYL HYDROLASE: STUDIES ON ITS REGULATION AND FUNCTION IN *MUS MUSCULUS*

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To my brother, Minglun

ACYLOXYACYL HYDROLASE: STUDIES ON ITS REGULATION AND FUNCTION IN *MUS MUSCULUS*

by

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ACYLOXYACYL HYDROLASE: STUDIES ON ITS REGULATION AND FUNCTION IN *MUS MUSCULUS*

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Acyloxyacyl hydrolase (AOAH) is an enzyme that detoxifies Gram-negative bacterial lipopolysaccharides (LPS) by selectively removing secondary acyl chains from the lipid A moiety. Originally found in neutrophils, it is also produced by monocyte-macrophages and renal proximal tubule cells. In the studies described here, I found that both immature dendritic cells (DCs) of the XS52 cell line and bone marrow-derived DCs produce AOAH. AOAH expression decreased when DCs were incubated with IL-4, IL-1β, TNFα and an agonistic CD40 antibody (maturation cocktail), and increased following treatment with microbial agonists that engage 3 distinct Toll-like receptors (LPS, TLR4; CpG oligodeoxynucleotides, TLR9; and a Gram-positive bacterium (*Micrococcus luteus*), TLR2). Maturation cocktail treatment also diminished, while LPS treatment enhanced or

maintained, the cells' ability to kill *E. coli*, deacylate LPS, and degrade bacterial proteins. Enzymatic deacylation of LPS is thus an intrinsic, regulated mechanism by which DCs may modulate host responses to this potent bacterial agonist.

To study the biological functions of AOAH, AOAH-deficient mice were generated by targeted gene disruption. AOAH did not protect mice from lethal doses of LPS or Gram-negative bacterial challenge. In response to subcutaneous injections of LPS, however, AOAH-deficient mice produced significantly higher levels of non-specific (polyclonal) IgM and IgG_3 than did wild type mice. Anti-double-stranded DNA and anti-nucleosome IgM and IgG antibody levels were also higher in LPS-immunized AOAH-deficient mice than in wild type control mice. In addition, the partially-deacylated LPS product (dLPS) induced lower polyclonal antibody responses *in vivo* than did mock-treated LPS, yet the anti-LPS specific responses to dLPS and LPS were equivalent. These results suggest that AOAH may diminish potentially harmful polyclonal antibody responses to Gram-negative infection but maintain the protective anti-LPS specific response. Since B cells do not produce the enzyme, my results also point to an important role for macrophages and DCs in modulating B-cell responses to LPS antigens. In addition, the absence of AOAH did not alter the ability of LPS to function as an adjuvant, indicating that this activity is mechanistically distinct from stimulation of polyclonal antibody production. Finally, the ability of a bacterial lipopeptide to stimulate polyclonal antibody production only in AOAH -/- mice suggests that the enzyme may also regulate immune responses to non-LPS bacterial agonists.

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LIST OF ABBREVIATIONS

ANA	antinuclear antibody
AOAH	acyloxyacyl hydrolase
APC	antigen presenting cell
APRIL	a proliferation-inducing ligand
BAFF	B cell activation factor belonging to the TNF family
BCR	B cell receptor
B-D	Bligh-Dyer
BLP	Braun's lipoprotein
BLyS	B lymphocyte stimulator, also called BAFF
BMDC	bone marrow derived dendritic cell
BPI	bactericidal/permeability-increasing protein
CFA	complete Freunt's adjuvant
CFU	colony forming unit
CMC	critical micellar concentration
DC	dendritic cell
dLPS	deacylated LPS

DNP	dinitrophenyl
ds	double-stranded
ECA	enterobacterial common antigen
ELISA	enzyme linked immunosorbent assay
FACS	fluorescent activated cell sorter
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GPIdeAC	*Trypanosoma brucei* inositol deacylase
HEL	hen egg lysozyme
Hep	heptose
HMG-1	high mobility group 1 protein
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICF	incomplete Freund's adjuvant
IL	interleukin
IL-1R	IL-1 receptor
IRAK	IL-1R-associated kinase
KDO	2 keto-3-deoxyoctulosonic acid

KO (-/-)	knock out
LBP	LPS binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
mBSA	methylated BSA
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIIC	MHC class II-rich vesicular compartment
MPL	monophosphoryl lipid A
MyD88	myeloid differentiation factor 88
MZ	marginal zone
NK	natural killer
NNP	4-hydroxy-3, 5-dinitrophenyl
ODN	oligodeoxynucleotide
OMP	outer membrane protein
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PC	phosphorycholine

PCR	polymerase chain reaction
PE	R-phycoerythrin
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RF	rheumatoid factor
SD	standard deviation
SE	standard error
SLE	systemic lupus erythematosus
ss	single stranded
TACI	transmembrane activator and calcium modulator cycophilin ligand interactor
TAP	transporter associated with antigen processing
TCA	trichloroacetic acid
TD / TI	thymus-dependent / thymus-independent
TIR	toll / IL-1 receptor domain
TIRAP/ MAL	TIR domain-containing adapter protein / myd88-adapter-like
TLC	thin layer chromatography
TLR	Toll-like receptor
TNF	tumor necrosis factor
WT (+/+)	wild type

12:0	lauric acid
14:0	myristic acid
3-OH 14:0	3-hydroxy myristic acid
16:0	palmitic acid
16:1	palmitoleic acid
18:0	stearic acid
18:1	oleic acid

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

A. Structure and function of Gram-negative bacterial lipopolysaccharides

1. A glimpse at LPS

Discovered more than a century ago, bacterial LPSs (endotoxins) have been the most intensively studied Gram-negative bacterial molecules. The interest in LPSs is mainly based on the powerful and diverse impact that they have on infected hosts. LPS contains several well-conserved regions and is vital to the integrity of the Gram-negative bacterial outer membrane. Animal hosts sense the presence of LPS as an alarm of infection, and mount immune responses to eliminate invading microbes. However, when a host is exposed to LPS excessively, uncoordinated responses may damage the host. These unwanted reactions can include disturbance of coagulation, multiple organ failure, septic shock and even death.

LPS resides in the outer leaflet of the outer membrane of all Gram-negative bacteria found in natural environments (Fig. 1). One bacterial cell contains about 1-4×10^6 molecules of LPS, which occupy about 75% of the bacterial surface [1]. LPS comprises 3% of the total cell dry weight in rough strains used in the laboratory, such as *E. coli* K-12, and more than 10% in smooth *E coli* isolated from clinical sources. (Rough and smooth strains will be discussed later.)

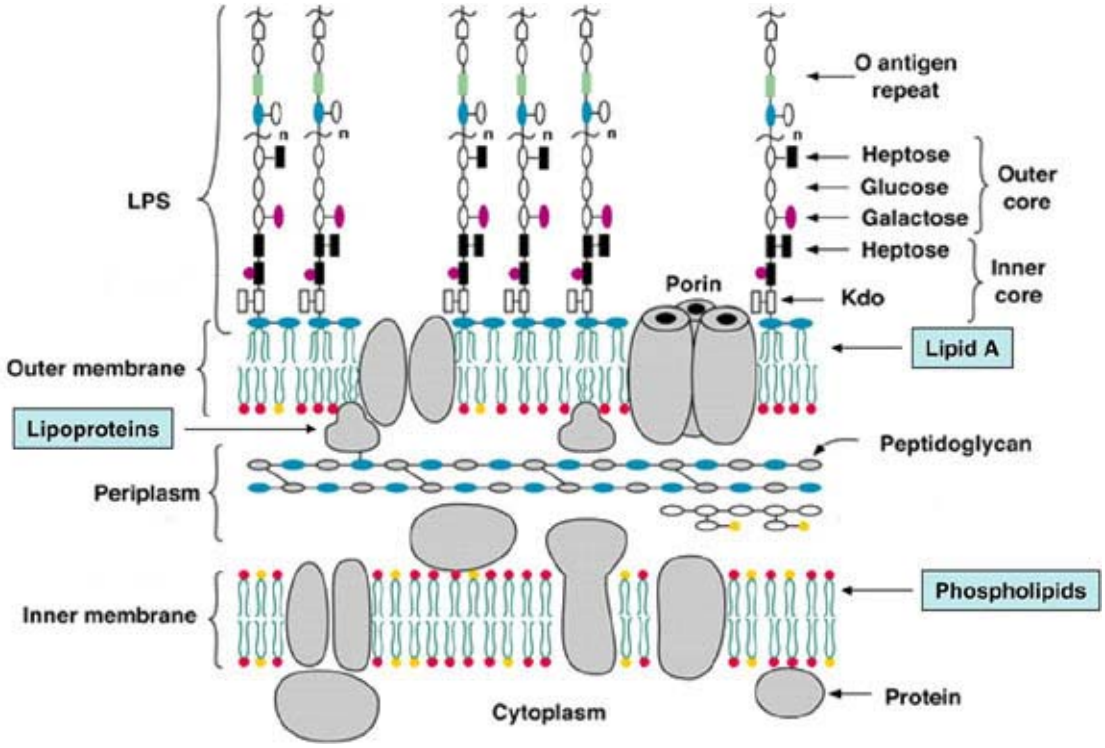


Figure 1. Model of the enteric bacterial membrane.

The enteric bacterial cell envelope consists of three layers: inner membrane, periplasm, and outer membrane. The outer membrane is constructed extremely asymmetrically: the outer leaflet is mainly composed of LPS and the rest of the surface is filled with outer membrane proteins and enteric bacterial common antigen (not shown), whereas the inner leaflet consists of phospholipids and bacterial lipoproteins [2].

2. Chemical structure of LPS

a. Chemical structure of enterobacterial LPS

Satisfactory LPS purification techniques were developed in the 1950's and 1960's. Traditional chemical techniques, which were used to study sugar and lipid composition, and recently NMR and mass spectrometry technology, have been applied to determine

LPS structures. For historical reasons, most of our understanding of LPSs has come from the Enterobacteriaceae. Enterobacterial LPS consists of three regions: lipid A, core and O-antigen. Lipid A anchors LPS to the bacterial membrane. The Core and O-antigen are hydrophilic polysaccharides that extend outward from bacterium (Fig. 1, 2).

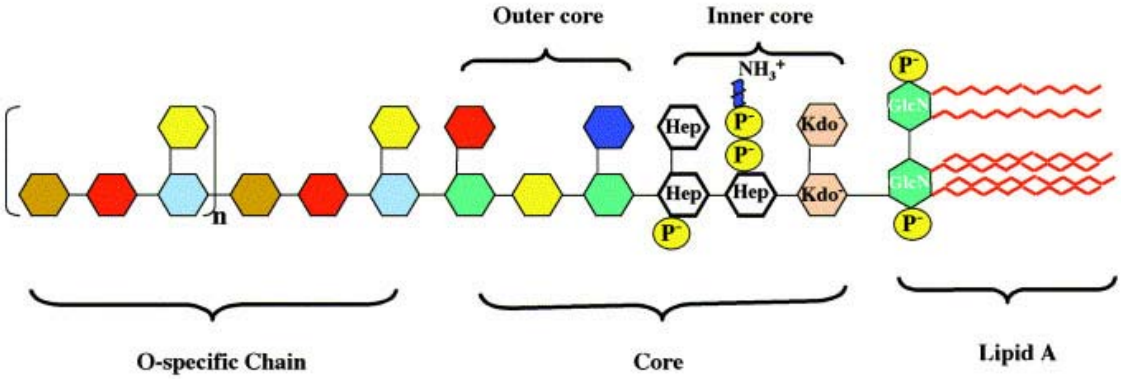


Figure 2. Schematic diagram of the chemical structure of enteric bacterial LPS.
LPS contains lipid A, core and O-antigen. Highly hydrophobic lipid A inserts LPS into the outer membrane of the Gram-negative bacterial cell envelope. Lipid A is the endotoxic principle of LPS. The inner core, covalently linked to lipid A, contains unusual sugars, KDO and Hep. The distal outer core is mainly composed of common sugars such as hexoses and hexosamines. The O-antigen, which extends further from the bacterial surface, consists of polymer of repeating saccharide units [3].

Lipid A

In the 1950s, Luderitz and Westphal prepared a saccharide-free portion of LPS, termed lipid A, by treating LPS with acid, and they proposed that lipid A is the endotoxic center of LPS. This hypothesis was supported by the observation that Re LPS, with only 2 keto-3-deoxyoctulosonic acid (KDO) and lipid A, exhibited endotoxic activity. In 1975, Galanos et al. showed that free lipid A was endotoxic and pyrogenic [4]. This result was

confirmed by Shiba et al., who synthesized lipid A and demonstrated that synthetic lipid A had identical bioactivities to natural free lipid A [5;6]. Lipid A has a conserved architecture (Fig. 3A). Enterobacterial lipid A has a β-D-GlcN-(1-6)-α-D-GlcN disaccharide backbone carrying two phosphoryl groups, at position 1 (position 1 of the reducing glucosamine residue, GlcN I) and position 4' (position 4 of the non-reducing glucosamine, GlcN II). Four molecules of 3-hydroxytetradecanoate (3-OH-14:0) attach directly to this backbone at positions 2 and 3 as well positions 2' and 3' by ester or amide linkage. The hydroxyl groups of two of the 3-OH-14:0 residues (at position 2' and 3') are substituted with secondary acyl chains (laurate, myristate) to form acyloxyacyl groups. An acyloxyacyl group is sometimes also found at position 2, with palmitate as the secondary acyl chain.

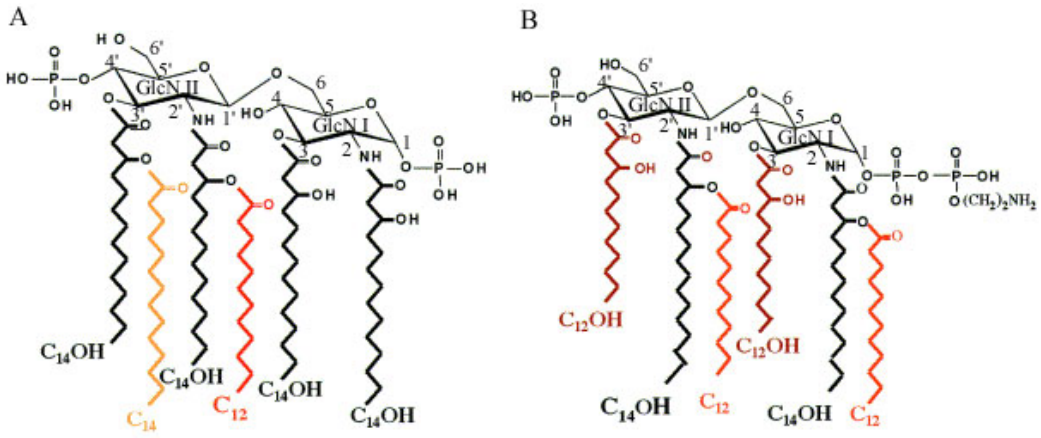


Figure 3. The chemical structure of lipid A moiety of *E. coli* LPS (A) and *N. meningitidis* LOS (B).

Lipid A consists of a glucosamine disaccharide backbone which carries two substituted phosphate groups. Primary fatty acyl chains (3-OH fatty acids) link directly to hydroxyl and amino groups on the backbone. Secondary fatty acyl chains (usually non-hydroxylated fatty acids) attach to primary fatty acyl chains by forming acyloxyacyl groups. The structure of *N. meningitidis* LOS lipid A (B) differs from that of *E. coli* LPS lipid A (A) mainly in acylation pattern ([3+3] versus [4+2]) and the length of the fatty acids (average of 12:0 versus 14:0).

Core

The core region of enterobacterial LPS consists of 10-12 sugars which can be subdivided into an inner and an outer portion. The inner core is covalently bound to lipid A, usually through KDO. The outer core is composed of neutral or amino hexoses, such as D-glucose, D-galactose, D-glucosamine, D-galactosamine or of N-acetyl derivatives. The saccharide residues of both inner and outer core can be decorated with negatively-charged groups like phosphate or pyrophosphate, which may interact with Ca^{2}+ and Mg^{2}+ ions that are required for normal membrane structure and function [1]. The core is structurally rather uniform, with a little diversity primarily being found in the outer core

region. Five different core-types have been found for all *E. coli* serotypes (R1-R4, K12), and only one for all *Salmonella* serotypes.

O-antigen

The O-antigen, or O-specific chain, covalently attaches to the outer core. The O-antigen is a long-chain polysaccharide comprised of repeating units of one to eight sugars. The structure of the repeating units (saccharide constituents, sequence, ring form, and type of chemical linkage, substitution) exhibits enormous variability among strains, and confers serotype specificity on a species or strain of bacteria. Compared to the relatively conserved core structure, more than 160 O-antigens have been identified for *E. coli*. An O-antigen may contain up to 50 or more units, and the number of O-antigen repeating units is heterogeneous in a single bacterial culture [7] and even on a single organism. This gives rise to the ladder pattern of molecular weights when LPS is visualized by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Smooth versus rough LPS

Wild-type enterobacteria with an O-antigen are called "smooth" because of the morphology of their colonies. The hydrophilic O-antigen renders enteric bacteria resistant to solubilization by intestinal enzymes and the lipids in bile. The O-antigen also protects bacteria from phagocytosis, the fusion of phagosomes containing bacteria with lysosomes [8] and the attack of complement [9] so that it is important for bacterial survival *in vivo*. Enterobacterial strains that lack LPS O-antigen are termed "rough", and their LPSs are denoted as Ra, Rb, Rc, Rd, Re, in the order of decreasing core length. For example,

Ra LPS contains lipid A and a complete core but no O-antigen, while Re LPS is composed of lipid A and two or three molecules of KDO. Re LPS appears to be the minimum requirement to maintain the integrity of the enterobacterial outer membrane [10]. Although rough mutants are viable *in vitro*, they are not pathogenic to animals since without O-antigen, they cannot persist and survive in tissues or body fluids.

b. Chemical structure of non-enterobacterial LOS

The chemical structures of LPSs from Gram-negative bacteria that colonize respiratory and urogenital mucosal surfaces have also been studied, such as *Haemophilus influenzae*, *N. meningitidis*, and *N. gonorrhoeae* [11]. They are different from enteric bacterial LPS in several aspects [12]. First, like rough mutants of enterobacteria, they lack O-antigens, and they express only short (about 10 sugar residues), non-repeating oligosaccharides attached to lipid A. For this reason, their LPSs are termed lipooligosaccharides (LOSs). Without O-antigen, non-enterobacteria have developed strategies to escape host defenses; for example, the attachment of N-acetyl neuraminic acid to the outer core region protects the genus *Neisseria* from the bactericidal activity in serum[13]. It has been hypothesized that this allows *Neisseria* to evade host immune responses by mimicking Neu5Nac-carrying host antigens. Second, the lipid A structure of LOS is different from that of enteric bacterial LPS (Fig. 3). For enteric bacterial LPS, the acylation pattern is asymmetric [4+2], i.e., both of the acyloxyacyl groups are at the non-reducing GlcN-residue (GlcN II) whereas none is on the reducing sugar (GlcN I). In contrast, a symmetrical fatty acid distribution [3+3] was found in mucosal bacteria like *Neisseria meningitidis*. GlcN I and GlcN II each carries three fatty acids with one

acyloxyacyl group. Third, *N. meningitidis* LOSs have shorter fatty acyl chains, with an average of 12 carbon atoms, while *E. coli* LPSs have fatty acids varying from 12 to 16 carbon atoms. Although LPS is essential for viability of enterobacteria, LOS is not indispensable for *N. meningitidis*. It has been shown that a *N. meningitidis* mutant strain devoid of LOS is viable, and its outer membrane is not visibly altered in spite of its slow growth rate[14].

3. Physical structure of LPS (LPS in bacterial outer membrane)

Many studies on LPS have been done with purified LPS. LPS molecules, like other amphiphilic molecules, will aggregate into larger supra-molecular structures above the critical micellar concentration (CMC). Below the CMC, or in the presence of chelators like EDTA, LPS is present as monomers. However, neither of these preparations represents natural LPS. In the native bacterial outer membrane, or in released bacterial debris, LPS is tightly associated with some of the outer membrane proteins, such as Braun's lipoprotein, outer membrane protein A and peptidoglycan-associated lipoprotein [15]. Some of the outer membrane proteins (TLR2 agonists, see below) are also bioactive and they can synergize with LPS to induce biological responses [16;17]. Correspondingly, Katz et al. have shown that LPS, when presented as purified agent, or as a component in bacterial outer membrane, induces different host responses [18].

4. Bioactivities of LPS

a. Recognition of LPS by animal cells

The interaction of LPS with animal cells has been studied extensively in the past few years. LPS binding protein (LBP), a plasma lipid transfer protein, delivers LPS monomers from aggregates or bacterial membranes to CD14. CD14, mainly expressed on mononuclear phagocytes, can bind and retain LPS on the cell surface. LBP also transfers LPS to a soluble form of CD14, which makes CD14-negative cells (such as endothelial cells and fibroblasts) become LPS responsive. However, glycosylphosphatidylinositol (GPI)-anchored CD14 itself cannot transduce the LPS signal because it lacks transmembrane and intracytoplasmic domains with which to facilitate intracellular signaling. In 1998, genetic analysis demonstrated that Toll-like receptor 4 (TLR4) is a critical signal tranducer for LPS. Mice that have a missense point mutation in TLR4 gene (C3H/HeJ) or that have a chromosomal deletion in the TLR4 genomic locus (C57BL/10ScCr) are hyporesponsive to LPS [19]. Examination of TLR4 null mice has confirmed the function of TLR4 as an essential component of the LPS recognition receptor [20]. One year later, it was found that MD-2, a secreted protein that is retained on the cell surface through interaction with the extracellular domain of TLR4, is also indispensable for LPS signaling [21].

The mechanisms by which the TLR4-MD2 complex recognizes LPS are still elusive. Many studies have shown that CD14 is not essential for LPS binding and signaling. CD14-deficient human cells are still responsive to LPS even in serum free medium lacking soluble CD14 [22]. Other LPS delivery and signal transduction pathways

may be present. Expression of other cell surface LPS-binding protein such as CD18 and the scavenger receptor also allows LPS signaling [23-25]. It seems that these LPS binding molecules' function is to bring LPS to the TLR4-MD2 complex, by inserting LPS either into the cell membrane or directly into the complex. In addition, dimerization of TLR4 seems to be a critical step in LPS signaling. Chimeric constructs of TLR4/CD4 have been shown to dimerize at their cytosolic domains and lead to cellular activation [26;27]. However, it is not clear how LPS induces dimerization of TLR4.

b. Other TLRs

When an animal host encounters invasive microbes, innate immune responses provide immediate protection. A critical role of the innate immune system is to detect and recognize microbial invaders. Innate immune recognition of invading pathogens is mediated by a set of germline-encoded receptors (pattern recognition receptors, PRRs) that have evolved to recognize conserved pathogen-associated molecular patterns (PAMP) [28]. Janeway et al. hypothesized that PAMPs must be not produced by the host organism; must be conserved, invariant structures shared by various microorganisms; and must be essential for the microbe's survival [29]. Although these invariant structures are called PAMPs, they are not unique to pathogens, and are actually found in all microorganisms, pathogenic or not. The recognition of PAMPs by mammalian cells can be attributed mainly to TLRs, a protein family homologous to the *Drosophila* host defensive protein, Toll. At least 10 TLRs have been found and the PAMP ligands for some of them have been identified. As discussed in the preceding part, TLR4 recognizes the best characterized PAMP, LPS. TLR2 recognizes a variety of PAMPs, such as Gram-positive

cell wall components peptidoglycan, lipoteichoic acids and bacterial lipoproteins or lipopeptides. Interestingly, LPSs *from Porphyromonas gingivalis* and *Leptospira interrogans* have both been shown to be recognized by TLR-2 instead of TLR4, since their lipid A structure is different from that of the typical *E. coli* or Salmonella LPSs [30;31]. The reason that TLR2 can recognize such a wide variety of PAMPS is probably due to TLR2 pairing with other TLR members such as TLR1 and TLR6 [26;32;33]. PAMP ligands for TLR3, TLR5, and TLR9 are double-stranded RNA, flagellin and unmethylated DNA with the CpG motif, respectively [34-36].

c. TLR signaling pathways

TLRs contain an extracellular leucine-rich domain and a cytoplasmic Toll / interleukin-1 receptor (TIR) domain. The TLR4 signaling pathway shows striking similarity to that used by IL-1 receptor. Activation of TLR4 leads to recruitment of MyD88 (myeloid differentiation factor 88), a cytoplasmic adapter protein which interacts with the TIR domain of TLR4. MyD88 then recruits IRAK-1 (IL-1 receptor-associated kinase-1) and activates a signaling cascade, which leads to NF-κB and AP-1 activation. In addition, a protein called TIRAP (TIR domain-containing adapter protein) / MAL (MyD88-adapter-like), able to interact with both TLR4 and IRAK2, can supplement MyD88 in TLR4 signaling [37;38], which explains the observation that in MyD88 null mice, although the cytokine response to LPS was abolished, activation of NF-κB was retained [39].

LPS activation of TLR4 on mammalian cells triggers the production of diverse mediators of inflammation, such as TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12, upregulates costimulatory and MHC molecules on antigen-presenting cells to promote antigen presentation to T cells, and enhances antibody production. An appropriate response to LPS protects the host by destroying the invading microbes and neutralizing their virulence factors. However, over-stimulation of cytokines, together with LPS-induced clotting and complement cascades, may have detrimental effects on the host.

5. Structure and function of LPS

The conserved lipid A moiety is the endotoxic principle. It seems that over millions years of evolution, the recognition of lipid A has been finely tuned. Any change involving the constituents and structural features of lipid A may have a tremendous impact on its bioactivity [1;3;40]. The understanding of the structure-function relationships of lipid A mainly comes from synthetic lipid A and its analogs with various modifications. For enterobacterial LPS, full bioactivity requires the presence of a β(1-6)-linked D-GlcN disaccharide backbone phosphorylated at positions C-1 and C-4' and substituted with six fatty acyl chains with two 3-acyloxyacyl groups. Structures with only one phosphate at either C-1 or C4' are about 100-fold less active than *E. coli* lipid A. GlcN monosaccharide structures phosphorylated and acylated at various positions have much lower bioactivity, suggesting that the disaccharide backbone is indispensable for recognition by mammalian receptors. Alteration of the fatty acyl chains also has dramatic effects. Subtraction, addition, or dislocation of a single acyl group reduces activity by 100-fold. Absence of the 2 secondary fatty acyl chains totally abolishes lipid

A activity on human cells [1]. Lengthening or shortening the fatty acyl chains may also decrease LPS activity. In addition, the chemical linkage by which the secondary fatty acyl chains attaches to the primary fatty acyl chains also has an effect on endotoxicity. Matsuura et al. have shown that when the ester-branched acyl group of monosaccharide lipid A is replaced with alkyl-branched type, this synthetic structure has higher toxicity (lower LD_{50} value in mice) but lower adjuvanticity [41].

These structure-function relationships derived from synthetic lipid A and its analogs tend to hold true when the natural occurring LPSs are studied. For example, penta-acylated lipid A of *Chlamydia trachomatis*, *Rhodobacter sphaeroides*, or *Rhodobacter capsulatus* has little or no endotoxic activity. *L. pneumophila*, containing hexa-acylated lipid A with long acyl residues of at least 18 carbon atoms in length, has weak or no activities.

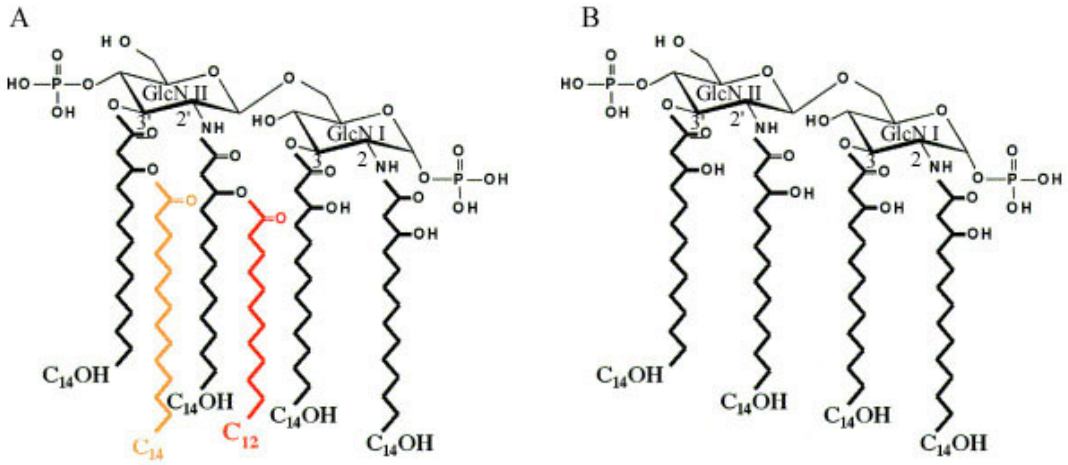


Figure 4. Schematic structure of Lipid A (A) and lipid IVA (B).

The bioactivity of synthetic lipid A analogs also depends on the host species. Human cells are distinct from mice cells in recognition of lipid IVA, a tetra-acylated biosynthetic precursor of lipid A (Fig. 4). Human cells discriminate lipid A and lipid IVA, and only respond to lipid A, whereas mouse cells respond to both of them [42-44]. The differential responses are due to both TLR4 and MD-2 molecules [45]. *Pseudomonas aeruginosa* grown in conventional bacterial culture medium has a penta-acylated lipid A. In contrast, LPS isolated from the airway of cystic fibrosis patients has hexa-acylated lipid A structure. Human cells but not murine cells discriminate between penta-acylated and hexa-acylated lipid A structures. Hexa-acylated LPS induces robust responses in human cells whereas penta-acylated LPS does not. This differentiation is dependent on a hypervariable region in the extracellular domain of human TLR4 [45].

Although recognition of LPS is mainly triggered by its lipid A moiety, the polysaccharide chain of LPS, which includes core and O-antigen, also plays a major role the physical presentation of LPS. Optimal lipid A activity is expressed only if at least one KDO residue is covalently attached [46]. In the Limulus assay, various strains of *E. coli* have over 600-fold different activities [47]. LPS with O-antigens composed of poly-mannose, such as *Klebsiella pneumoniae* O3, *E. coli* O8 and O9, have significantly increased adjuvanticity. These observations may be explained by increased adherence of the "mannan-type" LPS to antigen-presenting cells via mannose receptors [48].

6. Disposal and inactivation of LPS by the host

Following exposure to LPS, the host senses the presence of infection and mounts immune responses against microbial invaders. To reduce detrimental perturbations caused by LPS, the host uses various means to inactivate LPS and eventually remove it.

Circulating LPS can be inactivated by binding to host plasma lipoproteins. High-density, low-density, very-low density lipoprotein and chylomicons all can sequester and inactivate LPS. Several lipid or LPS binding proteins can transfer LPS to lipoproteins; these include LBP, CD14, phospholipid transfer protein, and cholesterol ester transfer protein [49;50]. In addition to inactivating LPS, lipoproteins also play a role as a transporter to remove LPS from the circulation and deliver it to the liver and eventually to dispose of LPS in bile and intestine. It has also been shown that even LPS attached on the surface of monocytes can be transferred to lipoproteins, and therefore the stimulatory effects on monocytes are diminished. Soluble CD14 accelerates this process [51].

Lipid A and the inner core of LPS are extremely negatively charged. A group of cationic proteins or peptides can bind to this region and deactivate LPS. These include bactericidal/permeability-increasing protein (BPI), CAP18 peptide/LL-37, lactoferrin, serum amyloid P-derived peptides and lysozyme [52]. Most of these proteins are generated by phagocytes and other cell types. Remarkably, bacteria can modulate their LPS structure to evade the attack of cationic antimicrobial proteins. Such modifications include the addition of ethanolamine, aminoarabinose and palmitate to lipid A, which promotes resistance to cationic LPS-binding proteins or peptides [53;54].

LPS can also be deactivated by internalization. CD18 on macrophages binds to LPS present on bacteria and promotes engulfment of bacteria [55;56]. Cells expressing CD14 and scavenger receptor can bind and endocytose LPS monomers and aggregates, whereas whole bacteria may be internalized by phagocytosis. After internalization, LPS is sequestered intracellularly. In addition, internalized LPS can be degraded and deactivated by a host enzyme, acyloxyacyl hydrolase (AOAH).

B. Dendritic cells and infection

1. Innate and adaptive immune responses

When an animal encounters a microbe, various defense mechanisms can be mobilized to combat the microbial invader. Rapidly after infection, the innate immune system is activated. The innate defense system includes phagocytes, NK cells, antimicrobial peptides and the alternative complement pathway, which are available immediately to fight against a variety of pathogens without requiring prior exposure. The innate immune system uses germline-coded receptors for the recognition of microbes, reacts nonspecifically to pathogens, and generates no immune memory. When infection cannot be controlled by innate host defenses, the adaptive immune response is required. The adaptive defense system is composed of T and B lymphocytes, which have an extremely diverse repertoire of cell surface receptors that are generated somatically and can potentially bind to any foreign antigen. Recognition of microbial antigens by certain T or B cell receptors triggers activation and proliferation of these cells (known as clonal expansion), pathogen-specific cellular or antibody responses can be mounted, and

immune memory develops. Although B and T lymphocytes are absolutely necessary for the generation of an efficient immune response, they rely on the innate immune system to decipher and integrate the "danger signals" carried by pathogens and to respond appropriately (Th1 versus Th2). Antigen-presenting cells (APCs) express receptors for recognition of microbial signature molecules, present microbial antigens to T cells and provide costimulatory molecules for T cell activation [57;58].

Dendritic cells, members of the innate immune system, are the most potent APCs. They have evolved to sense pathogens and tune the immune response. DCs are derived from bone-marrow progenitors. Moving through blood and lymphatic channels, they populate all lymphoid and most nonlymphoid tissues and organs. DCs have heterogeneous lineages, but they all possess similar morphology (dendrites) and function (ability to present antigen to T cells and activate naïve T cells). Immature DCs reside in peripheral tissues, such as the epidermis, dermis and submucosae, where the host usually encounters pathogens. They express pattern recognition receptors (PRRs), which detect the presence of infection by recognizing microbe-associated molecule patterns. Recently characterized TLRs are prime examples of PRRs and a series of TLRs recognize various conserved microbial molecules, as discussed in the preceding section. Different dendritic cells express different TLRs, which may allow DCs to discriminate between diverse microbial stimuli. Following an encounter with bacteria or bacterial components, immature DCs capture bacteria, process bacterial antigens and migrate via lymphatic channels to the T cell areas of regional lymph nodes, where they mature, lose their antigen-capturing ability, present antigenic peptides on MHC molecules on their cell

surfaces, upregulate costimulatory molecules, and become specialized for presenting antigens to T cells [59;60]. Recently, it has been found that induction of costimulatory molecule expression on APCs is not the only mechanism that controls T cell activation. In response to microbial stimuli, DCs block the suppressive effect of regulatory T cells, allowing activation of pathogen-specific T cells [61].

2. Antigen uptake

Because immature DCs express few cell-surface MHC, adhesion and costimulatory molecules, they are unable to stimulate T cells. The location of immature DCs and their ability to phagocytose microbes suggest that, like other phagocytes, they may play an important role in innate immunity. Indeed, studies have shown that immature DCs can efficiently take up and kill microbes [62-65].

Immature DCs take up antigens in several ways. First, they take up particles, microbes and apoptotic and necrotic cell fragments by phagocytosis. Second, they sample extracellular fluid and solutes by macropinocytosis. Third, they utilize membrane receptor-mediated endocytosis to internalize antigens. C-type lectin receptors (mannose receptor, DEC205, scavenger receptors), Fcγ receptors (CD64 and CD32) and complement receptors (CD11b, CD11c) all mediate antigen uptake and make antigen presentation more efficient [59]. After DCs have captured antigens and received maturation signals, their capacity for antigen uptake rapidly declines and they start antigen processing and presentation.

3. Antigen processing

Antigen processing by DCs occurs mainly through two major pathways, the endocytic and proteosomal pathways. To activate antigen-specific CD4 helper T cells, DCs present antigenic peptides in MHC class II molecules, which can be loaded through the endocytic pathway. Antigens internalized by DCs gain access to early and late acidic endosomes, where some proteolysis occurs. Peptide fragments then associate with MHC class II molecules in the MHC class II-rich vesicular compartment (MIIC) with the aid of an accessory molecule, HLA-DM, and are transported to the cell surface. Both the formation of peptide-MHC II complexes and the transport of the complexes to the cell surface are tightly controlled. Turley et al. have reported that immature DCs sequester intact antigen in endosomes, and process and convert antigens into peptide-MHC II complexes upon induction of DC maturation by LPS [66]. In addition, Kleijmeer et al. have demonstrated that immature DCs store MHC II molecules in internal vesicles of MIIC, and, in response to LPS, transfer them to the limiting membrane of the MIIC, where HLA-DM is located, and then transport them to the plasma membrane [67].

MHC class I molecules display peptide antigens to CD8 T cells. DCs degrade cytosolic proteins in proteosomes and transport the resultant peptides via the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). In the ER, peptide antigens are loaded to MHC I/ βM_2, and MHC I/βM_2/peptide complexes are transported in exocytic vesicles to the DC plasma membrane. It was originally thought that this pathway only processed cytoplasmic proteins. However, endocytic antigens can also be presented by MHC I molecules; this process is termed cross-presentation. After

phagocytosis of bacteria, bacterial antigens can be cross-presented in DCs. A model antigen, ovalbumin, expressed on the surface of recombinant *Streptococcus gordonii* or in the cytoplasm of *E. coli* and *S. typhimurium*, is processed and presented on MHC I molecules. This presentation requires TAP, proteosomes, and newly synthesized MHC I molecules and is 10^6 times more efficient than that of soluble OVA protein [68-70]. Both elicited peritoneal macrophages and bone marrow-derived macrophages can also cross-prime CD8 T cells, but they use an alternate MHC I presentation pathway that does not require TAP or proteosomes [71].

In addition to taking up bacterial antigens directly, DCs can also ingest apoptotic material from neighboring macrophages induced to undergo apoptosis by *Salmonella* infection and then process and present *Salmonella* peptides on MHC I and MHC II. In contrast, bystander macrophages fail to do so [72]. Furthermore, when macrophages are induced to become necrotic by higher concentrations of *Salmonella,* bystander DCs show reduced antigen presentation. These studies suggest that bacteria may induce phagocyte apoptosis, leading to abrogation of direct antigen presentation by the phagocytes, and that bystander DCs are able to take up the apoptotic material and present bacterial antigens for T cell scrutiny.

DCs are able to present antigens containing lipid on a non-classical antigen presenting molecule, CD1, to a restricted set of T cells as well as to NK T cells [73;74]. Like MHC I molecules, CD1 molecules are associated with β2M. However, presentation on CD1 requires an acidic compartment but not TAP. The crystal structure of CD1 molecule revealed that CD1 has a deep hydrophobic groove, into which the lipid portion

of microbial glycolipids may insert. Because LPS is also a glycolipid, partial deacylation of LPS by AOAH might be able to trim the bulky lipid moiety of LPS so that LPS can be presented by CD1 or CD1-like molecules.

4. Activation of DCs by LPS

The impact of LPS on DC activation and maturation has been intensively studied *in vivo* and *in vitro*. *In vitro* studies have shown that LPS induces human or murine DCs to down-regulate endocytosis and phagocytosis, up-regulate MHC and costimulatory molecules (CD40, CD80 and CD86), secret cytokines (IL-12, IL-10, IL-6, TNF-α), rearrange adhesion molecules and increase antigen-presenting capacity [71;75-84].

Systemic administration of LPS induces the migration of splenic DC from the marginal zone to the T cell area within 4-6 h, and this movement parallels a maturation process, diminished antigen-capturing ability and increased T cell stimulatory function. 48 h after LPS injection, the number of DCs in the spleen is reduced markedly, suggesting that, after maturation and interaction with T cells, DCs die *in situ* to turn off antigen presentation [85]. In another study, to measure antigen presentation directly, Reis e Sousa et al. made a monoclonal antibody that specifically recognizes a processed peptide of hen egg lysozyme (HEL) bound to the murine MHC class II I-A^K. Following *s.c.* immunization of HEL by itself, this protein was not efficiently presented by lymph node DCs. When LPS was coinjected, DCs presenting HEL peptide rapidly accumulated in the outer periarteriolar lymphoid sheaths and in follicular areas, suggesting that LPS enhances peptide loading onto MHC class II and promotes DC migration to the T cell area [86;87]. Furthermore, in response to LPS, DCs in skin, intestine, heart and kidney all

migrate out of the peripheral tissues, and the depleted DC pool can soon be replenished by DC precursors [88-90].

5. DCs sense the presence of LPS via cell surface TLR4

LPS cannot activate BMDCs from TLR4 deficient or C3H/HeJ mice, suggesting that TLR4 is indispensable for LPS signaling on DCs [91]. TLR4 expression has been found on human PBMC-derived DCs as well as their precursors, monocytes, which have a higher expression level [92;93]. Since TLR4 is expressed at a very low level on immature DC (a few hundred molecules or fewer), it might transduce the LPS signal very efficiently. When immature DCs were induced to mature with LPS, expression of TLR4 transiently increased and then nearly disappeared. Correspondingly, stimulation of immature DCs, but not mature DC, with LPS resulted in the activation of IRAK, a downstream molecule of TLR signaling cascade [92]. Human PBMC-derived DCs do not express CD14 molecules on their cell surface, and they rely on soluble CD14 to respond to LPS [80].

LPS-induced DC activation or maturation is regulated by different and sometime overlapping pathways. Kaisho et al. have shown that LPS induced DC cytokines production and costimulatory molecule upregulation via MyD88-dependent and -independent pathways [91]. LPS may cause activation of several transcription factors, such as NF-κB and AP-1. Studies have suggested that the NF-κB pathway accounts for upregulation of MHC and costimulatory molecules on the cell surface, while the MAP kinase pathway is responsible for cell survival [94]. Ardeshna et al. showed that inhibiting

the NF-κB pathway significantly reduced the LPS-induced upregulation of MHC as well as CD80, CD83, and CD86; inhibiting p38 SAPK reduced the LPS-induced upregulation of CD80, CD83, CD86, but not of MHC and CD40, while the PI-3 kinase /Akt pathway, was responsible for maintaining survival of LPS-stimulated DCs [84].

6. Other maturation stimuli for DCs

In addition to LPS, other conserved microbial molecules such as bacterial lipoprotein, peptidoglycan, CpG DNA, and double-stranded DNA can also induce DC maturation through recognition by respective TLRs. Furthermore, proinflammatory cytokines (TNF-α, IL-1β), produced by DCs and other cells in response to microbes, and T cell derived signals (CD40 ligand) are able to trigger DC maturation. Different maturation stimuli drive distinct developments events. Although TNF-α, LPS and CD40 ligand generated mature DCs in terms of their surface marker profiles (MHC, CD40, CD80, CD86), detailed cell biological analysis in terms of both morphology and MHC II localization revealed that TNF-α only induced the early step of DC maturation, while LPS drove more advanced maturation and CD40 caused the most typical full maturation [82]. It has also been shown that LPS and CD40 both increase IL-6, IL-12 p40, IL-15 and TNF-α mRNA / protein levels in murine BMDCs. However, only LPS up-regulates IL-1α, IL-1β, IL-12p35 and MIF mRNA[95]. In another study, Kelleher et al found that although either LPS or CD40 activated DCs (antigen-presentation to CD4 T cells, expression of costimulatory molecules, secretion of cytokines and chemokines), only in the presence of CD40 ligand were CTLs generated from naïve polyclonal CD8+ T cells

in vitro [96]. Multiple stimuli may act synergistically on activation of DCs. Lapointe et al. have shown that a combination of LPS and CD40 ligand had a positive impact on the ability of DC to secrete IL-12 and IL-10 and to activate tumor antigen-specific CD8 T cells [83].

The effects of purified LPS and whole Gram-negative bacteria on DCs have been compared using oligonucleotide microarray analysis [97]. LPS was able to mimic and account for about 88% of the entire bacterial response, suggesting that LPS is the major and most potent immune stimulatory molecule in Gram-negative bacteria. In another study, the responses of PBMC-derived DCs to *Neisseria meningitidis*, an LPS-deficient isogenic mutant, and purified LPS from the same strain were investigated. The wild type strain induced higher level of TNF-α, IL-1α, and IL-6 than either the LPS deficient strain or purified LPS. Only the wild type strain stimulated IL-12 production. Remarkably, adding exogenous LPS to the LPS deficient mutant strain only partially restored TNF-α, IL-1α, and IL-6 production and did not restore IL-12, suggesting that LPS in the context of bacterial membrane is required for high-level cytokine production [98]. Accordingly, it has been found that although both bacteria and LPS can activate NF-κB translocation in DCs 30 min after treatment, bacteria-induced NF-κB activation was more pronounced [99].

