CONSTITUTIVE OVEREXPRESSION OF ACYLOXYACYL HYDROLASE IN *MUS MUSCULUS*

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CONSTITUTIVE OVEREXPRESSION OF ACYLOXYACYL HYDROLASE IN *MUS MUSCULUS*

by

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Acyloxyacyl hydrolase (AOAH) is a highly conserved host lipase that selectively removes the secondary acyl chains from lipid A, the bioactive center of Gram negative bacterial lipopolysaccharide (LPS). Deacylated LPS has a marked reduction in bioactivity and antagonizes the LPS signaling pathway. Thus, AOAH deacylation of LPS may represent a mechanism by which animals control responses to Gram-negative bacteria. Prior to the experiments described in this study, mice deficient in AOAH were found to be susceptible to the long-term effects of LPS. *Aoah*$^{-/-}$ mice developed long-lasting hepatomegaly, exaggerated

antibody responses, and prolonged immunosuppression in response to small doses of LPS. In the studies described here, AOAH was overexpressed in mice by using CD68 promoter sequences which have been shown by others to drive transgene expression in macrophages. CD68p-AOAH transgenic mice had constitutive overexpression of AOAH in macrophages, dendritic cells and tissues rich in these cells (liver, spleen and lung). They also secreted the enzyme into blood and deacylated LPS at a faster rate both *in vitro* and *in vivo*. Importantly, constitutive overexpression of AOAH did not interfere with the initial pro-inflammatory responses to LPS, in keeping with prior observations that AOAH-mediated inactivation of LPS occurs over several hours and does not moderate acute reactions to LPS *in vivo*.

The protective role of constitutive AOAH overexpression was determined by two test systems. First, after an intraperitoneal dose of LPS, CD68p-AOAH transgenic mice returned to their pre-challenge weights more rapidly than did the wildtype mice. Secondly, CD68p-AOAH transgenic mice were less susceptible to LPS and Gram-negative bacteria induced hepatosplenomegaly. These results suggest that overexpression of AOAH in macrophages could accelerate recovery from Gram-negative bacterial infections in animals, including humans.

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PRIOR PUBLICATIONS

Shao, B., Lu, ., Katz, S.C., Varley, A.W., Hardwick, J., Rogers, T.E., **Ojogun**, **N.**, Rockey, D.C., Dematteo, R.P., and Munford. R.S. 2007. A host lipase detoxifies bacterial lipopolysaccharides in the liver and spleen. *J Biol Chem* 282:13726-13735.

Ojogun, N., Varley, A.W., Kuang, T., Shao, B., Greaves, D.R., Munford, R.S. 2008. Constitutive overexpression of acyloxyacyl hydrolase prevents prolonged reactions to bacterial lipopolysaccharide *in vivo* (Submitted).