7. DCs and B cells

DCs can stimulate B cells indirectly by presenting antigen to T helper cells, which provide membrane bound CD40 ligand and secreted cytokines for B cell proliferation and differentiation. DCs can also activate naïve and memory B cells directly. DCs induce

the differentiation of CD40-activated memory B cells to IgG and IgA plasma cells by secreting IL-6R α-chain, which associates with IL-6 and binds to the IL-6 receptor on B cells. DCs also contribute to naïve B cell differentiation into IgM-secreting cells and IL-12 is essential for the differentiation of naïve B cells while IL-6R signaling promotes antibody secretion [100;101]. DCs can also stimulate activated B cells to undergo isotype switching. DCs capture and transfer unprocessed antigen to B cells and induce an IgG class switch [102]. Human tonsil interdigitating DCs produce IL-13, which is essential for IgM production in CD40-activated naïve B cells, and they direct naïve B cells toward isotype switching (IgG and IgA) [103].

Recently, it has been reported that DCs interact with splenic marginal zone B cells and promote a TI-2 immune response. In response to a systemic infection, blood-derived $CD11c^{lo}$ immature DCs efficiently capture and transport bacteria *(S. pneumoniae)* to the spleen, provide essential survival signals to antigen-specific marginal zone B cells and promote them to differentiate into IgM-secreting plasmablasts. This DC-B cell interaction, shown both *in vivo and in vitro*, relies on TACI (transmembrane activator and calcium modulator cycophilin ligand interactor) molecules, which are receptors for the DC-derived TNF-family ligands, BLyS (B lymphocyte stimulator, also called BAFF) and APRIL (A proliferation-inducing ligand) [104].

C. Deacylation of LPS by AOAH

Since LPS has a broad spectrum of bioactivities, and host reactions to it may be deleterious, can animals degrade and thereby detoxify LPS? As reviewed in the preceding section, fatty acyl chains and phosphate groups are required for LPS

recognition by animal cells, and the polysaccharide chain also plays an important role in bacterial virulence. Do animals have enzymes that can degrade each of these components?

Deacylation of the lipid A moiety of LPS was first described in a primitive phagocyte, *Dictyostelium discoideum* (a slime mold), which uses bacteria as a carbon source [105]. It was not until 1983 that Hall and Munford first reported that human neutrophils can remove the secondary fatty acyl chains from the lipid A moiety of LPS (Fig. 5) [106]. The enzyme was then purified and was named acyloxyacyl hydrolase (AOAH). Although catalytic activities towards phosphate groups of lipid A have been found in murine macrophages, no corresponding enzyme has been identified [107]. Even less is known about how mammalian hosts degrade the polysaccharide chain of LPS. The only reported LPS polysaccharide degradative enzymes, deacetylases, were found in phages specific for smooth *Salmonella* strains; deacetylation is probably required for phage adsorption [52].

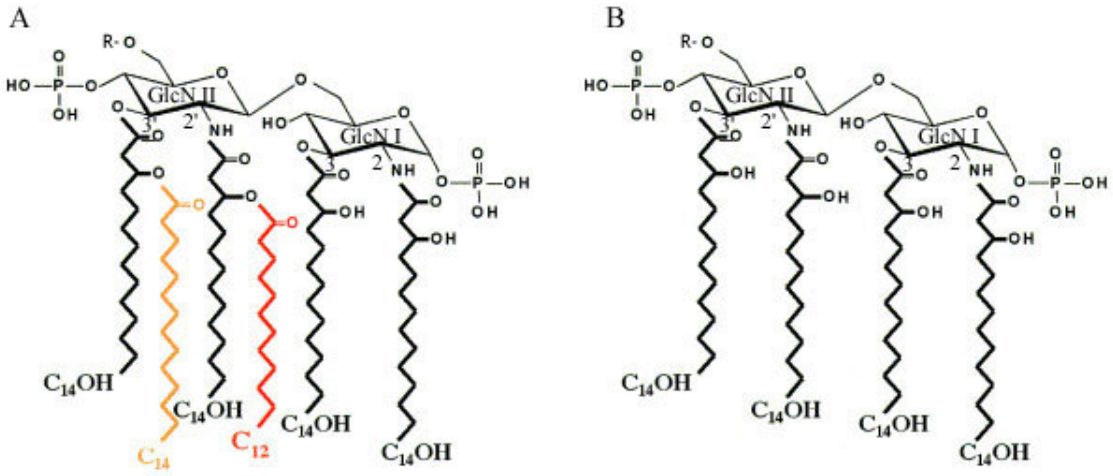


Figure 5. AOAH releases secondary fatty acyl chains from the lipid A moiety of LPS.

(A) AOAH cleavage sites are shown by arrows. (B) Enzymatically deacylated LPS, which is similar to lipid IVA. R represents the polysaccharide chain of LPS.

1. Characteristic molecular structure of AOAH

AOAH is the only LPS degrading enzyme that has been purified, cloned and characterized. Molecular structure studies have revealed that AOAH is an unique enzyme composed of two disulfide-linked two subunits encoded by a single mRNA [108]. AOAH is synthesized as a single chain peptide precursor and is processed post-translationally [109]. The large subunit contains a G-X-S-X-G lipase consensus motif and the central serine is a part of a D-H-S catalytic triad found in many lipases [110]. The small subunit, with six cysteines and an N-linked glycosylation site, is homologous to the human sphingolipid activator proteins (saposins), and to saposin-like polypeptides (surfactant protein B, NK-lysin, acid sphingomyelinase and amoebopores) [111;112].

Intriguingly, saposins are cofactors for glycohydrolases that act on sphingolipids, which resemble LPS by having a relatively constant lipid core and a carbohydrate chain of variable size and structure. Saposin and saposin-like proteins constitute a family of small proteins that function at lipid-water or lipid-air interfaces. Analysis of AOAH amino acid and DNA sequences reveals no homology to any of the other proteins known to have lipid A binding sites [113]. In 2001, Guther et al. purified and characterized the *Trypanosoma brucei* inositol deacylase (GPIdeAC), an enzyme that cleaves an acyl chain from the inositol group of GPI anchors. It has been shown that GPIdeAC has significant amino acid sequence and hydropathy similarity to AOAH (identity, 35%, similarity, 54%) [114]. However, recombinant GPIdeAC has no activity as an LPS-deacylating enzyme (X.-H. Li and R. S. Munford, unpublished data). AOAH has been highly conserved over evolution. Mouse, rabbit and human AOAHs have 77% amino acid sequence identity and 89% similarity. The AOAHs of *Mus musculus* and *Dictyostelium discoideum* have about 30% amino acid similarity with identity in four of the five sequence motifs that characterize GDSL lipase family.

Structure-function analysis of the AOAH protein has shown that both large and small subunits are required for AOAH activity. Elimination of the S^{263} in the G-X-S-X-G lipase motif in the large subunit reduced LPS-deacylating activity by more than 99%. The large subunit by itself had 100-fold less activity toward LPS and phospholipids. Deletion of an internal 33-amino acid region of the small subunit reduced the enzymatic activity by more than 50%. In addition, the small subunit plays an important role in AOAH intracellular localization. Recombinant AOAH with truncated or no small

subunit did not target to intracellular vesicles. Thus, it was proposed that large subunit performs catalytic cleavage while small subunit confers the recognition of its unique substrate and facilitates the intracellular targeting of the enzyme [109].

2. Intracellular localization of AOAH

AOAH activity has been found in phagocytic leucocytes such as neutrophils and monocyte-macrophages. Due to the low abundance of this enzyme (about 2500 molecules per HL-60 human promyelocyte) [108], immunolocalization has been very difficult. It has been suggested that AOAH is contained in an acidic granule because neutrophil granules (but not azurophilic granules) have enzyme activity and ammonium chloride can inhibit LPS deacylation in these cells [109;115]. Using indirect immunoflurescence, intracellular AOAH localization was studied in BHK570 cells transfected with a plasmid containing recombinant AOAH cDNA. Bright fluorescence showed in large vesicular structures [109].

3. AOAH deacylates LPS and phospholipids.

AOAH specifically removes the secondary fatty acyl chains from the lipid A moiety of LPS, while it leaves the primary fatty acyl chains intact. Therefore, the lipid A moiety of AOAH deacylated LPS (dLPS) resembles lipid IVA, an intermediate in the lipid A biosynthesis pathway [116] (Fig. 5) .

AOAH can release secondary fatty acyl chains from LPSs that have substantially different lipid A structure, such as LPSs from *S. typhimurium*, *E. coli*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae*.

It seems that this enzyme has a preference for shorter, saturated and non-hydroxylated secondary fatty acids, but little preference for the position of the fatty acids on the lipid A backbone [117].

In addition to LPS, AOAH releases fatty acyl chains from other substrates, such as glycerophospholipids, lysophospholipids and diacylglycerol. Similarly, AOAH preferentially removes saturated (or short) fatty acids from the above substrates regardless of the position of the fatty acids (sn-1 or sn-2) on the glycerol moiety. AOAH also can catalyze fatty acyl chain transfer from LPS to, a model acceptor, monooleoylglyceryl ether [118]. These various *in vitro* activities raise the possibility that AOAH may play other biological roles in addition to degrading LPS.

In vitro LPS deacylation by AOAH protein requires detergent, Triton X-100, and low pH [106;118]. Since purified LPS represents a different physical state from the LPS residing in a bacterial outer membrane, it was uncertain whether phagocytes bearing AOAH could act on the LPS that was a constituent of intact bacteria. Katz et al. have shown that mononuclear cells and neutrophils, isolated from inflammatory exudates elicited in the peritoneal cavity of a rabbit, could deacylate the LPS in whole bacteria, while cell free ascitic fluid could not in spite of its ability to deacylate purified LPS in the absence of detergent and low pH [119]. Mononuclear cells were more active than neutrophils, probably due to their higher content of AOAH-like enzymatic activity [120]. Only the secondary fatty acyl chains were specifically released from LPS in whole bacteria, which suggested an AOAH-like reaction. These studies presented clear evidence that in relatively natural conditions (no detergent, pH 7.4), phagocytes can

degrade bacterial LPS and that the phagocytic machinery is required for deacylation of LPS residing in bacterial membranes. The deacylation kinetics of LPS in whole bacteria by mononuclear cells and neutrophils was slow, with 50% and 20-30% deacylation, respectively, at 20 hours. It is uncertain whether the slow deacylation can counteract fast LPS signaling (which occurs within minutes) and whether the dLPS can be released from phagocytes and antagonize intact LPS.

4. AOAH-deacylated LPS has reduced bioactivity.

Treatment of LPS with AOAH diminishes the biopotency of various LPSs. In the rabbit dermal Shwartzman reaction [121], enzymatically deacylated LPSs (dLPSs) had reduced tissue toxicity by at least 100-fold. Deacylation abolishes the ability of LPS to stimulate human endothelial cells, neutrophils and monocytes [122-125]. In addition, dLPS is also a LPS-specific inhibitor that antagonizes intact LPS activity by competing for LBP, CD14, and probably TLR4 -MD-2 [126]. However, due to the different structures of murine TLR4 and MD2 [42-45], dLPS, like lipid IVA, has partial agonistic activity toward murine cells. In the murine splenocyte mitogenesis assay, dLPS has only 10- fold less activity than control LPS [121]. While *S. typhimurium* or *E. coli* LPSs and dLPS were mainly studied in these mouse experiments, Erwin et al. also studied the effects of AOAH on the bioactivities of several LPSs that differ from *Salmonella* LPS, including LPSs from *Haemophilus influenzae*, *Neisseria meningitidis*, *and Neisseria gonorrhoeae.* Surprisingly, the potency of *Neisseria* (*meningitidis* and *gonorrhoeae*) LOS in the murine splenocyte mitogenicity test was reduced over 100-fold by AOAH deacylation and dLOS could antagonize the mitogenic activity of *Neisseria* and *Salmonella* LPS [127]. This study

has offered us a valuable tool (*Neisseria* LOS) to investigate how AOAH functions in mice.

5. Regulation of AOAH activity.

Cellular and extracellular AOAH activity can be regulated. Although AOAH has not been detected in human plasma, AOAH activity increased in rabbits and mice plasma in response to LPS injection, and LPS stimulated the release of AOAH from rabbit leucocytes *in vitro* [128]. Cody et al have demonstrated that in isolated murine peritoneal macrophages, LPS upregulated AOAH mRNA expression by 10-20 fold, and LPS increased the LPS-deacylating activity of cell lysates by 3-5 fold. IFN-γ also induced AOAH mRNA expression. In addition, after intraperitoneal challenge of mice with LPS, AOAH mRNA expression in lung and liver was induced by 3- and 6- fold, respectively [129].

D. Antibody responses to LPS

1. Thymus-dependent (TD) and thymus-independent (TI) antigens

For a protein antigen to activate a B cell, two signals are required. Signal one comes from the engagement of B cell receptors with the antigen, and the second signal is delivered by helper T cells, which recognize antigen peptides presented by MHC II molecules on B cells, interact with B cells by CD40 ligand-CD40 association, and secret cytokines. Because the antibody response to protein antigens is dependent upon helper T cells, protein antigens are called TD antigens. Conversely, TI antigens are able to

stimulate antibody responses in athymic animals. TI antigens fall into two classes, which stimulate B cells by different mechanisms. TI-1 antigens, such as LPS and bacterial DNA containing the CpG motif, have intrinsic B cell activating activity. It is now clear that the intrinsic B cell-stimulating activity of TI-1 antigens is due to B cell expression of TI-1 antigen recognition and signaling molecules, mainly of the TLR family. TI-2 antigens are molecules that have highly repetitive structures, such as bacterial capsular polysaccharides and polymerized flagellin. TI-2 antigens do not possess an intrinsic activity that can directly stimulate mature B cells. Instead, they induce B cell proliferation by extensive crosslinking of specific B cell receptors (BCRs). A second signal, such as engagement of TLRs, ligation of CD21 (CR2) and various cytokines, is required for TI-2 antibody secretory responses. In addition, T cells ($\alpha\beta$, $\gamma\delta$ T cells and NK T cells), NK cells, DCs and macrophages are probably all involved with the delivery of a second signal, directly or indirectly [130].

2. LPS-specific and polyclonal antibody responses.

LPS can induce both specific and polyclonal B cell responses. *In vitro* experiments with murine splenocytes in serum-free medium have shown markedly different dose-response profiles for LPS-induced specific and polyclonal responses [131]. A hapten, 4-hydroxy-3, 5-dinitrophenyl (NNP), was conjugated to LPS so that the LPS-specific response (to NNP) could be studied. The optimal concentrations (2.5-12 ng/ml) required for induction of specific anti-NNP cells were several orders of magnitude lower than the concentrations (10-40 μg/ml) required for polyclonal (nonspecific) splenocyte activation. Low concentrations of LPS failed to induce non-specific B cells, and when

NNP receptors on B cells were blocked with free NNP, optimal specific B cell stimulating concentrations were completely non-activating. The following four hypotheses were based on these studies: (1) At low concentrations of LPS, only B cells with specific B cell receptors for antigenic determinants on LPS can concentrate sufficient LPS on the surface to become activated. (2) The binding or crosslinking (NNP determinants were repeated on the LPS molecules.) of BCRs may deliver signals to B cells and then decrease the threshold for B cell activation. (3) The LPS-NNP molecule may bring BCR and LPS receptor to close vicinity and they then cooperate to activate B cells. (4) Since engagement of antigen results in BCR cluster formation and a rapid translocation to lipid raft domains in the B-cell membrane, the LPS receptor is then brought to the same region, where LPS signaling is facilitated. In this study, because whole splenocytes were used, it is not known whether the other types of cells present (macrophages, DCs, T cells, NK cells) contributed to the specific and polyclonal antibody responses to LPS. In addition, whether LPS has similar dose-response profiles *in vivo* was not shown.

3. How does LPS signal B cells?

The TLR4-MD2 complex was first identified to be able to deliver a LPS signal in B cells. C3H/HeJ or TLR4 mull mice do not make anti-LPS specific antibodies or show a polyclonal antibody response after LPS immunization *in vivo***.** Splenocytes from these mice do not proliferate and differentiate into plasma cells and secret antibodies *in vitro* [19;20;132;133]. In addition to the TLR4-MD2 complex, B cells require another member of the TLR family, RP105 protein, for optimal LPS recognition. RP105 was first identified as a

cell surface molecule that contributes to protecting B cells from irradiation-induced apoptosis, up-regulation of cell surface expression of CD86, and induction of B-cell proliferation [134]. Interestingly, RP105 is associated with a MD-2 homologous molecule, MD-1. Similar to MD-2's role vis a vis TLR4, MD-1 plays an important role in RP105 intracellular traffic, stability and cell surface expression [135]. Although RP105 and TLR4 share similar extracellular leucine-rich repeats, RP105 does not have an intracellular TIR domain, indicating that RP105 has a different signal transduction pathway from TLR4. *In vitro* experiments have shown functional cooperation between TLR4 and RP105 in LPS-induced NF-κB activation [136]. RP105 -/- and MD-1 -/- mice both have dramatically impaired LPS-induced antibody production *in vivo*, but unlike C3H/HeJ or TLR4 -/- mice, the antibody responses are not completely abolished [135;136]. These observations suggest that RP105-MD-1 plays a supporting but important role in B cell responses to LPS. Although RP105-MD-1 complexes are also expressed on macrophages and DCs, these cells do not require RP105-MD-1 for LPS recognition [137-139]. How RP105-MD-1 interact with TLR4-MD-2 and why B cells exclusively utilize this additional LPS signaling pathway remain to be understood.

Although some studies have indicated that activation of B cells by LPS occurred independently of other types of cells [140;141], other investigations have shown that cytokines derived from macrophages and T cells may play an important role in specific or polyclonal B cell responses to LPS [142-144]. Recently, it has been shown that B cells express TACI and BCMA receptors, which recognize DC-derived soluble TNF-family ligands APRIL and/or BAFF. Expression of these ligands by DCs is promoted by innate

immune signals and the binding of these ligands to receptors on B cells promotes B cell survival and activation [145]. Blood DCs capture TI-II antigen (PC) and interact with splenic marginal zone (MZ) B cells to initiate T-Independent immune responses in a TACI-dependent way. Similarly, peritoneal macrophages support antigen (PC)-specific B-1 cell activation [104]. It is possible that DCs or macrophages take up LPS or gram-negative bacteria, secret soluble factors and interact with B cells in a similar way.

Whether the direct B cell-DC interaction can enhance B cell responses to LPS is not clear. The fate of LPS in other phagocytes has been studied. Forestier et al. studied the intracellular pathway of *B. abortus* LPS (a nonclassical LPS) in macrophages and they found that LPS migrate from endosomes to lysosomes and reached the cell surface with very slow kinetics (more than 3 days). LPS present at the cell surface is found in large clusters with the O-Ag facing the extracellular medium and it is not cleared by macrophages after 3 months [146]. When Wuorela et al. fed blood peripheral monocytes with heat-killed *Yersinia enterocolitica* O:3 bacteria, both the core region and the O-Ag persisted in cytoplasmic vacuoles and on the plasma membrane for at least 7 days [147]. The persistence of LPS on phagocytes' surface may provide a constant source of LPS, as in the joints of arthritic patients [148]. Duncan et al. have found that after mouse peritoneal macrophages ingested bacteria, LPS was exocytosed from cells at a rate that was considerably lower than that of other bacterial constituents. After 3 days' incubation, about 40% of LPS was released, while 60% remained inside cells [149]. Although structural alterations of both exocytosed and intracellular LPSs were detected, those LPSs still retained bioactivity in mice [149;150].

4. Antibody responses to LPS

a. Antibodies that recognize the polysaccharide chain

In general, gram-negative bacterial infections induce more vigorous antibody responses to strain-specific O-antigens of smooth LPS and outer core of rough LPS than to the inner core and lipid A regions [151-154]. Antibodies directed against exposed epitopes on the LPS O-antigen and outer core are more protective to animals than are antibodies to the less-exposed inner core and lipid A region [155-157].

b. Antibody against lipid A and core

Immunization of mammals with rough mutants of gram-negative bacteria induces antibodies against the core and lipid A regions [158;159]. Because antigenic epitopes from the core and lipid A are shared among different strains of Gram-negative bacteria and lipid A is the bioactive center of LPS, it was hypothesized that antibodies against these conserved regions would protect animals from infection by a variety of bacteria and would neutralize endotoxin. Pollack et al. found that patients with higher levels of circulating antibody (both IgM and IgG) to *E. coli* LPS core had a better chance of surviving *Pseudomonas aeruginosa* septicemia [160]. Some studies have also found that antiserum elicited passively or actively against core and lipid A regions protects mice against experimentally-induced Gram-negative bacterial sepsis [151;161;162]. By contrast, other studies have shown that antisera to LPS core determinants do not protect animals from a broad range of pathogens [163-166]. Pollack et al. have demonstrated that monoclonal antibodies prepared against core and lipid A regions did not recognize their epitopes on

smooth LPS, due to epitope concealment by the overlying polysaccharide chain [167]. These results suggest that the usage of these putative cross-reactive antibodies against Gram-negative bacterial infection may be limited by their inability to react with the epitopes on native LPS.

c. Isotypes of anti-LPS antibodies

In mice, LPS specific and polyclonal responses are mainly restricted to IgM, IgG_3 and IgG_{2b} although the other isotypes are also generated [154;168-171]. Pollack et al. have found that antibodies against lipid A were all IgMs, while antibodies to the core region were mainly IgM and IgG_3 [167]. However, regarding-LPS induced polyclonal antibody responses, lipid A-rich, polysaccharide-free LPS from *Salmonella minnesota* R595 induced elevated serum levels of IgM, IgG_3 and IgG_{2b} [171]. IgG_3 is also the principle IgG isotype represented in immune responses to TI-II antigens [169;172].

Cytokines can affect isotype expression. For example, in the presence of IL-4, LPS induces splenocytes to produce IgG_1 and IgE while IgG_3 and IgG_{2b} production is diminished [173]. IFN-γ selectively increases IgG_{2a} production [174] and IL-5 enhances IgA production in B cells cultured with LPS [175]. In addition, the antibody isotype(s) depends upon the type of LPS and the substance(s) with which the LPS is mixed. Mixture of LPS with phospholipids or hydrophobic proteins induced mainly an IgG_1 response, while mixture of LPS with more hydrophilic proteins induced predominantly IgG_2 responses [176;177].

d. Protective functions of anti-LPS antibodies

LPS-specific antibodies act against Gram-negative bacterial infection and sepsis. (1) Many studies have shown that anti-LPS antibodies are able to inhibit LPS induced proinflammatory cytokine production, such as TNF-α and IL-6 *in vitro* and *in vivo* [178-184]. (2) Anti-LPS antibodies act as opsonins, both by themselves and in conjunction with complement. The constant region of IgG is recognized by Fc receptors on macrophages, DCs and neutrophils. There are no corresponding Fc receptors for IgM antibodies. However, IgM antibodies are able to activate the complement cascade efficiently and complement proteins (C3b, iC3b and C4b) can bind to complement receptors (CR1, CR3 and CR4) on phagocytes [170]. Anti-LPS antibodies and the complement they activate are then able to tag bacteria, bacterial debris and LPS, facilitating the uptake and clearance of these bacterial components by phagocytes. (3) Terminal components of complement may directly kill bacteria by forming pores in their membranes. However, although complement can bind to O-antigen on the bacterial cell surface, the long O-antigen may prevent the access of the C5b-C9 pore-forming complex to deeper bacterial membrane structures; this explains why rough mutants are much more susceptible to serum than smooth strains [185].

5. Natural antibodies.

Natural antibodies play an important role in combating Gram-negative bacterial infection. Natural antibodies are present in the serum of normal, non-immunizied animals. Most natural antibodies are of the IgM isotype, but IgG and IgA natural

antibodies have also been found [186]. A major source of natural antibodies seems to be the peritoneal CD5+ B1 cells [187-189]. Natural antibodies are encoded by germline variable genes without extensive somatic mutation and they have low affinity but broad specificities to common bacterial and self antigens, such as phosphorycholine (PC), LPS and polysaccharides [190;191]. Natural antibodies contain binding activity to LPS, especially to its O-antigen and outer core regions [189]. Natural antibodies are likely to be the first line of defense against microbial infection. Recombinase-activating gene-2 null (RAG-2 -/-) mice, which have no serum antibodies due to arrested B cell development at the pro-B stage, and Bruton's tyrosine kinase (Btk)-deficient mice, which have no CD5+ B1 cells and reduced serum levels of IgG_3 and IgM, demonstrated increased sensitivity to LPS that correlated with impaired LPS clearance. Reconstitution of RAG2-/- mice or Btk-/- mice with non-immune normal mouse serum and mouse IgM respectively before LPS challenge significantly improved the clearance of LPS from the circulation as well as mouse survival [192]. Natural antibodies not only confer resistance to purified LPS, but also to bacterial infection. In the mouse cecal ligation and puncture (CLP) model, a infectious disease model that resembles a clinical situation of systemic microbial infection, mice deficient in natural secretory IgM showed much higher mortality than did control mice, and reconstitution with IgM purified from normal mouse serum completely restored resistance to CLP [193].

6. B1 cells and marginal zone B cells.

The antibody response to LPS is mainly derived from B-1 cells. Su et al have shown that splenic B cells depleted of Ly-1 (CD5) + B-1 cells had diminished antibody response to O-Ag/core, suggesting that the antibody response to O-Ag/core was restricted to B-1 cells. In contrast, the anti-lipid A antibody response came from conventional CD5- B2 cells [154]. By using the ELISPOT assay, Reid et al. demonstrated that peritoneal B cells, of which the majority are B1 cells, had many more responders to smooth LPS than did splenocytes, which are primarily B2 cells (less than 2-5% B1 cells) [57]. Splenic marginal zone B cells are possibly another source of anti-LPS antibodies, since their ability to proliferate and differentiate into plasma cells in response to LPS is much higher than that of follicular B cells [194]. Considering the fact that B1 cells and splenic marginal zone B cells are the primary sources of antibody to TI-2 antigens such as polysaccharide, it is not a surprise that both cell populations are the major responders to LPS, especially to its polysaccharide region.

B-1 cells can be distinguished from recirculating follicular cells (conventional B-2 cells) by several features. B-1 cells are IgM^{hi}, IgD^{lo} and CD23 $(Fc\varepsilon R)^{-}$, while B-2 cells are IgM^{lo}, IgD^{hi} and $CD23^{+}$. Originally, B-1 cells were identified by their expression of CD5. Subsequently, CD5- peritoneal B cells, with similar properties, have also been found. CD5+ B-1 cells are then referred to as B-1a cells, while CD5- B-1 cells are called B-1b cells. B-1 cells reside predominantly in the peritoneal and pleural cavities. Developed early in ontogeny, B-1 cells have a long half-life and self renewal capacity.

B1 cells utilize a limited repertoire of germline V-region genes for recognition of ubiquitous bacterial and self antigens, and the V-region genes usually do not undergo somatic mutations. B-1 cells are refractory to activation through B cell receptor ligation, and they are mainly involved in response to TI antigens and autoimmunity. Splenic marginal zone (MZ) B cells share similar phenotypical and functional characteristics with B-1 cells. They are also IgM^{hi}, IgD^{lo} and $CD23^{-}$. Both MZ B cells and B-1 cells but not follicular B cells express CD9, a member of the TM4SF (transmembrane 4 super family) that plays a role in cell adhesion, cell migration, and signal transduction [195]. MZ B cells do not recirculate in lymph, they have a long half-life and high responsiveness to TI antigens, and like B-1 cells, they produce mainly IgM and IgG_3 antibodies [196;197]. The compartmentalization of B-1 cells and MZ B cells implies that they may have functions that are related to the locations they occupy in the immune system. It is proposed that B-1 cells are primarily responsible for early immune responses against microbial invasion through the gut epithelium, while MZ B cells are poised to react rapidly to blood-born microbes [196].

7. LPS and autoimmunity.

Multiple mechanisms are essential for the generation and maintenance of B cell tolerance, which include central tolerance due to developmental arrest in the bone marrow and receptor editing, as well as peripheral tolerance due to deletion, anergy and ignorance [198]. Breaking B cell tolerance may depend on exposure to bacterial molecules and T cell help. Studies have shown that some anergic B cells can be stimulated by LPS

to proliferate and differentiate into antibody-secreting cells, similar to the response of non-anergic B cells [199;200]. Some anergic and ignorant B cells with specificity for components of the cell nucleus, which are believed to be the initial antigens of systemic lupus erythematosus (SLE), would also be stimulated by LPS [201]. Thus, T cell-independent activation of B cells via TLRs may be a critical initial step in breaking B cell tolerance. Furthermore, in response to LPS, B cells or DCs upregulate the expression of costimulatory molecules, which allows B cells and DCs to present antigens to T cells and activate T cells. If T cell tolerance is also impaired (such as reduced activation-induced T cell death, AICD), these activated T cells would then drive somatic mutation and affinity maturation of self-reactive B cells to produce higher affinity auto-antibodies.

After injection of LPS in mice, DNA appeared in the circulating blood, anti-DNA antibodies were produced, and DNA-anti-DNA immune complexes were then detected in renal glomeruli, as is characteristic of SLE. The formation of immune complexes is due to the potent polyclonal B cell-activating activity of LPS but not to the release of DNA by LPS [202-204]. LPS also induced rheumatoid factor (RF) IgM in several stains of mice, including C57BL/6, BALB/c and C3H/St, but not in C3H/HeJ mice. The kinetics of the RF response to LPS is similar to that to a unrelated hapten dinitrophenyl (DNP), and to that of total IgM production, suggesting that production of IgM-RF results from polyclonal activation of B cells by LPS [205]. Although the anti-DNA antibodies are involved in the deposition of immune complex in kidneys, the presence of anti-DNA antibodies is not necessarily associated with tissue injury. Studies have shown that B-1a cells produce antoantibodies, including anti-DNA and RF antibodies [191;206]. However, it

has been established that high affinity autoantibodies to double-stranded DNA in SLE, as well as RF in rheumatoid arthritis (RA), are produced by B-2 cells but not B-1 cells [189;207].

8. B-1 cells, IL-5 and mucosal IgA

The development of peritoneal B-1 cells depends on IL-5. IL-5, a dimeric glycoprotein mainly produced by activated T cells and mast cells, promotes B-1 cell proliferation and differentiation and enhances IgA isotype switching and production [208;209]. In IL-5 -/- mice or IL-5 receptor α chain (IL-5Rα) -/- mice, the number of peritoneal B-1 cells is reduced by 50-80% [210;211]. IL-5Rα -/- mice showed lower serum IgM and IgG_3 levels than did normal mice (probably due to the decreased B-1 cell population), whereas IL5 -/- mice had normal serum antibody concentrations. Furthermore, although IL-5Rα -/- mice had normal levels of IgA in serum, IgA levels in mucosal secretions (saliva and fecal extracts) were reduced when compared with wild type mice, and, consistently, the number of IgA-producing cells in the mucosa-associated tissue were decreased [212].

IgA plays an important role in attenuating local inflammatory responses to bacteria or LPS in the gut [213;214]. Both B-1 and B-2 lineages contribute to the IgA plasma cells in the gut. 60% of intestinal IgA plasma cells derive from B-2 cell precursors in Peyer's patches, and 40% of IgA plasma cells originate from peritoneal B-1 cells, which have undergone isotype switching to IgA and populate the intestinal lamina propria [215]. B-1 and B-2 cells require different cytokines for activation and differentiation into IgA + cells in the intestine. B-2 lineage IgA+ cells are IL-6 dependent and IL-5 independent. In

contrast, B-1 derived IgA+ cells are IL-5 dependent and IL-6 independent. In the intestine of IL-5 -/- mice, although total IgA + cell numbers are slightly reduced, there is a significant depletion of B-1 lineage IgA + cells, and correspondingly, the IgA response to a B-1 antigen (phosphorycholine) but not to a B-2 antigen (ovalbumin) is decreased [216].

9. LPS and adjuvant activity.

An adjuvant is any substance that enhances immune responses to antigens that are mixed with it. It was recognized that LPS was able to serve as an adjuvant several decades ago, but the mechanisms were not fully understood [217]. The mechanisms of the adjuvant activity of LPS may have three aspects:

(1) LPS enhances DC's ability to present antigens to T cells. LPS augments almost every step of antigen presentation, including antigen processing and loading peptides onto MHC molecules [66;218]; neo-biosynthesis, stabilization and surface expression of MHC molecules [70;81;219], surface expression of costimulatory molecules [220;221]; and migration of DCs to the T cell area in lymphoid tissue [85;86]. LPS also greatly increases antigen presentation by the other two antigen presenting cells, macrophages and B cells [222-226].

(2) LPS induces cytokine production, which enhances APCs, B, T and NK cell functions. LPS stimulates DCs, macrophages, epithelial cells and other cells to secret TNF-α, IL-1β, IL-6, IL-12 and IFN-γ [227;228].

3) LPS abolishes the suppressor activity of CD4+, CD25+ regulatory T cells. The immune response to bacterial polysaccharide is greatly amplified by the use of lipid A analog monophosphoryl lipid A (MPL), which is partially due to the inactivation of regulatory T cell activity [229-231]. Recently, Pasare and Medzhitov have shown that IL-6 production by DCs in response to LPS allows pathogen-specific T cells to overcome the suppressive effect of T regulatory cells [61].

Matsuura et al. have synthesized monosaccharide lipid A analogs with alkyl-branched acyl chains instead of the usual ester-branched acyl chains. The alkyl-bonded fatty acid is presumably resistant to cleavage by AOAH. They found that, *in vitro*, analogs with alkyl branches had lower adjuvant activity than analogs with ester branches despite that they expressed higher lethal toxicity to mice. These results indicate that recognition of the ester bond or its cleavage by AOAH may be required for the adjuvant activity of LPS [41].

The adjuvant activity of LPS has been explored for vaccine development. LPS itself is excluded due to its high toxicity and pyrogenicity. Because the adjuvant activity of LPS resides in the lipid A moiety, lipid A derivatives and analogs have been developed and tested with the intention of uncoupling its toxic effects from its potentially useful immunomodulatory activity. MPL is obtained by exposing LPS from *S. minnesota R595* to acid hydrolysis, which removes the glycosidic phosphate at the 1 position and the inner core (KDO) residues at the 6' position, and subsequently to mild alkaline treatment, which causes the loss of the base-labile fatty acyl chain attached to the 3 position. The

resulting product, MPL, displays greatly reduced toxicity but retains the immunostimulatory activities of the parent lipid A [232;233]. MPL has been used as an adjuvant in numerous human vaccine trials for infectious disease and cancer indications.

In response to MPL, B cells from BALB/c mice showed antibody responses, while B cells from C3H/HeJ mice did not. Consistently, IFN-γ was produced following intravenous administration of MPL in C57BL/6 mice but not in C3H/HeJ mice [234]. These results suggest that MPL is recognized by TLR4 in mammalian cells. MPL can induce maturation of DCs, as measured by increased cell surface expression of MHC, CD80, CD86, CD40 and CD83 and production of IL-12. Although maturation induced by MPL was weaker than that induced by LPS, it seemed to be sufficient to support optimal T cell activation. Furthermore, it has been shown that MPL acts directly on T cells by increasing their intracellular calcium and up-regulating their CD40 ligand expression [235]. The dissociation of toxicity and adjuvanticity of MPL can be explained if the toxic activity of LPS requires a strong and persistent signal through TLR4 while a weak and transient signal is enough for its adjuvant activity. Alternatively, MPL may act on other cell types, such as T cells, or induce different signaling events downstream of TLR4.

E. General introduction to my dissertation research

The research to be described in this thesis was designed to improve understanding of the role of acyloxyacyl hydrolase in the inflammatory and immune responses to LPS. In chapter II, I shall present evidence showing that AOAH is present in dendritic cells, where it can deacylate the LPS in ingested *E. coli*, and that its activity in dendritic

cells can be regulated by exogenous stimuli. In chapter III, I present the initial characterization of the AOAH-null mouse, with particular attention to the impact of AOAH on antibody responses to LPS. The final chapter describes my attempts to demonstrate a role for AOAH in the deacylation of two non-LPS bacterial molecules, enterobacterial common antigen (ECA) and outer membrane lipoproteins.

CHAPTER II. STIMULUS-REGULATED DEACYLATION OF LPS BY DENDRITIC CELLS

A. Introduction

DCs, the most potent antigen-presenting cells, sense potentially dangerous microbes and initiate adaptive immune responses to them. Immature DCs reside in non-lymphoid tissues, such as skin, where they are poised to capture and process microbial invaders. Following 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 ability, start antigen-processing and becoming specialized for presenting antigens to T cells [59;60;236]. 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, peptidoglycan, bacterial lipoproteins, DNA that contains unmethylated CpG motifs, and viral double-stranded RNA) [59;60;236]. Different agonists may drive distinct maturation events and act synergistically to induce the maturation of DCs [82;83;237].

In addition to inducing adaptive immune responses to microbial antigens, DCs also contribute to innate immunity by ingesting and killing microbes [62-64;238] and by secreting mediators that recruit macrophages, natural killer cells, and eosinophils to sites of infection [59;60]. At the present time, 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, I 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 primary fatty acids (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 12:0, myristate 14:0) to form acyloxyacyl groups. Animals have an enzyme, acyloxyacyl hydrolase (AOAH), that specifically 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* [239]. 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 [122;125;126]. Mice that are unable to produce AOAH due to targeted gene disruption have exaggerated antibody responses to LPS (see chapter III).

Although AOAH had previously been detected only in neutrophils and monocyte-macrophages, we found that immature DCs also produce the enzyme. We then asked whether DCs can alter their expression of AOAH, and their ability to deacylate the LPS in ingested *E. 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 anti-bacterial responses, increasing or decreasing their ability to process this important bacterial molecule in response to environmental cues.

B. Materials and Methods

1. Reagents

Unless otherwise indicated, reagents were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. Phosphorothioate-modified CpG (5'-TCCATGACGTTCCTGATGCT-3') and GpC (5'-TCCATGAGCTTCCTGATGCT-3') oligodeoxynucleotides (ODNs) were obtained from Invitrogen, Grand Island, NY.

2. Purification of smooth LPS (*E. coli* O9)

The procedure used for extraction of LPS from *E. coli* O9 was that described by Westphal and Jann in 1965 [240]. *E. coli* O9 were collected by centrifugation, washed once with PBS, resuspended in water and incubated at 68-70°C. Equal volume of 90% phenol solution (68-70°C) was added to bacterial suspension and extracted by swirling in a 68-70°C water bath. The extraction mixture was rapidly cooled in an ice bath, and two phases were separated by centrifugation at 10,000 g for 15 min. The water phase (top phase) which contains LPS was transferred a clean tube. The phenol phase was re-extracted with water twice. All the water phases were pooled and treated with ribonuclease and deoxyribonuclease, then with proteinase K to get rid of nucleic acid and protein contamination. LPS was concentrated by precipitation with ethanol at -20°C. Then LPS was resuspended in 0.1% (v/v) thiethanolamine and stored at -20°C.

3. 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

AOAH exon, eliminating a 705 bp region that encodes untranslated mRNA, the translation start site, the leader and pro-peptide sequences, and 41 amino acids of the small subunit of the enzyme [108]. Mouse DNA was screened by Southern blot analysis using an EcoRI – BamHI probe derived from the 5' genomic sequence upstream of the long arm of the targeting vector. C57BL/6 (B6) mice were obtained from the Jackson laboratory. AOAH null B6 mice were derived by breeding AOAH null 129S6/SvEvTac mice with B6 mice for 8 generations, and then selecting progeny that carried the disrupted AOAH gene by Southern blot analysis. All mice used were bred and housed in a specific pathogen free utility at UT Southwestern Medical Center Department of Animal Resource.

4. Cell lines

XS52 is an immature DC line that was derived from newborn BALB/c mouse epidermis [241]. XS52 cells were maintained in XS medium (complete RPMI [cRPMI] supplemented with 0.5 ng/ml recombinant murine GM-CSF (rmGM-CSF, R&D Systems, Minneapolis, MN) and culture supernatant (5%, v/v) from a NS47 stromal cell line. cRPMI is RPMI 1640 (Cellgro, Herndon, VA) with 10% heat-inactivated fetal bovine serum (FBS, endotoxin < 0.06 EU/ml, Hyclone, Logan, UT), 2 mM L-glutamine (Gibco, Rockville , MD), 100 μM nonessential amino acids (Gibco), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Gibco), 10 μM sodium pyruvate, 25 mM HEPES, pH 7.4, and 50 μM 2-mercaptoethanol. NS47 cells [241] were cultured in cRPMI medium. XS106, a mature DC line established from A/J mice [242], was also carried in XS medium [242]. Both XS52 cells and XS106 cells were harvested by pipetting. The BALB/c–derived

macrophage line RAW264.7 was maintained in Dulbecco-modified Eagle medium (DMEM, Cellgro) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Murine macrophage cell lines P388D1 and J774 were cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

5. Bone marrow-derived dendritic cells (BMDCs) and peritoneal macrophages

DCs were generated from bone marrow as described by Inaba et al. [243] with minor modifications. 5-8 weeks old, male or female mice were used. Femurs and tibias were removed and kept in cRPMI on ice until all mice have been processed. The bones were immersed in a petri dish containing 70% ethanol for 2 min, and then washed twice with ice-cold cRPMI. Both ends of each bone were cut with a razor blade and shafts were transferred to a separate dish. Marrow was flushed out of the shafts with 10ml of ice-cold cRPMI using a syringe with a 25G needle. The clumps were broken up with a 10 ml syringe with a 22 G needle (5 times, up and down) in petri dish and transferred into a 50ml culture tube. The dish was washed with 10 ml cRPMI and transferred to the above tube. Cells were centrifuged for 10 min at 800 g. To lyse RBCs, cell pellets were resuspended in RBC lysing buffer (3ml per mouse) and incubated at room temperature for 5 min 5ml heat inactivated FCS were underlaid. Cells were centrifuged for 10min at 800 g, and cell pellets were resuspended in 50 ml of cRPMI. 10 μl of cell were counted, and cells were resuspended at 2×10^6 / ml in cRPMI medium containing 10ng/ml rmGM-

CSF and seeded in 6-well-plate, 3 ml/well. About 30-40 $\times 10^6$ bone marrow cells (excluding RBC) can be obtained from each mouse.

On day 3 of culture, non-adherent cells were removed and 2 ml of fresh medium containing rmGM-CSF were added. On day 5, additional 2 ml of fresh medium or medium with treatment were added. On day 7, non-adherent CD11c+ cells were purified using anti-CD11c monoclonal antibody N418 coupled to magnetic microbeads (Miltenyi Biotech Inc., Auburn, CA) (see below). Flow cytometric analysis (FACScan, Becton Dickinson, Franklin Lakes, NJ) showed that more than 95% of the sorted cells were CD11c+. These purified CD11c+ cells had DC morphology (veiled and dendritic processes) when viewed using light microscopy after cytospinning. Around 10×10^6 BMDCs can be obtained from each mouse.

Thioglycollate-elicited peritoneal macrophages were prepared as described [244]. Two months or older mice were injected with 3 ml of aged thioglycollate intraperitoneally. Five days later, mouse peritoneal cavity was flushed with 6 ml of RPMI medium and cells were collected by centrifugation. After wash with RPMI, cells were resuspended in 5 ml of cRPMI medium and seeded in 6-well-plate. 16-24 hours later, the floating cells were washed away and the adherent cells were used as peritoneal macrophages.

All cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2 and air.

6. MACS purification of CD11c+ cells from BMDC culture

The manufacturer's instructions were followed (Miltenyi Biotech Inc., Auburn, CA). On day 7 of BMDC culture, floating cells were collected, washed once with buffer

A (PBS, 0.5% heat inactivated FBS, 2mM EDTA, PH=7.2) and resuspended in buffer A at 10^7 cells/80ul. Fc receptors were blocked with 0.5 mg/ml of normal mouse IgG (Caltag Laboratories, Burlingame, CA) on ice for 45 min. 20 μl of anti-CD11c monoclonal antibody N418 conjugated magnetic microbeads was added to every 80 μl of cells. Cells were incubated with beads at 4°C for 30 min. During the time of incubation, the MS separation column was attached to the MACS MultiStand and balanced with 1 ml of buffer A. After incubation of cells with beads, 400 μl of buffer A was added to cells and total volume of 500 μl of cell suspension was applied to column. The column was washed 3 times with 500 μl of buffer. 1 ml of cRPMI was applied to the column, the column was removed from the magnetic field and cells were flushed out with a plunger. The purity of positive selected cells was analyzed by flow cytometry.

7. Flow cytometry

XS52 cells were washed with PBS and harvested with PBS containing 2 mM EDTA. BMDCs were purified by magnetic cell sorting. About $2\text{-}5\times10^5$ cells were transferred to 1.5 ml microcentrifuge tubes and washed once with 1ml/tube of FACS staining medium (Hanks Balanced Salt Solution, 2% BSA, 10mM Hepes, pH 7.4). To block Fc binding, cells were resuspended in 45 μl of staining medium with 0.5 mg/ml normal mouse IgG and incubated on ice for 45 min. 5 μl of 1:10 diluted antibodies [anti-CD14-PE (rmC5-3); anti-CD40-PE (3/23); anti-CD86-PE (GL1), anti-CD11c-FITC (HL3) and corresponding isotype control antibodies] were added to each tube. These antibodies were all purchased from PharMingen and used at 5 μg/ml. After incubation on ice for 1

hour, cells were washed with 1 ml of staining medium twice and resuspended in 250 μl of staining medium. The samples were transferred to 12×75 Falcon tubes and analyzed by FACScan, gating on cells that excluded propidium iodide. Data were analyzed using Cell Quest (Becton Dickinson) software.

8. Induction of DC differentiation

We followed the method of Yamada and Katz [245]. Briefly, XS52 cells were cultured in cRPMI medium supplement with rmGM-CSF and IL-4 (10 ng/ml each) for 6 days; the medium was replaced with fresh medium containing rmGM-CSF and IL-4 on day 3. The cells were then cultured for 3 additional days in the presence of GM-CSF, IL-4, TNF-α (10 ng/ml each, all from R&D Systems), IL-1β (10 ng/ml, PharMingen, Franklin Lakes, NJ), 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 hours or they were incubated for 9 days; 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, 10^5 bacterial 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 37Cº for 1hour. The cells then were washed with cRPMI medium without antibiotics and cultured in XS medium that contained 50 μg/ml gentamicin to kill extracellular bacteria. The cells were harvested at 24 hours. When incubation was

continued for 9 days, this procedure was repeated on days 3 and 6, and cells were harvested on day 9.

To induce the maturation of BMDCs, 10 ng/ml LPS, 1 μM CpG ODN and control GpC ODN, 40 μg/ml *Micrococcus luteus* or a cocktail of IL-4, TNFα, IL-1β and agonistic anti-CD40 antibody were added to day 5 BMDC cultures. On day 7, the non-adherent cells were harvested and CD11c+ cells were sorted by CD11c monoclonal antibody-conjugated magnetic micro-beads.

9. Deacylation of purified LPS by cell lysates.

Double-labeled LPS ([^{14}C]-glucosamine backbone and [^{3}H]-fatty acyl chains) [246] was incubated with cell lysates for 16 hrs at 37°C in AOAH reaction mixture (fatty acid-free bovine serum albumin [1mg/ml], 5 mM $CaCl_2$, 0.05% Triton X-100, and 20 mM Tris-citrate, pH 5.0). The reaction was terminated by adding 2.5 volumes of 100% ethanol. After centrifugation to precipitate intact LPS and partially deacylated LPS, the [^{3}H]-fatty acids in the supernatant were quantitated by β-scintillation counting (Packard Instrument Company, Downers Grove, IL). Confirmation that the deacylating activity was AOAH-like (releasing only 12:0 and 14:0 from the LPS) was obtained using TLC [106].

10. Deacylation of purified LPS by DCs

LPS with [^{3}H]-labeled fatty acyl chains was purified from *E. coli* LCD25. 1 μg of [^{3}H]-labeled LPS was added to 5×10^5 DC culture, and incubated with cells for 6 and 24 hours. After incubation, both cells and medium were harvested, and 2.5 volume of ethanol was added. The amount of released fatty acids, which was ethanol soluble, was

measured by β-scintillation counting. The amount of fatty acids released from cell culture medium only (no cells) at 6 and 24 hours was used as background deacylation.

11. Deacylation of LPS in whole *E. coli*

E. coli LCD25, an aceEF, gltA strain of *E. coli* K12, is unable to produce its own acetate or use acetate as carbon or energy source [247]. When LCD25 cells are cultured in 2 × minimal medium with sodium 2-[^{14}C]acetate (New England Nuclear Life Science Product, Boston, MA), ^{14}C is exclusively incorporated into fatty acyl chains. The method of Katz *et al* [119] was followed to [^{14}C]-label fatty acyl chains in LCD25. LCD25 were cultured for 16 hours at 30°C in 2 × minimal medium containing 0.8 mM sodium acetate. 2 × minimal medium is composed of 0.8% glucose, 0.08% L-glutamate, 2% of L-leucine, L-isoleucine, L-valine, L-methionine each, 0.02% thiamine, 0.04% $MgSO_4.7H_2O$, 0.4% citric acid·H_2O, 2% K_2HPO_4, 0.7% $NaNH_4HPO_4·4H_2O$, 50μg/ml of kanamycin. LCD25 were then centrifuged and washed once with PBS and subcultured at a starting concentration of 2.4× 10^7 bacterial CFU/ml in 2× minimal medium containing 0.3 mM sodium 2-[^{14}C]acetate (17 μCi/ml) at 30°C for 20 hours. Fatty acyl chains in bacterial LPS and phospholipids were then [^{14}C] labeled, yielding about 20,000 dpm /10^6 bacterial CFUs. ^{14}C-labeled LCD25 bacteria were added to XS52 cells (cell: bacteria ratio = 1:50) in XS medium without antibiotics and incubated for 1 hr at 37ºC. The cells were then washed and incubated for 6 or 24 hrs in XS medium with 50 μg/ml gentamicin. For BMDCs, non-adherent cells were harvested on day 7 of culture, mixed with [^{14}C]-labeled LCD25 (cell: bacteria ratio = 1:50) in cRPMI medium without antibiotics, and incubated

at 37°C for 1hr. Then the CD11c+ cells were purified by magnetic micro-beads and the CD11c- cells and unbound bacteria were washed away. The sorted CD11c+ cells were cultured in cRPMI supplemented with 10 ng/ml rmGM-CSF and 50 μg/ml gentamicin for 6 or 24 hours. The cells and culture media were pooled to measure LPS deacylation. At the 0 hr time point, samples were harvested immediately after [^{14}C]-labeled LCD25 bacteria were added to DCs.

12. Bligh-Dyer (B-D) extraction

To isolate LPS, partially deacylated LPS and fatty acids, method of Bligh and Dyer's was used [248]. Briefly, 0.5 ml of sample was mixed with 10 μl of 25% triton X-100, 2 ml of methanol, 1 ml of chloroform and 5 μl of glacial acetic acid. After incubation at room temperature for at least 1 hour, 1.5 ml of 0.05 M potassium chloride and 1 ml of chloroform were added. The mixture then was centrifuged and 2 phases were separated. Hydrophobic fatty acids were present in chloroform phase, while amphipathic LPS or partially deacylated LPS stayed at the interface of chloroform and water/methanol phases.

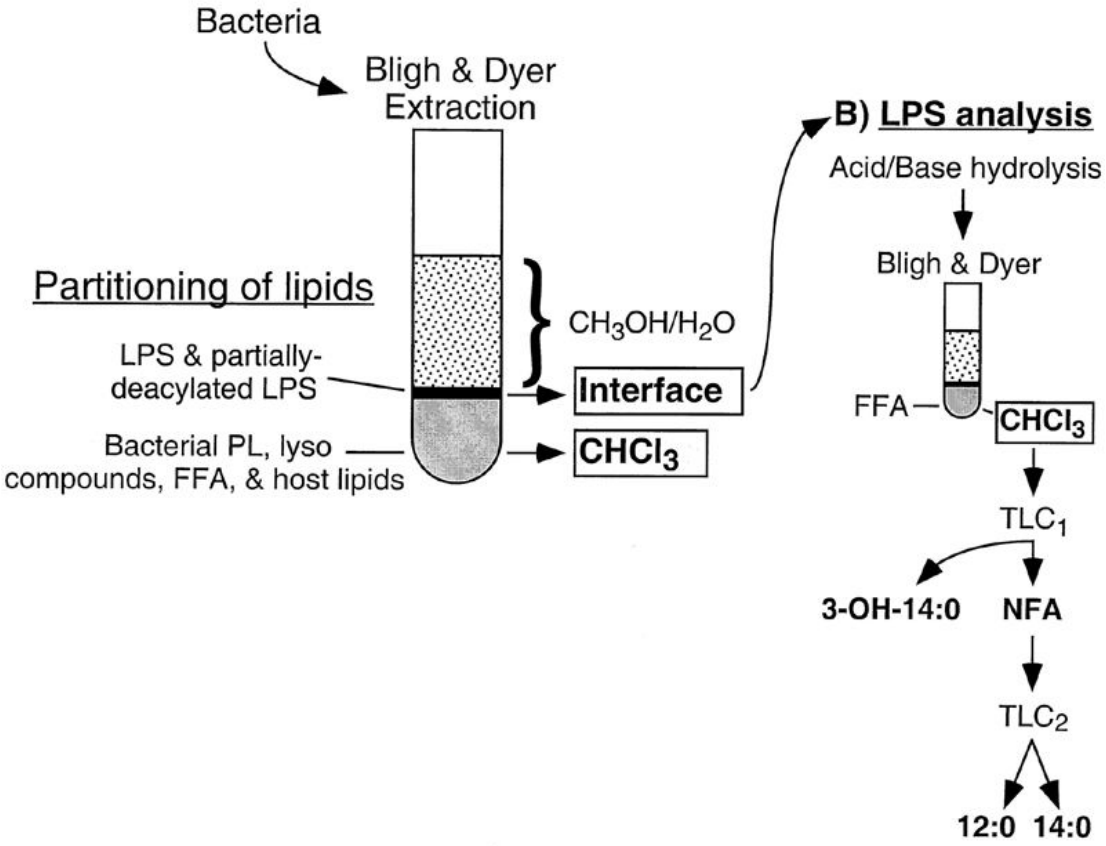
13. Assay of deacylation of LPS in whole bacteria

LPS deacylation was quantitated by calculating the loss of the fatty acyl chains from LPS [119] (Fig. 6A). The interface of a B-D extraction, which contains LPS and partially deacylated LPS, was washed 3 times with chloroform and dried under argon. The interface was then hydrolyzed with 4 M HCl and 4 M NaOH [244]. The hydrolyzed interface was extracted again and the chloroform phase, which contains released fatty acids, was recovered, dried and resuspended in 100 μl of methanol: chloroform 1:1 (v:v).

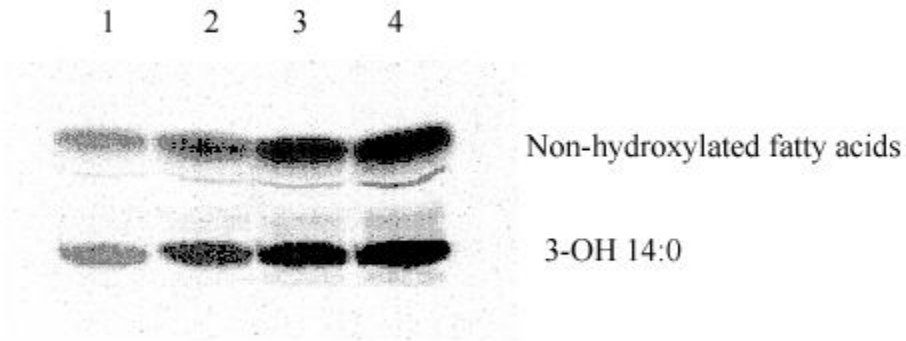
The interface-derived fatty acids were resolved by thin layer chromatograph (TLC). First, the primary (3-OH-14:0) and secondary (12:0, 14:0) fatty acids were separated using TLC system 1 (Silica gel G, petroleum ether: diethyl ether: acetic acid =70:30:1) (Fig. 6B). The two bands containing 3-OH-14:0 and the non-hydroxylated fatty acids were visualized by phosphorimager (Molecular Dynamics, Sunnyvale, CA). Then the 3-OH-14:0 band was scraped and the radioactivity was quantitated by β-scintillation counting. The non-hydroxylated 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) (Fig. 6C); the plates were developed, dried and developed again to get better separation of fatty acids. The bands corresponding to each species of non-hydroxylated fatty acid (12:0, 14:0, 16:0) were scraped and quantitated. The fatty acyl constituents in each band on TLC plate were identified by comparing with fatty acid standards using HPLC and TLC (see below). The dpm recovered from each band was normalized to the total dpm measured for each sample before extraction. The values at t=0 hr were set as 100%, and the values at other time points were converted to percentages by comparing them to the t=0 hr value. For example, 12:0 attached to LPS at 24 hrs (%) = (12:0 recovered at 24 hrs/total dpm of 24 hr sample before extraction) / (12:0 recovered at 0 hr/ total dpm of 0 hr sample before extraction) ×100. The distribution of the radioactivity in 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.

A


Bacteria
Bligh & Dyer Extraction
Partitioning of lipids
LPS & partially-deacylated LPS
Bacterial PL, lyso compounds, FFA, & host lipids
CH_3OH/H_2O
Interface
$CHCl_3$
B) LPS analysis
Acid/Base hydrolysis
Bligh & Dyer
FFA
$CHCl_3$
TLC_1
3-OH-14:0
NFA
TLC_2
12:0
14:0

B


1
2
3
4
Non-hydroxylated fatty acids
3-OH 14:0

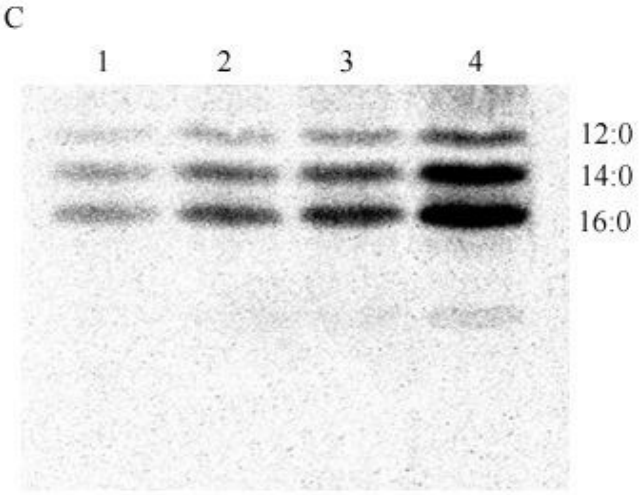


Figure 6. Analysis of the deacylation of LPS in whole bacteria.

(A) Schematic representation of procedure for lipid analysis [119]. Samples were subjected to B-D extraction. LPS and partially deacylated LPS, which is present at the interface of chloroform and water/methanol phases, were isolated and hydrolyzed using acid and subsequently base. The released fatty acids were then purified by another step of B-D extraction and this time the chloroform phase, which contained the hydrolyzed free fatty acids, was collected and concentrated. Two steps of TLC were performed to separate and quantitate fatty acyl constituents. (B) TLC system 1. The primary (3-OH-14:0) and secondary (12:0, 14:0) fatty acids were separated by using silica gel G plate and solvent system I: petroleum ether:diethyl ether:acetic acid = 70:30:1. (C) TLC system 2. The non-hydroxylated fatty acids were extracted from the silica gel and resolved using reverse-phase KC18 plate and solvent system II: acetic acid: acetonitrile = 3:7. The plates were developed, dried and developed again. Lanes 1-4 in (B) and (C) represent fatty acids derived from the B-D extraction interface of 2.5, 5, 10, and 20 μl [^{14}C]-labeled-LCD25 bacteria.

14. Identification of fatty acid constituents in TLC bands by HPLC

To identify fatty acyl constituents in TLC bands, bands corresponding to 12:0, 14:0 and 16:0 on TLC II plate were scraped, extracted using B-D method and applied on HPLC (Waters 840 HPLC instrument equipped with a β-RAM radioactive flow detector). The retention time of fatty acyl chains in each band was compared with fatty acid standards. The retention times are shown in the table 1. The 12:0 band on TLC contains only 12:0, while 14:0 band contains 14:0 and a small amount of 16:1, 16:0 band contains mainly 16:0 and a little 18:1.

Table 1. Comparison of fatty acids extracted from TLC plates with fatty acid standards by HPLC.

Fatty acid	12:0	14:0	16:1	16:0	18:1	18:0
Retention time (min) Standards	10.412	16.888	19.734	29.285	34.803	45.732
Major peak derived from TLC plates	10.153	16.535		28.703		

Stationary phase, C_8 column, 4.6mm×250mm (Whatman); mobile phase, 84.9% methanol, 0.1% acetic acid, 15% water, room temperature, flow rate = 1ml/min.

In addition, fatty acid standards and fatty acids hydrolyzed from LCD25 B-D extraction interface of were loaded on parallel lanes on TLC plate and developed. The plate was dried and visualized by Phosphorimager (Fig. 7).

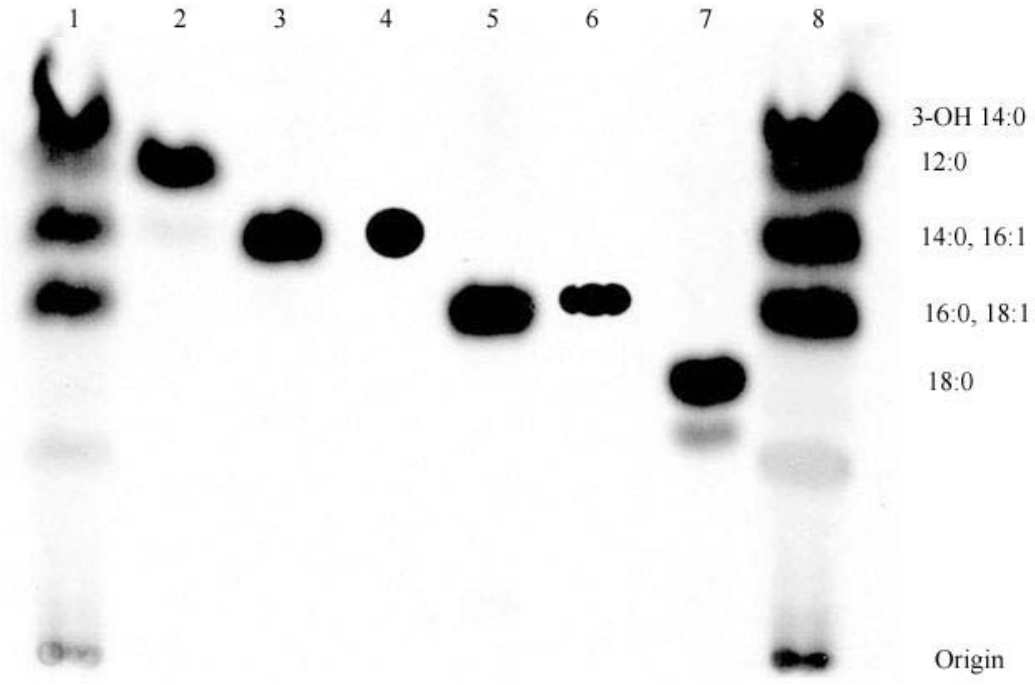


Figure 7. Identification of fatty acids derived from the interface of Bligh-Dyer extraction by TLC.

Lanes 1 and 8, fatty acids released from B-D extraction interface; Lane 2, [^{14}C]-labeled 12:0; Lane 3, [^{14}C]-labeled 14:0; Lane 4, non-radiolabeled 16:1; Lane 5, [^{14}C]- labeled 16:0; Lane 6, non-radiolabeled 18:1; Lane 7, [^{14}C]-labeled 18:0. After the TLC plate was developed and dried twice, the plate was exposed to iodine vapor, and non-saturated fatty acids (16:1 and 18:1) were stained brown and those brown bands were marked with [^{14}C]-labeled 12:0 so that the migration position of 16:1 and 18:1 could be visualized with Phosphorimager. TLC system 2, reverse-phase KC18 plate, and solvent system II (acetic acid/acetonitrile (3:7, v:v)) were used in this system.

15. Validation of the lipid analysis method

In the above assay, there are multiple steps of B-D extraction and separation of fatty acids by TLCs. To test whether the interface fatty acids recovered at the end correlate with those present at the beginning of the analysis, 2.5, 5, 10, and 20 μl of [^{14}C]-

labeled *E. coli* LCD25 were analyzed, and the recovered fatty acids were plotted against the amount of bacteria added at the beginning (Fig. 8). The recovered fatty acids (3-OH 14:0, 12:0, 14:0 and 16:0) showed good proportionality with the amount of bacteria added (r^2=0.99). All of the lipid analyses I have done are within this linear range.

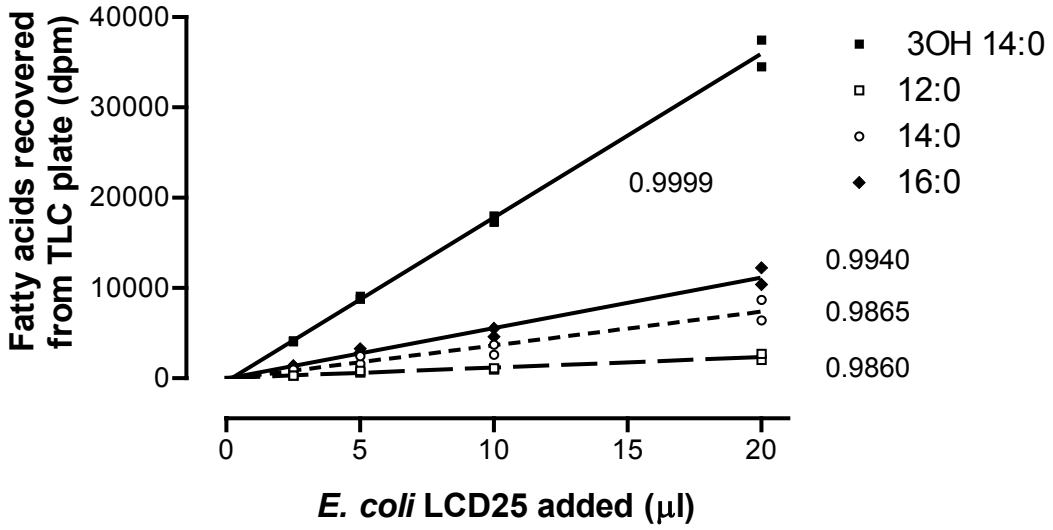


Figure 8. Validation of the lipid analysis method.

In the lipid analysis described above, the recovered fatty acids from 2.5, 5, 10, and 20 µl of [^{14}C]-labeled *E. coli* LCD25 correlate with the amount of bacteria added. Each data point was assayed in duplicate. The numbers shown are r^2.

16. Bacterial protein degradation

To label bacterial proteins, LCD25 cells were cultured in 2×minimal medium plus 1 mM sodium acetate with 0.04 µM [^{3}H]-arginine (New England Nuclear Life Science Product) and 10 µM non-radiolabeled L-arginine, yielding about 3,500 [^{3}H] dpm /10^6 bacterial CFUs. [^{3}H]-arginine-labeled LCD25 were added to XS52 cells (bacteria:cell ratio = 50:1). Incubation, washing, and harvesting were performed as in the LPS

deacylation protocol described above. The 0 hr time-point samples were harvested immediately after the [^{3}H]-arginine-labeled LCD25 bacteria were added to DCs. 0.5 ml of each sample was mixed with an equal volume of 20% trichloroacetic acid (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 hr was considered to represent 100%, and the values at later time points were converted to percentages by comparing them to the t=0 hr value. Each experimental condition was assayed in duplicate.

17. Phagocytosis

1×10^6 DCs were washed with PBS and suspended in 0.2 ml cRPMI medium. BODIPY-*E. coli* (Molecular Probes, Eugene, OR) (0.05 ml of 10^9 bacteria/ml) were then added to the cells, which were then incubated at 37Cº in the dark for 1 hr 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 hr before adding BODIPY *E. coli* or maintained on ice during the incubation.

18. Bactericidal activity

LCD25 bacteria, labeled with ^{3}H-arginine as described above (Bacterial Protein Degradation), were added to XS52 cells in XS medium without antibiotics. Bacteria were added at a cell: bacteria ratio of 1:50. After incubation at 37ºC for 0.5 hr, the cells were then washed and incubated for 0.5 or 2 hrs in XS 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 ^{3}H dpm at this time and dividing by the ^{3}H dpm/CFU.

19. mRNA analysis

Total RNA was isolated from untreated or treated XS52 cells (RNAqueous Kit, Ambion, Austin, TX) according to manufacturer's instruction. A region of the AOAH cDNA was amplified using primers Seq_mAOAH-ex12F (CCAACTCTCTGGTGTAACTGGATTT) and Seq_mAOAH-ex12R (TCTCAAACGATGGTAAATGGATTTT). TaqMan® MGB probe (FAM™ dye-labeled) ACGAGTGGAATTGAAG and primers were designed and synthesized by Applied Biosystems (Foster City, CA). Plasmid PMF612, which contains murine AOAH cDNA, was used as a reference molecule for the standard curve calculation. TaqMan® 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 ABI PRISM® 7700 Sequence Detection System.

C. Results

1. Immature dendritic (XS52) cells and bone marrow-derived DCs express AOAH.

We first measured LPS-deacylating 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 deacylating activity that was similar to that of murine peritoneal macrophages and greater than that of the other cell lines tested (Fig. 9). Mature DCs (XS106) expressed much lower activity than did immature DCs (XS52).

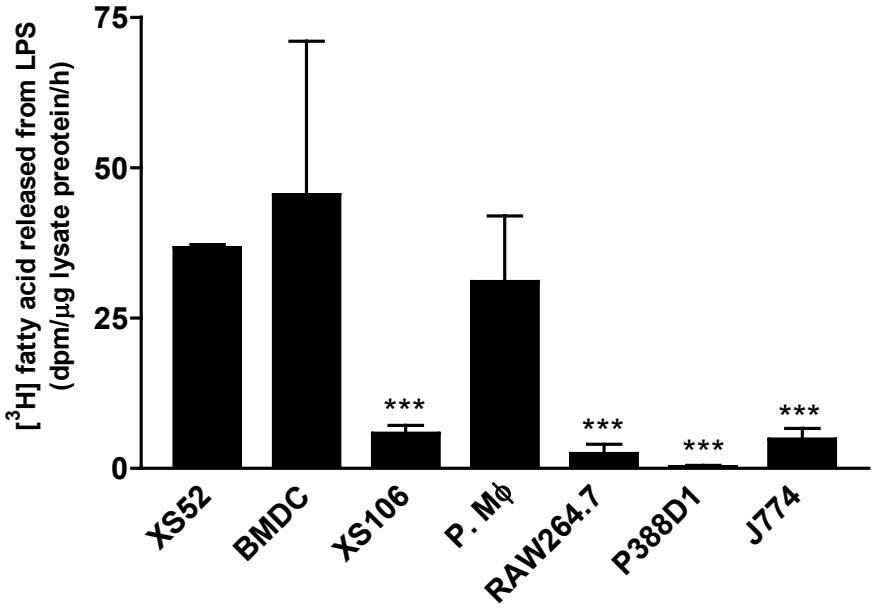


Figure 9. LPS deacylation by cell lysates.

Lysates of cultured cells were assayed for their ability to deacylate [^{3}H] / [^{14}C] labeled purified LPS as described in Materials and Methods. Each bar represents mean of three and more independent experiments. Error bars represent 1 SE. ***, significantly different from XS52 cells, P<0.001 (Student's *t* test).

2. BMDCs derived from AOAH +/+ mice can deacylate LPS while BMDCs from AOAH -/- cannot.

In the above assay, we measured purified LPS deacylation by cell lysates in the acidic buffer (pH=5.0) with detergent (0.1% Triton X-100). We then tested whether BMDCs can deacylate purified LPS in physical condition (pH=7.4, no detergent), and whether the AOAH is required for LPS deacylation. We found XS52 cells (data not shown) and BMDCs derived from AOAH +/+ mice released fatty acids from purified LPS while BMDCs from AOAH -/- mice could not (Fig. 10), indicating that DCs can deacylate purified LPS in natural condition, and that AOAH plays an essential role.

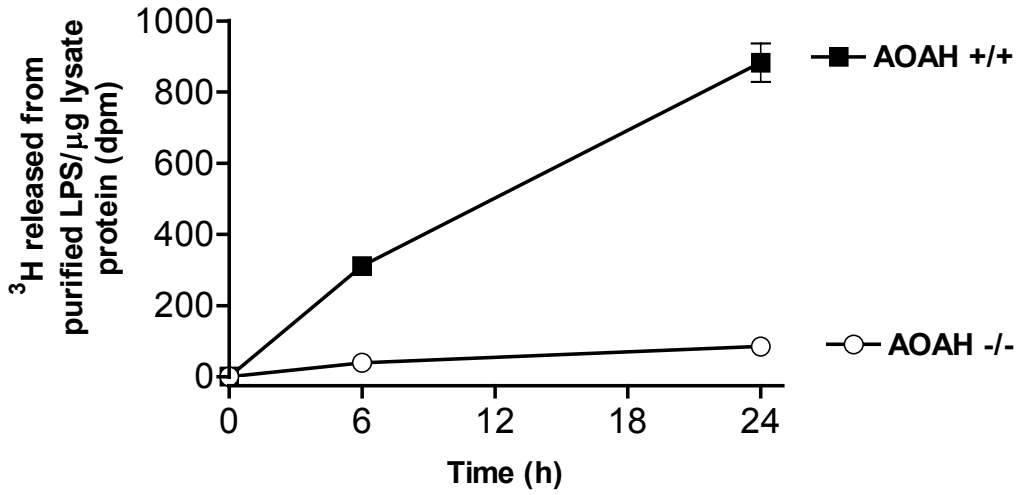


Figure 10. Deacylation of purified LPS by BMDCs

Purified LPS with ^{3}H-labeled fatty acids were added to BMDCs AOAH +/+ or -/-. After 6 and 24 hours incubation, cells and medium were harvested and the released fatty acids were measured. Data shown are means ± 1 SD.

3. XS52 cells deacylate the LPS in rough and smooth bacteria in an AOAH-like manner.

We next asked whether DCs can deacylate LPS in its natural setting, the outer membrane of Gram-negative bacteria. Since 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 XS52 cells deacylated the LPS in *E. coli* LCD25 in an AOAH-like manner (Fig. 11A). 3-OH-14:0, the primary fatty acyl chain of lipid A, was not removed from the LPS backbone, whereas the non-hydroxylated (secondary) fatty acids (12:0, 14:0) were cleaved over time. Because *E. coli* LCD25 is a Ra (no O-Ag) mutant strain, then we tested whether DCs can deacylate LPS in smooth bacteria. A plasmid containing O9-antigen synthesis genes [249] was introduced into *E. coli* LCD25 and the expression of O9-antigen in the transfected bacteria was confirmed. The transfected bacteria were lysed and treated with protease K. Analysis by SDS-PAGE and subsequent silver staining showed a typical "ladder pattern" of regularly spaced bands. We found that XS52 cells were able to deacylate smooth LPS in whole bacteria in an AOAH-like manner (Fig. 11B).

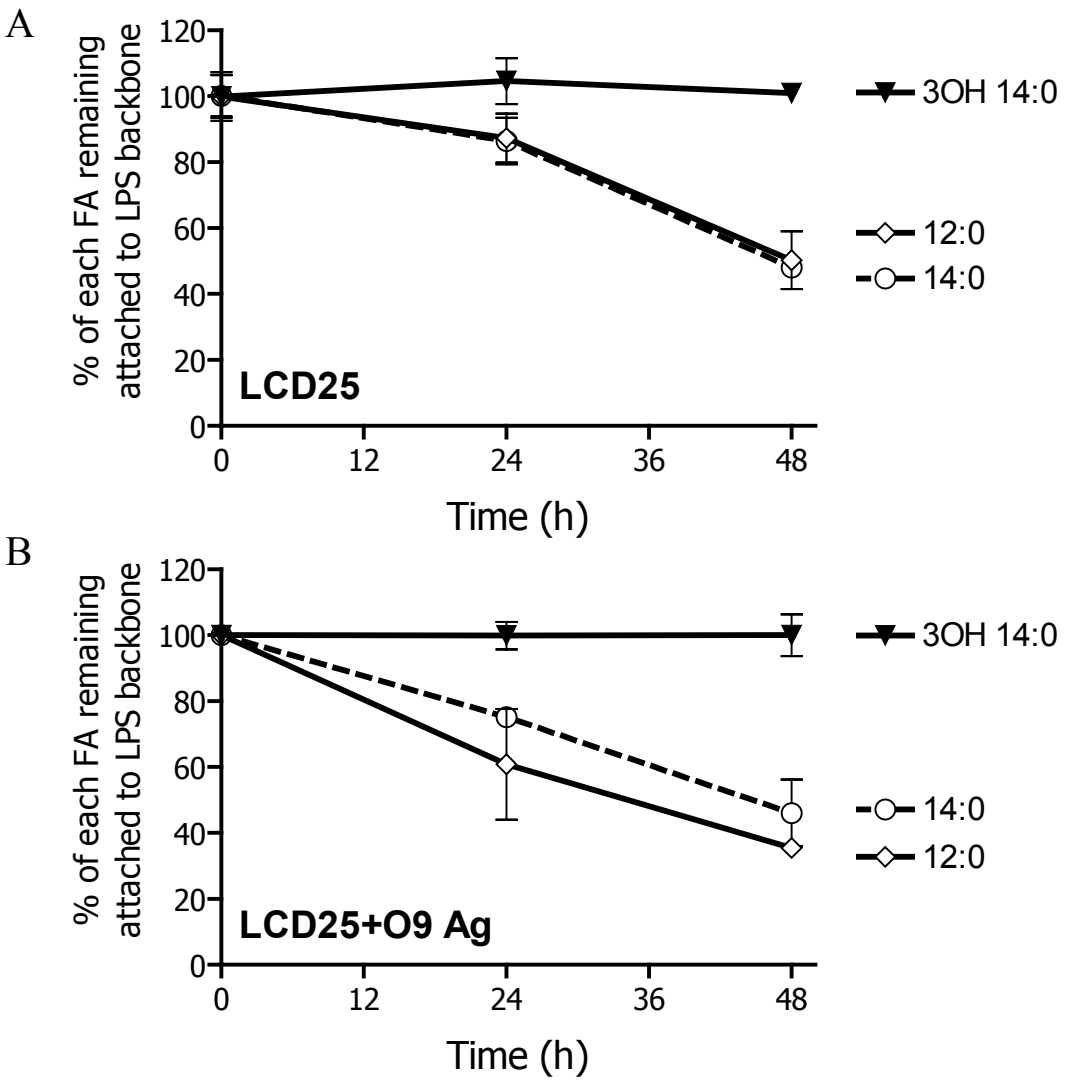


Figure 11. Deacylation of LPS in *E. coli*.

(A) XS52 cells deacylate LPS in whole bacteria in an AOAH-like manner. The secondary fatty acyl chains (12:0 and 14:0) were released while primary fatty acid (3-OH 14:0) remained attached. (B) XS52 cells deacylate LPS-O9 in whole bacteria in an AOAH-like manner.

4. AOAH plays an essential role in deacylating LPS in whole bacteria.

Similar to XS52 cells, BMDC from AOAH +/+ mice deacylated LPS in *E. coli* LCD25 in an AOAH-like manner. However, BMDC from AOAH -/- mice deacylated significantly less LPS than did their wild type counterparts (Figure 12); the apparent removal of 20-30% of the 12:0 by the -/- BMDC is unexplained, since they were unable to deacylate purified LPS (Fig. 10) and peritoneal macrophages from AOAH -/- mice did not deacylate the LPS in *E. coli* (Fig. 12).

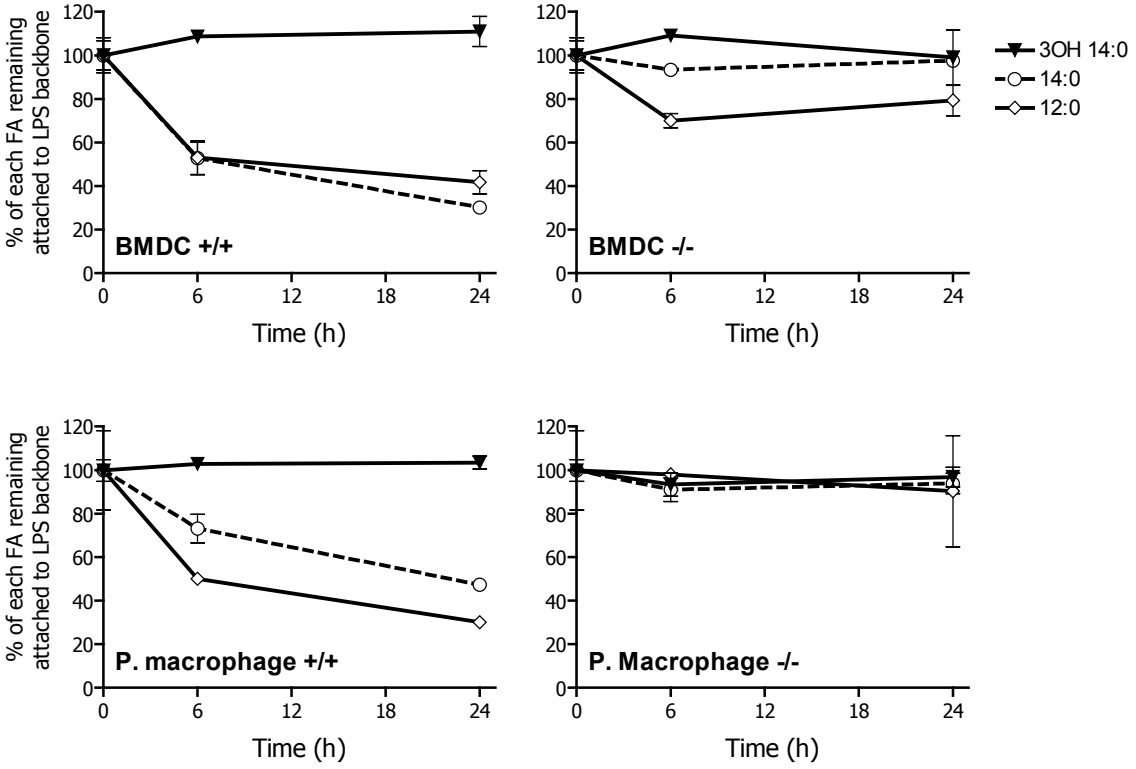


Figure 12. Deacylation of LPS in *E. coli* LCD25 by BMDCs or P. macrophages from AOAH +/+ or -/- mice.

BMDCs and P. macrophages deacylate LPS in whole *E. coli* in an AOAH-like manner. LPS deacylation was totally abolished in P. macrophages from AOAH -/- mice. However, BMDCs from AOAH -/- mice could deacylate small amount of LPS.

5. 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 [245], we treated XS52 cells with 10 ng/ml of IL-4 for 6 days and for an additional 3 days 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. 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 [75;237].

After treatment with IL-4 for 6 days and the cytokine cocktail for 3 more days, CD40 expression on XS52 cells had increased a little (Fig. 13A), while CD14 expression (Fig. 13A) and phagocytic activity (Fig. 13B) had decreased. These changes were accompanied by a 6-fold decrease in AOAH activity (Fig. 13C).

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 days, their surface expression of CD40 increased, while CD14 expression, phagocytic activity and AOAH activity were maintained or slightly increased (Figure 13A-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 hours, 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 (data not shown).

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 the panel D of Figure 13, AOAH mRNA abundance decreased 8-fold after treatment with the maturation cocktail and increased 2-fold after treatment with LPS.

Maturation of XS52 cells, when induced by pro-inflammatory cytokines and an agonistic CD40 antibody, was thus associated with decreases in CD14 expression, phagocytic activity, and AOAH activity, while CD40 expression was enhanced. Although treatment with LPS or Gram-negative bacteria was also followed by increased expression of the co-stimulatory molecule, CD40, the cells maintained their ability to recognize LPS, internalize bacteria and deacylate LPS.

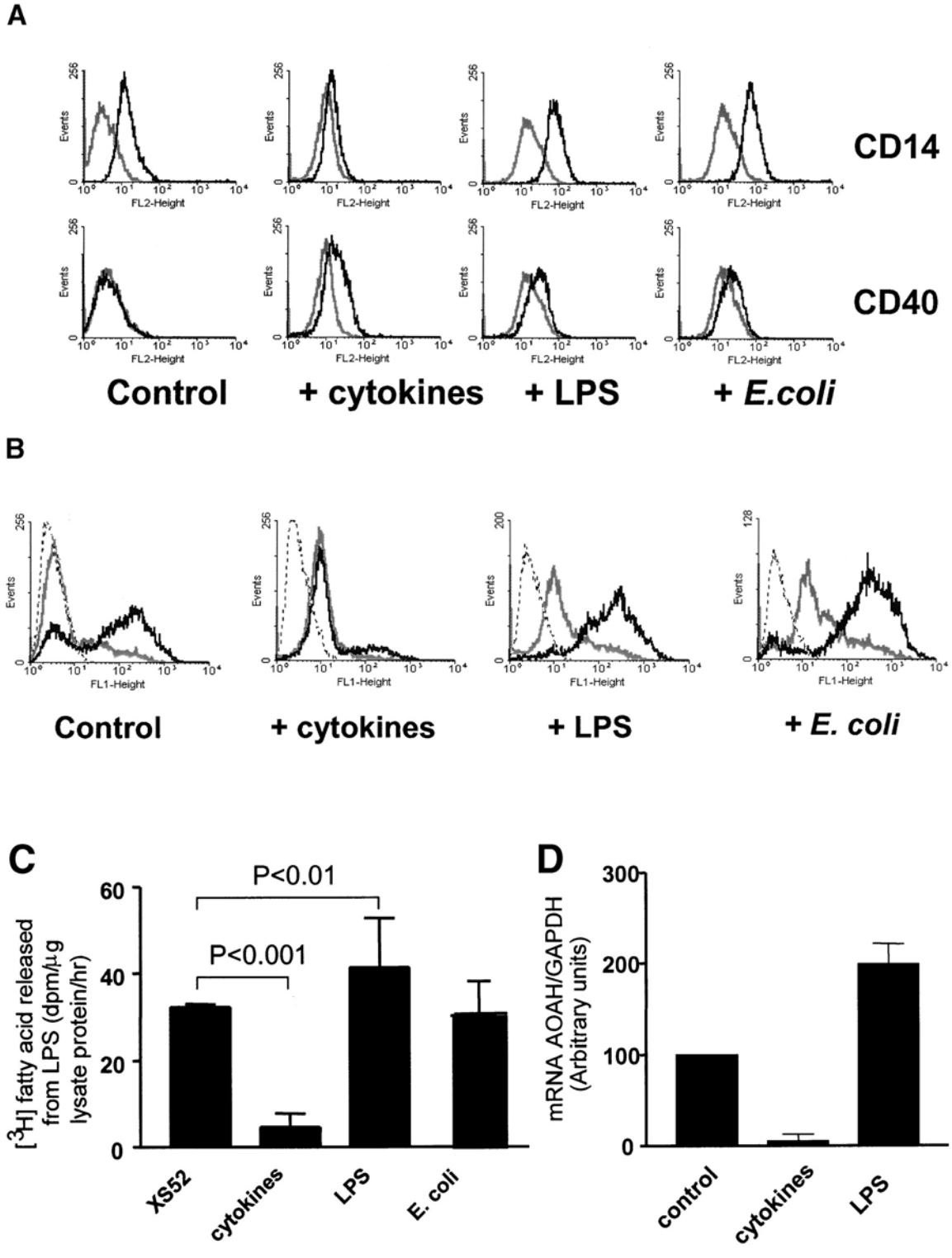

A
CD14
CD40
Control
+ cytokines
+ LPS
+ E.coli
B
Control
+ cytokines
+ LPS
+ E. coli
C
P<0.01
P<0.001
[3H] fatty acid released from LPS (dpm/μg lysate protein/hr)
XS52
cytokines
LPS
E. coli
D
mRNA AOAH/GAPDH (Arbitrary units)
control
cytokines
LPS

Figure 13. 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), LPS (10 ng/ml) or *E. coli* (10^5 CFU/ml) for 9 days (see Methods). Black line, specific antibody; grey 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*; grey 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 9 independent experiments. (D) AOAH mRNA abundance as assessed by real-time PCR measurements of AOAH and GAPDH mRNA. The data are combined from 2 independent experiments. In C and D, the bars represent means ± 1 SD.

6. Regulation of AOAH activity in BMDCs.

We treated BMDCs with IL-4 and the same maturation cocktail for 2 days. CD40 and CD86 expression increased (Fig. 14A), while phagocytic activity decreased (Fig. 14B). Immature BMDCs expressed very little CD14, and treatment with maturation cocktail did not change its expression (data not shown). Cytokine-induced maturation decreased AOAH activity by 6-fold (Fig. 14C).