LIST OF FIGURES

LIST OF ABBREVIATIONS

LPS- lipopolysaccharide

SDS PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis

Kdo- 2-keto-3 deoxyoctonic acid

NMR- nuclear magnetic resonance

LOS- lipooligosaccharide

GlN- glucosamine

LBP- LPS binding protein

TLR4- toll like receptor 4

TNF-α- tumor necrosis factor- alpha

MD-2- myeloid differentiation protein-2

TIR- toll-interleukin-1 receptor

MyD88- myeloid differentiation factor 88

Mal- MyD88 adaptor protein

TRIF- TIR-containing adaptor molecule

TRAM- TRIF-related adaptor molecule

NFκB- nuclear factor κB

IRAK- IL-1 receptor-associated kinase

TRAF-6- TNF receptor factor-6

MAPK- mitogen activated protein kinase

RIP1- receptor-interacting protein 1

IRF3- interferon regulatory factor 3

MBL- mannose binding lectin

BPI- bactericidal permeability-increasing protein

AOAH- acyloxyacyl hydrolase

dLPS- deacylated LPS

NOD- nucleotide oligomerization domain

NAFLD- non-alcoholic fatty liver disease

NASH- non-alcoholic steatohepatitis

VAT-visceral adipose tissue

HSL- hormone sensitive lipase

IRS- insulin receptor substrate

ROS- reactive oxygen species

SREBP-1c- sterol response element binding protein 1c

ChREBP- carbohydrate response element binding protein

VLDL- very low density lipoprotein

ob/ob- leptin deficient mice

MTT- microsomal triglyceride transfer protein

PPAR-α- peroxisome proliferator activated receptor alpha

Tlr4$^{-/-}$- TLR4 knockout

ELISA- enzyme liked immunosorbent assay

BMDM- bone marrow derived macrophages

BMDC- bone marrow derived macrophages

IP macs- intraperitoneal macrophages

Aoah$^{-/-}$- AOAH knockout

Aoa$^{+/+}$- AOAH wildtype

H&E- hematoxylin and eosin

DK- TLR4 and AOAH double knockout

IPIST- intraperitoneal insulin sensitivity test

CHAPTER ONE: LITERATURE REVIEW

A. STRUCTURE, FUNCTION AND BIOACTIVITY OF LIPOPOLYSACCHARIDE

1. Introduction to Gram-negative bacteria and LPS

Gram negative bacteria, which include common pathogens like *Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae*, are so called because of the inability of their cell wall to retain the crystal violet dye during Gram's staining procedure. The cell wall of gram negative bacteria is multilayered: an inner membrane, a thin peptidoglycan layer, and an outer membrane which contains proteins, phospholipids and importantly, lipopolysaccharide (LPS) (Fig 1). The outer membrane is involved in nutrient transport and prevents toxic substances like antibiotics from penetrating the cell. It also mediates the physiological interaction between the bacterial cell and its host. Therefore, the integrity of the membrane and of the LPS is crucial to the survival and viability of the bacteria. LPS is a heat stable molecule that occupies about 75% of the surface of the bacterium (1, 2) . When bacterial cells divide, a small amount of LPS is liberated from the cells into the medium. When they are killed by antibiotics, phagocytosis or the complement complex, a larger amount of LPS is released from the surface of the cell.

Interest in LPS stems from its ability to potently stimulate the immune system and initiate morbidity and mortality due to gram-negative sepsis. The LPS structure serves as a molecular pattern which the host recognizes as non-self, then mounts an

immune response to get rid of invading microorganisms (3). Therefore recognizing small amounts of LPS can be protective to the host. However when present in large quantities, excessive responses by the host can lead to intravascular coagulation, tissue damage, systemic inflammatory response, multiple organ failure, shock and death.