When BMDCs were treated with 10 ng/ml LPS for 2 days, they expressed more surface CD40 and CD86 (Fig. 14A) and slightly more CD14 (as previously reported [250]; data not shown), while their phagocytic activity decreased (Fig. 14B). AOAH activity increased by 3-fold (Fig. 14C). Up-regulation of co-stimulatory molecules has also been described in Salmonella-infected BMDCs *in vitro* [71] and in response to LPS *in vivo* [85].

Both maturation cocktail and LPS thus augment cell surface CD40 and CD86 expression in BMDCs and decrease their phagocytic activity. Maturation cocktail decreases BMDC AOAH activity while LPS increases it.

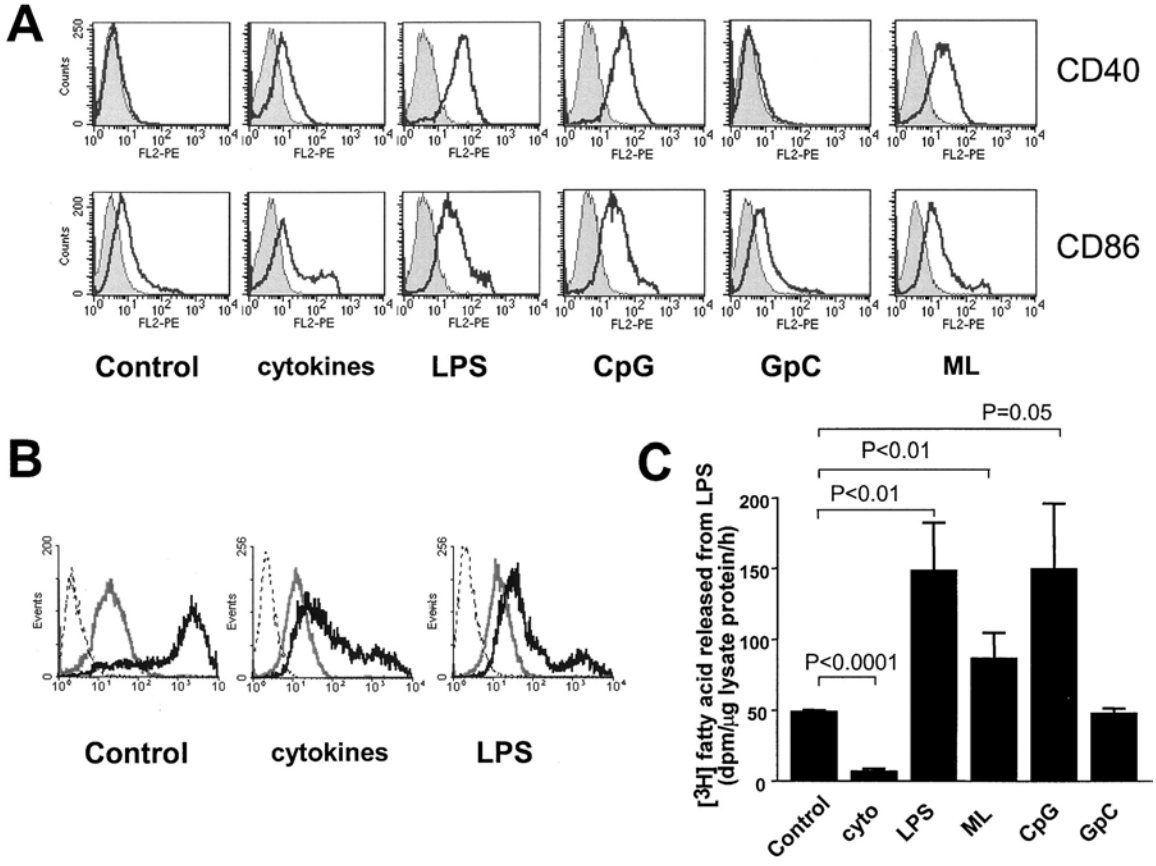


Figure 14. Maturation-induced changes in BMDC.

(A) Cell-surface expression of CD40 and CD86 on BMDCs and on BMDCs treated with maturation cocktail (see Methods), LPS (10 ng/ml), CpG ODN (1 μM), GpC ODN (1 μM), and *Micrococcus luteus* (40 μg/ml) for 2 days. Black line, specific antibody; grey solid histogram, control antibody of the same isotype. (B) Phagocytosis of BODIPY-labeled *E. coli*. Dotted line, cells only; black line, cells with BODIPY-*E. coli*; grey 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 2 or more independent experiments. In C, the bars show the means ± 1 SE.

7. Other bacterial agonists also increase AOAH expression in BMDCs.

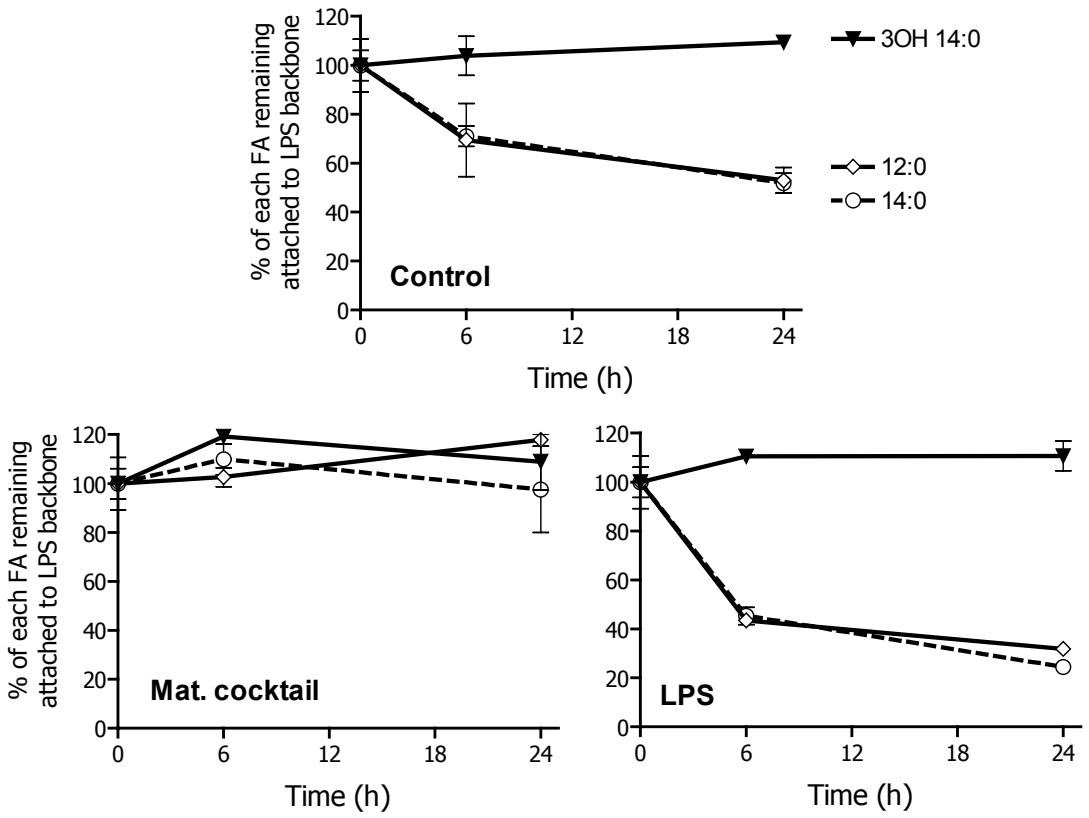
We next asked whether the augmentation of AOAH activity is LPS-specific, since LPS is an AOAH substrate. LPS is thought to activate DCs by interacting with the TLR4-MD-2 signaling complex. To find out if cell activation *via* other TLRs can increase or decrease AOAH expression, we incubated BMDCs with bacterial CpGs [251;252] (TLR9 agonist) and *Micrococcus luteus* (Gram-positive bacterium; agonist for TLR2 [253], possibly also other TLRs). As is shown in Figure 14C, CpG and ML both increased AOAH activity. These results indicate that BMDC AOAH can be regulated *via* signals downstream of TLRs other than TLR4.

8. After exposure to maturation cocktail or LPS, XS52 cells alter their ability to deacylate LPS in whole bacteria.

We then asked how the different stimuli affect the ability of XS52 cells to deacylate LPS in whole bacteria. XS52 cells that had been treated with IL-4 for 6 days and maturation cocktail for 3 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 deacylate LPS in *E. coli* was abolished in the maturation cocktail-treated group and was increased by LPS treatment (Fig. 15A). In parallel experiments, we studied the ability of the cells to degrade bacterial protein. Protein degradation was 4-fold lower in maturation cocktail-treated XS52 cells than in untreated cells, while LPS-treatment maintained the protein degradation rate (Fig. 15B). Under the conditions

studied here, maturation thus altered the cells' ability to degrade bacterial LPS and protein in a similar fashion.

A



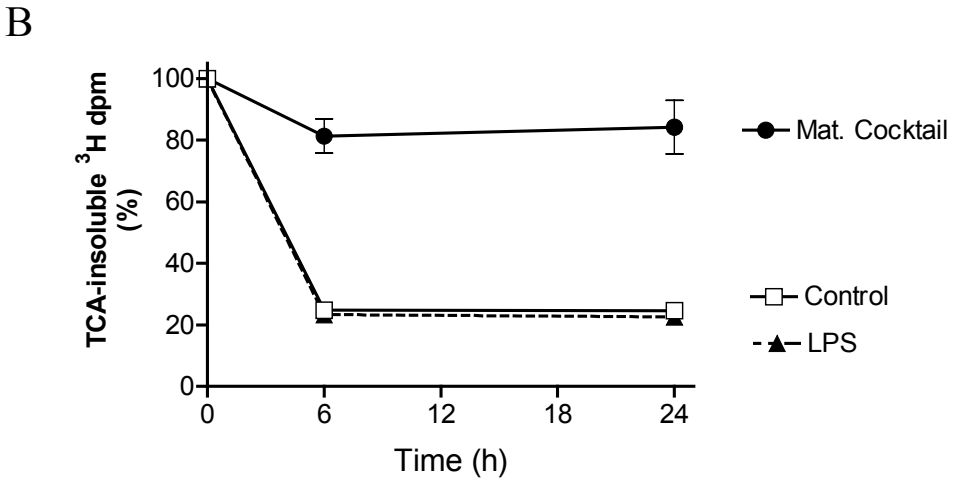


Figure 15. **Impact of exogenous stimuli on the deacylation of LPS and degradation of bacterial protein in phagocytosed *E. coli.***

(A) XS52 cells, untreated or treated for 9 days with IL-4 and maturation cocktail or LPS (see Methods), were allowed to take up [^{14}C]-labeled *E. coli* for 1 hr. After washing to remove unattached cells and further incubation for 6 hours, 30% of the 12:0 and 14:0 had been removed from the LPS backbone in untreated cells, while 60-70% was removed in LPS-treated cells ($p < 0.05$). Cytokine treatment abolished the ability of the cells to deacylate the LPS in *E. coli*. (B) XS52 cells, untreated or treated with IL-4 and maturation cocktail or LPS, were incubated with [^{3}H]-arginine-labeled *E. coli* for 1 hour. IL-4 and maturation cocktail treatment decreased protein degradation while LPS treatment maintained it. Treatment with IL-4 and maturation cocktail also reduced phagocytosis (Fig. 14B); this likely contributed to the decrease in LPS deacylation and protein degradation seen in these cells. The data shown in each panel are from 1 of 3 experiments with similar results. Error bars indicate SD.

9. Differentiation also influences the bactericidal activity of DCs.

We then studied whether treatment with IL-4 and maturation cocktail or LPS can change DCs' bactericidal activity. IL-4- and maturation cocktail-treated XS52 cells were

less able (by 50%) to kill *E. coli*, while LPS treatment maintained or slightly increased killing (Fig. 16.).

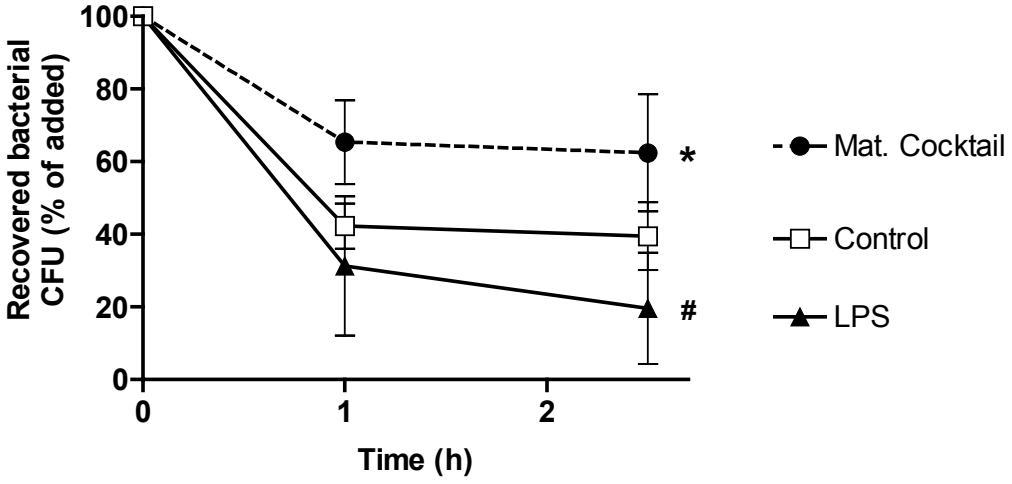


Figure 16. Impact of cell maturation on bactericidal activity.

IL-4 and maturation cocktail treatment decreased the cells' ability to kill *E. coli* by ~50%,*, $P<0.05$), whereas LPS maintained bactericidal activity or increased it by approximately 20% (#, $P=0.1$). Data shown are means ± 1 SD from 3 independent experiments.

10. AOAH is not required for bactericidal activity of DCs.

AOAH deacylation of LPS in bacterial outer membrane may contribute to killing bacteria by disintegrating bacterial membrane or increase its permeability. The ability of BMDCs from AOAH -/- mice to kill bacteria was compared with that of BMDCs from +/+ mice. BMDCs -/- killed bacteria at similar efficiency to BMDCs +/+ (Fig. 17), suggesting that AOAH does not play a role in bactericidal activity. Similarly, bone

marrow-derived macrophages from AOAH +/+ and -/- mice did not show a difference in bactericidal activity either (data not shown).

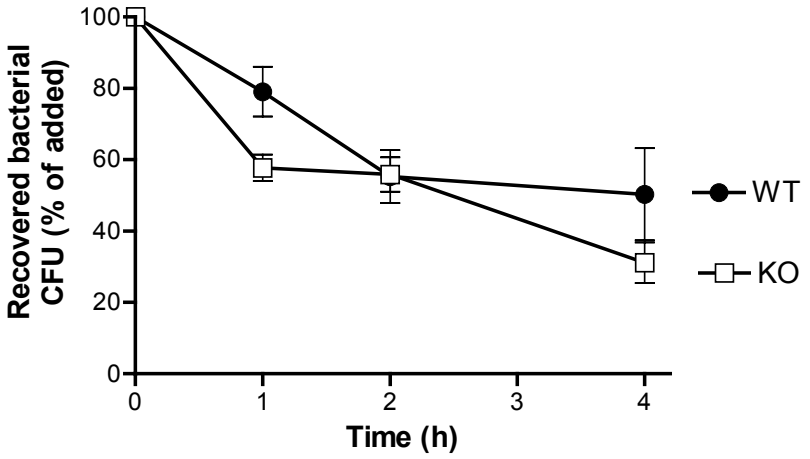


Figure 17. Bactericidal activity of BMDCs from AOAH +/+ and -/- mice.
BMDCs were prepared from either AOAH +/+ or -/- mice, and tested for their bactericidal activity. Data shown are means ± 1SD.

D. Discussion

Deacylation of the lipid A moiety of LPS was first described [105] in Dictyostelium discoideum, a slime mold that digests internalized bacteria as a foodstuff. The discovery that human neutrophils can also carry out LPS deacylation was reported in 1983 [106], 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 [254], 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 [110](A.W.

Varley, C. Johnson, R.S. Munford, 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 deacylates the LPS contained in phagocytosed bacteria. We also identified a previously unsuspected role in LPS deacylation 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 [52].

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 (chapter III); because the ability of extracellular AOAH to deacylate LPS is quite limited [120] 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. [129]; 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 [255] 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 by diminishing their ability to phagocytose, kill, and digest *E. coli*, exposure to either *E. coli* or to LPS maintained the cells' phagocytic ability, CD14 expression, AOAH activity, and the ability to kill and digest internalized bacteria. Expression of a costimulatory molecule (CD40), used here as a marker of maturation,

increased slightly during the response to all of the stimuli studied. These results are consistent with the currently accepted paradigm in which immature DCs can internalize and process bacterial antigens [68], whereas mature DCs lose these capabilities as they gain in antigen-presenting ability [59;60;76]. Together with the results of others [238;256;257], 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 [78]) 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 [91]. 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 macropinocytosis or HLA-DR and CD40 expression [84]. Furthermore, Rescigno et al. 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 [94]. In contrast, Sallusto et al. 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 macropinocytosis and Fc receptors,

disappearance of the class II compartment, and up-regulation of adhesion and costimulatory molecules [79].

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 [238].

In all phagocytes studied *in vitro* to date, LPS deacylation has occurred over many hours [115;119]. 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 [146;147], or after the LPS has been released into the phagocyte's environment [258]. 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* [122;126]. 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.

CHAPTER III. CHARACTERIZATION OF AOAH NULL MICE

A. Introduction

AOAH specifically removes the secondary fatty acyl chains from the lipid A moiety of LPS. Many studies have shown that enzymatically deacylated LPS (dLPS) not only has significantly reduced bioactivity, but it also has an antagonistic activity towards LPS. To understand the biological function(s) of AOAH *in vivo*, mice with a disrupted AOAH gene have been generated. The following hypotheses have been tested:

First, AOAH may contribute to LPS detoxification in *vivo*. Munford and Hall found that in the rabbit dermal Shwartzman reaction, an assay for tissue toxicity of LPS, AOAH-deacylation decreases LPS potency by about 100-fold [121]. In keeping with this observation, Matsuura et al. demonstrated that lipid A analogs with alkyl-branched secondary fatty acyl chains (which are resistant to AOAH cleavage) were significantly more toxic (lower LD_{50}) in mice than were lipid A analogs with ester-branched fatty acids[41]. However, because *in vitro* studies have shown that macrophages and DCs deacylate LPS very slowly [65;119], it is not known whether deacylation can counteract an animal's deleterious responses to LPS, which may often be induced in a relatively fast manner.

Second, AOAH may decrease LPS-induced antibody responses *in vivo*. LPS is a TI-1 antigen; it can induce B cell proliferation and differentiation without help from T cells. LPS can stimulate both LPS-specific and polyclonal (nonspecific) antibody

responses *in vitro* and *in vivo*. The *in vitro* mouse splenocyte mitogenicity assay has demonstrated that dLPS, with 10-fold decreased potency, is a partial agonist [121]. This reduction of LPS potency for stimulating mouse cells by AOAH treatment is not as dramatic as the 100- to 500- fold reduction seen in human cells, a difference that is due to differences in human and mouse TLR4 and MD2 [42;45]. However, these observations were based on studies that used enterobacterial LPS. Erwin et al. compared the impact of AOAH-deacylation on the bioactivity of LPSs from bacteria other than enteric bacteria, and they found that the potency of *Neisseria* lipooligosaccharide (LOS) in the murine splenocyte mitogenicity assay was reduced over 100-fold by AOAH deacylation [127]. In addition, deacylated Neisseria LOS could block the splenocyte mitogenicity of *Neisseria* and *Salmonella* LPS. Differences in the structure of the lipid A moiety may account for the different behavior of AOAH-deacylated Neisseria LOS and enterobacterial LPS toward murine cells [117]. This difference allows us to use *Neisseria* LOS to detect a significant impact of LPS deacylation *in vivo* and *in vitro*.

Animals are constantly exposed to their commensal flora as well as to microorganisms in their environment. It has been shown that at least some of the natural antibodies in blood and intestine are induced by specific antigens that animals have been exposed to [213;259;260]. By deacylating LPS and decreasing its immunostimulating activity, AOAH might play a role in reducing natural antibody production.

Third, AOAH might prevent antoantibody formation. As a consequence of polyclonal B cell activation following immunization with LPS, the concentrations of autoimmune antibodies such as rheumatoid factor [205] and anti-DNA antibodies [261]

increase in mouse sera. Izui et al. found immune complexes deposited in renal glomeruli three days after LPS injection [261]. Whether AOAH plays a role in modulating antibody responses to LPS and in preventing autoimmune diseases *in vivo* is not clear. Because the antibody response is a relatively long-term event (serum antibody usually peaks at about 7-10 days after immunization), the slow rate of LPS deacylation may have an influence on antibody production. However, AOAH expression has been found predominantly in myeloid cells, such as neutrophils, monocytes-macrophages and DCs, and not in B cells. Whether these myeloid cells can present the deacylated LPS to B cells, directly or indirectly, is not known.

Fourth, AOAH may regulate the adjuvant activity of LPS. Because enzymatic deacylation reduces the bioactivity of LPS, the adjuvanticity of LPS may also be diminished. In contrast, Matsuura et al. found that lipid A analogs with alkyl-branched secondary fatty acyl chains had significantly lower adjuvant activity *in vitro* than had lipid A analogs with ester-branched fatty acids, suggesting that the deacylation of LPS may be required for its adjuvanticity.

B. Materials and Methods

1. Reagents

Unless otherwise indicated, reagents were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO.

2. Mice

The generation of AOAH null mice by targeted disruption of AOAH gene and the maintenance of mice were described in chapter II.

3. Lethal toxicity test

Male and female mice, from 6 to 8 weeks old, were tested. AOAH wild type (WT) or knock out (KO) 129S6/SvEvTac (129) mice (8 mice per group) were challenged with 1×10^{9} bacterial CFU of living avirulent *E. coli* LCD25 per 25 gram of body weight *i.p.* 129 and C57BL/6 (B6) mice (AOAH WT or KO, 15-20 mice per group) were injected with 1.2 and 0.7 mg of purified *E. coli* LPS 0111:B4 / 25 gram of body weight respectively *i.p.* The administered doses of bacteria and LPS were lethal for 50 % of the animals (LD_{50}s), as determined in WT mice in pilot experiments. Mice were checked twice a day for 10 days and the percentage of survivors was calculated.

4. Serum and fecal pellet collection

Blood samples were collected from the tail vein and kept at 4°C for 16 hours. The sera were then isolated by centrifugation and stored at 4°C with 0.02% NaN_3. Fresh

fecal pellets were collected, weighed and suspended in PBS with a protease inhibitor cocktail (Roche) and 0.02% NaN_3 (1 ml per 100 mg fecal pellet). After the tubes were vortexed at room temperature for at least 15 minutes and centrifuged, the supernatant was transferred to fresh tubes. Samples were assayed immediately or stored at -80°C until analysis.

5. *In vitro* cytokine production by DCs in response to LPS

BMDCs from AOAH WT and KO 129 mice were prepared and seeded at 5×10^5/well in 6-well-plates. Cells were treated with 0, 3, 10, and 30 ng/ml of LPS 0111:B4, and cell culture media were collected at 6 and 24 hours. Nine cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and TNF-α) in the media were assayed using SearchLight Mouse Cytokine Array at Pierce Boston Technology Center.

6. *In vitro* BMDC maturation with LOS treatment

BMDCs from AOAH WT or KO 129 mice were seeded at 5×10^5/well in 6-well-plates and treated with 10^{-3}, 10^{-2}, 10^{-1} and 1 ng/ml of LOS or 1, 10, 10^2 and 10^3 ng/ml of dLOS for 24 hours, and the CD40 expression (mean fluorescence intensity, MFI) was determined by FACS as described in chapter II.

7. Immunization of mice

Highly purified *N. meningitidis* LOS (which will be referred to as LOS) was kindly provided by Drs. M. Apicella and J. Weiss at the University of Iowa. Both LOS and LPS (*E. coli* LPS 0111:B4) were diluted in PBS at 50 μg/ml and sonicated or

vortexed vigorously before usage. B6 or 129 mice were injected with 10 μg (200 μl) of LOS or LPS subcutaneously on the back, and then 5 μg (100 μl) was given *via* the same route 2 and 4 weeks later. Blood samples were collected from the tail veins at day 0 (before injection), 7 days after each injection, and one and two months after the third injection.

In addition to LOS or LPS, mice were immunized with other adjuvants, such as synthetic lipopeptide (tripalmitoylglycerylcysteine-Gly-Ser-Ser-His-His was synthesized corresponding to the N terminus of the T. *pallidum* subspecies *pallidum* 47-KDa lipoprotein [262]), which was generously provided by Dr. M. Norgard (UT Southwestern Medical Center), and complete Freund adjuvant (CFA, Sigma). Three groups of B6 mice were injected with 10 μg of LOS, 10 μg of lipopeptide, or 100 μl of CFA:PBS emulsion (1:1, v/v) *s.c.*, respectively. Mice were bled before and 1, 2, and 3 weeks after the first injection. Mice were boosted with the same adjuvant at week four and bled one week after the second injection.

To assay the adjuvant activity of LPS, three groups of mice received LOS (5 μg), ovalbumin (50 μg, Sigma, grade V), or LOS (5 μg) mixed with ovalbumin (50 μg) respectively. Mice were given a total of three injections *s.c.* at 2 week intervals and bled 7 days after each injection.

In the above studies, male mice from 4 to 8 weeks old (5 to 7 mice per group) were immunized, and both AOAH WT and KO mice were studied and compared.

8. Enzyme-linked immunosorbent assay (ELISA)

a. Polyclonal (total) antibody

96-well plates were coated with polyvalent goat anti-mouse IgG, IgM and IgA antibody, which was diluted 1:1000 in buffer that contained 15 mM Na_2CO_3, 30 mM $NaHCO_3$, 3 mM NaN_3, pH 9.55. After 16 hours incubation at 4°C, the plates were washed and blocked with 10% FBS PBS at 37°C for 1 hour. Samples and standards were then diluted in blocking buffer and added to the plates. After incubation for 2 hours at room temperature, the plates were washed and the detection antibodies, conjugated with HRP (anti-mouse IgM, total IgG, IgG_3, IgG_1, IgG_{2a} and IgA), were added and incubated for 2 hours at room temperature. Anti-mouse IgM, IgA and total IgG were purchased from Sigma, and anti-mouse IgG_3, IgG_1, IgG_{2a} were obtained from Southern Biotechnology Associates (Birmingham, AL). The plates were washed, and HRP substrate (PharMingen) was added. After about 10-20 minutes development, the reaction was stopped by adding 1M of sulfuric acid and plates were read at test filter 450 nm, reference filter 570 nm using a MRX Microplate Reader (Dynex Technologies Incorporation, Chantilly, VA).

b. Anti-LPS or anti-LOS antibody

E. coli 0111:B4 LPS diluted in the above coating buffer at 10 μg/ml and *N meningitidis* LOS diluted in PBS containing 10 mM $MgSO_4$ at 5 μg/ml were used to coat the wells of 96-well plates. The subsequent steps were the same as for polyclonal antibody determination.

In each step of the ELISA, 100 μl of reagents or diluted samples were added. For polyclonal antibody determination, a standard curve was generated from sera with known Ig concentrations on the same 96-well-plate and the sample concentrations were derived from the standard curve. For LOS- or LPS-specific antibody determinations, sera from mice that had received multiple LOS or LPS injections were used as the standard and arbitrary units were derived.

c. Antinuclear antibodies (ANAs)

Measurement of anti-ssDNA (single stranded DNA), anti-dsDNA (double stranded DNA), anti-histone and anti-histone/DNA were performed as described previously by Mohan et al. [263]. To determine anti-dsDNA antibody concentration, 96-well plates were precoated with 100 μg/ml of methylated BSA (mBSA) at 37°C for 1 h, and after washing, the same plates were coated with 50 μg/ml of dsDNA (Sigma, dissolved in PBS, sonicated and filtered through 0.8 μm cellulose acetate) at 37°C for 1 h. For anti-ssDNA ELISA, the same concentration of dsDNA was boiled for 10 min and chilled on ice for 10 min before adding to mBSA-precoated plates. For the anti-histone ELISA, 96-well plates were coated with 10 μg/ml of total histone (Boehringer Mannheim) at 37°C for 1 h. When the anti-histone/DNA concentration was measured, the plates precoated with histone were washed, 50 μg/ml of dsDNA was added and the then the plates were incubated at 37°C for 1 h. After the plates were blocked, serial dilutions of mouse serum were added and incubated at 37°C for 1 h. The following detection steps

were performed as described above. Fifty μl of reagents or samples were added to each well.

9. Enzymatic deacylation of LOS

Two hundred μg of *N meningitidis* LOS (non-radiolabeled LOS mixed with LOS containing [^{14}C]-labeled fatty acids) was resuspended in reaction buffer, which contains sodium acetate 30 mM, pH 5.5; Triton X-100 (0.1%); sodium chloride (0.9%), and incubated at 37°C with 1 μg of purified human AOAH for 2 or 3 days. The LOS was then precipitated with ethanol and the released fatty acids were measured. The LOS pellet was then resuspended in the above reaction mixture and the deacylation reaction was repeated until about 30% of the [^{14}C]-fatty acids were released. Mock-treated LOS was subjected to the same incubation as for enzyme treatment, in reaction buffer without AOAH.

10. Splenocyte proliferation assay and *in vitro* antibody production assay

Mice were anesthetized and then sacrificed by cervical dislocation. Their spleens were removed aseptically and transferred to a 6 cm Petri dish containing RPMI medium. The spleens were mashed into a single cell suspension by using the frosted ends of microscope slides. The cell suspension was transferred to a 15 ml Falcon tube and centrifuged. After the RBCs in the preparation were lysed (RBC lysis buffer), the cells were washed and resuspended in 10 ml of RPMI. The tube was then allowed to stand for 10 min on ice so that debris and large clumps of cells settled to the bottom. The upper 9 ml of cell suspension was transferred to a different tube and the cells were washed twice

with RPMI and counted. Splenocytes were then diluted in cRPMI medium and seeded at 3×10^5/100 μl /well in 96-well plates. Various concentrations of LOS treated with AOAH (dLOS) or mock treated LOS (LOS) or the mixture of both were diluted in cRPMI medium and added to plates (100 μl /well). The plates were incubated at 37°C for 24 hours, and 1 μCi of [^{3}H] thymidine (ICN Biomedicals, Inc., Irvine, CA, diluted in 50 μl cRPMI) was added to each well. The plates were returned to the incubator for an additional 16 hours. Cells were lysed by adding water and the DNA was harvested onto glass-fiber discs using a PHD cell harvester (Model 200A, Cambridge Technology Inc. Watertown, MA). The discs were transferred to scintillation vials and soaked in SDS-EDTA (0.2 ml) for 16 hours to elute the radioactivity before adding scintillation fluid. Each sample was assayed in quadruplicate and the stimulation index, which is the ratio of [^{3}H] incorporation into treated cells to the [^{3}H] incorporation into untreated control cells, was calculated.

To assay *in vitro* antibody production, splenocytes were incubated with LOS or dLOS for 7 days, the plates were centrifuged and the total IgM antibody concentration in the supernatant was measured.

C. Results

1. AOAH does not protect mice from LPS or gram-negative bacterial challenge.

LD_{50}s of gram-negative bacteria or LPS for AOAH WT 129 and B6 mice were first determined by challenging mice with graded doses of *E. coli* LCD25 or purified *E.*

coli 0111:B4 LPS *i.p.* These doses were then used to challenge both AOAH WT and KO mice and their survival curves were compared.

AOAH WT or KO 129 mice were injected with living avirulent *E. coli* LCD25 *i.p.* (Fig. 18A). An avirulent bacterial strain was used because bacterial virulence determinants would complicate the analysis. The survival of the WT and KO mice was similar. The absence of AOAH thus did not render mice more susceptible to gram-negative bacteria.

In whole gram-negative bacteria, in addition to LPS, there are other bacterial components that may cause sepsis by themselves or by acting synergistically with LPS, such as bacterial lipoproteins and DNA containing CpG. These components may obscure the effects of enzymatic LPS deacylation. We thus decided to challenge mice with purified LPS. 129 and B6 mice, AOAH WT or KO, were injected with purified *E. coli* LPS 0111:B4 *i.p.* and the results showed that AOAH KO mice were not more susceptible to LPS than were AOAH WT mice (Fig. 18 B and C).

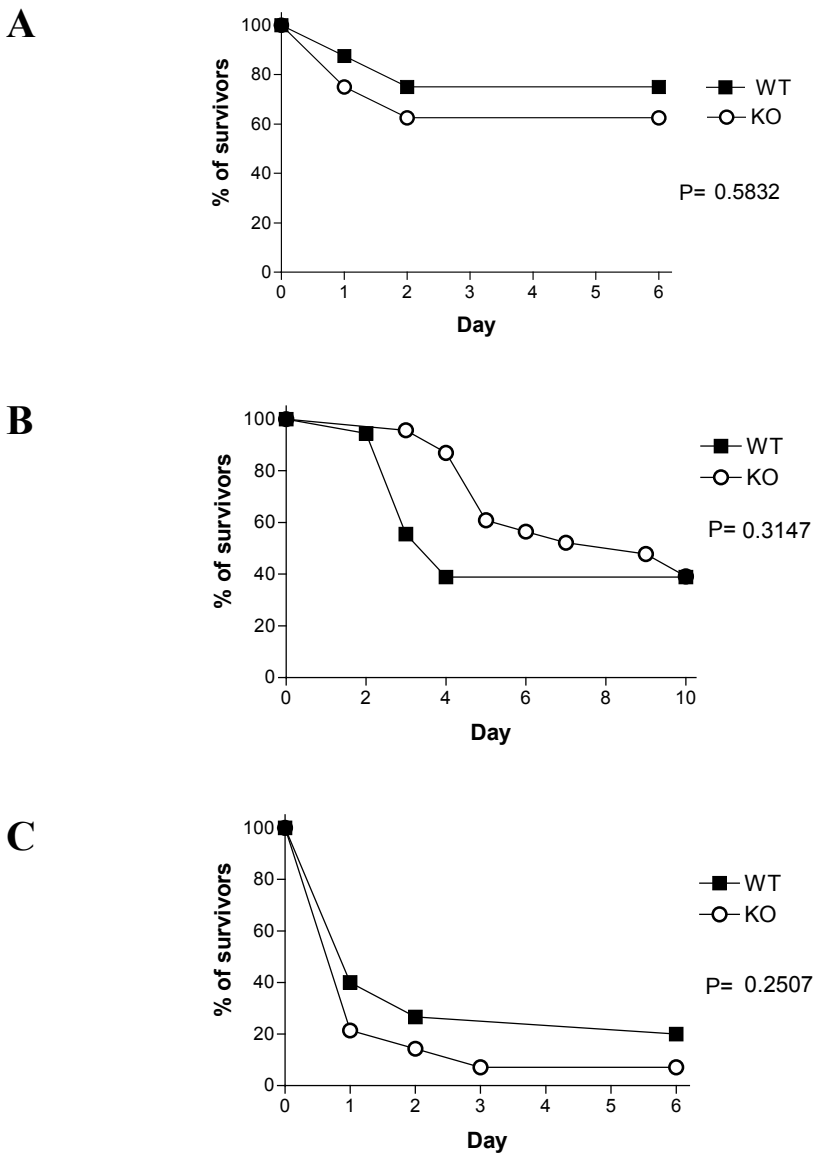


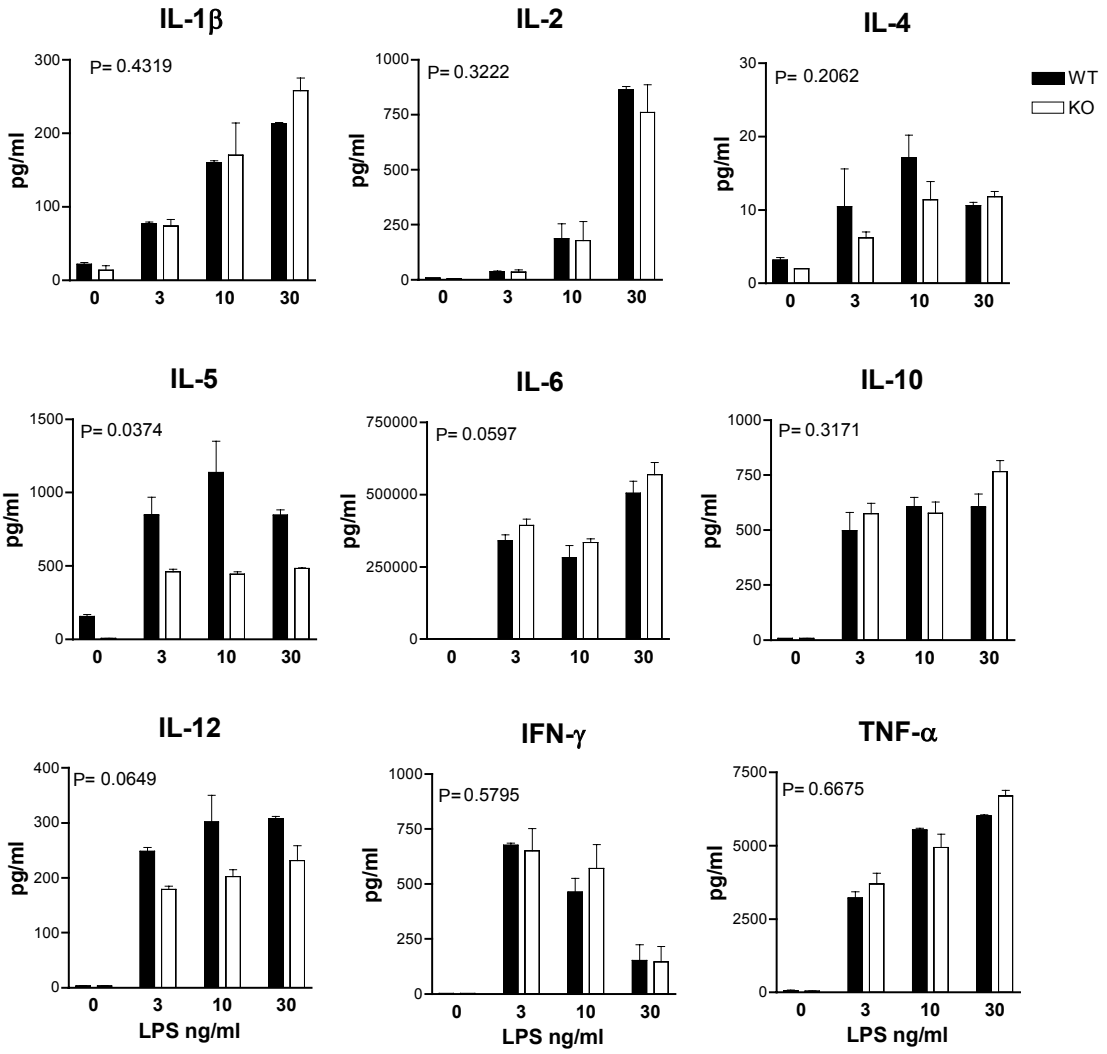
Figure 18. Comparison of susceptibility of AOAH WT and KO mice to gram-negative bacteria and purified LPS.

(A) 129 mice, 8 in each group, were challenged with living *E. coli* LCD25 *i.p.*, 1×10^9/25 g body weight. (B) 129 mice, 20 in each group, were injected with 1.2 mg *E. coli* 0111:B4 LPS/ 25 g body weight, *i.p.* (C) B6 mice, 15 in each group, were given 0.62 mg *E. coli* 0111:B4 LPS/ 25 g body weight, *i.p.* Mice were checked twice a day for 10 days and the differences between WT and KO survival curves were analyzed by the log-rank test.

2. BMDCs from AOAH KO 129 mice secret less IL-5 before and after LPS treatment than do BMDCs from AOAH WT mice.

When DCs are exposed to LPS, various cytokines are produced and secreted. It is known that DCs express quite high amounts of AOAH. Whether the presence of AOAH can diminish cells' responses to LPS (cytokine secretion) was tested. BMDCs from AOAH WT and KO 129 mice were untreated or treated with various doses of *E. coli* 0111: B4 LPS for 6 and 24 hours, then the secreted cytokines in the culture medium were determined by SearchLight Mouse Cytokine Array. For the nine cytokines I tested, AOAH WT and KO BMDCs secreted similar levels of cytokines except for IL-5 (Fig. 19), suggesting that the deacylation of LPS by BMDCs cannot in turn decrease LPS-induced cytokine secretion. Unexpectedly, BMDCs from AOAH KO mice secreted significantly less IL-5, both with and without LPS treatment, than did BMDCs from AOAH WT mice. This observation raises the possibility that AOAH influences IL-5 production by DCs and other cells.

A


IL-1β
P= 0.4319
IL-2
P= 0.3222
IL-4
P= 0.2062
WT
KO
IL-5
P= 0.0374
IL-6
P= 0.0597
IL-10
P= 0.3171
IL-12
P= 0.0649
IFN-γ
P= 0.5795
TNF-α
P= 0.6675
pg/ml
LPS ng/ml
0
3
10
30

B

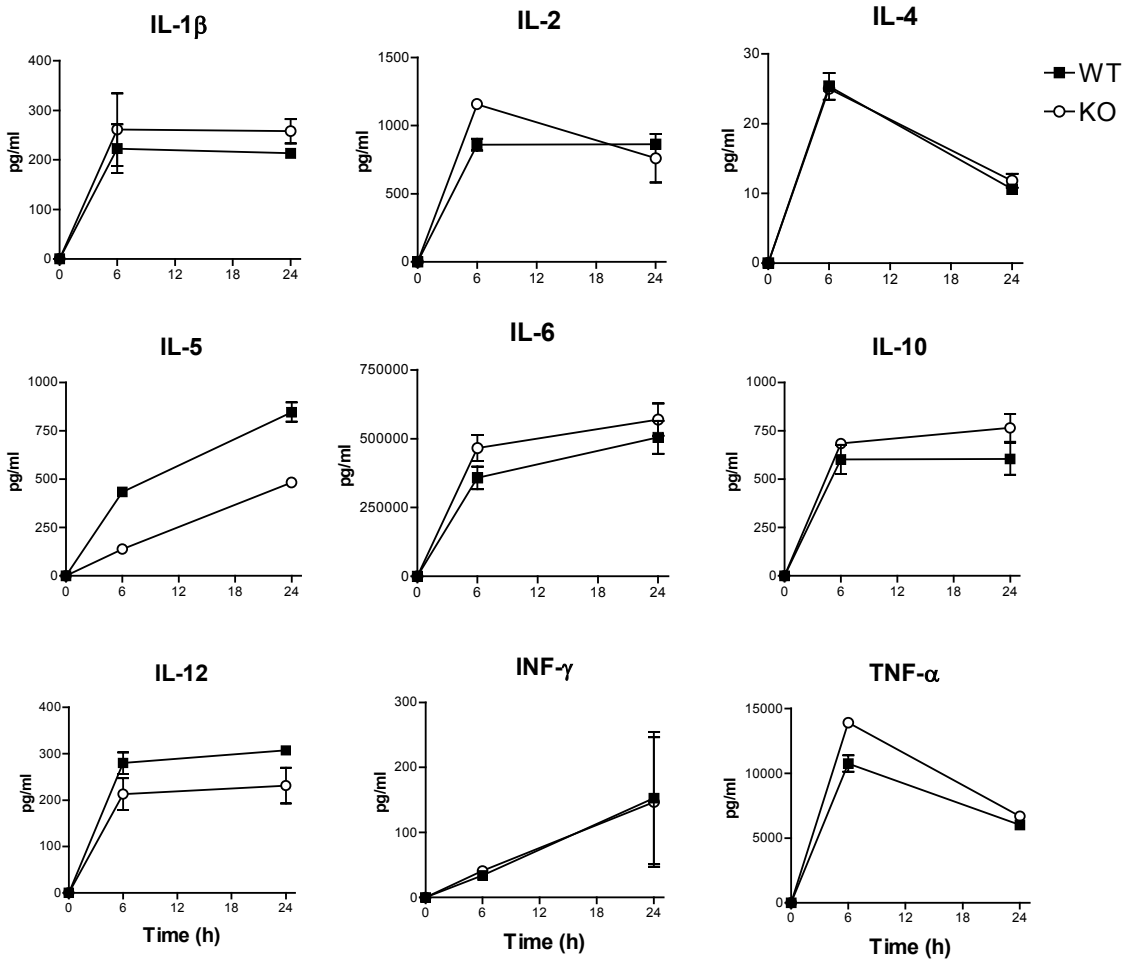


Figure 19. Cytokine production by BMDCs from AOAH WT or KO 129 mice with and without LPS treatment.

BMDCs were prepared from AOAH WT and KO mice as described in chapter II. BMDCs were untreated or treated with 3, 10 and 30 ng/ml of *E. coli* LPS 0111:B4. Cell culture media were harvested at 6 and 24 hours after incubation, and concentrations of nine cytokines were determined by SearchLight Mouse Cytokine Array at Pierce Boston Technology Center. (A) Cytokine production in response to graded dose of LPS at 24 hours. P values were obtained using paired Student's *t* test. (B) Cytokine production by BMDCs treated with 30 ng/ml of LPS at 6 and 24 hours. Cytokine levels at 0 hour were designated as 0.

3. LOS induces a similar level of CD40 expression on BMDCs from AOAH WT and KO mice.

I then tested whether the absence of LPS deacylation may cause exaggerated up-regulation of costimulatory molecules on DCs in response to LPS. BMDCs from AOAH WT and KO mice were prepared and treated with various doses of LOS or dLOS. Untreated BMDCs did not express CD40 on the cell surface. Deacylation of LOS reduced LOS's potency to stimulate CD40 expression by about 100 fold. However, the CD40 expression on AOAH KO BMDCs was not higher than that of AOAH WT BMDCs after LOS treatment (Fig. 20), indicating that the presence of AOAH does not diminish LPS-induced DC activation or maturation.

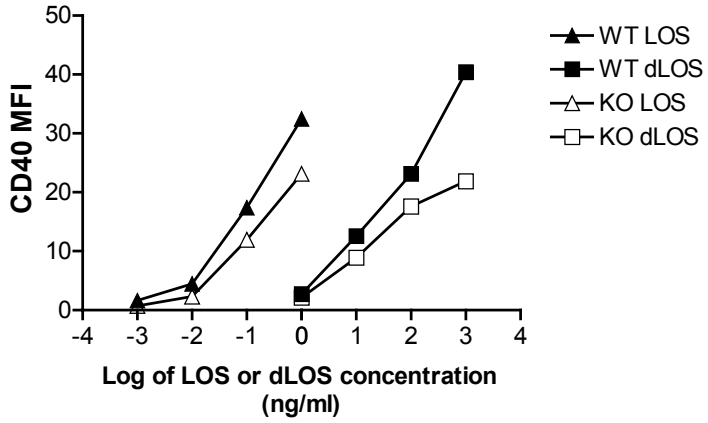


Figure 20. LOS induces similar levels of CD40 expression on AOAH WT and KO BMDCs.

BMDCs from AOAH WT and KO mice were treated with 10^{-3}, 10^{-2}, 10^{-1} and 1 ng/ml of LOS or 1, 10, 10^2 and 10^3 ng/ml of dLOS for 24 hours, and the CD40 expression (mean fluorescence intensity, MFI) was determined by FACS. The difference in activity between LOS and dLOS were quantified graphically by determining the horizontal distance between their dose-response curves and calculating the ratios of corresponding LOS or dLOS concentrations.

4. Comparison of natural antibody levels in sera and intestine of AOAH WT and KO mice.

Some natural antibodies in serum and in intestinal fluid are produced in response to specific antigens. Because mice and humans are constantly exposed to commensal and environmental microbes, AOAH might down-regulate natural antibody production by decreasing specific and/or polyclonal B cell responses to LPS. To test this hypothesis, serum samples were collected from non-immunized 129, B6 or C3H/HeN mice and serum concentrations of IgM, IgG_3, IgG_1 and IgG_{2a} were determined by ELISA (Fig. 21). For both B6 and 129 mice, AOAH KO mice had similar levels of IgM, IgG_3 and IgG_{2a}, but a lower level of IgG_1 than did AOAH WT mice. AOAH KO HeN mice had significantly higher levels of IgM, IgG_3 and IgG_1 serum natural antibodies than the wild type controls. These results suggest that AOAH may play a role in regulating natural antibody production.

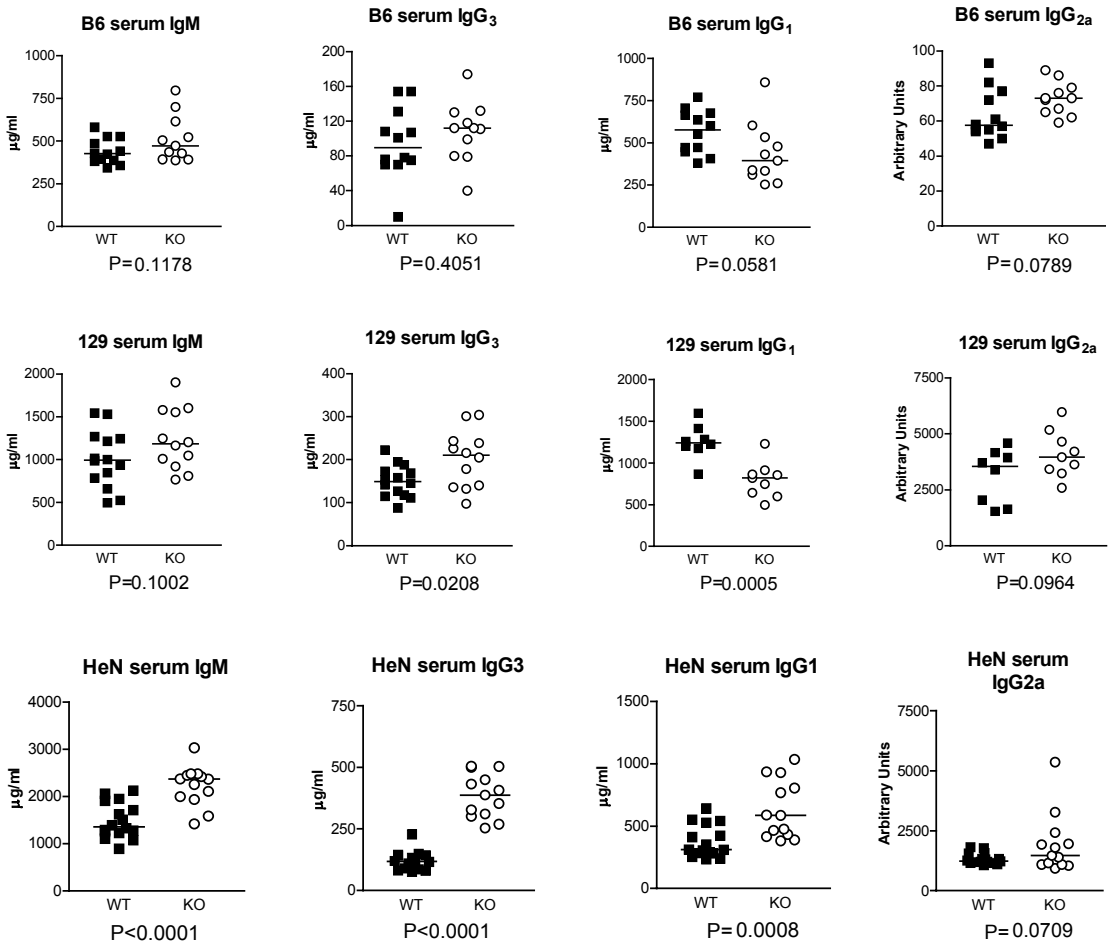


Figure 21. Natural antibodies in AOAH WT and KO mice sera.

The concentrations of IgM, IgG_3, IgG_1 and IgG_{2a} in sera of non-immunized mice were determined by ELISA. Student's *t* test was used and the lines represent medians of the samples.

The antibody levels at the intestinal mucosal surface were assessed by measuring antibody levels in fecal extracts. As shown in Figure 22, AOAH KO 129 mice have significantly lower IgA levels but slightly higher levels of IgG and IgM in fecal extracts than do AOAH WT mice. The impaired IgA secretion in the guts of AOAH KO mice is

probably due to reduced IL-5 secretion in those mice (Fig. 19), because studies have shown that IL-5 is indispensable for B-1 cell development and their differentiation into IgA-secreting cells in the gut. However, on the B6 background, the absence of AOAH was not associated with decreased fecal IgA levels.

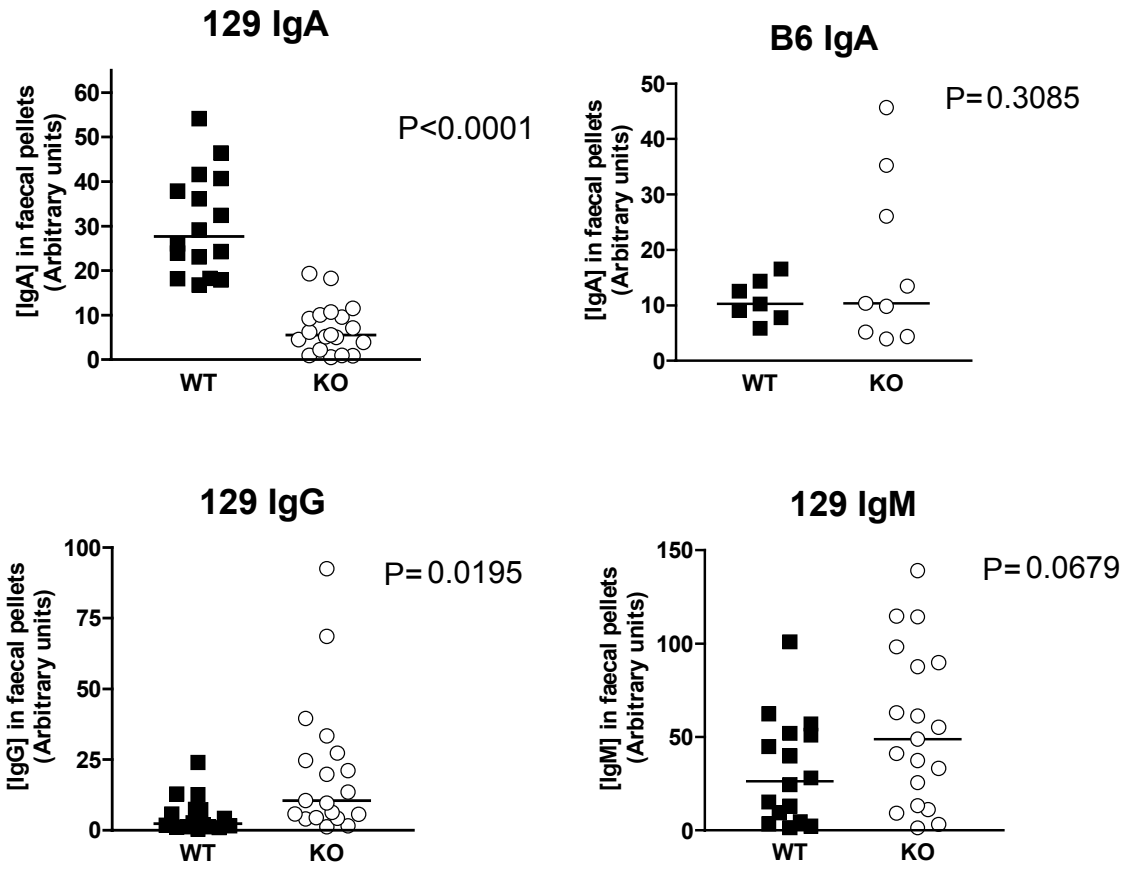


Figure 22. Antibody concentrations in extracts of fecal pellets from AOAH WT and KO mice.

Fresh fecal pellets were collected and resuspended in PBS with a proteinase inhibitor cocktail and NaN_3. Total IgA, IgM and IgG concentrations were then measured by ELISA. The difference between WT and KO was analyzed using Student's *t* test. The medians of the samples are shown.

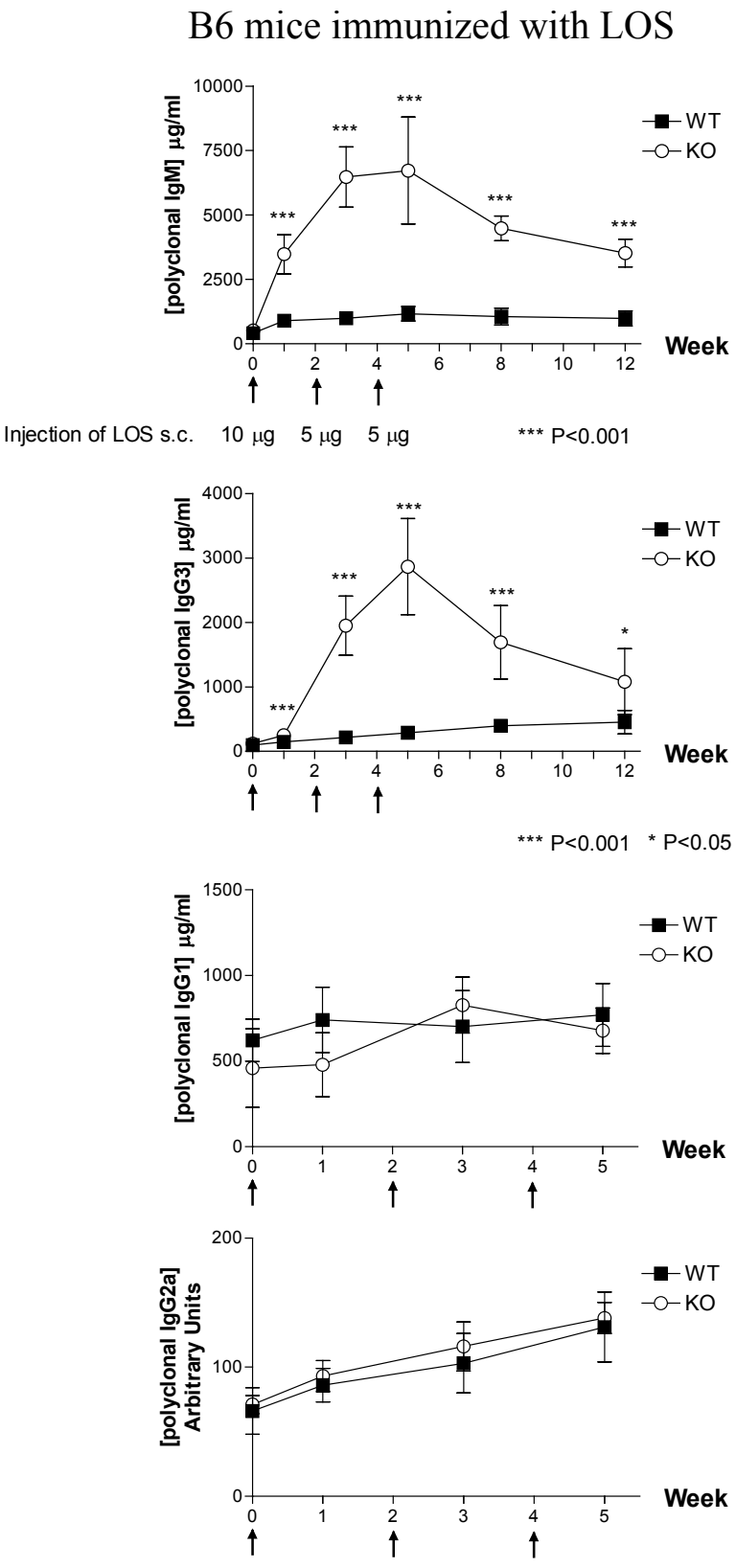
5. After immunization with LPS, AOAH -/- mice produce higher levels of specific and polyclonal IgM and IgG_3 than do AOAH +/+ mice.

In vitro studies have shown that enzymatic deacylation of LPS by AOAH decreases the ability of LPS to stimulate B cells. AOAH may thus play a role in decreasing antibody responses to LPS. For mouse B cells, AOAH treatment has a more dramatic impact on the stimulating activity of *Neisseria* LOS than it has on the potency of enterobacterial LPS. We compared the specific and polyclonal antibody responses in AOAH WT and KO mice after immunization with *Neisseria* LOS or *E. coli* 0111:B4 LPS. B6 mice were immunized with purified LOS or LPS via subcutaneous injection on the back. One week after the first injection with either LOS (Fig. 23A) or LPS (Fig. 23B), AOAH KO mice had significantly higher levels of polyclonal IgM than did WT mice. AOAH KO mice immunized with LOS also produced higher levels of polyclonal IgG_3 than did WT mice at one week after the first injection. After the second and the third injections of either LOS or LPS, KO mice made about 7-10-fold more IgM and IgG_3 than did WT mice. One and two months after the last injection, although the total antibody titers dropped, KO mice still had significantly higher levels of IgM and IgG_3 in their sera than did WT mice. In contrast, the polyclonal IgG_1 and IgG_{2a} levels were comparable between WT and KO mice. Similar results were observed in 129 mice: AOAH KO mice produced higher levels of polyclonal IgM and IgG_3 but not IgG_1 or IgG_{2a} than did AOAH WT mice after immunization with LOS or LPS (data not shown).

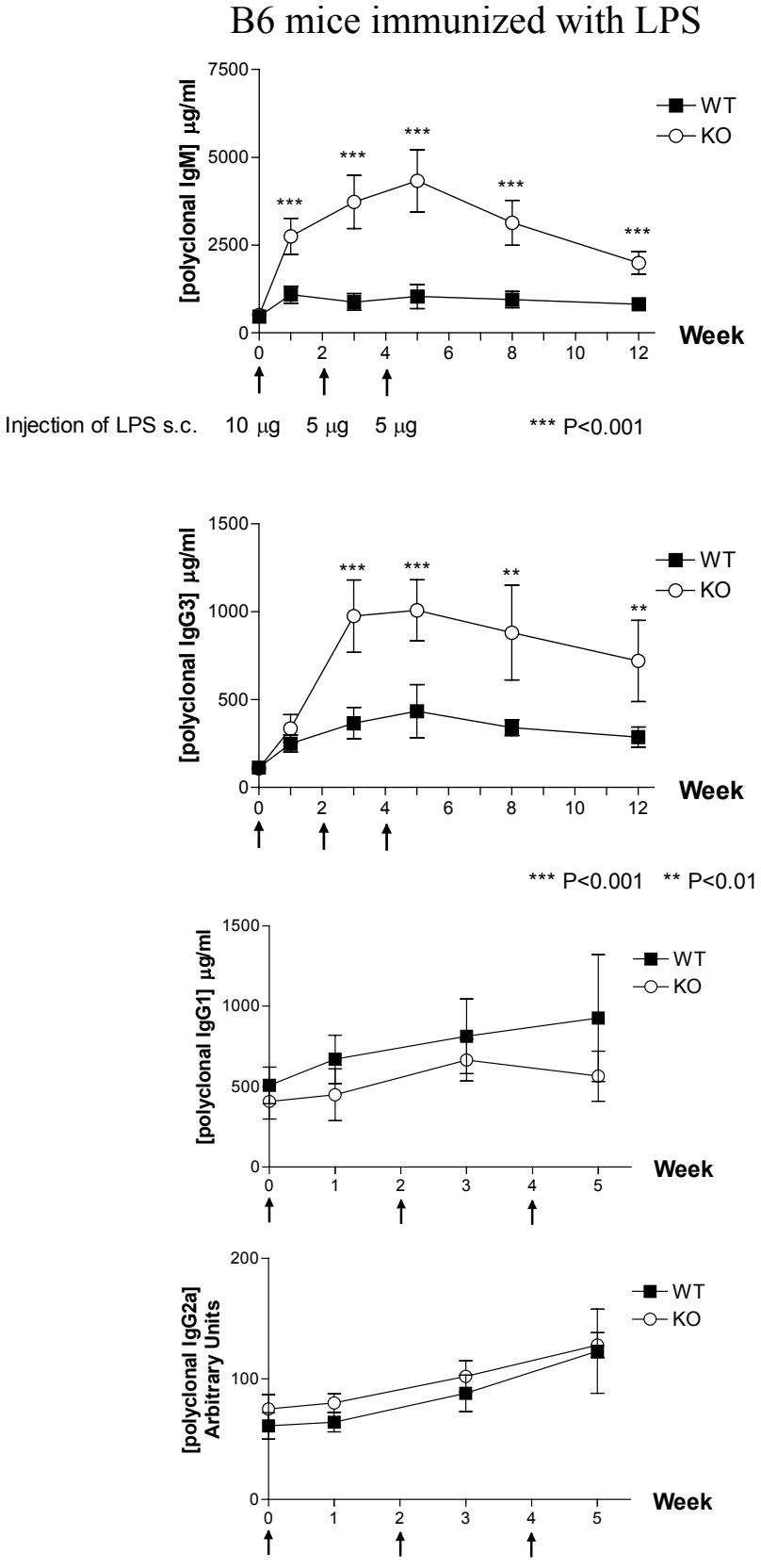
AOAH KO B6 mice also made significantly higher levels of anti-LOS- or LPS-specific IgM (Fig. 23C) and IgG_3 (data not shown) than did AOAH WT mice. Anti-LOS or LPS IgG_1 or IgG_{2a} were not detectable.

These results suggest that AOAH may play a role in negative regulation of polyclonal and specific antibody responses to LPS *in vivo*.

23A


B6 mice immunized with LOS
[polyclonal IgM] µg/ml
10000
7500
5000
2500
0
WT
KO
***
Week
0 2 4 6 8 10 12
Injection of LOS s.c. 10 µg 5 µg 5 µg
*** P<0.001
[polyclonal IgG3] µg/ml
4000
3000
2000
1000
0
*
*** P<0.001 * P<0.05
[polyclonal IgG1] µg/ml
1500
1000
500
0 1 2 3 4 5
[polyclonal IgG2a] Arbitrary Units
200
100

23B


B6 mice immunized with LPS
[polyclonal IgM] µg/ml
7500
5000
2500
0
WT
KO
***
Week
0 2 4 6 8 10 12
Injection of LPS s.c. 10 µg 5 µg 5 µg
*** P<0.001
[polyclonal IgG3] µg/ml
1500
1000
500
0
**
*** P<0.001 ** P<0.01
[polyclonal IgG1] µg/ml
0 1 2 3 4 5
[polyclonal IgG2a] Arbitrary Units
200
100

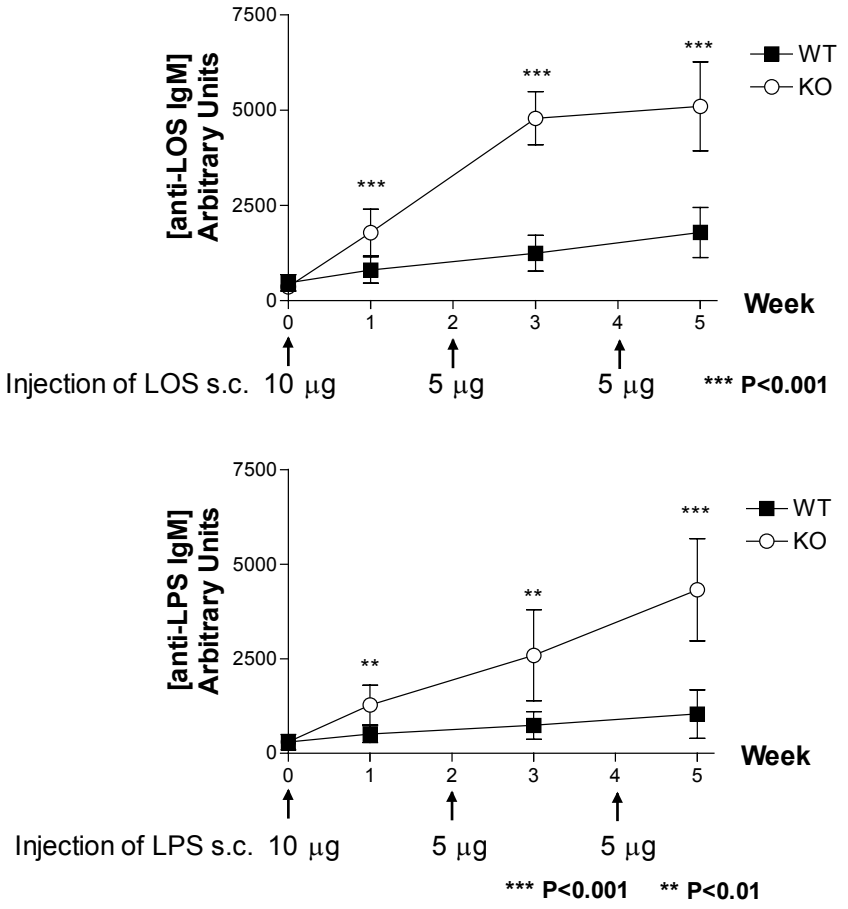


Figure 23. AOAH KO mice make higher polyclonal and specific IgM and IgG_3 antibody responses to LOS or LPS immunization than do AOAH WT mice.

AOAH KO and WT male mice (5 or 6 in each group) were immunized with purified *Neisseria* LOS or *E. coli* LPS 0111:B4 three times at two weeks intervals, *s.c.* on the back. The three injection times are indicated by arrows. 10 μg of LOS or LPS was given with the first injection, and 5 μg with the second and the third injections. Mice were bled before the first injection, one week after each injection and 1 and 2 months after the third injection. Sera were isolated and assayed for polyclonal and specific antibodies by ELISA. (A) Polyclonal antibody concentrations in B6 mice immunized with LOS. (B) Polyclonal antibody concentrations in B6 mice immunized with LPS. (C) Anti-LOS or LPS antibody concentrations in B6 mice immunized with LOS or LPS. Means ± 1 SD are shown. Student's *t* test was used to compare the increased serum Ig concentrations, which were obtained by subtracting Ig concentrations in the pre-immunization sera from Ig concentrations in the post-immunization sera.

6. Deacylated LOS maintains its ability to induce anti-LOS specific response.

AOAH diminishes polyclonal antibody responses to LPS, suggesting that dLPS has reduced ability to induce polyclonal antibody production. B6 WT mice were immunized with 0.5 μg of LOS or dLOS *s.c.* Three weeks after the primary injection, mice were boosted with a second dose of 0.5 μg of LOS or dLOS *s.c.* and one week after the second injection, mice were bled. Both total IgM and anti-LOS specific IgM concentrations in serum samples were measured by ELISA. LOS induced significantly higher level of polyclonal IgM response than did dLOS (Fig. 24A), suggesting that deacylation abolishes the B cell mitogenicity of LPS. However, dLOS stimulated similar anti-LOS specific IgM as did LOS (Fig. 24B), suggesting that deacylation may maintain the ability of LOS to induce antibody to itself.

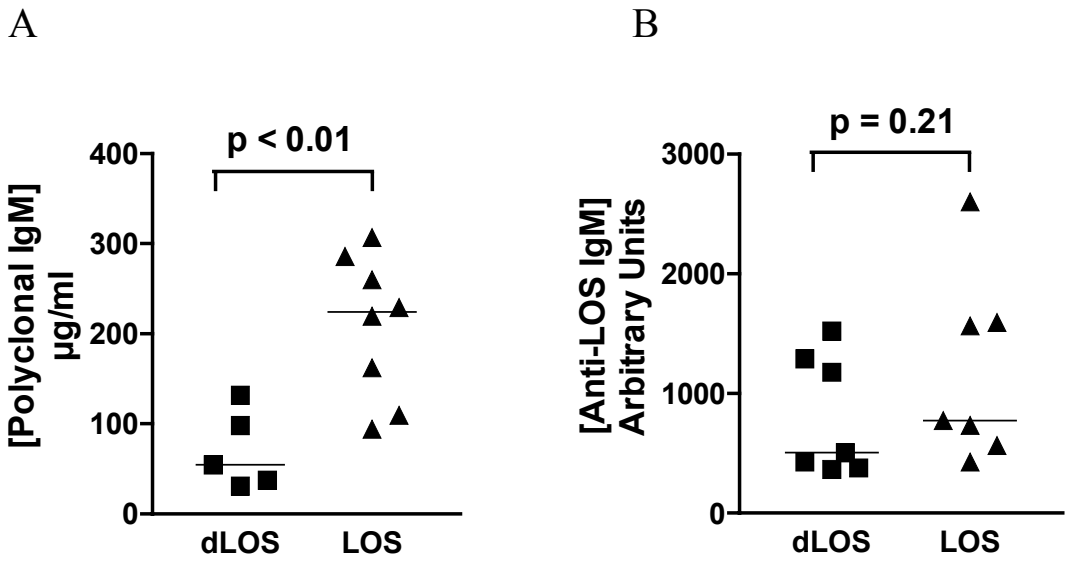


Figure 24. dLOS has decreased ability to induce polyclonal antibody response while preserving the ability to generate anti-LOS specific response.

B6 mice were immunized with 0.5 µg of LOS or dLOS *s.c.* 3 weeks later, mice received a second injection of LOS or dLOS and blood samples were then collected one week later. (A) Polyclonal IgM concentrations in mouse sera. (B) Anti-LOS specific IgM concentrations. Student's *t* test was used to compare the increased serum Ig concentrations, which were obtained by subtracting Ig concentrations in the pre-immunization sera from Ig concentrations in the post-immunization sera. The medians of the samples are shown.

6. Splenocytes from AOAH WT and KO mice respond similarly to LOS.

We then tested whether macrophages and DCs in spleen can deacylate LPS, and therefore diminish LPS's ability to stimulate B cell mitogenesis. Splenocytes from AOAH WT and KO mice were isolated and treated with graded doses of LOS or dLOS *in vitro*. For both AOAH WT and KO splenocytes, dLOS showed much less ability to stimulate splenocyte proliferation (Fig. 25A); dLOS inhibited LOS's activity in the same assay (Fig. 25B), in keeping with the ability of deacylated Neisseria LOS to act as an antagonist. Similarly, deacylation decreased LOS's activity to stimulate splenocyte

secretion of IgM by about 25 fold (Fig. 25C). However, LOS did not induce exaggerated proliferation or differentiation responses in splenocytes from AOAH KO mice, suggesting that, in such an *in vitro* system, deacylation of LOS by myeloid cells is not able to decrease B cell responses to LOS.

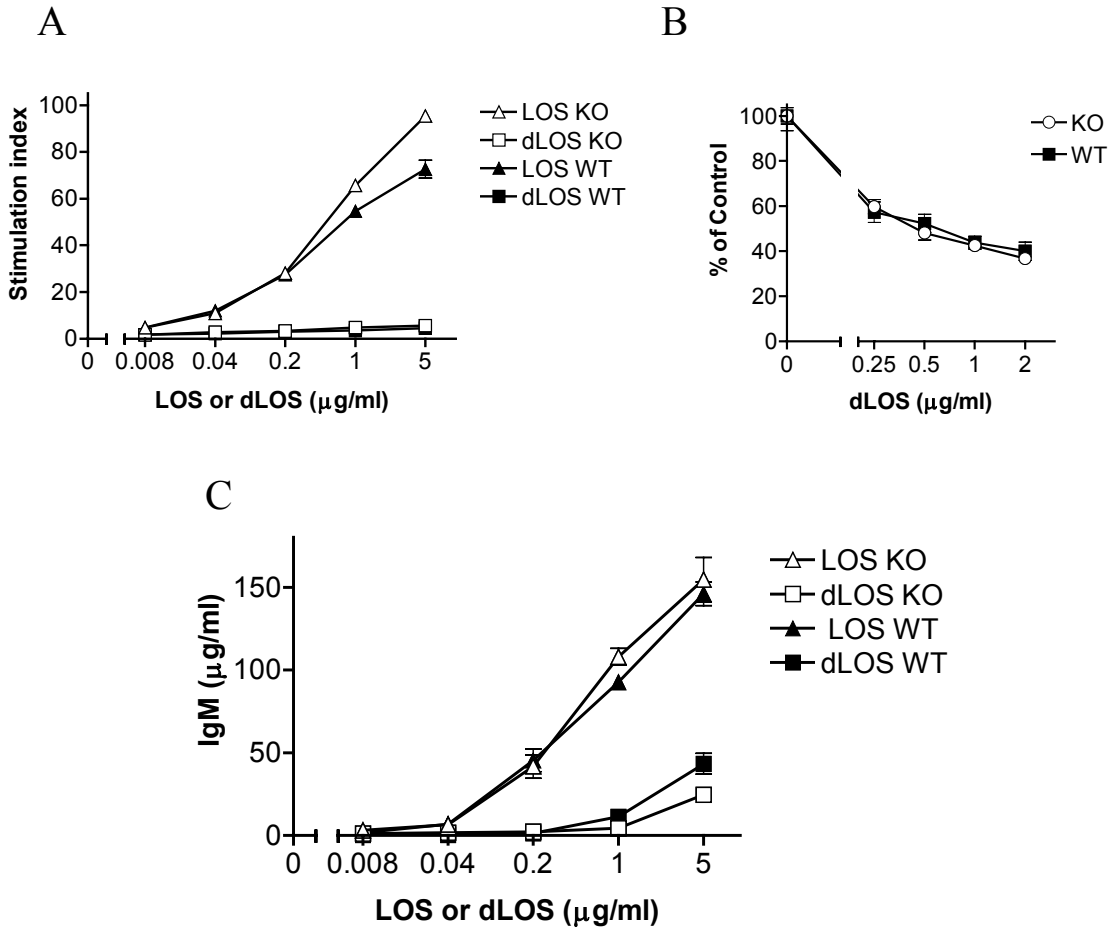


Figure 25. Splenocytes from AOAH WT and KO mice have similar dose-response profiles to LOS or dLOS.

(A) Splenocytes were treated with 0.008, 0.04, 0.2, 1 and 5 μg/ml of LOS or dLOS and the proliferation of splenocytes was measured by [^{3}H]-thymidine incorporation. (B) Splenocytes were treated with 0.2 μg/ml of LOS mixed with 0.01, 0.2, 0.5, 1 and 2 μg/ml of dLOS and splenocyte proliferation was measured. (C) Splenocytes were treated as in (A) for 7 days, and the IgM in the culture supernatant was measured by ELISA.

7. After exposure to LOS or LPS, AOAH KO mice produce higher levels of anti-nuclear antibodies than do WT mice.

Since AOAH KO mice made much more polyclonal antibody than AOAH WT mice after LOS or LPS immunization, we then asked whether autoimmune antibodies such as anti-nuclear antibodies were also elevated in AOAH KO mice. Anti-ssDNA, anti-dsDNA, anti-DNA/histone and anti-histone IgM and IgG were measured in the sera of B6 mice after the second injection of LOS (Fig. 23). I found that AOAH KO mice made significantly higher levels of anti-nuclear IgM and IgG than did WT mice (Fig. 26). The anti-nuclear antibody responses highly correlate with polyclonal antibody responses, suggesting that elevated anti-nuclear antibody levels in KO mice may be caused by exaggerated polyclonal antibody response to LOS.

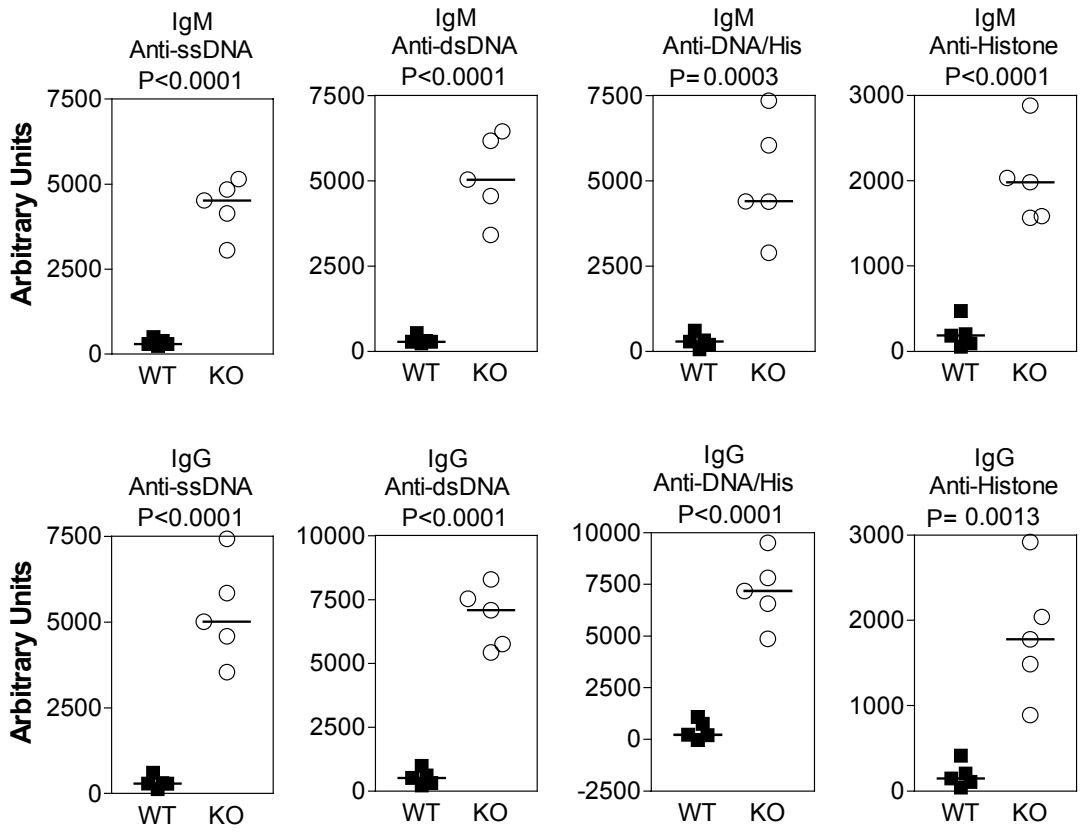


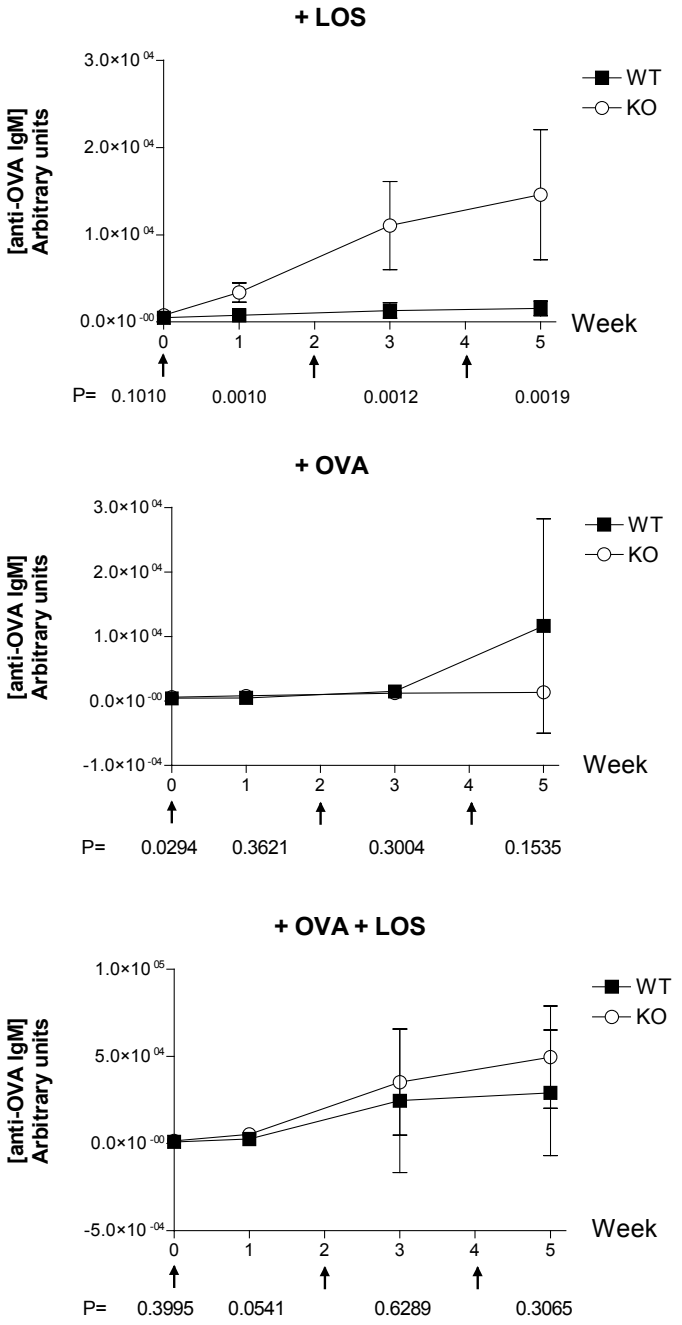
Figure 26. Anti-nuclear antibodies in sera from LOS-immunized AOAH WT and KO mice.

Sera from B6 mice, obtained after the second injection of LOS, were assayed for anti-nuclear antibodies. Student's *t* test was used to compare antibody levels in WT and KO mice, and the medians of the samples are shown.

8. AOAH does not alter the adjuvant activity of LPS.

We then tested whether AOAH plays a role in regulating the adjuvant activity of LPS. B6 mice were immunized with LOS, OVA or LOS mixed with OVA *s.c.* Anti-OVA antibodies in mouse sera were measured by ELISA (Fig. 27). Both AOAH WT and KO mice that received OVA mixed with LOS made moderately higher amounts of anti-OVA IgM and dramatically higher levels of IgG_1 than did mice that received LOS or OVA only. Anti-OVA IgG_{2a} and IgG_3 were not detectable. However, AOAH KO mice did not make substantially more or less anti-OVA antibodies than did WT mice, whether OVA was injected with LOS or not, indicating that AOAH does not regulate the adjuvant activity of LOS.

27A


+ LOS
[anti-OVA IgM] Arbitrary units
3.0×10 04
2.0×10 04
1.0×10 04
0.0×10 -00
WT
KO
Week
0 1 2 3 4 5
P= 0.1010 0.0010 0.0012 0.0019
+ OVA
[anti-OVA IgM] Arbitrary units
3.0×10 04
2.0×10 04
1.0×10 04
0.0×10 -00
-1.0×10 -04
WT
KO
Week
0 1 2 3 4 5
P= 0.0294 0.3621 0.3004 0.1535
+ OVA + LOS
[anti-OVA IgM] Arbitrary units
1.0×10 05
5.0×10 04
0.0×10 -00
-5.0×10 -04
WT
KO
Week
0 1 2 3 4 5
P= 0.3995 0.0541 0.6289 0.3065

27B

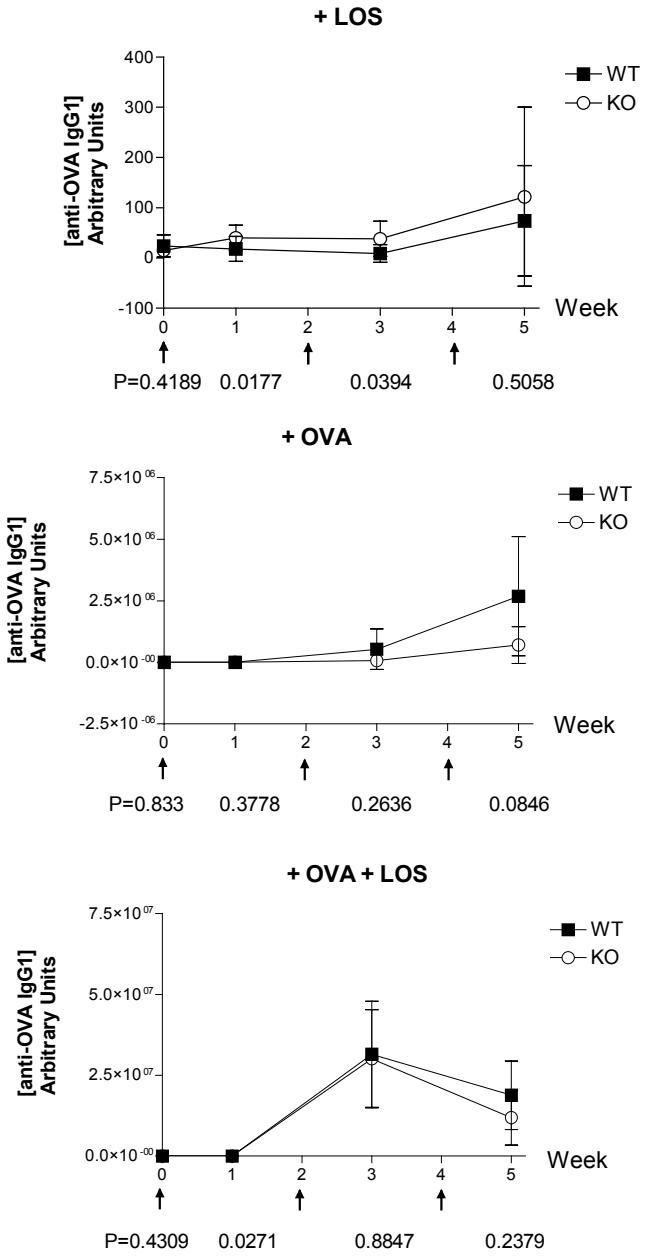


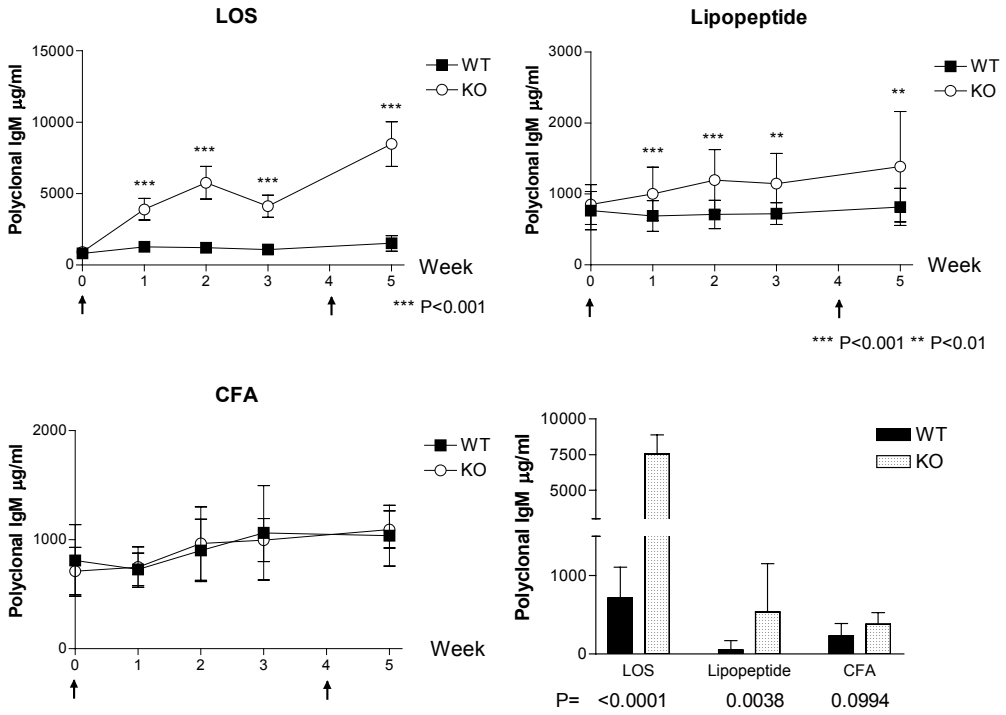
Figure 27. The adjuvant activity of LPS in AOAH WT and KO mice.