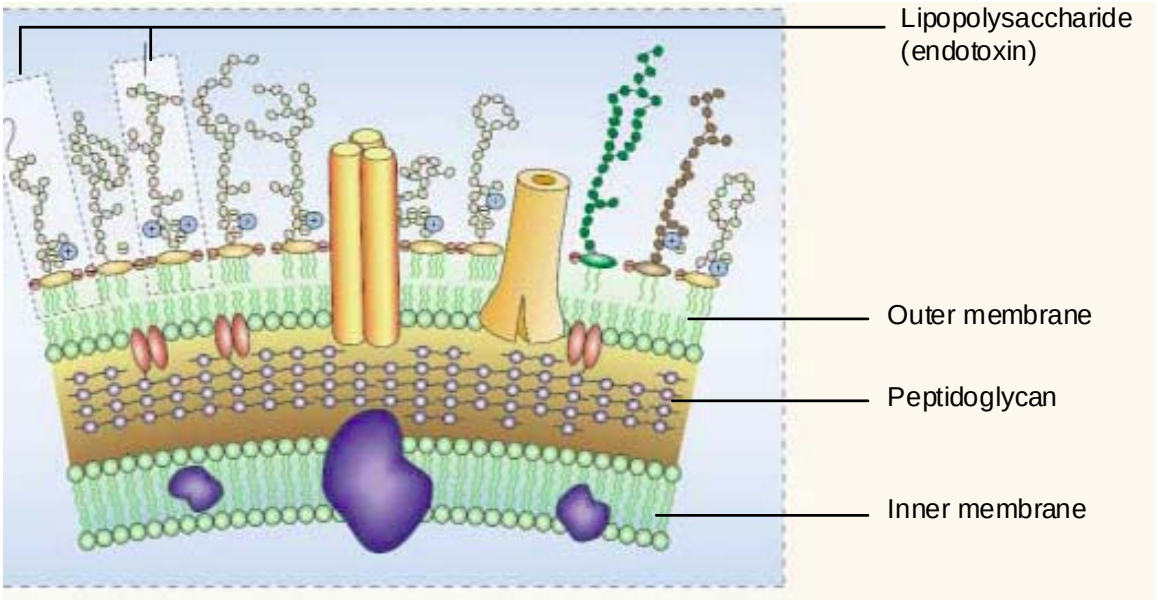


Figure 1: Cell wall of Gram negative bacteria.
Inner and outer membrane separated by a peptidoglycan layer. The LPS is located in the outer membrane (4) (Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Imm* 3:169-176, copyright 2003).

1. History of LPS/endotoxin

Richard Pfeiffer (1858-1945), who is considered the father of endotoxin, made two major observations that led him to formulate the endotoxin hypothesis. First, in a bid to identify the toxins involved in cholera pathogenesis, he observed that guinea pigs that were previously immunized with *V.cholerae* died after being inoculated with the bacteria. However no living V.cholerae could be detected in the abdominal cavities of

the animals. The V.cholerae cells had been lysed suggesting that bacterial viability was not essential for V.cholerae toxicity. Second, he observed that heat killed bacteria maintained their toxic potential proving that it was not a classical protein toxin. He reasoned that since dead cells lost their cytoplasm, the toxin was not present in the cytoplasm but was in the insoluble part of the bacterium. Pfeiffer came to the conclusion that V.cholerae had a toxin that was heat stable and associated with the insoluble part of the bacteria. He called it endotoxin. However he failed to realize that the toxin was not positioned towards the inside of the cell, rather, it was on the cell surface and exposed to the outside (5).

2. Chemical structure of LPS

After identification of endotoxin, techniques to isolate a fairly pure preparation were developed rather slowly. Otto Luderitz and Otto Westphal used the phenol-water extraction method to isolate and chemically characterize endotoxin. This method of extraction is still in use today. Due to the presence of polysaccharide and lipid components, they named the product lipopolysaccharide (LPS) (4). Once a pure LPS preparation was obtained, its chemical and structural properties were elucidated.

LPS from enterobacterial species like *Salmonella enterica* and *Escherichia coli* have been studied in depth. The propensity of the molecule to form aggregates made determination of its molecular weight challenging but, mass spectrometry has proved useful in obtaining accurate molecular masses of native LPS molecules.

LPS has a common structure which consists of two to three defined regions: a lipid-rich region called lipid A, a core region, and in some bacteria an O antigen or specific chain (Fig 2).

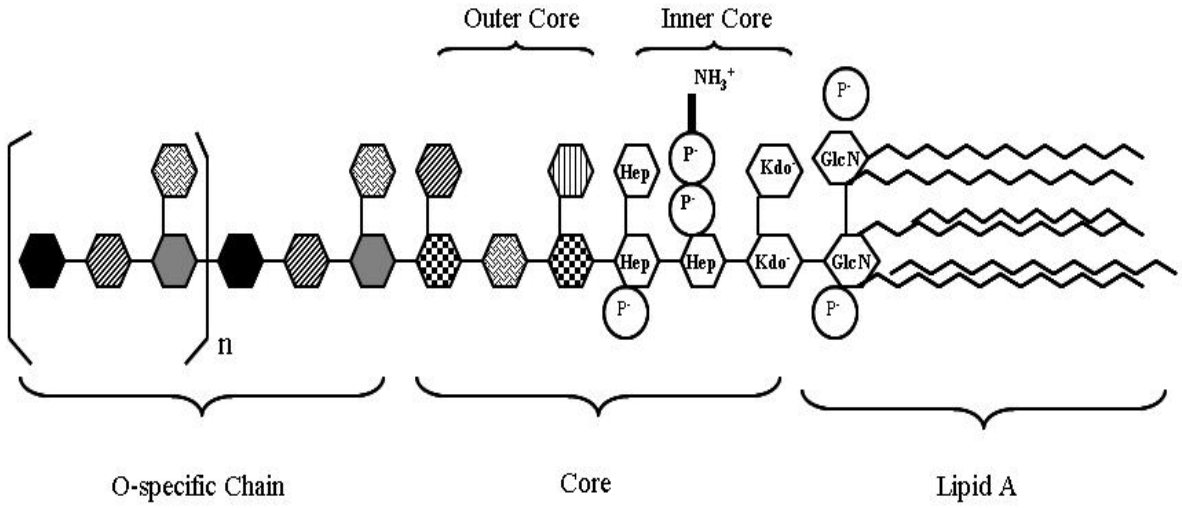


Fig 2: Schematic diagram of the chemical structure of smooth LPS.
LPS has three defined regions: the O-antigen consisting of repeating glycosyl residues, the core which is further subdivided into the outer core and inner core, and the highly hydrophobic lipid A which inserts into the bacterial outer membrane of gram negative bacteria. Abbreviations GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-mannose-heptose; P, phosphate; EtN, ethanolamine, zig-zag lines denote fatty acids (6) (Modified from Caroff M et al. *Microbes Infect* 4:915-926).

The O-antigen (O-specific chain)

The O-antigen of LPS consists of repeating units of one to eight glycosyl residues which make polyoligosaccharides extending out from the surface of bacteria. An O-antigen may have up to 50 identical subunits and the number of subunits differs in a single culture (6). The variation in subunit length is responsible for the ladder-like pattern of LPS molecules observed on SDS polyacrylamide electrophoresis gels (7). The structure of the repeating units differs between strains and confers serological specificity

to the LPS and bacteria. Differences in the O-antigen could be in the nature of the monosaccharide residues, their sequence, ring form, substitution and chemical linkage(1). During infection, the O-antigen of pathogenic bacteria allows it to escape the complement complex and resist some antibiotics (8).

The presence of an O-antigen gives colonies of bacteria that have it a smooth appearance on agar media. These are called smooth type bacteria. On the other hand, bacteria with LPS that lack an O-antigen are referred to as rough type bacteria because the edges of their colonies appear irregular and rough. Rough type bacteria are able to grow and multiply in vitro indicating that the O-antigen dispensable for viability.

The Core region

The core region is structurally more uniform than the O-antigen. It can be subdivided into an inner core and an outer core, with the latter being closer to the O-antigen. E.coli and Proteus serotypes have five different core types R1 to R4 and K-12. The outer core contains common hexoses like D-glucose, D-galactose and N-acetyl-D-glucosamine while the inner core is composed of the LPS specific heptose and 2-keto-3deoxyoctonic acid (Kdo). The Kdo and heptose resides are usually substituted by charged residues like phosphate or pyrophosphate resulting in a concentration of charged resides in this region of the inner core. The negatively charged residues attract to the cell surface of the bacteria the divalent cations that are necessary for maintaining the structure and function of the outer membrane (9)**.**

Studies of LPS mutants of Salmonella minnesota and Salmonella typhimurium identified mutations in two gene clusters called rough A (rfa) and rough B (rfb). These mutants were defective in producing specific glycosyl or phosphoryl transferases and

they resulted in truncated LPS molecules since omission of a sugar in the core will prevent the addition of subsequent sugars, including the O-antigen. The oligosaccharide core length of the mutants ranges from the Ra phenotype (longest) to the Re phenotype (shortest). Ra LPS consists of a complete core and lipid A but lacks O-antigen. Re LPS consists of only two or three Kdo molecules and lipid A. One Kdo residue linked to lipid A is the minimal requirement for bacteria to grow and multiply. In general, LPS from most enteric bacteria have a complete core (9) (7).