B6 mice received three injections of LOS, OVA or OVA+LOS *s.c.* at two weeks interval. The arrows indicate times of injection. Each group had 6 mice, male and female. Mice were bled before and one week after each injection. The serum anti-OVA IgM and IgG_1 concentrations were determined and compared between AOAH WT and KO mice. (A) Anti-OVA IgM concentrations in mice that injected with LOS, OVA or OVA mixed with LOS. (B) Anti-OVA IgG_1 concentrations in mouse sera. The data shown represent means ± 1SD. Student's *t* test was used.

9. Polyclonal responses to adjuvants other than LPS.

I then asked whether the exaggerated antibody response to LPS in AOAH KO mice is specific for LPS immunization, since LPS is the substrate of AOAH. I immunized mice with two other adjuvants, lipopeptides and complete Freunt's adjuvant (CFA), and compared the antibody responses between AOAH WT and KO mice. Synthetic lipopeptides retain the TLR2-stimulating activity of bacterial lipopeptides. CFA is essentially made of killed *Mycobacterium* bacilli suspended in incomplete Freund's adjuvant (ICF), which is a mixture of paraffin oil and mannide monooleate (85:15, v:v). Killed mycobacteria can activate TLR2 and ICF induces TLR2 but not TLR4 expression in the livers of mice [264]. Both lipopeptide and CFA induced slightly increased polyclonal IgM (much less than that induced by LOS) after the first immunization, while polyclonal IgG_3 was not elevated. When the mice were boosted with the second dose of adjuvant, the serum polyclonal IgG_3 levels were dramatically increased. In response to lipopeptide, AOAH KO mice made more IgM (but not more IgG_3) than did AOAH WT mice (Fig. 28), suggesting that AOAH may also release the ester-bound fatty acyl chains from lipopeptide, therefore reducing its biopotency *in vivo* (See Chapter IV). The polyclonal responses to CFA did not reveal a difference between AOAH WT and KO mice.

A


LOS
Lipopeptide
CFA
Polyclonal IgM µg/ml
WT
KO
Week
*** P<0.001
*** P<0.001 ** P<0.01
LOS
Lipopeptide
CFA
P= <0.0001 0.0038 0.0994

B

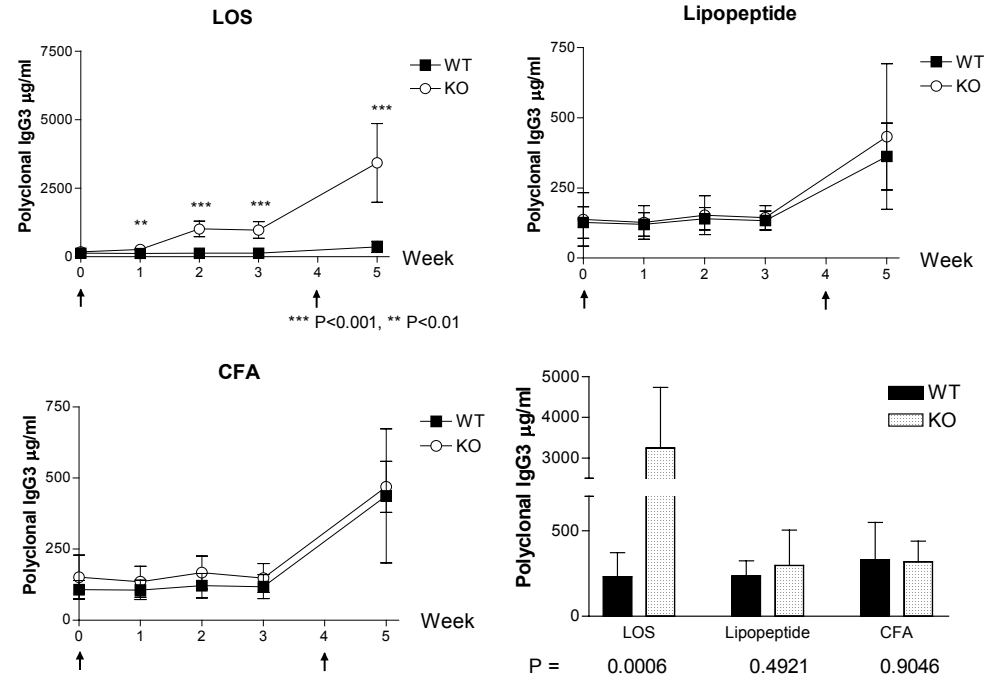

LOS
Lipopeptide
CFA
Polyclonal IgG3 µg/ml
WT
KO
Week
*** P<0.001, ** P<0.01
LOS
Lipopeptide
CFA
P = 0.0006 0.4921 0.9046

Figure 28. Polyclonal antibody responses to lipopeptides and CFA.

B6 mice were given LOS, lipopeptide, 10 μg/mouse or CFA: PBS emulsion (1:1, v:v) 100 μl, *s.c.* on the back. There were six or seven male mice in each group. Four weeks after the first injection, mice received the second injection of the same adjuvants, same dose, and *s.c.* Arrows indicate the times of the injections. Blood samples were collected before the first injection, 1, 2, and 3 weeks after the first injection, and 1 week after the second injection. Polyclonal IgM and IgG_3 concentrations were determined by ELISA. (A) Serum polyclonal IgM concentrations in mice immunized with LOS, lipopeptide or CFA. The bar graph shows the serum IgM concentrations after the second injection. (B) Serum polyclonal IgG_3 concentrations. The bar graph represents the serum IgG_3 level after the second immunization. Means ± 1 SD are shown and the P values were obtained by Student's *t* test.

D. Discussion

Treatment of LPS with AOAH diminishes the biopotency of LPSs, as shown most dramatically in the rabbit dermal Shwartzman reaction [121]. Deacylation also abolishes the ability of LPS to stimulate human endothelial cells, neutrophils and monocytes [122-125]. In addition, dLPS is a LPS-specific inhibitor that can antagonize intact LPS activity by competing for LBP, CD14, and probably TLR4 -MD-2 [126]. The generation of AOAH KO mice made it possible to study the biological functions of AOAH *in vivo*.

First, I tested whether AOAH can diminish LPS toxicity *in vivo*. AOAH WT and KO mice were challenged with either purified LPS or living Gram-negative bacteria and their survival curves were compared. The results showed that AOAH did not protect mice from LPS or Gram-negative bacterial challenge. This result was not totally unexpected. First, LPS is deacylated at a very slow rate (over many hours), while cytokine responses to LPS are much faster. For example, TNF-α and IL-1 are released

within minutes after LPS exposure. However, Wang et al. have reported that a late mediator, high mobility group-1 (HMG-1) protein, which was found in mouse serum from 8 to 32 hours after LPS exposure, causes lethality in mice [265]. It will be interesting to find out whether AOAH can diminish HMG-1 production in mice exposed to LPS. Second, in the toxicity studies described above, *E. coli* LPS 0111:B4 or *E. coli* LCD25 was used to challenge mice. For mouse cells, deacylation of enteric bacterial LPS decrease its bioactivity to a lesser extent than does deacylation of *Neisseria* LOS. By using LOS instead of LPS to challenge mice, we may be able to see a more convincing difference if AOAH has a protective role *in vivo*.

When BMDCs from AOAH WT and KO mice were treated with LPS, KO BMDCs had comparable cytokine responses to WT BMDCs, despite the fact that WT BMDCs are able to deacylate LPS. Similarly, the absence of AOAH did not increase CD40 expression in response to LOS, although dLOS has much less ability to stimulate CD40 expression than does LOS. These *in vitro* results suggest that the real time deacylation of LPS by DCs cannot in turn influence their responses to LPS, probably due to the slow deacylation rate.

BMDCs from AOAH WT 129 mice produced a small amount of IL-5 at 24 hours in the absence of LPS treatment, probably caused by the spontaneous secretion or the presence of trace amount of stimuli although low endotoxin FBS (<0.06 EU/ml) was used to prepare the culture medium. However, unstimulated BMDCs from AOAH KO mice did not produce IL-5. In addition, after exposure to LPS, WT BMDCs secreted about twice as much IL-5 as did KO BMDCs. It has been found that HMG-1, the late mediator

of LPS responses, binds to the conserved lymphokine elements-0 (CLE0) in the IL-5 promoter, which is essential for the expression of IL-5 [266]. It is conceivable that LPS may induce more HMG-1 protein secretion in AOAH KO BMDCs, which depletes the nuclear pool of HMG-1 so that IL-5 production is decreased. However, HMG-1 also binds to CLE0 in the IL-4 promoter, and IL-4 production was not significantly lower in the culture medium of AOAH KO BMDCs than that of WT BMDC. HMG-1 thus seems unlikely to account for the observed difference in IL-5 production.

LPS induces specific and polyclonal antibody responses *in vivo* and *in vitro*. Enzymatically deacylated LPS, especially enzymatically deacylated *Neisseria* LOS, has much lower activity in stimulating proliferation of murine splenocytes. I studied whether AOAH plays a role in modulating antibody responses to LPS. After the mice were injected with LOS or LPS *s.c.*, AOAH KO mice produced higher levels of LOS- or LPS-specific and polyclonal IgM and IgG_3 than did AOAH WT mice, suggesting that AOAH deacylates LOS or LPS *in vivo* and diminishes their stimulatory activity toward B cells. AOAH is found predominantly expressed in myeloid leucocytes, such as DCs, monocytes-macrophages and neutrophils, but not in B cells (data not shown). LPS would presumably be deacylated by myeloid cells first and then the deacylated LPS would be presented to B cells directly or indirectly. An interaction of B cells with DCs or macrophages has been shown by Balazs et al, who found that blood-derived $CD11c^{lo}$ DCs capture and transport the TI-II antigen PC to marginal zone B cells, and that this process promotes B cell differentiation. Similarly, peritoneal macrophages provide support to PC-specific B-1 cells. In addition, this DC-B cell interaction requires the

interaction of DC-derived soluble ligands, BAFF and APRIL with their B cell receptors [104]. Whether DCs or macrophages can transport LPS to B cells, interact and activate B cells, whether the deacylation of LPS by DCs modulates B cell activation, and whether DCs and B cells interact directly or via BAFF and APRIL remain to be understood. Because LPS deacylation occurs intracellularly, how macrophages and DCs would present dLPS to B cells or other macrophages and DCs is not clear. dLPS may be recycled to phagocyte surface, be exocytosed, or be released when the phagocytes die. In the study described here, mice were immunized with LPS *s.c.*, so epidermal or dermal DCs might take up LPS, deacylate it, and regulate B cell function in the draining lymph nodes.

Although the concentration of LPS required to stimulate a specific LPS antibody response is about 1000 times lower than the concentration needed to induce polyclonal responses *in vitro*, the LPS dose (10 or 5 μg) I used in these *in vivo* studies increased both specific and polyclonal antibodies (especially in KO mice) and the specific responses correlated very well with polyclonal antibody responses, indicating that the increased anti-LPS antibody is quite likely a consequence of increased polyclonal responses.

The amplified polyclonal antibody responses to LPS in AOAH KO mice led to the augmented activation of autoimmune B cells and the elevated secretion of autoantibodies. Anti-DNA antibody and RF production following LPS immunization in mice was reported in the 1970's, and the deposition of immune complexes in the kidney was also observed [261]. In AOAH KO mice, probably as a consequence of amplified polyclonal antibody responses, several autoimmune antibodies (anti-nuclear antibodies,

ANA) were elevated after LPS immunization. ANAs of both IgM and IgG isotypes were significantly higher in the AOAH KO mice than in the AOAH WT mice; pathogenic antoantibodies in SLE are predominantly of IgG isotype [267]. Both the early anti-DNA/histone ANA in SLE and the putatively pathogenic anti-dsDNA ANAs were elevated in AOAH KO mice. The renal histology or pathology of those mice will be studied. Because autoimmunity may be caused by more than one defective gene, impaired T cell function may be required to induce organ damage, as shown by Shi et al. [268].

Self-reactive B cells are deleted in the bone marrow and those self-reactive B cells that make their way to the periphery are either deleted or rendered anergic. Studies have shown in some cases that anergic B cells can be stimulated by LPS to divide and differentiate into antibody-secreting cells. To avoid autoimmunity caused by polyclonal activation of B cells by TI-1 antigens, mechanisms that negatively regulate TI-1 antigen-induced polyclonal antibody production must be involved. Degradation of TI-1 antigens might be one of these mechanisms. AOAH deacylates LPS and diminishes LPS bioactivity, and therefore may prevent the overstimulation of autoreactive B cells.

When mice were immunized with low dose (0.5 μg) of LOS or dLOS, dLOS induced less polyclonal IgM than did LOS. However, dLOS maintains the ability to induce anti-LOS specific IgM. Thus, deacylation of LPS by AOAH may diminish unwanted polyclonal antibody responses while maintaining the potentially protective anti-LPS specific antibody response.

Surprisingly, although *in vitro* the deacylation of *E. coli* LPS decreases its bioactivity to a much less extent (by about 10-fold) than does the deacylation of Neisseria LOS (by about 100-fold) [127], AOAH seems to have similar impact on the antibody responses to LPS and LOS immunization *in vivo*. This result suggests that the way that LPS is deacylated and presented to B cells or other cells may be different *in vivo* from that *in vitro*, or that AOAH may have functions other than deactivating LPS; for example, AOAH may regulate LPS trafficking inside cells, the exocytosis of LPS, or the efflux of LPS from the cell surface [51].

The effects of AOAH on *in vivo* antibody responses to LPS have not been reproduced *in vitro*. Although dLOS has much reduced activity in stimulating splenocyte proliferation and IgM secretion, AOAH deficiency did not influence splenocyte responses (proliferation and IgM secretion) to LOS. *In vivo*, the DCs in the periphery probably take up and deacylate some of LPS before they reach the regional lymph nodes and interact with B cells or other cells. Pilot experiments have been performed to allow BMDCs to capture and deacylate LOS first and then the loaded BMDCs were added to splenocytes in culture. However, BMDCs from AOAH KO mice did not induce higher levels of splenocyte proliferation or antibody secretion than did WT BMDCs. The specific microenvironment or the architecture of lymphoid organs may thus be important.

AOAH deficiency has an impact on the IgM and IgG_3 responses to LPS, but not on IgG_1 and IgG_{2a} responses. IgM and IgG_3 are the predominant antibody isotypes induced by LPS and decreased degradation of LPS leads to persistent B cell stimulation. The restriction of exaggerated antibody responses to IgM and IgG_3 in AOAH KO mice

may be caused by over-stimulation of B-1 or marginal zone B cells since they are high responders to LPS and preferentially secrete IgM and IgG_3 [196]. Whether the LPS injected *s.c.* can reach the spleen or peritoneal cavity and whether the lymph nodes have B cells similar to B-1 cells or marginal zone B cells are not clear. B-2 cells, which can also be activated by LPS [194;269] may be responsible for part of the IgM response.

Over the two-month period after the last immunization, the IgM and IgG_3 titers in AOAH KO mice dropped by about 30-60%, but they were still significantly higher than those in AOAH WT mice, despite the fact that the IgM and IgG_3 titers in AOAH WT mice declined relatively slowly. The half-lives of mouse IgM and IgG_3 are about 0.5 and 4 days respectively. These results suggest that in both AOAH WT and KO mice, new IgM and IgG_3 were synthesized even two months after immunization. The slowly declining antibody titer in AOAH KO mice suggests that some other mechanisms may be involved in the elimination or degradation of LPS.

In the serum of AOAH KO HeN mice, the levels of natural antibodies of IgM, IgG_3 and IgG_1 are higher than those of wild type control mice. For 129 and B6 mice, the KO and WT mice have comparable serum levels of IgM and IgG isotypes, except for IgG_1, which is less abundant in AOAH KO 129 ($p=0.0005$) and possibly also in AOAH KO B6 ($p=0.0581$) mice. These results suggest that AOAH may play a role in modulating natural antibody production. Unexpectedly, AOAH KO 129 mice had about 6-fold less IgA in their fecal pellet extracts than did WT control mice. This result might be explained by the impaired IL-5 secretion observed in AOAH KO DCs *in vitro*. Studies using either IL-5 or IL-5 receptor α deficient mice have shown that IL-5

signaling is required for B-1 cell development and IgA secretion at mucosal surfaces by B-1 cells [212;216]. However, Corcoran et al. have reported that 129/Sv mice have an IL-5 signaling defect in B cells and reduced number of B-1 cells in the peritoneal cavity [270]. Similar levels of IgA were detected in fecal pellet extracts from AOAH WT and KO B6 mice.

I also studied the role that AOAH plays in LPS's ability to be an adjuvant. In both AOAH WT and KO mice, the presence of LOS slightly augmented the anti-OVA IgM response to OVA compared with the response to OVA by itself. With the adjuvant, anti-OVA IgG_1 production was dramatically induced especially after the second injection, suggesting that antigen specific T cells were activated and promoted B cell differentiation and isotype switching. However, LOS showed similar adjuvant activity in AOAH WT and KO mice, indicating that AOAH does not play an essential role in regulating LOS adjuvanticity. This is interesting since AOAH can regulate LPS-induced B cell responses but not the ability of LPS to enhance antibody production to a protein. The lipid A analog MPL has provided good evidence that the toxicity and adjuvanticity of LPS can be separated [232;233]. The adjuvant activity of LPS may require much less TLR4-stimulation than does B cell activation and more TLR4 signaling does not increase the adjuvant effect. Thus, enzymatically deacylated LOS or partially deacylated LOS may be good enough to induce adjuvant activity, while they cannot induce B cell activation.

Whether other adjuvants can induce the different antibody response in AOAH WT and KO mice has also been investigated. CFA contains TLR2 agonists, including lipoarabinomannan. Lipopeptides are also TLR2 agonists. CFA and lipopeptide induced

lower levels of polyclonal antibody secretion than did LOS. IgM and IgG_3 polyclonal antibodies were comparable in AOAH WT and KO mice immunized with CFA. Lipopeptide induced significantly higher polyclonal IgM in AOAH KO mice than in WT mice, while the polyclonal IgG_3 levels were similar in those mice. These results suggest that AOAH may release fatty acyl chains from bacterial lipoproteins, and possibly decrease their bioactivity (Chapter IV). However, *in vitro* evidence that AOAH can deacylate bacterial lipoproteins is still missing. The similar polyclonal IgG_3 levels probably reflect the fact that, unlike LPS, lipopeptide may not preferentially induce IgG_3 responses.

LPS is not the only substrate of AOAH. AOAH also is enzymatically active towards certain phospholipids and is highly homologous to trypanosomal inositol deacylase, which releases fatty acyl chains from an intermediate in GPI anchor biosynthesis. It is thus possible that AOAH may have other functions *in vivo*. AOAH KO mice have begun to help us understand the biological roles of AOAH and will continue to do so.

CHAPTER IV. OTHER POTENTIAL AOAH SUBSTRATES: BACTERIAL LIPOPROTEINS AND ENTEROBACTERIAL COMMON ANTIGEN

A. Introduction

In the outer membrane of gram-negative bacteria, in addition to LPS, there are outer membrane proteins (OMP), including bacterial lipoproteins. Bacterial lipoproteins are generally very active substances immunologically and some of them have been found to be tightly bound to LPS [271]. Enteric gram-negative bacteria also possess a unique cell surface glycolipid, the enterobacterial common antigen (ECA). Both bacterial lipoproteins and ECA have ester-linked fatty acyl chains, which makes them potential substrates for AOAH.

The N-terminus of lipoproteins is a cysteine residue to which a diacylglycerol and an amide-bound fatty acyl chain are covalently attached (Fig. 29). The most abundant bacterial lipoprotein is Braun's lipoprotein (BLP), which has 58 amino acid residues. BLP exists in two forms, a free form and a form that is covalently linked to peptidoglycan (murein) by the C-terminal domain [272]. About 65-75% of the fatty acids hydrolyzed from purified BLP were 16:0 and 18:1 [273;274].

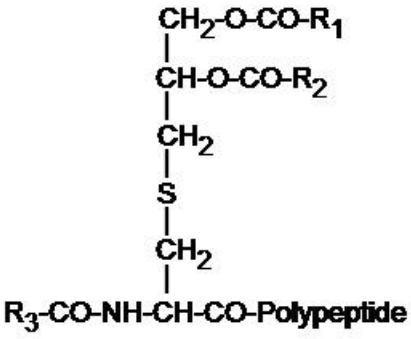


Figure 29. Schematic diagram of the chemical structure of N-terminus of bacterial lipoproteins.

The N-terminal glycerylcysteine contains two ester-linked and one amide-linked fatty acid. R1, R2 and R3 represent fatty acyl chains.

Bacterial lipoproteins are agonists for TLR2, and recently, TLR1 and TLR6 have been shown to heterodimerize with TLR2 and modulate its ligand recognition and signaling efficiency [26;275]. Bacterial lipoproteins or synthetic lipopeptide analogs have been shown to activate mouse B cells, mouse and human macrophages and DCs and even to induce lethal shock [257;276;277]. Furthermore, bacterial lipoproteins have been shown to act synergistically with LPS to induce proinflammatory cytokine production and lethal shock [16]. Lipoproteins lose their bioactivity when the ester-linked fatty acids are removed by alkaline hydrolysis. The two ester-linked fatty acyl chains of lipoproteins might also be released by AOAH, with a resulting decrease in biopotency.

Discovered 40 years ago by Kunin et al., ECA is found in all members of the family enterobacteriaceae. ECA contains a long linear polysaccharide chain, which is composed of a conserved trisaccharide repeat. The trisaccharide unit consists exclusively of N-acetyl-D-glucosamine, N-acetyl-D-mannosaminuronic acid and 4-acetamido-4, 6-

dideoxy-D-galactose. Three forms of ECA have been found, phosphoglyceride-linked ECA (ECA_{PG}), LPS-linked ECA (ECA_{LPS}) and cyclic ECA. ECA_{PG} is formed by covalent linkage of the polysaccharide chain to phosphoglyceride (PG) and it is nonimmunogenic. The PG-derived fatty acids are predominantly 16:0, 16:1 and 18:1. Immunogenic ECA_{LPS} occurs only in bacterial rough mutants with appropriate cores. ECA_{LPS} consists of ECA chains linked to the complete R1, R4 or K12 core region of LPS that lacks O-antigen; only about 5% of the cores are substituted with ECA polysaccharide chains [278]. The O-translocase, which is responsible for the transfer of O-antigen from the lipid carrier to the LPS core, was found to be required for the occurrence of ECA_{LPS} [279]. The third form of ECA occurs as the cyclic form (ECA_{cyc}), which consists of four to six trisaccharide repeat units. ECA_{cyc} has not been found in *E. coli*. Both ECA_{PG} and ECA_{LPS} are found on the outer leaflet of the bacterial outer membrane, and on SDS-PAGE, they have a ladder-like pattern characteristic of polymers made of a homologous series of repeat units. Because the PG moiety of ECA_{PG} has two ester-linked fatty acids, ECA_{PG} might also be an AOAH substrate.

When the deacylation of LPS in whole bacteria by DCs or macrophages was studied, I found that not only were fatty acids derived from LPS released over time, but fatty acids derived from other bacterial components were also lost from the interface of B-D extraction. In this study, I demonstrated that both bacterial lipoproteins and ECA are present at the interface of a B-D extraction of *E. coli* cells and I tested whether they can be deacylated by AOAH. In addition, I found that ECA contributes to preventing bacteria from being phagocytosed by DCs.

B. Materials and methods

1. Treatment of E coli LCD25 with proteinase K

E. coli LCD25 were cultured in 2 × minimal medium that contained [^{14}C] sodium acetate so that the fatty acyl chains in the bacteria were [^{14}C]-labeled (chapter II). The bacteria were then treated with 75 μg/ml of proteinase K in buffer which contained 1mM $CaCl_2$, 0.5% SDS and 0.01 M Tris, pH 7.8, at 37 °C for 16 hours. Mock-treated bacteria were incubated in buffer without proteinase K.

2. Purification of murein-BLP complexes

A bacterial pellet was resuspended in PBS and sonicated. The bacterial suspension was then added dropwise to a 10 × volume of boiling 4% SDS; the mixture was stirred while it cooled and centrifuged at 22,000 g for 2 h. The sediment was resuspended in water by vigorous vortexing.

3. Treatment of BLP with lysozyme

Some of the BLP is bound to murein; treatment with lysozyme can digest the murein and free the BLP. Purified murein-BLP complexes were incubated with 100 μg/ml of lysozyme in 0.01 M Tris, pH 7.6 at 37 °C for 16h.

4. Western blotting

Western blotting was used to test whether BLP is present in the interface of a B-D extraction of bacteria. *E. coli* LCD25 or a BLP-deficient strain, JE5505 [280], were subjected to B-D extraction and the interface materials were isolated and treated with or without lysozyme. The resulting materials were electrophoresed on a 8-16% gradient SDS-polyacrylamide gel and transferred to nitrocellulose (Immobilon-P, Millipore, Bedford, MA). All of the samples were prepared in sample buffer (2.5% SDS, 22% glycerol in Tris base and trace bromophenol blue) and were boiled before loading onto gels. After blocking of the nitrocellulose for 16 hours at 4°C with 10% nonfat milk in TTBS (150 mM NaCl, 50 mM Tris, 0.1% Tween-20, pH 7.5), the blots were incubated with a purified monoclonal mouse anti-BLP antibody, 1C7 (T505), diluted 1:1000 in blocking buffer, at room temperature for 1 hour. The blots were then washed with TTBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody at room temperature for 1 hour. After washing with TTBS, peroxidase substrate was added (Western Lighting Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, Inc., Boston, MA) and film (Kodak) was exposed to the blots for 5 to 30 seconds and developed. The monoclonal anti-BLP antibody was generously provided by J. Hellman (Massachusetts General Hospital, Boston). Bacterial strain JE5505 was kindly provided by our collaborator, P. D. Rick (Uniformed Services University of the Health Sciences, Bethesda, MD).

4. Generation of ECA deficient *E coli* LCD25

WecA (rfe) and WecG (rffM) genes encode transferases that catalyze the synthesis of lipid I and lipid II respectively, and which are the first two intermediates in the biosynthesis of the ECA trisaccharide unit [281]. The WecA gene is also essential for the synthesis of LPS O-antigens in *E. coli* O8, O9 and O16. The disrupted WecA and WecG gene was introduced into LCD25 using P1-transducion as described [281]. First, bacterial strains 21548 and 21568, which contain WecA::Tn10 and WecG::Tn10 genes respectively, were resuspended in TB with 5 mM $CaCl_2$, and a P1 bacterial phage lysate was added at 1:1000 dilution. After 5 hours incubation at 37 °C with shaking, the bacteria were killed by chloroform, and the culture medium which contained the P1 phage with WecA::Tn10 or WecG::Tn10 was harvested by centrifugation. *E. coli* LCD25 were resuspended in 10 mM $MgSO_4$ and 5 mM $CaCl_2$, and serial dilutions of P1 phage with WecA::Tn10 or WecG::Tn10 medium were added and incubated at 31°C for 30 min without shaking. Sodium citrate (final concentration of 0.5 M) was added to stop transduction. LB medium was added and after 1 hour of incubation at 30 °C with shaking, the bacteria were spread on LB plates that contained tetracycline (selecting bacteria with WecA::Tn10 or WecG::Tn10 genes) and kanamycin (selecting LCD25). The successful disruption of ECA production was demonstrated by the lack of the ladder-like pattern when the [^{14}C]-labeled bacteria were resolved on SDS-PAGE and subjected to autoradiography. Bacterial mutant strains 21548, 21568 and P1 lysate were kindly provided by P. D. Rick.

5. Label bacteria with BODIPY

E. coli LCD25, LCD25 WecA-, LCD25 WecG-, LCD25 O16, LCD25 WecG-, O16 were cultured, washed and resuspended at 1×10^8 CFU/ml in medium that contained 0.15 M NaCl and 0.1M Na_2CO_3, pH 8.3. 10 μl of BODIPY solution (10 mg/ml, Molecular Probes, Inc., Eugene, OR) was added to 1 ml bacterial suspension, and incubated at room temperature for 1 hour. The bacteria were collected by centrifugation at 3000 g for 5 minutes and washed twice with the above medium. After the bacteria were resuspended in 1 ml of PBS, they were examined using a fluorescent microscope.

C. Results

1. After bacteria are incubated with DCs, fatty acids derived from bacterial components other than LPS are lost from the Bligh-Dyer interface.

As described in chapter II, to study the deacylation of LPS in whole bacteria, I actually measured the loss of 3-OH 14:0, 12:0 and 14:0 from the interface of a B-D extraction. Katz et al. have shown that LPS contributes almost all of the 12:0 and about 75% of the 14:0 at the B-D interface [119]. I found that 16:0 was also lost from the interface when *E. coli* were incubated with DCs, with a rate similar to those observed for 12:0 and 14:0 (Fig. 30). The bacterial molecules that contribute this 16:0 and whether AOAH can release fatty acids from these molecules are not known.

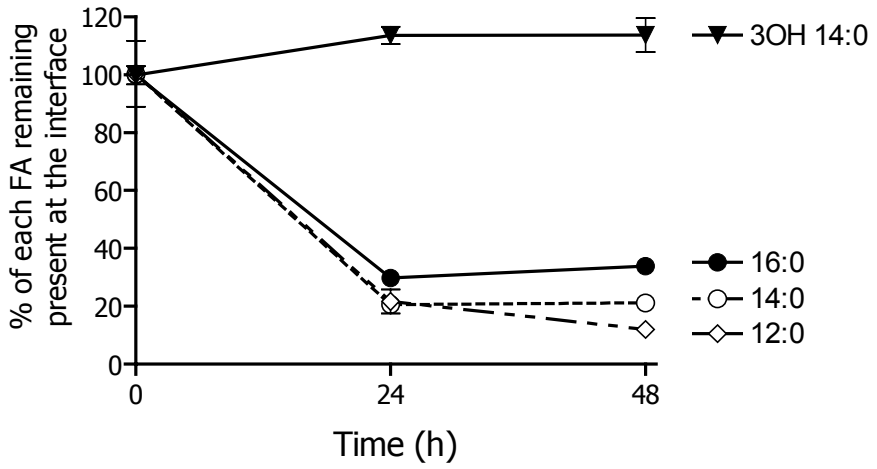


Figure 30. Palmitate (16:0) is lost from the interface of the Bligh-Dyer extraction.
E. coli LCD25 with [^{14}C]-labeled fatty acids were incubated with BMDCs as described in chapter II. The loss of 12:0, 14:0 and 16:0 from the interface of B-D extraction was determined.

The fatty acid compositions of purified LPS and the interface of bacterial B-D extraction were then compared. Because 3-OH 14:0 is exclusively derived from LPS, the relative amount of non-hydroxylated fatty acids was obtained by comparing with the amount of 3-OH 14:0 (Table. 2). In the B-D extraction interface, almost all of the 12:0, the majority (78%) of 14:0 and part of 16:0 are derived from LPS, while the minority of 14:0, some of 16:0, and almost all of the 16:1 and 18:1 are derived from bacterial components other than LPS. Since molecules present at the interface of B-D extraction are presumably amphiphilic, bacterial lipoproteins, which have hydrophilic protein portion and hydrophobic fatty acids, may be present at the interface. Degradation of the protein portion of bacterial lipoprotein may lead to the loss of the amphiphilic property of

lipoprotein molecules and therefore their disappearance from interface. I compared the fatty acid composition of B-D interface of bacteria treated with proteinase K with that of mock- treated bacteria by HPLC (described in Chapter II). Proteinase K treatment decreased 16:1, 16:0 and 18:1 at the interface (Table 3), suggesting that bacterial lipoproteins are present at the interface of the B-D extraction and contribute mainly to 16:1, 16:0 and 18:1.

Table 2. The relative amount of fatty acids derived from the interface of Bligh-Dyer extraction of purified LPS and whole *E. coli* LCD25.

	Purified LPS	IF of bacteria
3-OH 14:0	1.00	1.00
12:0	0.23	0.24
14:0	0.25	0.32
16:1	0	0.06
16:0	0.17	0.31
18:1	0	0.06

Interface material was subjected to acid/base hydrolysis and the amount of each species of fatty acids was measured by HPLC. The amount of non-hydroxylated fatty acids was expressed relative to the content of 3-OH 14:0 in the same preparation.

Table 3. Some of the fatty acids in the interface of Bligh-Dyer extraction are derived from bacterial lipoproteins.

	IF of bacteria	IF of bacteria + PK
3-OH 14:0	1.00	1.00
12:0	0.16	0.14
14:0	0.20	0.18
16:1	0.02	0.005
16:0	0.10	0.04
18:1	0.02	0.00

E. coli LCD25 with [^{14}C]-labeled fatty acids was treated or untreated with proteinase K 75 μg/ml at 37 °C for 16 hours. The treated or untreated bacteria were subjected to B-D extraction and the interface material was collected and hydrolyzed with acid/base. The released fatty acids were purified by B-D extraction and analyzed by using HPLC. The ratios of non-hydroxylated fatty acids to 3-OH 14:0 in the same preparation are shown. Note that there was less 16:1, 16:0 and 18:1 in the proteinase K-treated interface.

2. Braun's lipoprotein is present at the interface of the B-D extraction.

The most abundant bacterial lipoprotein, BLP, occurs in a free form and a murein-bound form. Treatment of the murein-bound form BLP with lysozyme, which can digest peptidoglycan, releases the free form of BLP. The B-D interfaces prepared from *E. coli* LCD25 and from a BLP-deficient strain, JE5505, were untreated or treated with lysozyme, and then resolved on SDS-PAGE and detected with an anti-BLP monoclonal antibody. The untreated interface contained both the free form (about 6 KDa) and

murein-bound form (which is present at the top of the stacking gel because of the large molecular weight) of BLP (Fig. 31 lane 1). The treatment with lysozyme decreased the molecule weight of the murein-bound form of BLP, and a ladder-pattern of BLP was detected because of the incomplete digestion of murein (Fig. 31 lane 2). The specificity of this monoclonal antibody is shown by its inability to recognize the B-D extraction interface of JE5505. Similar results are shown in Figure 33. The B-D interface was isolated from LCD25 with [^{14}C]-labeled fatty acids, resolved on SDS-PAGE, and then the bacterial components that contained fatty acids were detected by autoradiography. Murein-bound BLP was present at the top of the gel; after lysozyme treatment, its molecular weight dropped to about 14.3 KDa (Fig. 33, comparing lane 1 and 2, 3 and 4). This change was more obvious in the ECA deficient mutant (Fig. 33, lane 3 and 4). ECA deficient mutants will be described later.

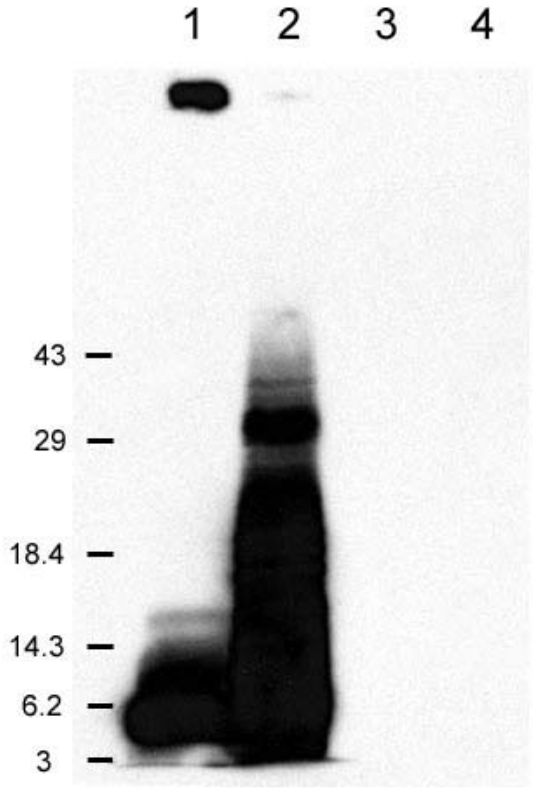


Figure 31. Western blotting analysis shows that BLP is present at the interface of the Bligh-Dyer extraction of *E. coli*.

E. coli LCD25 and a BLP deficient strain, JE5505, were subjected to B-D extraction and the interface material was isolated and treated or untreated with 100 μg/ml of lysozyme at 37°C for 24 h. The interface materials were then resolved on 8-16% gradient SDS-PAGE, transferred to nitrocellulose, and developed using monoclonal anti-BLP antibody as primary antibody. Lanes 1, 2, interface material of E. coli LCD25 untreated or treated with lysozyme; lanes, 3, 4, interface material of BLP deficient mutant JE5505 untreated or treated with lysozyme. Markers shown at the left are the apparent molecular weights (KDa).

I also analyzed the fatty acids attached to BLP. After purified murein-bound BLP is hydrolyzed with acid/base, the released fatty acids are purified and resolved on TLC system II. The majority of fatty acids attached to BLP are 16:0 or 18:1 or both (Fig. 32).

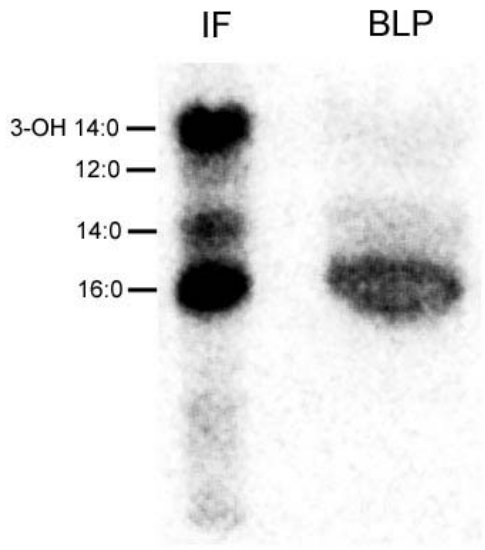


Figure 32. The fatty acids derived from BLP comigrate with 16:0 on TLC.

Fatty acids hydrolyzed from BLP by acid/base treatment were analyzed on TLC system 2 as described in chapter II. The relative positions of the fatty acids derived from the interface of B-D extraction were shown on the left.

3. ECA is present at the interface of B-D extraction.

Because ECA has a hydrophobic diacylglyceride moiety and a hydrophilic polysaccharide chain, we reasoned that it might also be present at the interface of the B-D extraction. WecA::Tn10 was introduced into *E. coli* LCD25 so that ECA synthesis was abolished and the fatty acids in the resulting bacterial strain (LCD25, WecA-) could be highly radiolabeled. The B-D interface of LCD25 and LCD25, WecA- were isolated and resolved on SDS-PAGE (Fig. 33). ECA is present at the interface and shows the expected ladder-like pattern on the gel; this pattern is missing in the ECA deficient strain LCD25, WecA-. Because LCD25 LPS has K12 core, which is an acceptor for ECA polysaccharide chains, the ladder pattern could be ECA_{LPS} or ECA_{PG}. However, the former form was excluded because when the gel was stained by silver, the ladder-like pattern was absent (data not shown), while the LCD25 LPS can be stained with silver on the same gel. The O-fatty acyl substituent at the position 1 of PG is mainly 16:0, and at position 2, 18:1, 16:1, and 16:0 are attached [278]. ECA thus contributes to some of the 16:1, 16:0 or 18:1 at the B-D interface. Because the amount of ECA is about one tenth of that of LPS, and each ECA_{PG} has 2 fatty acyl chains, ECA may contribute less than one tenth of nonhydroxylated fatty acids at the B-D interface.

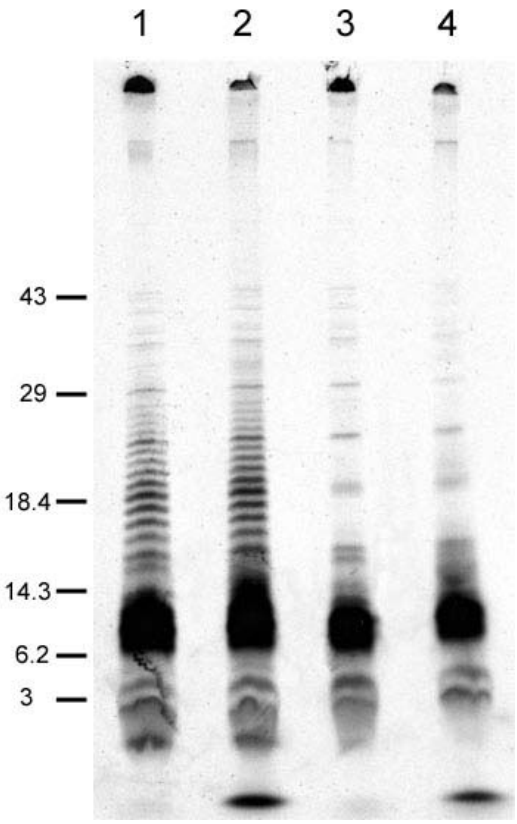


Figure 33. ECA is present at the interface of the Bligh-Dyer extraction.

[^{14}C]-labeled *E. coli* LCD25 with or without ECA was subjected to B-D extraction, and the interface material was isolated, untreated or treated with lysozyme, and then resolved on 15% SDS-PAGE. The bacterial components with fatty acyl chains in the interface were shown by autoradiography. Lanes 1, 2, interface of *E. coli* LCD25, untreated (1) or treated with lysozyme (2); lanes 3,4, interface of ECA deficient *E. coli* LCD25 (WecA-) untreated (3) or treated with lysozyme (4). The markers shown at the left are apparent molecule weights (KDa).

4. AOAH does not deacylate bacterial lipoproteins or ECA under conditions that are optimized for LPS deacylation *in vitro*.

Then I tested whether AOAH was able to deacylate other bacterial components in the interface *in vitro*. The B-D interface of [^{14}C]-labeled *E. coli* LCD25 was incubated with purified human AOAH in the reaction mixture used for measuring LPS deacylation (see Materials and Methods in Chapter II), and the released fatty acids were separated by TLC (Fig. 34). 12:0 and 14:0 were cleaved by AOAH treatment, while only a very small amount of 16:0 was released, suggesting that under such a *in vitro* condition, bacterial lipoprotein and ECA cannot be deacylated by AOAH.

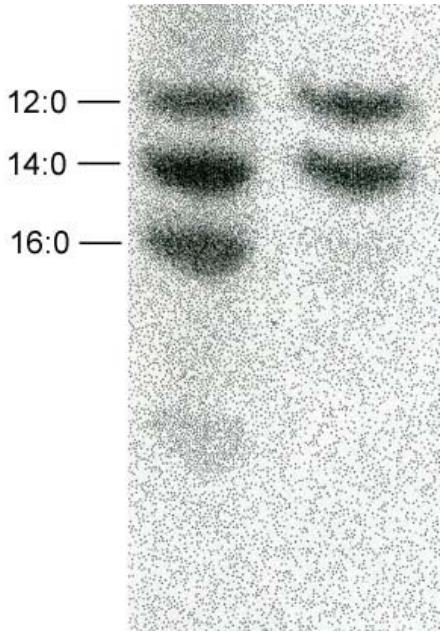


Figure 34. AOAH does not release 16:0 from the interface of Bligh-Dyer extraction *in vitro*.