Lipid A

For over fifty years, lipid A has been identified as the toxic center of LPS. This assignment was supported in part by the finding that chemically synthesized lipid A has identical biological activity to that of bacterial lipid A (9). Chemical analyses, NMR techniques and mass spectrometry revealed that it is an unusual glycophospholipid with distinctive features. In general enterobacterial lipid A is a biphosphorylated β-1,6 linked glucosamine disaccharide with fatty acid residues at position 2,3, 2’ and 3’ (Fig. 3A) (10). The fatty acids at positions 2 and 2’ are amide-linked to the disaccharide while those at the 3 and 3’ positions are ester-linked. Two of the glucosamine-linked fatty acids are often acylated at their 3-hydroxy groups with the so-called secondary acyl chains, forming acyloxyacyl linkages (11). Though the lipid A moiety is the most conserved region of LPS, variations exist in the degree of phosphorylation, type of hexosamine, and the chain length, position, number and type of acyl chains.

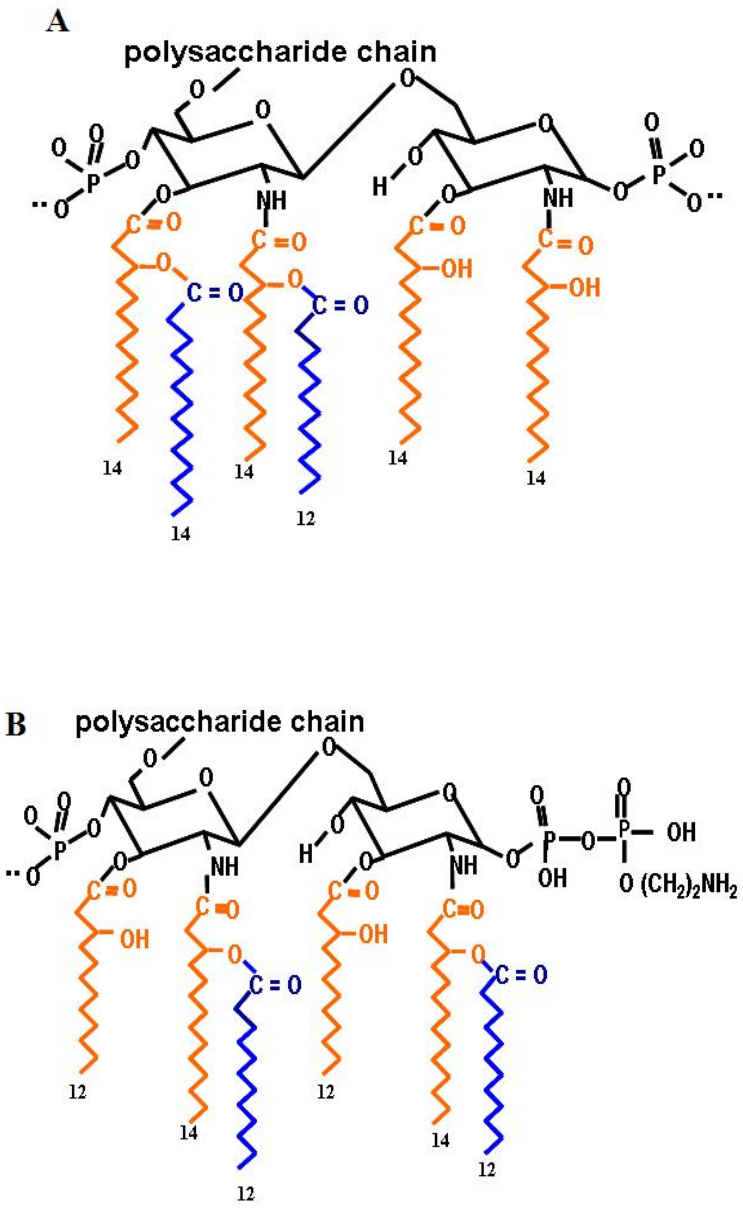


Fig. 3: Lipid A structure of *E.coli* and *N.meningitidis*

General architecture of lipid A consists of a β-1,6 linked glucosamine disaccharide backbone. Primary fatty acid chains are directly attached to the backbone via amide and ester linkages. Secondary or "piggy-back" fatty acid chains are attached to the primary chains at the 3 hydroxyl groups forming acyloxyacyl linkages. *E.coli* lipid A (A) differs from *N.meningitidis* lipid A (B) in the average length of fatty acids (14 vs. 12), the acylation pattern (symmetrical vs. non-symmetrical) and substitution of the phosphate groups (6) (Modified from Caroff M et al. *Microbes Infect* 4:915-926).

3. Structure of LPS from non-enteric bacteria

Mucosal pathogens like *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Haemophilus influenzae* are all examples of non-enteric bacteria that have been studied extensively (12). Like rough mutants of enteric bacteria, their LPSs lack O-antigens and have shorter non-repeating oligosaccharides (less than 10 sugar residues). Hence they are referred to as lipooligosaccharide or LOS (13). However unlike rough type LPS of enteric bacteria, LOSs have structural and antigenic similarities to human glycolipids and can potentially be modified in vivo by host substances or secretions (14). Such host cell mimicry can camouflage the bacterial surface from the host and contribute to pathogenesis. For example *Neisseria meningitidis* is able to add N-acetylneuraminic (sialic) acid to its LOS structure, which making it resistant to the lytic effects of serum antibody and complement (15).

The lipid A of *N.meningitidis* differs from classical E.coli lipid A in the location and chain lengths of acyl groups. Whereas the fatty acids are asymmetrically distributed in E.coli lipid A (the terminal GlNII has four acyl chains and the reducing GlNI residue has two acyl chains) they have a symmetrical distribution pattern in *N.meningitidis* (Fig.3B). Furthermore, the fatty acids in lipid A from enteric bacteria have an average chain length ranging from 12 to 16 carbon atoms while LOS have an average chain length of 12 carbon atoms. Finally the phosphate groups of meningococcal lipid A are substituted by O-phosphorylethanolamine (16).

4. Recognition of LPS by host cells

Due to the amphipathic nature of LPS, it was initially hypothesized that it interacts nonspecifically with responsive host cells by hydrophobic insertion into their cell membrane (17). However over the last couple of decades much progress has been made in identifying the specific molecules that animals use to recognize and respond to LPS.

The lipid A moiety of LPS binds to LPS binding protein (LBP) an acute phase protein that is produced by the liver and circulates in the bloodstream. LBP facilitates the transfer of LPS to membrane-bound CD14 (mCD14) or soluble CD14 (sCD14) (18, 19). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein that was once thought to be the LPS receptor. However it was unlikely that CD14 alone could transduce the LPS signal because it lacks a transmembrane domain. The use of positional cloning to identify the nature of LPS hyporesponsiveness in C3H/HeJ mice led to the discovery of TLR4 as the signaling receptor for LPS in 1998 (20, 21). Furthermore, studies in TLR4 knockout mice confirmed the role of TLR4 in LPS signaling (22). One year later Shimazu et al discovered that a soluble glycoprotein, myeloid differentiation protein-2 (MD-2), associates with the extracellular domain of TLR4 and is indispensable for LPS signaling (23). Binding of LPS to the MD-2—TLR4 complex also results in TLR4 oligomerization on the cell surface (24).

Besides the MD-2—TLR4 complex, other molecules might be involved in LPS recognition. Heat shock proteins 70 and 90, CXCR4, CD55 and CD11/CD18 have all been suggested to be part of the LPS activation cluster and may possibly act as LPS transfer molecules (25-27). The significance of these signaling proteins is still uncertain.

5. TLR4 signal transduction

When LPS is recognized by the MD-2—TLR4 complex, downstream adaptors are recruited through the Toll-interleukin-1 receptor (TIR) domain of TLR4. These downstream adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also called TIRAP), TIR-containing adaptor molecule (TRIF, also called TICAM-1) and TRIF-related adaptor molecule (TRAM, also called TICAM-2) (28). Studies in MyD88 knockout mice showed that they were resistant to LPS induced septic shock. Furthermore MyD88 deficient macrophages were unable to proliferate and produce pro-inflammatory cytokines in response to LPS even though they were able to activate nuclear factor κB (NF-κB) (29). However MyD88 deficient macrophages were still able to produce type I interferons and interferon inducible genes (30). These studies demonstrated the presence of MyD88 dependent and MyD88 independent TLR4-mediated response to LPS (Fig. 4).