The B-D interface of LCD25 with [^{14}C]-labeled fatty acids was isolated and incubated with purified human AOAH in a reaction mixture as described in chapter II, at 37 °C for 16 hours. The released fatty acids were then extracted and resolved by two steps of TLC, as described in chapter II. Shown above is the second step of TLC. AOAH-released non-hydroxylated fatty acids (right lane) are compared with acid/base hydrolyzed non-hydroxylated fatty acids (left lane).

5. 16:0 is lost from the interface of Bligh-Dyer extraction after the bacteria are incubated with AOAH -/- macrophages.

Then I asked whether peritoneal macrophages from AOAH -/- mice can release 16:0 from the interface and found that although KO macrophages were unable to release 12:0 or 14:0, they released 16:0 at about the same rate as did WT macrophages (Fig. 35). Because the loss of bacterial lipoproteins from the interface can be due to either the degradation of the protein portion or the release of lipids from of bacterial lipoprotein, I can not conclude that AOAH cannot deacylate bacterial lipoprotein based on this experiment alone. For a similar reason, because it is not clear whether the polysaccharide chain of ECA can be degraded, and whether the disappearance of 16:0 from the interface is caused by the deacylation of ECA is not known.

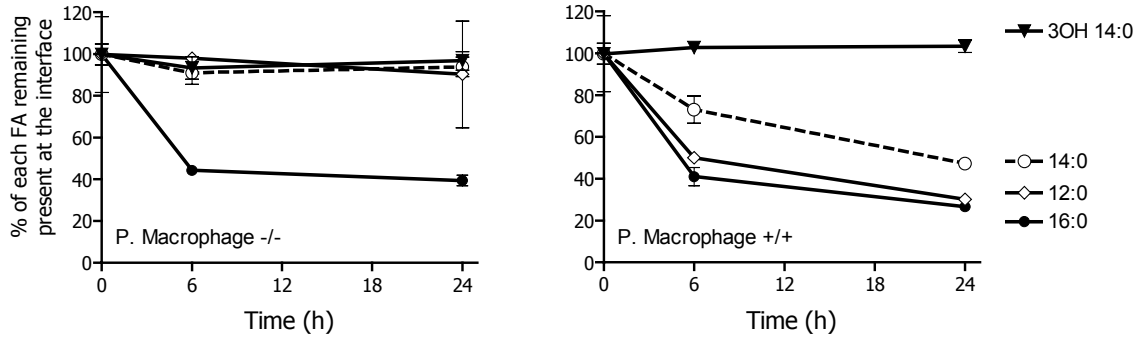


Figure 35. Peritoneal macrophages from AOAH -/- mice release 16:0 from the interface of a B-D extraction.

Peritoneal macrophages, prepared from AOAH -/- and +/+ mice as described in Chapter II, were allowed to take up [^{14}C]-labeled *E. coli* LCD25 and digest them. The cells and medium were harvested at 0, 6 and 24 hours and then subjected to B-D extraction. The percentages of fatty acids remaining at the interface of the B-D extraction were measured and calculated. AOAH -/- macrophages were unable to remove 12:0 and 14:0 from the LPS in the interface, yet they did deplete the 16:0.

6. ECA helps bacteria evade phagocytes.

The functions of ECA are not clear. The growth and physiology of ECA-deficient mutants appear unaltered *in vitro*. Studies have shown that ECA may increase bacterial survival in mice and therefore render them more virulent [282;283]. After [^{14}C]-labeled LCD25 or LCD25, WecA- were incubated with BMDCs, about 4 or 5 times more LCD25, WecA- were captured by BMDCs than were the parental strain LCD25 (Fig. 36). This result suggests that, similar to O-antigen, ECA may prevent bacteria from being captured by phagocytes.

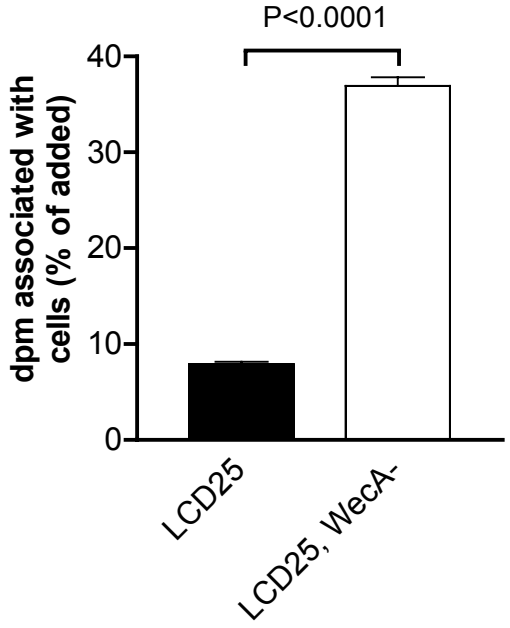


Figure 36. BMDCs capture more ECA-deficient bacteria than control bacteria.
[^{14}C]-labeled bacteria were added (bacteria:cell ratio = 50: 1) to BMDCs, which were prepared from B6 mice and seeded at 5×10^5/ well in a 6-well-plate. After incubation for 1 hour, the medium was aspirated, the cells were washed twice with cRPMI to get rid of unbound bacteria, and the cells were then harvested. The radioactivity associated with the cells was measured by scintillation counting and the percentage of the total dpm that bound to the cells was calculated. Experiments were done in quadruplicate and means ± 1SD are shown. P values were obtained using Student's *t* test.

To further determine and compare the roles of ECA and O-antigen in bacterial anti-phagocytosis, I transfected a plasmid (pPR1461) that contains genes for expressing an O-antigen, O16, into either LCD25 or LCD25, WecG-. The WecG gene encodes an enzyme that catalyzes the second step in ECA unit synthesis; LCD25, WecA- could not be used in this study because the WecA gene is also important for the synthesis of many O antigens, including O16. The successful abolition of ECA synthesis by disrupting the WecG gene and the introduction of O16 antigen expression is shown in Figure 37.

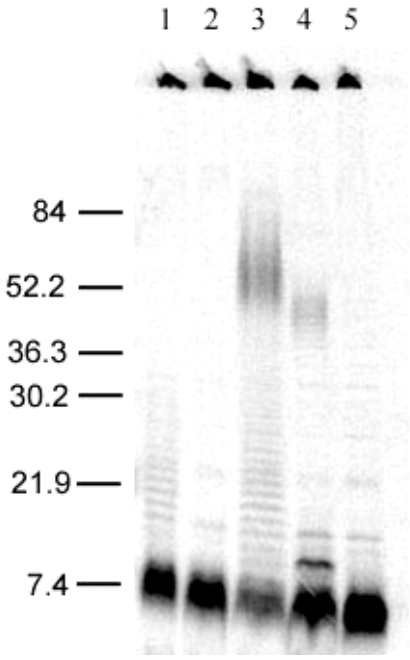


Figure 37. Disruption of WecG gene and introduction of O16 synthesis genes into LCD25.

WecG::Tn10 was introduced into LCD25 by P1 phage, and a plasmid containing O16 synthesis genes was transfected into LCD25 or LCD25, WecG- by electroporation. Bacterial fatty acids were labeled with [^{14}C]-acetate, the B-D interfaces of the labeled bacteria were resolved on SDS-PAGE and bacterial components containing fatty acyl chains were detected by autoradiography. Lane 1, LCD25; 2, LCD25, WecG-, lacking the ECA-ladder; 3, LCD25, O16, which has both ECA and the high molecular weight O16 ladder ; 4, LCD25, WecG-, O16, which lacks ECA but possesses O16 antigen; 5, LCD25, WecA-.

I then incubated BMDCs with the various bacteria and, after washing off the unbound bacteria, I stained the cells and bacteria and counted the number of bacteria that associated with the BMDCs under the microscope (Fig. 38). Each BMDC captured a median of one LCD25, about four WecA- and about eight WecG-. Unexpectedly, in this study, O16 did not prevent bacteria from binding to BMDCs.

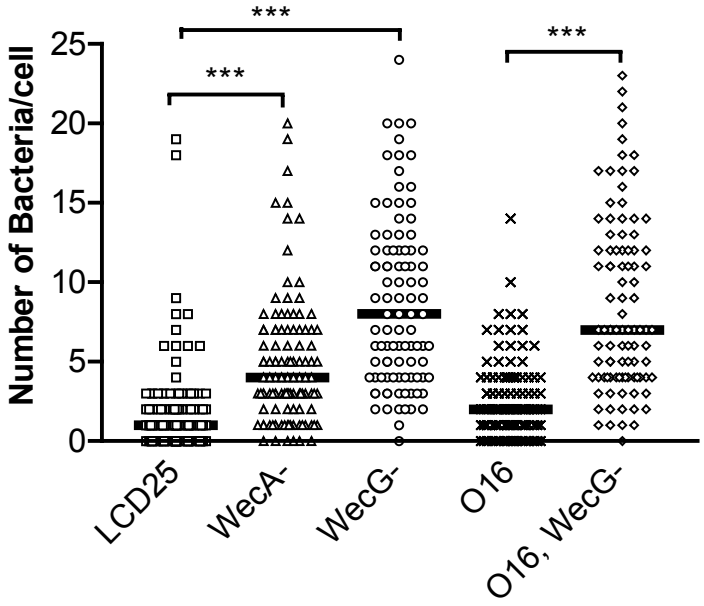


Figure 38. ECA-deficient bacteria are more susceptible to being captured by BMDCs.

BMDCs were seeded at 5×10^5/ well onto cover slips in a 6-well-plate, and after incubation for 16 hours at 37°C, *E. coli* LCD25 with or without ECA or O16 were added at a bacteria:cell ratio of 50:1. After incubation for 1 hour at 37 °C, the unbound bacteria were washed away, and the cells and bacteria were stained with the HEMA 3 Staining Set (Biochemical Sciences Inc). The number of bacteria that associated with each cell was counted; a total of 100 cells were evaluated and the medians are shown.

Because the method used for this study did not distinguish intracellular bacteria from extracellular ones, I next studied whether ECA contributes to evading phagocytosis by labeling the various bacterial strains with BODIPY and measuring the intracellular fluorescence by FACS (Fig. 39). The results showed that BMDCs phagocytosed substantially more WecA- and WecG- bacteria than control bacteria. Expression of O16 also helped bacteria escape BMDC phagocytosis.

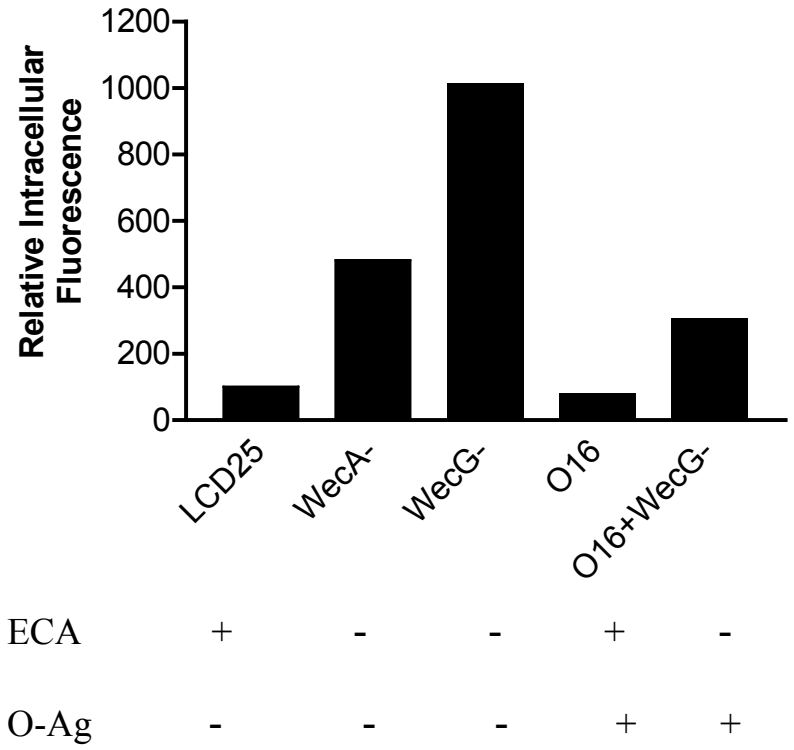


Figure 39. ECA and O-antigen protect bacteria from phagocytosis by BMDCs.

LCD25 with and without ECA or O16 were labeled with BODYPY and the MFI of bacteria were measured by FACS. Bacteria were added to BMDCs at a bacteria:cell ratio of 50:1 and incubated at 37 °C for 1 hour. Then the intracellular bacterial fluorescence of BMDCs was measured by FACS after quenching the extracellular fluorescence with trypan blue. As controls, BMDCs were incubated with bacteria at 4 °C. The relative phagocytic activity was calculated as (intracellular MFI at 37 °C - intracellular MFI at 4 °C) ÷ bacterial MFI.

D. Discussion

Lipoproteins have been found in both Gram-positive and Gram-negative bacteria. Bacterial lipoproteins differ from mammalian lipoproteins in that bacterial lipoproteins contain a N-terminal triacylated cysteine, a unique structure that can be recognized as a PAMP by mammals. Similar to LPS, bacterial lipoproteins have profound immunoregulatory functions. Lipoproteins induce proinflammatory cytokine production in macrophages and DCs and lethal shock in LPS-responsive and –nonresponsive mice [16]. It has been shown that microbial lipoproteins activate cells in a TLR2 dependent manner, requiring the lipid element for optimal activity. Little is known how bacterial lipoproteins can be degraded or inactivated by animals so that their immunostimulatory potency can be diminished. Conceivably, the peptide portion of lipoproteins can be degraded by proteinases. To decrease biopotency, the peptide bonds close to N-terminus have to be cleaved because the triacylated cysteine with a few attached amino acids is still bioactive. Alternatively, the lipid portion of the lipoprotein may be cleaved by lipases. AOAH is a candidate lipase because it cleaves ester-linked fatty acyl chains from LPS and phospholipids.

When I began to study the deacylation of LPS in whole bacteria, I found that, in addition to 12:0 and 14:0, which are derived mainly from LPS, 16:0 was also lost from the interface of the B-D extraction. I found that bacterial lipoproteins, such as Braun's lipoprotein, were present at the interface and contributed to the content of 16:0, 16:1 and 18:1. However, purified AOAH failed to deacylate the lipoproteins at the interface in the reaction buffer that is used for measuring LPS deacylation. Furthermore, BMDCs from

AOAH null mice could also release 16:0 from the interface, suggesting that the loss of 16:0 from the interface may be caused by the degradation of the protein part of lipoproteins or deacylation of lipoproteins by other lipases.

In contrast, there is evidence suggesting that AOAH may deacylate bacterial lipoproteins *in vivo* and thereby reduce their B-cell stimulatory activity. After AOAH WT or KO mice were immunized with synthetic lipopeptide (tripalmitoylglycerylcysteine-Gly-Ser-Ser-His-His), significantly more polyclonal IgM response was induced in AOAH KO mice than in WT mice (chapter III), which is similar to what has been observed in LPS-immunized mice. Purified bacterial lipoprotein will now be used to immunize AOAH WT and KO mice to test if AOAH influences the production of antibodies specific for the protein moiety. If it can deacylate bacterial lipoproteins, AOAH could have a broad spectrum of functions, regulating host responses not only to gram-negative bacterial but also to gram-positive bacteria.

ECA was also found to be present at the interface of the B-D extraction. This was shown by resolving the B-D interface from [^{14}C]-labeled bacteria on SDS-PAGE. Because ECA-derived fatty acids are mainly 16:0 and 18:1, ECA contributes to the 16:0 band on TLC. It is not known whether ECA can be degraded by mammalian cells. *In vitro*, purified AOAH did not release 16:0 from the interface, indicating that ECA is not deacylated under such conditions.

The functions of ECA are not known. ECA_{PG} is not immunogenic and does not possess stimulatory activity towards mammalian cells. Valtonen et al. have found that the absence of ECA reduced the mouse virulence of *S. typhimurium* inoculated *i.p.* by

about 10 fold [284]. Nnalue et al. have shown that ECA contributes to the increased survival of Salmonella species in mice *in vivo* [285]. In this study, I found that ECA renders bacteria able to evade phagocytes, which may partially explain these results.

BMDCs captured 4- or 5-fold more ECA-deficient *E. coli* LCD25 than their parental strain, as determined by measuring cell-associated bacterial radioactivity or direct counting of cell-associated bacteria. The phagocytosis experiments showed that both ECA and O16 prevent phagocytosis. Like the O-antigen of LPS, the long polysaccharide chain of ECA may protect bacteria from phagocytosis, the fusion of phagosome-containing bacteria with lysosomes [8] and complement attack [9]. Furthermore, ECA may render enteric bacteria resistant to solubilization by intestinal enzymes and the lipids in bile, which may explain why ECA is unique to enteric bacteria.

CHAPTER V. CONCLUDING REMARKS AND FUTURE DIRECTIONS

AOAH is a unique mammalian enzyme that specifically removes the secondary fatty acyl chains from the lipid A moiety of LPS [106]. AOAH-treated LPS has dramatically reduced tissue toxicity, as shown in the rabbit dermal Shwartzman reaction [121]. *In vitro* experiments with human cells revealed that enzymatic deacylation not only decreases LPS bioactivity but also converts LPS to its own antagonist [122-126]. So far, no enzymatic activity that releases the primary fatty acyl chains or degrades the lipid A backbone has been identified. Partial deacylation of LPS might be an economical and effective way to diminish animals' responses to LPS.

A. Stimulus-regulated deacylation of LPS by DCs.

AOAH activity has been found primarily in myeloid cells, such as neutrophils and monocytes-macrophages. Immature DCs, which are mainly distributed in tissues that interface with the external environment, also can capture, kill and digest microbial invaders with high efficiency. In this study, I found that, in addition to their ability degrade bacterial protein, immature DCs express high levels of AOAH and deacylate the LPS in *E. coli* they have phagocytosed in an AOAH-like manner. These results underscore the point that DCs are not mere "mobile sentinels" to initiate adaptive immunity, but they also play a pivotal role in innate immunity. More importantly, in addition to processing and presenting protein antigens, they degrade one of the most bioactive bacterial molecules, LPS, and may regulate immune responses to it.

The expression of AOAH in DCs is also regulated by exogenous stimuli. Maturation cocktail (IL-4, TNF-α, IL-1β and agonistic CD40 antibody) induced the "typical" maturation of DCs, which decreased their phagocytic activity and increased cell surface expression of costimulatory molecule(s). AOAH expression was dramatically reduced in maturation cocktail-treated DCs, suggesting that the ability to deacylate LPS is a feature of immature DC. In contrast, when they were treated with LPS or whole *E. coli*, DCs upregulated costimulatory molecule(s) yet they maintained or increased AOAH expression. The regulation of AOAH expression occurred at the mRNA level, as shown by real-time PCR. In keeping with this finding, Cody et al. reported that LPS treatment could increase AOAH mRNA abundance in murine macrophages (10–20-fold) and *in vivo* in mouse lung and liver (3–6-fold). Furthermore, upregulation of AOAH expression was not restricted to stimulation by LPS, the AOAH substrate. Other TLR agonists, such as CpG ODN (TLR9) and *M. luteus* (TLR2), also increased AOAH expression, indicating that the ability to deacylate LPS is regulated by multiple conserved microbial molecular "patterns".

In addition, I have shown that XS52 cells treated with maturation cocktail have decreased ability to phagocytose, to kill bacteria, to degrade bacterial proteins and to deacylate LPS, whereas exposure to LPS maintains or increases the DCs' ability to take up and kill bacteria and to digest bacterial components. Thus, in response to different environmental stimuli, DCs coordinate AOAH expression and LPS deacylation with many other anti-bacterial responses, increasing or decreasing their ability to degrade this important bacterial molecule.

In the future, whether AOAH plays a role in presenting bacterial antigens will be studied. Various studies have shown that LPS molecules can interfere with the presentation of bacterial protein antigens. Forestier et al studied the intracellular trafficking of *Brucella* LPS and found that the LPS initially accumulated within the lysosomes of macrophages, followed by recycling to the plasma membrane [146]. The same authors also demonstrated that *Brucella* LPS specifically associates with MHC II molecules in APCs [258]; at the cell surface of macrophages, LPS clusters are highly enriched in MHC II molecules, so that the LPS molecules might interfere with antigen presentation to $CD4^+$ T cells [286]. In addition, studies suggest that the presence and the length of the O-antigen of LPS may influence the presentation of bacterial protein antigens to T cells [287;288]. AOAH might alter the physical properties of LPS and modulate its influence on antigen presentation. In the bacterial outer membrane, LPS is tightly associated with bacterial outer membrane proteins, presumably by hydrophobic interactions between the fatty acyl chains of LPS and the hydrophobic regions of membrane proteins. Deacylation may decrease the hydrophobicity of LPS and induce disaggregation of complexes of LPS and outer membrane proteins, thus facilitating membrane protein processing and presentation.

B. Characterization of AOAH null mice.

Although AOAH can detoxify LPS *in vitro*, the absence of AOAH did not make mice more susceptible to LPS or Gram-negative bacterial challenge. This is probably because LPS deacylation occurs at a relatively slow rate and is unable to counteract the (quicker) cytokine responses to LPS. In keeping with this observation, *in vitro*

experiments showed that AOAH deficiency did not cause exaggerated cytokine responses or surface expression of costimulatory molecules by DCs. The slow rate of LPS deacylation is probably due to the nature of the reaction, the low abundance of AOAH and the slow traffic of LPS to the intracellular "deacylation" compartment.

AOAH-deficient mice showed expanded polyclonal antibody responses to LPS, indicating that AOAH plays an important role in negatively regulating mitogenicity of LPS. Because B cells do not express AOAH and AOAH is not very active in extracellular fluids, the ability of AOAH to decrease polyclonal antibody formation suggests that some of the injected LPS may be deacylated by AOAH-bearing phagocytes, such as DCs, macrophages and neutrophils, prior to interacting with B cells. DCs are of special interest because they can take up antigen from peripheral sites and migrate to lymphoid tissues, interacting with T or B cells. DCs have been suggested to facilitate B cell function [289], and to transport antigen to B cells in initiating and modulating TD responses [102]. Balazs et al. have shown that a DC subset promotes TI-II Ag-specific plasma cell differentiation *in vivo* and *in vitro* [104]. My results suggest that DCs or macrophages also modulate B cell responses to a TI-1 Ag, LPS, *in vivo*.

Correlating with the exaggerated polyclonal responses, autoimmune antibodies, such as anti-nuclear antibodies, were dramatically elevated in AOAH-null mice immunized with LPS. For a TD-antigen to activate B cells, cognate T cell help is required; therefore, only when both T and B cell tolerance is broken, can autoimmune antibody be produced. However, little is known how animals remain tolerant after they are exposed to polyclonal B cell activators. In this study, I show that the ability to

degrade and deactivate LPS diminishes the production of autoimmune antibodies. Thus, AOAH may be important for avoiding infection-induced autoimmunity.

When mice were injected with a low dose of LPS or dLPS, dLPS had less ability to stimulate polyclonal responses *in vivo* than did LPS, yet it retained the ability to induce anti-LPS specific antibodies. AOAH may thus preserve the ability of LPS to induce antibodies to itself.

In addition to LPS, AOAH null mice also had increased antibody responses to lipopeptide. AOAH may be able to deacylate and deactivate bacterial lipoproteins. Because bacterial lipoproteins are present in both Gram-negative and –positive bacteria, AOAH may be capable of regulating antibody responses to a wide range of bacterial infections. However, as discussed in Chapter IV, *in vitro* evidence that bacterial lipoproteins can be AOAH substrates remains to be obtained.

LPS is also an adjuvant, i.e., it can enhance the immune response to a protein antigen. Adjuvants are known to be able to increase antigen presentation and provide costimulation to T cells. I found that AOAH deficiency did not alter the ability of LPS to act as an adjuvant, indicating that the deacylation of LPS by APCs, such as DCs and macrophages, does not have an effect on the cells' ability to activate T cells despite the fact that AOAH has such a dramatic impact on B cell function. Deacylation of LPS then maintains its beneficial adjuvant activity and antigenicity, while diminishing its nonspecific antibody stimulatory and potentially harmful activities.

In the future, whether AOAH null mice also have exaggerated antibody responses to whole Gram-negative bacteria will be studied. How DCs or macrophages present the deacylation information to B cells and whether BAFF or APRIL is required will be also be investigated. The kinds of B cells (B1, MZB, or B2 cells) that produce the polyclonal antibody will be evaluated. Histology or pathology of kidneys and other organs from LPS immunized AOAH KO and WT mice will be compared. Although AOAH may be critical in regulating B cell tolerance after LPS immunization, the induction of tissue lesions in AOAH null mice may require additional check points, such as breaking tolerance in T cells. Mice with the disrupted AOAH gene will be bred with an autoimmune-prone strain that has impaired T cell function, such as MLR *lpr/lpr* mice, which harbor a mutation in FAS gene and have reduced activation-induced T cell death. The epistatic interaction of AOAH -/- and *lpr* may lead to severe autoimmune diseases before or after LPS immunization.

REFERENCE LIST

1. Rietschel,E.T., T.Kirikae, F.U.Schade, U.Mamat, G.Schmidt, H.Loppnow, A.J.Ulmer, U.Zähringer, U.Seydel, F.Di Padova, M.Schreier, and H.Brade. 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB J.* 8:217-225.

2. Raetz,C.R. and C.Whitfield. 2002. Lipopolysaccharide endotoxins. *Annu.Rev.Biochem.* 71:635-700.

3. Caroff,M., D.Karibian, J.-M.Cavaillon, and N.Haeffner-Cavaillon. 2002. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes Infect.* 4:915-926.

4. Galanos,C. 1975. Physical state and biological activity of lipopolysaccharides. Toxicity and immunogenicity of the lipid A component. *Z.Immun.-Forsch.* 149:S214-S229.

5. Tanamoto,K.-I., U.Zahringer, G.R.McKenzie, C.Galanos, E.Th.Rietschel, O.Luderitz, S.Kusumoto, and T.Shiba. 1984. Biological activities of synthetic lipid A analogs: pyrogenicity, lethal toxicity, anticomplement activity, and induction of gelation of Limulus amoebocyte lysate. *Infect.Immun.* 44:421-426.

6. Galanos,C., O.Luderitz, E.T.Rietschel, O.Westphal, H.Brade, B.Brade, M.Freudenberg, U.Schade, M.Imoto, H.Yoshimura, S.Kusumoto, and T.Shiba. 1985.

Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. *Eur.J.Biochem.* 148:1-5.

7. Munford,R.S., C.L.Hall, and P.D.Rick. 1980. Size heterogeneity of Salmonella typhimurium lipopolysaccharides in outer membranes and culture supernatant membrane fragments. *J.Bacteriol.* 144:630-640.

8. Porte,F., A.Naroeni, S.Ouahrani-Bettache, and J.P.Liautard. 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. *Infect.Immun.* 71:1481-1490.

9. Grossman,N., M.A.Schmetz, J.Foulds, E.N.Klima, V.E.Jimenez-Lucho, L.L.Leive, K.A.Joiner, and V.Jiminez. 1987. Lipopolysaccharide size and distribution determine serum resistance in Salmonella montevideo. *J.Bacteriol.* 169:856-863.

10. Rietschel,E.T., L.Brade, B.Lindner, and U.Zahringer. 1992. Molecular biochemistry of lipopolysaccharides. *In* Bacterial Endotoxic Lipopolysaccharides. D.C.Morrison and J.L.Ryan, editors. CRC Press, Boca Raton,FL. 3-42.

11. Preston,A., R.E.Mandrell, B.W.Gibson, and M.A.Apicella. 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit.Rev.Microbiol.* 22:139-180.

12. Kulshin,V.A., U.Zähringer, B.Lindner, K.-E.Jäger, B.A.Dmitriev, and E.T.Rietschel. 1991. Structural characterization of the lipid A component of *Pseudomonas*

aeruginosa wild-type and rough mutant lipopolysaccharides. *Eur.J.Biochem.* 198:697-704.

13. Mandrell,R.E., A.J.Lesse, J.V.Sugai, M.Shero, J.M.Griffiss, J.A.Cole, N.J.Parsons, H.Smith, S.A.Morse, and M.A.Apicella. 1990. In vitro and in vivo modification of Neisseria gonorrhoeae lipooligosaccharide epitope structure by sialylation. *J.Exp.Med.* 171:1649-1664.

14. Steeghs,L., R.Den Hartog, A.Den Boer, B.Zomer, P.Roholl, and P.Van der Ley. 1998. Meningitis bacterium is viable without endotoxin. *Nature* 392:449-450.

15. Hellman,J., P.M.Loiselle, E.M.Zanzot, J.E.Allaire, M.M.Tehan, L.A.Boyle, J.T.Kurnick, and H.S.Warren. 2000. Release of gram-negative outer-membrane proteins into human serum and septic rat blood and their interactions with immunoglobulin in antiserum to *Escherichia coli* J5. *Journal of Infectious Diseases* 181:1034-1043.

16. Zhang,H.W., J.W.Peterson, D.W.Niesel, and G.R.Klimpel. 1997. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. *J.Immunol.* 159:4868-4878.

17. Sato,S., F.Nomura, T.Kawai, O.Takeuchi, P.F.Muhlradt, K.Takeda, and S.Akira. 2000. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J.Immunol.* 165:7096-7101.

18. Katz,S.S., K.Chen, S.Chen, M.E.Doerfler, P.Elsbach, and J.Weiss. 1996. Potent CD14-mediated signalling of human leukocytes by *Escherichia coli* can be mediated by interaction of whole bacteria and host cells without extensive prior release of endotoxin. *Infect.Immun.* 64:3592-3600.

19. Poltorak,A., X.He, I.Smirnova, M.-Y.v.H.C.Liu, X.Du, D.Birdwell, E.Alejos, M.Silva, C.Galanos, M.Freudenberg, P.Ricciardi-Castagnoli, B.Layton, and B.Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 282:2085-2088.

20. Hoshino,K., O.Takeuchi, T.Kawai, H.Sanjo, T.Ogawa, Y.Takeda, K.Takeda, and S.Akira. 1999. Cutting Edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the *Lps* gene product. *J.Immunol.* 162:3749-3752.

21. Shimazu,R., S.Akashi, H.Ogata, Y.Nagai, K.Fukudome, K.Miyake, and M.Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J.Exp.Med.* 189:1777-1782.

22. Akashi,S., H.Ogata, F.Kirikae, T.Kirikae, K.Kawasaki, M.Nishijima, R.Shimazu, Y.Nagai, K.Fukudome, M.Kimoto, and K.Miyake. 2000. Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. *Biochem.Biophys.Res.Commun.* 268:172-177.

23. Ingalls,R.R. and D.T.Golenbock. 1995. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. *J.Exp.Med.* 181:1473-1479.

24. Ingalls,R.R., B.G.Monks, R.Savedra, Jr., W.J.Christ, R.L.Delude, A.E.Medvedev, T.Espevik, and D.T.Golenbock. 1998. CD11/CD18 and CD14 share a common lipid A signaling pathway. *J.Immunol.* 161:5413-5420.

25. Ingalls,R.R., B.G.Monks, and D.T.Golenbock. 1999. Membrane expression of soluble endotoxin-binding proteins permits lipopolysaccharide signaling in Chinese hamster ovary fibroblasts independently of CD14. *J.Biol.Chem.* 274:13993-13998.

26. Ozinsky,A., D.M.Underhill, J.D.Fontenot, A.M.Hajjar, K.D.Smith, C.B.Wilson, L.Schroeder, and A.Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc.Natl.Acad.Sci.USA* 97:13766-13771.

27. Medzhitov,R., P.Preston-Hurlburt, and C.A.Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-397.

28. Medzhitov,R. and C.A.Janeway. 1997. Innate immunity: impact on the adaptive immune response. *Curr.Opin.Immunol.* 9:4-9.

29. Janeway,C.A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp.Quant.Biol.* 54:1-13.

30. Hirschfeld,M., J.J.Weis, V.Toshchakov, C.A.Salkowski, M.J.Cody, D.C.Ward, N.Qureshi, S.M.Michalek, and S.N.Vogel. 2001. Signaling by toll-like receptor 2 and

4 agonists results in differential gene expression in murine macrophages. *Infect.Immun.* 69:1477-1482.

31. Werts,C., R.I.Tapping, J.C.Mathison, T.H.Chuang, V.Kravchenko, G.Saint, I, D.A.Haake, P.J.Godowski, F.Hayashi, A.Ozinsky, D.M.Underhill, C.J.Kirschning, H.Wagner, A.Aderem, P.S.Tobias, and R.J.Ulevitch. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat.Immunol* 2:346-352.

32. Wyllie,D.H., E.Kiss-Toth, A.Visintin, S.C.Smith, S.Boussouf, D.M.Segal, G.W.Duff, and S.K.Dower. 2000. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. *J.Immunol.* 165:7125-7132.

33. Takeuchi,O., T.Kawai, P.F.Muhlradt, M.Morr, J.D.Radolf, A.Zychlinsky, K.Takeda, and S.Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int.Immunol.* 13:933-940.

34. Alexopoulou,L., A.C.Holt, R.Medzhitov, and R.A.Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll- like receptor 3. *Nature* 413:732-738.

35. Hayashi,F., K.D.Smith, A.Ozinsky, T.R.Hawn, E.C.Yi, D.R.Goodlett, J.K.Eng, S.Akira, D.M.Underhill, and A.Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll- like receptor 5. *Nature* 410:1099-1103.

36. Hemmi,H., O.Takeuchi, T.Kawai, T.Kaisho, S.Sato, H.Sanjo, M.Matsumoto, K.Hoshino, H.Wagner, K.Takeda, and S.Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.

37. Fitzgerald,K.A., E.M.Palsson-McDermott, A.G.Bowie, C.A.Jefferies, A.S.Mansell, G.Brady, E.Brint, A.Dunne, P.Gray, M.T.Harte, D.McMurray, D.E.Smith, J.E.Sims, T.A.Bird, and L.A.O'Neill. 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413:78-83.

38. Horng,T., G.M.Barton, and R.Medzhitov. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat.Immunol* 2:835-841.

39. Kawai,T., O.Adachi, T.Ogawa, K.Takeda, and S.Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.

40. Alexander,C. and E.T.Rietschel. 2001. Bacterial lipopolysaccharides and innate immunity. *J.Endotoxin Res.* 7:167-202.

41. Matsuura,M., S.-I.Shimada, M.Kiso, A.Hasegawa, and M.Nakano. 1995. Expression of endotoxic activities by synthetic monosaccharide lipid A analogs with alkyl-branched acyl substituents. *Infect.Immun.* 63:1446-1451.

42. Poltorak,A., P.Ricciardi-Castagnoli, S.Citterio, and B.Beutler. 2000. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proc.Natl.Acad.Sci.USA* 97:2163-2167.

43. Lien,E., T.K.Means, H.Heine, A.Yoshimura, S.Kusumoto, K.Fukase, M.J.Fenton, M.Oikawa, N.Qureshi, B.Monks, R.W.Finberg, R.R.Ingalls, and D.T.Golenbock. 2000. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J.Clin.Invest.* 105:497-504.

44. Akashi,S., Y.Nagai, H.Ogata, M.Oikawa, K.Fukase, S.Kusumoto, K.Kawasaki, M.Nishijima, S.Hayashi, M.Kimoto, and K.Miyake. 2001. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition. *Int.Immunol.* 13:1595-1599.

45. Hajjar,A.M., R.K.Ernst, J.H.Tsai, C.B.Wilson, and S.I.Miller. 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat.Immunol.* 3:354-359.

46. Haeffner-Cavaillon,N., M.Caroff, and J.-M.Cavaillon. 1989. Interleukin-1 induction by lipopolysaccharides: Structural requirements of the 3-deoxy-D-manno-2-octulosonic acid (KDO). *Mol.Immunol.* 26:485-494.

47. Koyama,S., E.Sato, H.Nomura, K.Kubo, M.Miura, T.Yamashita, S.Nagai, and T.Izumi. 2000. The potential of various lipopolysaccharides to release IL-8 and G-CSF. *Am.J.Physiol Lung Cell Mol.Physiol* 278:L658-L666.

48. Paeng,N., N.Kido, G.Schmidt, T.Sugiyama, Y.Kato, N.Koide, and T.Yokochi. 1996. Augmented immunological activities of recombinant lipopolysaccharide possessing the mannose homopolymer as the O-specific polysaccharide. *Infect.Immun.* 64:305-309.

49. Hailman,E., J.J.Albers, G.Wolfbauer, A.Y.Tu, and S.D.Wright. 1996. Neutralization and transfer of lipopolysaccharide by phospholipid transfer protein. *J.Biol.Chem.* 271:12172-12178.

50. Wurfel,M.M., E.Hailman, and S.D.Wright. 1995. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide (LPS) by LPS-binding protein and reconstituted high density lipoprotein. *J.Exp.Med.* 181:1743-1754.

51. Kitchens,R.L., P.A.Thompson, S.Viriyakosol, G.E.O'Keefe, and R.S.Munford. 2001. Plasma CD14 decreases monocyte responses to LPS by promoting the transfer of cell-bound LPS to plasma lipoproteins. *J.Clin.Invest.* 108:485-493.

52. Elsbach,P. 2000. Mechanisms of disposal of bacterial lipopolysaccharides by animal hosts. *Microbes and Infection* 2:1171-1180.

53. Guo,L., K.B.Lim, C.M.Poduke, M.Daniel, J.S.Gunn, M.Hackett, and S.I.Miller. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* 95:189-198.

54. Gunn,J.S. 2001. Bacterial modification of LPS and resistance to antimicrobial peptides. *J.Endotoxin.Res.* 7:57-62.

55. Wright,S.D. and M.T.C.Jong. 1986. Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide. *J Exp Med* 164:1876-1888.

56. Wright,S.D., S.M.Levin, M.T.C.Jong, Z.Chad, and L.G.Kabbash. 1989. CR3 (CD11b/CD18) expresses one binding site for Arg-Gly- Asp-containing peptides and a second site for bacterial lipopolysaccharide. *J.Exp.Med.* 169:175-183.

57. Medzhitov,R. and C.A.Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296:298-300.

58. Pulendran,B., K.Palucka, and J.Banchereau. 2001. Sensing pathogens and tuning immune responses. *Science* 293:253-256.

59. Banchereau,J., F.Briere, C.Caux, J.Davoust, S.Lebecque, Y.J.Liu, B.Pulendran, and K.Palucka. 2000. Immunobiology of dendritic cells. *Annu.Rev.Immunol* 18:767-811.

60. Banchereau,J. and R.M.Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.

61. Pasare,C. and R.Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science* 299:1033-1036.

62. Newman,S.L. and A.Holly. 2001. *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect.Immun.* 69:6813-6822.

63. Gildea,L.A., R.E.Morris, and S.L.Newman. 2001. *Histoplasma capsulatum* yeasts are phagocytosed via very late antigen-5, killed, and processed for antigen presentation by human dendritic cells. *J.Immunol.* 166:1049-1056.

64. Kolb-Mäurer,A., A.Unkmeir, U.Kämmerer, C.Hübner, T.Leimbach, A.Stade, E.Kämpgen, M.Frosch, and G.Dietrich. 2001. Interaction of *Neisseria meningitidis* with human dendritic cells. *Infect.Immun.* 69:6912-6922.

65. Lu,M., M.Zhang, R.L.Kitchens, S.Fosmire, A.Takashima, and R.S.Munford. 2003. Stimulus-dependent deacylation of bacterial lipopolysaccharide by dendritic cells. *J.Exp.Med.* 197:1745-1754.

66. Turley,S.J., K.Inaba, W.D.Garrett, M.Ebersold, J.Unternaehrer, R.M.Steinman, and I.Mellman. 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* 288:522-527.

67. Kleijmeer,M., G.Ramm, D.Schuurhuis, J.Griffith, M.Rescigno, P.Ricciardi-Castagnoli, A.Y.Rudensky, F.Ossendorp, C.J.Melief, W.Stoorvogel, and H.J.Geuze. 2001. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J.Cell Biol.* 155:53-63.

68. Svensson,M., B.Stockinger, and M.J.Wick. 1997. Bone marrow-derived dendritic cells can process bacteria for MHC-1 and MHC-II presentation to T cells. *J.Immunol.* 158:4229-4236.

69. Svensson,M. and M.J.Wick. 1999. Classical MHC class I peptide presentation of a bacterial fusion protein by bone marrow-derived dendritic cells. *Eur.J.Immunol.* 29:180-188.

70. Rescigno,M., S.Citterio, C.Thery, M.Rittig, D.Medaglini, G.Pozzi, S.Amigorena, and P.Ricciardi-Castagnoli. 1998. Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc.Natl.Acad.Sci.U.S.A* 95:5229-5234.

71. Yrlid,U., M.Svensson, C.Johansson, and M.J.Wick. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunol.Med.Microbiol.* 27:313-320.

72. Yrlid,U. and M.J.Wick. 2000. Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. *J.Exp.Med.* 191:613-624.

73. Burdin,N. and M.Kronenberg. 1999. CD1-mediated immune responses to glycolipids. *Curr.Opin.Immunol.* 11:326-331.

74. Porcelli,S.A. and R.L.Modlin. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu.Rev.Immunol.* 17:297-329.:297-329.

75. Svensson,M., C.Johansson, and M.J.Wick. 2000. *Salmonella enterica* serovar typhimurium-induced maturation of bone marrow-derived dendritic cells. *Infect.Immun.* 68:6311-6320.

76. Granucci,F., E.Ferrero, M.Foti, D.Aggujaro, K.Vettoretto, and P.Ricciardi-Castagnoli. 1999. Early events in dendritic cell maturation induced by LPS. *Microbes Infect.* 1:1079-1084.

77. Winzler,C., P.Rovere, M.Rescigno, F.Granucci, G.Penna, L.Adorini, V.S.Zimmermann, J.Davoust, and P.Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp.Med.* 185:317-328.

78. Lutz,M.B., N.A.Kukutsch, M.Menges, S.Rössner, and G.Schuler. 2000. Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy *in vitro*. *Eur.J.Immunol.* 30:1048-1052.

79. Sallusto,F., M.Cella, C.Danieli, and A.Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J.Exp.Med.* 182:389-400.

80. Verhasselt,V., C.Buelens, F.Willems, D.De Groote, N.Haeffner-Cavaillon, and M.Goldman. 1997. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells - Evidence for a soluble CD14-dependent pathway. *J.Immunol.* 158:2919-2925.

81. MacAry,P.A., M.Lindsay, M.A.Scott, J.I.O.Craig, J.P.Luzio, and P.J.Lehner. 2001. Mobilization of MHC class I molecules from late endosomes to the cell surface following activation of CD34-derived human Langerhans cells. *Proc.Natl.Acad.Sci.USA* 98:3982-3987.

82. Gatti,E., M.A.Velleca, B.C.Biedermann, W.Ma, J.Unternaehrer, M.W.Ebersold, R.Medzhitov, J.S.Pober, and I.Mellman. 2000. Large-scale culture and selective maturation of human Langerhans cells from granulocyte colony-stimulating factor-mobilized CD34+ progenitors. *J Immunol* 164:3600-3607.

83. Lapointe,R., J.F.Toso, C.Butts, H.A.Young, and P.Hwu. 2000. Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes. *Eur J Immunol* 30:3291-3298.

84. Ardeshna,K.M., A.R.Pizzey, S.Devereaux, and A.Khwaja. 2000. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* 96:1039-1046.

85. De Smedt,T., B.Pajak, E.Muraille, L.Lespagnard, E.Heinen, P.De Baetselier, J.Urbain, O.Leo, and M.Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J.Exp.Med.* 184:1413-1424.

86. Reis e Sousa and R.N.Germain. 1999. Analysis of adjuvant function by direct visualization of antigen presentation in vivo: endotoxin promotes accumulation of

antigen-bearing dendritic cells in the T cell areas of lymphoid tissue. *J.Immunol.* 162:6552-6561.

87. Manickasingham,S. and Reis e Sousa. 2000. Microbial and T cell-derived stimuli regulate antigen presentation by dendritic cells in vivo. *J.Immunol.* 165:5027-5034.

88. Roake,J.A., A.S.Rao, P.J.Morris, C.P.Larsen, D.F.Hankins, and J.M.Austyn. 1995. Dendritic cell loss from nonlymphoid tissue after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J.Exp.Med.* 181:2237-2247.

89. Roake,J.A., A.S.Rao, P.J.Morris, C.P.Larsen, D.F.Hankins, and J.M.Austyn. 1995. Systemic lipopolysaccharide recruits dendritic cell progenitors to nonlymphoid tissues. *Transplantation* 59:1319-1324.

90. MacPherson,G.G., C.D.Jenkins, M.J.Stein, and C.Edwards. 1995. Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence. *J.Immunol.* 154:1317-1322.

91. Kaisho,T., O.Takeuchi, T.Kawai, K.Hoshino, and S.Akira. 2001. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J.Immunol.* 166:5688-5694.

92. Visintin,A., A.Mazzoni, J.H.Spitzer, D.H.Wyllie, S.K.Dower, and D.M.Segal. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J.Immunol.* 166:249-255.

93. Kadowaki,N., S.Ho, S.Antonenko, R.W.Malefyt, R.A.Kastelein, F.Bazan, and Y.J.Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp.Med.* 194:863-869.

94. Rescigno,M., M.Martino, C.L.Sutherland, M.R.Gold, and P.Ricciardi-Castagnoli. 1998. Dendritic cell survival and maturation are regulated by different signaling pathways. *J.Exp.Med.* 188:2175-2180.

95. Morelli,A.E., A.F.Zahorchak, A.T.Larregina, B.L.Colvin, A.J.Logar, T.Takayama, L.D.Faio, and A.W.Thomson. 2001. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood* 98:1512-1523.

96. Kelleher,M. and P.C.L.Beverley. 2001. Lipopolysaccharide modulation of dendritic cells is insufficient to mature dendritic cells to generate CTLs from naive polyclonal $CD8^+$ T cells in vitro, whereas CD40 ligation is essential. *J.Immunol.* 167:6247-6255.

97. Huang,Q., D.Liu, P.Majewski, L.C.Schulte, J.M.Korn, R.A.Young, E.S.Lander, and N.Hacohen. 2001. The plasticity of dendritic cell responses to pathogens and their components. *Science* 294:870-875.

98. Dixon,G.L.J., P.J.Newton, B.M.Chain, D.Katz, S.R.Andersen, S.Wong, P.Van der Ley, N.Klein, and R.E.Callard. 2001. Dendritic cell activation and cytokine production induced by group B *Neisseria meningitidis*: Interleukin-12 production depends on lipopolysaccharide expression in intact bacteria. *Infect.Immun.* 69:4351-4357.

99. Hofer,S., M.Rescigno, F.Granucci, S.Citterio, M.Francolini, and P.Ricciardi-Castagnoli. 2001. Differential activation of NF-κB subunits in dendritic cells in response to Gram-negative bacteria and to lipopolysaccharide. *Microbes and Infection* 3:259-265.

100. Dubois,B., B.Vanbervliet, J.Fayette, C.Massacrier, C.van Kooten, F.Briere, J.Banchereau, and C.Caux. 1997. Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J.Exp.Med.* 185:941-951.

101. Dubois,B., C.Massacrier, B.Vanbervliet, J.Fayette, F.Briere, J.Banchereau, and C.Caux. 1998. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J.Immunol.* 161:2223-2231.

102. Wykes,M., A.Pombo, C.Jenkins, and G.G.MacPherson. 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J.Immunol.* 161:1313-1319.

103. Johansson,B., S.Ingvarsson, P.Bjorck, and C.A.Borrebaeck. 2000. Human interdigitating dendritic cells induce isotype switching and IL-13-dependent IgM production in CD40-activated naive B cells. *J.Immunol.* 164:1847-1854.

104. Balazs,M., F.Martin, T.Zhou, and J.Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity.* 17:341-352.

105. Nigam,V.N., D.Malchow, E.Th.Rietschel, O.Lüderitz, and O.Westphal. 1970. Die enzymatische abspaltung langkettiger fettsäuren aus bakteriellen lipopolysacchariden mittels extrakten aus der amöbe von Dictyostelium discoideum. *Hoppe-Seyler's Z Physiol Chem* 351:1123-1132.

106. Hall,C.L. and R.S.Munford. 1983. Enzymatic deacylation of the lipid A moiety of *Salmonella typhimurium* lipopolysaccharides by human neutrophils. *Proc.Natl.Acad.Sci.USA* 80:6671-6675.

107. Peterson,A.A. and R.S.Munford. 1987. Dephosphorylation of the lipid A moiety of Escherichia coli lipopolysaccharide by mouse macrophages. *Infect.Immun.* 55:974-978.

108. Hagen,F.S., F.J.Grant, J.L.Kuijper, C.A.Slaughter, C.R.Moomaw, K.Orth, P.J.O'Hara, and R.S.Munford. 1991. Expression and characterization of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides. *Biochemistry* 30:8415-8423.

109. Staab,J.F., D.L.Ginkel, G.B.Rosenberg, and R.S.Munford. 1994. A saposin-like domain influences the intracellular localization, stability, and catalytic activity of human acyloxyacyl hydrolase. *J.Biol.Chem.* 269:23736-23742.

110. Upton,C. and J.T.Buckley. 1995. A new family of lipolytic enzymes? *T.I.B.S.* 20:178-179.

111. Munford,R.S., P.O.Sheppard, and P.J.O'Hara. 1995. Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure. *J.Lipid Res.* 36:1653-1663.

112. Vaccaro,A.M., R.Salvioli, M.Tatti, and F.Ciaffoni. 1999. Saposins and their interaction with lipids. *Neurochem.Res.* 24:307-314.

113. Tobias,P.S., J.C.Mathison, and R.J.Ulevitch. 1988. A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. *J.Biol.Chem.* 263:13479-13481.

114. Güther,M.L.S., S.Leal, N.A.Morrice, G.A.M.Cross, and M.A.Ferguson. 2001. Purification, cloning and characterization of a GPI inositol deacylase from *Trypanosoma brucei*. *EMBO J* 20:4923-4934.

115. Luchi,M. and R.S.Munford. 1993. Binding, internalization, and deacylation of bacterial lipopolysaccharides by human neutrophils. *J.Immunol.* 151:959-969.

116. Raetz,C.R.H. 1992. Biosynthesis of lipid A. *In* Bacterial Endotoxic Lipopolysaccharides. D.C.Morrison and J.L.Ryan, editors. CRC Press, Boca Raton, FL. 67-80.

117. Erwin,A.L. and R.S.Munford. 1990. Deacylation of structurally diverse lipopolysaccharides by human acyloxyacyl hydrolase. *J.Biol.Chem.* 265:16444-16449.

118. Munford,R.S. and J.P.Hunter. 1992. Acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides, has phospholipase, lysophospholipase, diacylglycerollipase, and acyltransferase activities in vitro. *J.Biol.Chem.* 267:10116-10121.

119. Katz,S.S., Y.Weinrauch, R.S.Munford, P.Elsbach, and J.Weiss. 1999. Deacylation of lipopolysaccharide in whole *Escherichia coli* during destruction by cellular and extracellular components of a rabbit inflammatory peritoneal exudate. *J.Biol.Chem.* 274:36579-36584.

120. Weinrauch,Y., S.S.Katz, R.S.Munford, P.Elsbach, and J.Weiss. 1999. Deacylation of purified LPS by cellular and extracellular components of a sterile rabbit peritoneal inflammatory exudate. *Infect.Immun.* 67:3376-3382.

121. Munford,R.S. and C.L.Hall. 1986. Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. *Science* 234:203-205.

122. Pohlman,T.H., R.S.Munford, and J.M.Harlan. 1987. Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide *in vitro*. *J.Exp.Med.* 165:1393-1402.

123. Riedo,F.X., R.S.Munford, W.B.Campbell, J.S.Reisch, K.R.Chien, and R.D.Gerard. 1990. Deacylated lipopolysaccharide inhibits plasminogen activator inhibitor-1, prostacyclin, and prostaglandin E2 induction by lipopolysaccharide but not by tumor necrosis factor-alpha. *J.Immunol.* 144:3506-3512.

124. Dal Nogare,A.R. and W.C.Yarbrough, Jr. 1990. A comparison of the effects of intact and deacylated lipopolysaccharide on human polymorphonuclear leukocytes. *J.Immunol.* 144:1404-1410.

125. Kitchens,R.L., R.J.Ulevitch, and R.S.Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J.Exp.Med.* 1760:485-494.

126. Kitchens,R.L. and R.S.Munford. 1995. Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. *J.Biol.Chem.* 270:9904-9910.

127. Erwin,A.L., R.E.Mandrell, and R.S.Munford. 1991. Enzymatically deacylated *Neisseria* LPS inhibits murine splenocyte mitogenesis induced by LPS. *Infect.Immun.* 59:1881-1887.

128. Erwin,A.L. and R.S.Munford. 1991. Plasma lipopolysaccharide-deacylating activity (acyloxyacyl hydrolase) increases following lipopolysaccharide administration to rabbits. *Lab.Invest.* 65:138-144.

129. Cody,M.J., C.A.Salkowski, B.E.Henricson, G.R.Detore, R.S.Munford, and S.N.Vogel. 1997. Effect of inflammatory and anti-inflammatory stimuli on acyloxyacyl hydrolase gene expression and enzymatic activity in murine macrophages. *J.Endotoxin Res.* 4:371-379.

130. Vos,Q., A.Lees, Z.Q.Wu, C.M.Snapper, and J.J.Mond. 2000. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol.Rev.* 176:154-70.:154-170.

131. Coutinho,A., E.Gronowicz, W.W.Bullock, and G.Moller. 1974. Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses. *J.Exp.Med.* 139:74-92.

132. Coutinho,A., G.Moller, and E.Gronowicz. 1975. Genetical control of B-cell responses. IV. Inheritance of the unresponsiveness to lipopolysaccharides. *J.Exp.Med.* 142:253-258.

133. Qureshi,S.T., L.Larivière, G.Leveque, S.Clermont, K.J.Moore, P.Gros, and D.Malo. 1999. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (*Tlr4*). *J.Exp.Med.* 189:615-625.

134. Miyake,K., Y.Yamashita, M.Ogata, T.Sudo, and M.Kimoto. 1995. RP105, a novel B cell surface molecule implicated in B cell activation, is a member of the leucine-rich repeat protein family. *J.Immunol.* 154:3333-3340.

135. Nagai,Y., R.Shimazu, H.Ogata, S.Akashi, K.Sudo, H.Yamasaki, S.Hayashi, Y.Iwakura, M.Kimoto, and K.Miyake. 2002. Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood* 99:1699-1705.

136. Ogata,H., I.-H.Su, K.Miyake, Y.Nagai, S.Akashi, K.Rajewsky, M.Kimoto, and A.Tarakhovsky. 2000. The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *J.Exp.Med.* 192:23-29.

137. Miura,Y., R.Shimazu, K.Miyake, S.Akashi, H.Ogata, Y.Yamashita, Y.Narisawa, and M.Kimoto. 1998. RP105 is associated with MD-1 and transmits an activation signal in human B cells. *Blood* 92:2815-2822.

138. Zarember,K.A. and P.J.Godowski. 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J.Immunol.* 168:554-561.

139. Fugier-Vivier,I., O.de Bouteiller, C.Guret, F.Fossiez, J.Banchereau, M.G.Mattei, S.Ait-Yahia, E.Garcia, S.Lebecque, and Y.J.Liu. 1997. Molecular cloning of human RP105. *Eur.J.Immunol.* 27:1824-1827.

140. Wetzel,G.D. and J.R.Kettman. 1981. Activation of murine B lymphocytes. III. Stimulation of B lymphocyte clonal growth with lipopolysaccharide and dextran sulfate. *J.Immunol.* 126:723-728.

141. Vaux,D.L., B.L.Pike, and G.J.Nossal. 1981. Antibody production by single, hapten-specific B lymphocytes: an antigen-driven cloning system free of filler or accessory cells. *Proc.Natl.Acad.Sci.U.S.A* 78:7702-7706.

142. Martinez-Alonso,C., R.R.Bernabe, and F.Diaz-Espada. 1980. Different macrophage requirement in the specific and polyclonal responses induced by TNP-LPS and LPS. *Scand.J.Immunol.* 12:453-457.

143. Bucala,R. 1992. Polyclonal activation of B lymphocytes by lipopolysaccharide requires macrophage-derived interleukin-1. *Immunology* 77:477-482.

144. Corbel,C. and F.Melchers. 1983. Requirement for macrophages or for macrophage- or T cell-derived factors in the mitogenic stimulation of murine B lymphocytes by lipopolysaccharides. *Eur.J.Immunol.* 13:528-533.

145. MacLennan,I. and C.Vinuesa. 2002. Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. *Immunity.* 17:235-238.

146. Forestier,C., E.Moreno, J.Pizarro-Cerda, and J.P.Gorvel. 1999. Lysosomal accumulation and recycling of lipopolysaccharide to the cell surface of murine macrophages, an in vitro and in vivo study. *J.Immunol.* 162:6784-6791.

147. Wuorela,M., S.Jalkanen, P.Toivanen, and K.Granfors. 1993. *Yersinia* lipopolysaccharide is modified by human monocytes. *Infect.Immun.* 61:5261-5270.

148. Granfors,K., S.Jalkanen, R.Von Essen, R.Lahesmaa-Rantala, O.Isomäki, K.Pekkola-Heino, R.Merilahti-Palo, R.Saario, H.Isomäki, and A.Toivanen. 1989. Yersinia antigens in synovial-fluid cells from patients with reactive arthritis. *New Eng.J.Med.* 320:216-221.

149. Duncan,R.L., Jr. and D.C.Morrison. 1984. The fate of E. coli lipopolysaccharide after the uptake of E. coli by murine macrophages in vitro. *J.Immunol.* 132:1416-1424.

150. Duncan,R.L., Jr., J.Hoffman, V.L.Tesh, and D.C.Morrison. 1986. Immunologic activity of lipopolysaccharides released from macrophages after the uptake of intact E. coli in vitro. *J.Immunol.* 136:2924-2929.

151. Johns,M., A.Skehill, and W.R.McCabe. 1983. Immunization with rough mutants of Salmonella minnesota. IV. Protection by antisera to O and rough antigens against endotoxin. *J Infect Dis* 147:57-67.

152. Michael,J.G. and I.Mallah. 1981. Immune response to parental and rough mutant strains of Salmonella minnesota. *Infect.Immun.* 33:784-787.

153. Cross,A.S., H.Sidberry, and J.C.Sadoff. 1989. The human antibody response during natural bacteremic infection with gram-negative bacilli against lipopolysaccharide core determinants. *J.Infect.Dis.* 160:225-236.

154. Su,S., M.M.Ward, M.A.Apicella, and R.E.Ward. 1991. The primary B cell response to the O/core region of bacterial lipopolysaccharide is restricted to the Ly-1 lineage. *J.Immunol.* 146:327-331.

155. Dunn,D.L., W.C.Bogard, Jr., and F.B.Cerra. 1985. Efficacy of type-specific and cross-reactive murine monoclonal antibodies directed against endotoxin during experimental sepsis. *Surgery* 98:283-290.

156. Pennington,J.E. and E.Menkes. 1981. Type-specific vs. cross-protective vaccination for gram-negative bacterial pneumonia. *J.Infect.Dis.* 144:599-603.

157. Priest,B.P., D.N.Brinson, D.A.Schroeder, and D.L.Dunn. 1989. Treatment of experimental gram-negative bacterial sepsis with murine monoclonal antibodies directed against lipopolysaccharide. *Surgery* 106:147-155.

158. Galanos,C., O.Luderitz, and O.Westphal. 1971. Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. *Eur.J.Biochem.* 24:116-122.

159. Pollack,M., K.Oishi, J.Chia, M.Evans, G.Guelde, and N.Koles. 1990. Specificity and function of monoclonal antibodies reactive with discrete structural elements of bacterial lipopolysaccharide. *Adv.Exp.Med.Biol.* 256:331-40.:331-340.

160. Pollack,M., A.I.Huang, R.K.Prescott, L.S.Young, K.W.Hunter, D.F.Cruess, and C.M.Tsai. 1983. Enhanced survival in *Pseudomonas aeruginosa* septicemia associated with high levels of circulating antibody to *Escherichia coli* endotoxin core. *J.Clin.Invest* 72:1874-1881.

161. Ziegler,E.J., J.A.McCutchan, J.Fierer, M.P.Glauser, J.C.Sadoff, H.Douglas, and A.I.Braude. 1982. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. *New Eng.J.Med.* 307:1225-1230.

162. Marks,M.I., E.J.Ziegler, H.Douglas, L.B.Corbeil, and A.I.Braude. 1982. Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. *J.Clin.Invest* 69:742-749.

163. Ng,A.K., C.L.Chen, C.M.Chang, and A.Nowotny. 1976. Relationship of structure to function in bacterial endotoxins: serologically cross-reactive components and their effect on protection of mice against some gram-negative infections. *J.Gen.Microbiol.* 94:107-116.

164. Greisman,S.E. and C.A.Johnston. 1996. Evidence against the hypothesis that antibodies to the inner core of lipopolysaccharides in antisera raised by immunization with enterobacterial deep-rough mutants confer broad-spectrum protection during gram-negative bacterial sepsis. *J.Endotoxin Res.*

165. Greisman,S.E. and C.A.Johnston. 1988. Failure of antisera to J5 and R595 rough mutants to reduce endotoxemic lethality. *J.Infect.Dis.* 157:54-64.

166. Calandra,T., M.P.Glauser, J.Schellekens, and J.Verhoef. 1988. Treatment of gram-negative septic shock with human IgG antibody to *Escherichia coli* J5: A prospective, double-blind, randomized trial. *J.Infect.Dis.* 158:312-319.

167. Pollack,M., J.K.S.Chia, N.L.Koles, M.Miller, and G.Guelde. 1989. Specificity and cross-reactivity of monoclonal antibodies reactive with the core and lipid A regions of bacterial lipopolysaccharide. *J.Infect.Dis.* 159:168-188.

168. Yuan,D. and E.S.Vitetta. 1983. Structural studies of cell surface and secreted IgG in LPS-stimulated murine B cells. *Mol.Immunol.* 20:367-375.

169. Slack,J., G.P.Der-Balian, M.Nahm, and J.M.Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J.Exp.Med.* 151:853-862.

170. Oishi,K., N.L.Koles, G.Guelde, and M.Pollack. 1992. Antibacterial and protective properties of monoclonal antibodies reactive with *Escherichia coli* O111:B4 lipopolysaccharide: Relation to antibody isotype and complement-fixing activity. *J.Infect.Dis.* 165:34-45.

171. Izui,S., R.A.Eisenberg, and F.J.Dixon. 1981. Subclass-restricted IgG polyclonal antibody production in mice injected with lipid A-rich lipopolysaccharides. *J.Exp.Med.* 153:324-338.

172. Perlmutter,R.M., D.Hansburg, D.E.Briles, R.A.Nicolotti, and J.M.Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J.Immunol.* 121:566-572.

173. Paul,W.E. and J.Ohara. 1987. B-cell stimulatory factor-1/interleukin 4. *Annu.Rev.Immunol.* 5:429-59.:429-459.

174. Snapper,C.M. and W.E.Paul. 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236:944-947.

175. Coffman,R.L., B.Shrader, J.Carty, T.R.Mosmann, and M.W.Bond. 1987. A mouse T cell product that preferentially enhances IgA production. I. Biologic characterization. *J.Immunol.* 139:3685-3690.

176. Karch,H. and K.Nixdorff. 1983. Modulation of the IgG subclass responses to lipopolysaccharide by bacterial membrane components: differential adjuvant effects produced by primary and secondary stimulation. *J.Immunol.* 131:6-8.

177. Karch,H., J.Gmeiner, and K.Nixdorff. 1983. Alteration of the immunoglobulin G subclass responses in mice to lipopolysaccharide: effects of nonbacterial proteins and bacterial membrane phospholipids or outer membrane proteins of *Proteus mirabilis*. *Infect.Immun.* 40:157-165.

178. Burd,R.S., R.J.Battafarano, C.S.Cody, M.S.Farber, C.A.Ratz, and D.L.Dunn. 1993. Anti-endotoxin monoclonal antibodies inhibit secretion of tumor necrosis factor-α by two distinct mechanisms. *Ann.Surg.* 218:250-261.

179. Battafarano,R.J., R.S.Burd, C.S.Cody, T.A.Kellogg, C.S.Raymond, C.A.Ratz, and D.L.Dunn. 1993. Anti-lipopolysaccharide monoclonal antibodies inhibit macrophage TNF messenger RNA synthesis in vitro. *J.Surg.Res.* 54:342-348.

180. Battafarano,R.J., R.S.Burd, K.M.Kurrelmeyer, C.A.Ratz, and D.L.Dunn. 1994. Inhibition of splenic macrophage tumor necrosis factor alpha secretion in vivo by antilipopolysaccharide monoclonal antibodies. *Arch.Surg.* 129:179-180.

181. Fang,I.S., M.A.Wisniewski, C.C.Huntenburg, L.S.Knight, J.E.Bubbers, and M.J.Schneidkraut. 1993. Inhibition of lipopolysaccharide-associated endotoxin activities in vitro and in vivo by the human anti-lipid A monoclonal antibody SdJ5-1.17.15. *Infect.Immun.* 61:3873-3878.

182. Pollack,M., A.M.Espinoza, G.Guelde, N.L.Koles, L.M.Wahl, and C.A.Ohl. 1995. Lipopolysaccharide (LPS)-specific monoclonal antibodies regulate LPS uptake and LPS-induced tumor necrosis factor- α responses by human monocytes. *J.Infect.Dis.* 172:794-804.

183. Pollack,M., C.A.Ohl, D.Golenbock, F.Di Padova, L.M.Wahl, N.L.Koles, G.Guelde, and B.G.Monks. 1997. Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induced proinflammatory functions. *J.Immunol.* 159:3519-3530.

184. Baumgartner,J.D., D.Heumann, J.Gerain, P.Weinbreck, G.E.Grau, and M.P.Glauser. 1990. Association between protective efficacy of anti-lipopolysaccharide (LPS) antibodies and suppression of LPS-induced tumor necrosis factor α and interleukin 6. Comparison of O side chain- specific antibodies with core LPS antibodies. *J.Exp.Med.* 171:889-896.

185. Joiner,K.A., M.A.Schmetz, R.C.Goldman, L.Leive, and M.M.Frank. 1984. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for Escherichia coli O111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. *Infect.Immun.* 45:113-117.

186. Avrameas,S. 1991. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol.Today* 12:154-159.

187. Baumgarth,N., O.C.Herman, G.C.Jager, L.E.Brown, L.A.Herzenberg, and J.Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J.Exp.Med.* 192:271-280.

188. Herzenberg,L.A. and A.B.Kantor. 1993. B-cell lineages exist in the mouse. *Immunol.Today* 14:79-83.

189. Casali,P. and A.L.Notkins. 1989. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. *Immunol.Today* 10:364-368.

190. Ochsenbein,A.F. and R.Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol Today* 21:624-630.

191. Berland,R. and H.H.Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu.Rev.Immunol.* 20:253-300.

192. Reid,R.R., A.P.Prodeus, W.Khan, T.Hsu, F.S.Rosen, and M.C.Carroll. 1997. Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. *J Immunol* 159:970-975.

193. Boes,M., A.P.Prodeus, T.Schmidt, M.C.Carroll, and J.Chen. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp.Med.* 188:2381-2386.

194. Oliver,A.M., F.Martin, and J.F.Kearney. 1999. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J.Immunol.* 162:7198-7207.

195. Won,W.J. and J.F.Kearney. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J.Immunol.* 168:5605-5611.

196. Martin,F. and J.F.Kearney. 2001. B1 cells: similarities and differences with other B cell subsets. *Curr.Opin.Immunol.* 13:195-201.

197. Martin,F. and J.F.Kearney. 2002. Marginal-zone B cells. *Nat.Rev.Immunol.* 2:323-335.

198. Ohashi,P.S. and A.L.DeFranco. 2002. Making and breaking tolerance. *Curr.Opin.Immunol.* 14:744-759.

199. Mandik-Nayak,L., S.Seo, A.Eaton-Bassiri, D.Allman, R.R.Hardy, and J.Erikson. 2000. Functional consequences of the developmental arrest and follicular exclusion of anti-double-stranded DNA B cells. *J.Immunol.* 164:1161-1168.

200. Chu,Y.P., D.Taylor, H.G.Yan, B.Diamond, and L.Spatz. 2002. Persistence of partially functional double-stranded (ds) DNA binding B cells in mice transgenic for the IgM heavy chain of an anti-dsDNA antibody. *Int.Immunol.* 14:45-54.

201. Shlomchik,M.J., J.E.Craft, and M.J.Mamula. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat.Rev.Immunol.* 1:147-153.

202. Fournie,G.J., P.H.Lambert, and P.A.Meischer. 1974. Release of DNA in circulating blood and induction of anti-DNA antibodies after injection of bacterial lipopolysaccharides. *J.Exp.Med.* 140:1189-1206.

203. Izui,S., T.Kobayakawa, M.J.Zryd, J.Louis, and P.H.Lambert. 1977. Mechanism for induction of anti-DNA antibodies by bacterial lipopolysaccharides in mice; II. Correlation between anti-DNA induction and polyclonal antibody formation by various polyclonal B lymphocyte activators. *J.Immunol.* 119:2157-2162.

204. Izui,S., N.M.Zaldivar, I.Scher, and P.H.Lambert. 1977. Mechanism for induction of anti-DNA antibodies by bacterial lipopolysaccharides in mice. I. Anti-DNA induction by LPS without significant release of DNA in circulating blood. *J.Immunol.* 119:2151-2156.

205. Izui,S., R.A.Eisenberg, and F.J.Dixon. 1979. IgM rheumatoid factors in mice injected with bacterial lipopolysaccharides. *J.Immunol.* 122:2096-2102.

206. Broker,B.M., A.Klajman, P.Youinou, J.Jouquan, C.P.Worman, J.Murphy, L.Mackenzie, R.Quartey-Papafio, M.Blaschek, P.Collins, and . 1988. Chronic lymphocytic leukemic (CLL) cells secrete multispecific autoantibodies. *J.Autoimmun.* 1:469-481.

207. Victor,K.D., I.Randen, K.Thompson, O.Forre, J.B.Natvig, S.M.Fu, and J.D.Capra. 1991. Rheumatoid factors isolated from patients with autoimmune disorders are derived from germline genes distinct from those encoding the Wa, Po, and Bla cross-reactive idiotypes. *J.Clin.Invest* 87:1603-1613.

208. Takatsu,K., S.Takaki, and Y.Hitoshi. 1994. Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv.Immunol.* 57:145-90.:145-190.

209. Kantor,A.B. 1991. The development and repertoire of B-1 cells (CD5 B cells). *Immunol.Today* 12:389-391.

210. Kopf,M., F.Brombacher, P.D.Hodgkin, A.J.Ramsay, E.A.Milbourne, W.J.Dai, K.S.Ovington, C.A.Behm, G.Kohler, I.G.Young, and K.I.Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity.* 4:15-24.

211. Yoshida,T., K.Ikuta, H.Sugaya, K.Maki, M.Takagi, H.Kanazawa, S.Sunaga, T.Kinashi, K.Yoshimura, J.Miyazaki, S.Takaki, and K.Takatsu. 1996. Defective B-1 cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5R alpha-deficient mice. *Immunity.* 4:483-494.

212. Hiroi,T., M.Yanagita, H.Iijima, K.Iwatani, T.Yoshida, K.Takatsu, and H.Kiyono. 1999. Deficiency of IL-5 receptor alpha-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cell in mucosa-associated tissues. *J.Immunol.* 162:821-828.

213. Shroff,K.E., K.Meslin, and J.J.Cebra. 1995. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect.Immun.* 63:3904-3913.

214. Fernandez,M.I., T.Pedron, R.Tournebize, J.C.Olivo-Marin, P.J.Sansonetti, and A.Phalipon. 2003. Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity* 18:739-749.

215. Kroese,F.G., E.C.Butcher, A.M.Stall, P.A.Lalor, S.Adams, and L.A.Herzenberg. 1989. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int.Immunol.* 1:75-84.

216. Bao,S., K.W.Beagley, A.M.Murray, V.Caristo, K.I.Matthaei, I.G.Young, and A.J.Husband. 1998. Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. *Immunology* 94:181-188.

217. Seppala,I.J. and O.Makela. 1984. Adjuvant effect of bacterial LPS and/or alum precipitation in responses to polysaccharide and protein antigens. *Immunology* 53:827-836.

218. Cella,M., A.Engering, V.Pinet, J.Pieters, and A.Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388:782-787.

219. Kleijmeer,M.J., M.A.Ossevoort, C.J.H.van Veen, J.J.van Hellemond, J.J.Neefjes, W.M.Kast, C.J.M.Melief, and H.J.Geuze. 1995. MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells. *J.Immunol.* 154:5715-5724.

220. Liu,Y. and C.A.Janeway, Jr. 1991. Microbial induction of co-stimulatory activity for CD4 T-cell growth. *Int.Immunol.* 3:323-332.

221. Khoruts,A., A.Mondino, K.A.Pape, S.L.Reiner, and M.K.Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J.Exp.Med.* 187:225-236.

222. Verma,J.N., M.Rao, S.Amselem, U.Krzych, C.R.Alving, S.J.Green, and N.M.Wassef. 1992. Adjuvant effects of liposomes containing lipid A: enhancement of liposomal antigen presentation and recruitment of macrophages. *Infect.Immun.* 60:2438-2444.

223. McGhee,J.R., J.J.Farrar, S.M.Michalek, S.E.Mergenhagen, and D.L.Rosenstreich. 1979. Cellular requirements for lipopolysaccharide adjuvanticity. A role for both T lymphocytes and macrophages for in vitro responses to particulate antigens. *J.Exp.Med.* 149:793-807.

224. Gontijo,C.M. and G.Moller. 1991. Antigen processing and presentation by small and large B cells. *Scand.J.Immunol.* 34:207-213.

225. Krieger,J.I., S.F.Grammer, H.M.Grey, and R.W.Chesnut. 1985. Antigen presentation by splenic B cells: resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J.Immunol.* 135:2937-2945.

226. Harris,M.R., C.S.Kindle, A.F.Abruzzini, C.W.Pierce, and S.E.Cullen. 1984. Antigen presentation by the BCL1 murine B cell line: in vitro stimulation by LPS. *J.Immunol.* 133:1202-1208.

227. Pape,K.A., A.Khoruts, A.Mondino, and M.K.Jenkins. 1997. Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells. *J.Immunol.* 159:591-598.

228. Reisser,D., A.Pance, and J.F.Jeannin. 2002. Mechanisms of the antitumoral effect of lipid A. *BioEssays* 24:284-289.

229. Baker,P.J., J.R.Hiernaux, M.B.Fauntleroy, B.Prescott, J.L.Cantrell, and J.A.Rudbach. 1988. Inactivation of suppressor T-cell activity by nontoxic monophosphoryl lipid A. *Infect.Immun.* 56:1076-1083.

230. Ekwunife,F.S., C.E.Taylor, M.B.Fauntleroy, P.W.Stashak, and P.J.Baker. 1991. Differential effects of monophosphoryl lipid A on expression of suppressor T cell activity in lipopolysaccharide-responsive and lipopolysaccharide-defective strains of C3H mice. *Infect.Immun.* 59:2192-2194.

231. Baker,P.J., T.Hraba, C.E.Taylor, K.R.Myers, K.Takayama, N.Qureshi, P.Stuetz, S.Kusumoto, and A.Hasegawa. 1992. Structural features that influence the ability of lipid A and its analogs to abolish expression of suppressor T cell activity. *Infect.Immun.* 60:2694-2701.

232. Baker,P.J., J.R.Hiernaux, M.B.Fauntleroy, P.W.Stashak, B.Prescott, J.L.Cantrell, and J.A.Rudbach. 1988. Ability of monophosphoryl lipid A to augment the antibody response of young mice. *Infect.Immun.* 56:3064-3066.

233. Ulrich,J.T. and K.R.Myers. 1995. Monophosphoryl lipid A as an adjuvant. *In* Vaccine Design: The Subunit and Adjuvant Approach. M.F.Powell and M.J.Newman, editors. Plenum Press, NY. 495-524.

234. Persing,D.H., R.N.Coler, M.J.Lacy, D.A.Johnson, J.R.Baldridge, R.M.Hershberg, and S.G.Reed. 2002. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol.* 10:S32-S37.

235. Ismaili,J., J.Rennesson, E.Aksoy, J.Vekemans, B.Vincart, Z.Amraoui, F.Van Laethem, M.Goldman, and P.M.Dubois. 2002. Monophosphoryl lipid A activates both human dendritic cells and T cells. *J.Immunol.* 168:926-932.

236. Mellman,I. and R.M.Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-258.

237. Reis e Sousa,C. 2001. Dendritic cells as sensors of infection. *Immunity* 14:495-498.

238. Rescigno,M., M.Urbano, M.Rimoldi, B.Valzasina, G.Rotta, F.Granucci, and P.Ricciardi-Castagnoli. 2002. Toll-like receptor 4 is not required for the full maturation of dendritic cells or for the degradation of Gram-negative bacteria. *Eur.J.Immunol.* 32:2800-2806.

239. Munford,R.S. and C.L.Hall. 1989. Purification of acyloxyacyl hydrolase, a leukocyte enzyme that removes secondary acyl chains from bacterial lipopolysaccharides. *J.Biol.Chem.* 264:15613-15619.

240. Westphal,O. and K.Jann. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr.Chem.* 5:83-91.

241. Xu,S., K.Ariizumi, G.Caceres-Dittmar, D.Edelbaum, K.Hashimoto, P.R.Bergstresser, and A.Takashima. 1995. Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. *J.Immunol.* 154:2697-2705.

242. Matsue,H., K.Matsue, M.Walters, K.Okumura, H.Yagita, and A.Takashima. 1999. Induction of antigen-specific immunosuppression by CD95L cDNA- transfected 'killer' dendritic cells. *Nat.Med.* 5:930-937.

243. Inaba,K., M.Inaba, N.Romani, H.Aya, M.Deguchi, S.Ikehara, S.Muramatsu, and R.M.Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp.Med.* 176:1693-1702.

244. Munford,R.S. and C.L.Hall. 1985. Uptake and deacylation of bacterial lipopolysaccharides by macrophages from normal and endotoxin-hyporesponsive mice. *Infect.Immun.* 48:464-473.

245. Yamada,N. and S.I.Katz. 1999. Generation of mature dendritic cells from a $CD14^{+}$ cell line (XS52) by IL-4, TNF-α, IL-1β, and agonistic anti-CD40 monoclonal antibody. *J.Immunol.* 163:5331-5337.

246. Munford,R.S. and A.L.Erwin. 1992. Eucaryotic lipopolysaccharide deacylating enzyme. *Meth.Enzymol.* 209:485-492.

247. Munford,R.S., L.C.DeVeaux, J.E.Cronan, Jr., and P.D.Rick. 1992. Biosynthetic radiolabeling of bacterial lipopolysaccharide to high specific activity. *J.Immunol.Methods* 148:115-120.

248. Bligh,E.G. and W.J.Dyer. 1959. A rapid method of total lipid extraction and purification. *Can.J.Biochem.Physiol.* 37:911-917.

249. Jayaratne,P., D.Bronner, P.R.MacLachlan, C.Dodgson, N.Kido, and C.Whitfield. 1994. Cloning and analysis of duplicated rfbM and rfbK genes involved in the formation of GDP-mannose in Escherichia coli O9:K30 and participation of rfb genes in the synthesis of the group I K30 capsular polysaccharide. *J.Bacteriol.* 176:3126-3139.

250. Mahnke,K., E.Becher, P.Ricciardi-Castagnoli, T.A.Luger, T.Schwarz, and S.Grabbe. 1997. CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv.Exp.Med.Biol.* 417:145-159.

251. Sparwasser,T., E.S.Koch, R.M.Vabulas, K.Heeg, G.B.Lipford, J.W.Ellwart, and H.Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur.J.Immunol.* 28:2045-2054.

252. Askew,D., R.S.Chu, A.M.Krieg, and C.V.Harding. 2000. CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen-processing mechanisms. *J.Immunol.* 165:6889-6895.

253. Dziarski,R., Q.L.Wang, K.Miyake, C.J.Kirschning, and D.Gupta. 2001. MD-2 enables toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to gram-positive and gram-negative bacteria and their cell wall components. *J.Immunol.* 166:1938-1944.

254. Staab,J.F., S.Fosmire, M.Zhang, A.W.Varley, and R.S.Munford. 1999. Distinctive structural features are shared by human, lapine, and murine acyloxyacyl hydrolases. *J.Endotoxin Res.* 5:205-208.

255. Boldrick,J.C., A.A.Alizadeh, M.Diehn, S.Dudoit, C.L.Liu, C.E.Belcher, D.Botstein, L.M.Staudt, P.O.Brown, and D.A.Relman. 2002. Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc Natl.Acad.Sci.U.S.A* 99:972-977.

256. Unkmeir,A., U.Kämmerer, A.Stade, C.Hübner, S.Haller, A.Kolb-Mäurer, M.Frosch, and G.Dietrich. 2002. Lipooligosaccharide and polysaccharide capsule: Virulence factors of *Neisseria meningitidis* that determine meningococcal interaction with human dendritic cells. *Infect.Immun.* 70:2454-2462.

257. Bouis,D.A., T.G.Popova, A.Takashima, and M.V.Norgard. 2001. Dendritic cells phagocytose and are activated by *Treponema pallidum*. *Infect.Immun.* 69:518-528.

258. Forestier,C., E.Moreno, S.Méresse, A.Phalipon, D.Olive, P.J.Sansonetti, and J.P.Gorvel. 1999. Interaction of *Brucella abortus* lipopolysaccharide with major histocomopatibility complex class II molecules in B lymphocytes. *Infect.Immun.* 67:4048-4054.

259. Bos,N.A., C.G.Meeuwsen, P.Van Wijngaarden, and R.Benner. 1989. B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice. II. Analysis of antigen-binding capacities in relation to VH gene usage. *Eur.J.Immunol.* 19:1817-1822.

260. Macpherson,A.J., D.Gatto, E.Sainsbury, G.R.Harriman, H.Hengartner, and R.M.Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222-2226.

261. Izui,S., P.H.Lambert, G.J.Fournie, H.Turler, and P.A.Miescher. 1977. Features of systemic lupus erythematosus in mice injected with bacterial lipopolysaccharides: identificantion of circulating DNA and renal localization of DNA-anti-DNA complexes. *J.Exp.Med.* 145:1115-1130.

262. Weigel,L.M., M.E.Brandt, and M.V.Norgard. 1992. Analysis of the N-terminal region of the 47-kilodalton integral membrane lipoprotein of Treponema pallidum. *Infect.Immun.* 60:1568-1576.

263. Mohan,C., S.Adams, V.Stanik, and S.K.Datta. 1993. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J.Exp.Med.* 177:1367-1381.

264. Lim,S.K. 2003. Freund adjuvant induces TLR2 but not TLR4 expression in the liver of mice. *Int.Immunopharmacol.* 3:115-118.

265. Wang,H., O.Bloom, M.Zhang, J.M.Vishnubhakat, M.Ombrellino, J.Che, A.Frazier, H.Yang, S.Ivanova, L.Borovikova, K.R.Manogue, E.Faist, E.Abraham, J.Andersson, U.Andersson, P.E.Molina, N.N.Abumrad, A.Sama, and K.J.Tracey. 1999. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285:248-251.

266. Marrugo,J., D.G.Marsh, and B.Ghosh. 1996. The conserved lymphokine element-0 in the IL5 promoter binds to a high mobility group-1 protein. *Mol.Immunol.* 33:1119-1125.

267. Kewalramani,R. and A.K.Singh. 2002. Immunopathogenesis of lupus and lupus nephritis: recent insights. *Curr.Opin.Nephrol.Hypertens.* 11:273-277.

268. Shi,X., C.Xie, D.Kreska, J.A.Richardson, and C.Mohan. 2002. Genetic dissection of SLE: SLE1 and FAS impact alternate pathways leading to lymphoproliferative autoimmunity. *J.Exp.Med.* 196:281-292.

269. Snapper,C.M., H.Yamada, D.Smoot, R.Sneed, A.Lees, and J.J.Mond. 1993. Comparative in vitro analysis of proliferation, Ig secretion, and Ig class switching by murine marginal zone and follicular B cells. *J.Immunol.* 150:2737-2745.

270. Corcoran,L.M. and D.Metcalf. 1999. IL-5 and RP105 signaling defects in B cells from commonly used 129 mouse substrains. *J.Immunol.* 163:5836-5842.

271. Hellman,J., P.M.Loiselle, M.M.Tehan, J.E.Allaire, L.A.Boyle, J.T.Kurnick, D.M.Andrews, K.S.Kim, and H.S.Warren. 2000. Outer membrane protein A, peptidoglycan-associated lipoprotein, and murein lipoprotein are released by *Escherichia coli* bacteria into serum. *Infect.Immun.* 68:2566-2572.

272. Braun,V. and H.Wolff. 1970. The murein-lipoprotein linkage in the cell wall of *Escherichia coli*. *Eur.J.Biochem.* 14:387-391.

273. Braun,V. and K.Hantke. 1975. Characterization of the free form of murein-lipoprotein from the outer membrane of *Escherichia coli* B/r. *FEBS Lett.* 60:26-28.

274. Hayashi,S. and H.C.Wu. 1990. Lipoproteins in bacteria. *J.Bioenerg.Biomembr.* 22:451-471.

275. Takeuchi,O., S.Sato, T.Horiuchi, K.Hoshino, K.Takeda, Z.Dong, R.L.Modlin, and S.Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J.Immunol.* 169:10-14.

276. Melchers,F., V.Braun, and C.Galanos. 1975. The lipoprotein of the outer membrane of Escherichia coli: a B-lymphocyte mitogen. *J.Exp.Med.* 142:473-482.

277. Hertz,C.J., S.M.Kiertscher, P.J.Godowski, D.A.Bouis, M.V.Norgard, M.D.Roth, and R.L.Modlin. 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J.Immunol.* 166:2444-2450.

278. Kuhn,H.M., U.Meier-Dieter, and H.Mayer. 1988. ECA, the enterobacterial common antigen. *FEMS Microbiol.Rev.* 4:195-222.

279. Makela,P.H., G.Schmidt, H.Mayer, H.Nikaido, H.Y.Whang, and E.Neter. 1976. Enterobacterial common antigen in rfb deletion mutants of Salmonella typhimurium. *J.Bacteriol.* 127:1141-1149.

280. Gan,K., S.D.Gupta, K.Sankaran, M.B.Schmid, and H.C.Wu. 1993. Isolation and characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in prolipoprotein modification. *J.Biol.Chem.* 268:16544-16550.

281. Meier-Dieter,U., K.Barr, R.Starman, L.Hatch, and P.D.Rick. 1992. Nucleotide sequence of the Escherichia coli rfe gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the rfe-rff gene cluster. *J.Biol.Chem.* 267:746-753.

282. Nnalue,N.A. and B.A.Stocker. 1987. The effects of O-antigen character and enterobacterial common antigen content on the in vivo persistence of aromatic-dependent Salmonella sp. live-vaccine strains. *Microb.Pathog.* 3:31-44.

283. Valtonen,M.V., U.M.Larinkari, M.Plosila, V.V.Valtonen, and P.H.Makela. 1976. Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. *Infect.Immun.* 13:1601-1605.

284. Valtonen,M.V., U.M.Larinkari, M.Plosila, V.V.Valtonen, and P.H.Makela. 1976. Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. *Infect.Immun.* 13:1601-1605.

285. Nnalue,N.A. and B.A.Stocker. 1987. The effects of O-antigen character and enterobacterial common antigen content on the in vivo persistence of aromatic-dependent Salmonella sp. live-vaccine strains. *Microb.Pathog.* 3:31-44.

286. Forestier,C., F.Deleuil, N.Lapaque, E.Moreno, and J.P.Gorvel. 2000. *Brucella abortus* lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. *J.Immunol.* 165:5202-5210.

287. Braun,V., V.Bosch, E.R.Klumpp, I.Neff, H.Mayer, and S.Schlecht. 1976. Antigenic determinants of murein lipoprotein and its exposure at the surface of Enterobacteriaceae. *Eur.J.Biochem.* 62:555-566Craig Malloy.

288. Zirk,N.M., S.F.Hashmi, and H.K.Ziegler. 1999. The polysaccharide portion of lipopolysaccharide regulates antigen-specific T-cell activation via effects on macrophage-mediated antigen processing. *Infect.Immun.* 67:319-326.

289. Fayette,J., I.Durand, J.M.Bridon, C.Arpin, B.Dubois, C.Caux, Y.J.Liu, J.Banchereau, and F.Briere. 1998. Dendritic cells enhance the differentiation of naive B cells into plasma cells in vitro. *Scand.J.Immunol.* 48:563-570.

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