The MyD88 dependent pathway

Upon LPS stimulation, MyD88 recruits and associates with IL-1 receptor – associated kinase-4 (IRAK-4) through its death domain (31). IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1 dissociates from the receptor complex and associates with TNF receptor associated factor (TRAF-6) (32). TRAF-6 becomes activated and in turn activates transforming growth factor-β-activated kinase (TAK1). TAK1 then activates inhibitory-binding proteins κB kinase (IκB kinase) and mitogen activated protein kinase (MAPK) pathways (33). IKKα, IKKβ and IKKγ form a high molecular weight protein complex known as the signalosome. Phosphorylation of IκB

proteins by the signalosome leads to their degradation by the proteosome and translocation of the transcription factor NF-κB into the nucleus. NF-kB controls the expression of pro-inflammatory cytokines such as IL-1, IL-12, IL-8, IL-6 and TNF-α. Activation of the MAPK pathway leads to induction of AP-1, another transcription factor which also plays a role in expression of pro-inflammatory cytokines (34).

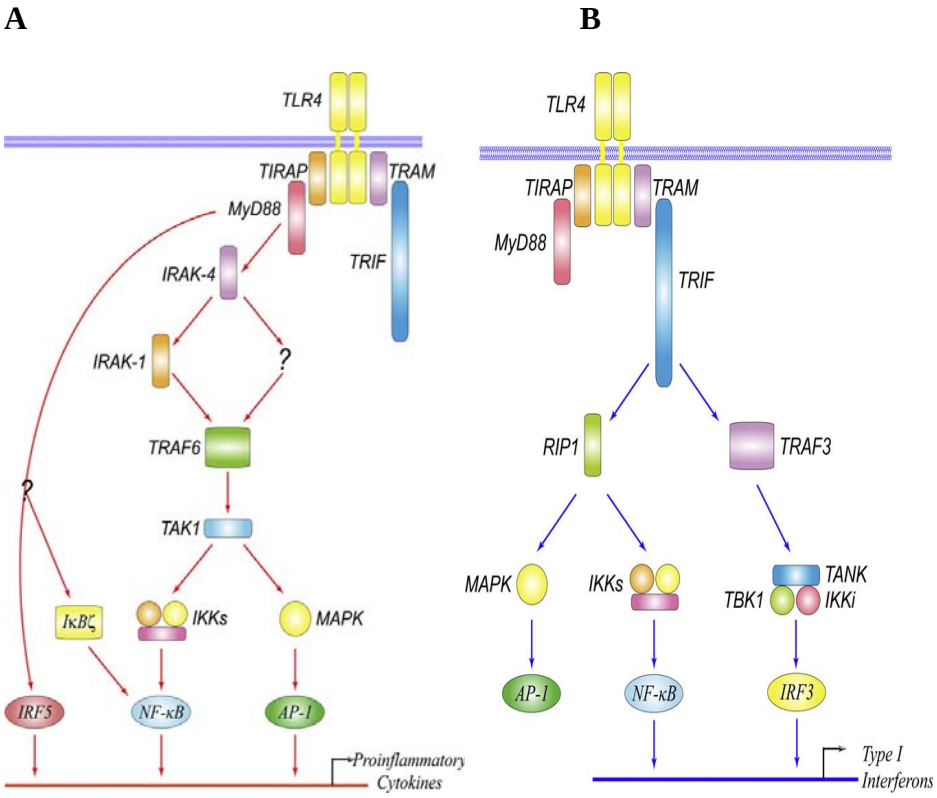


Fig 4: The MyD88-dependent and independent pathways. (A) The MyD88 dependent pathway involves activation and phosphorylation of IRAK which leads to activation of TRAF6 and the NF-κB and MAPK kinase pathways. This results in transcription of several pro-inflammatory cytokines. (B) The MyD88 independent pathway involves TRIF which induces transcription factors IRF3 and NF-κB by recruiting TRAF3 and RIP1. Activation of this pathway leads to production of type I interferon's (35) (Reprinted from Lu et al *Cytokine* 42:145-151 copyright 2008 wit h permission from Elsevier).

The MyD88 independent pathway

TRIF is a key molecule in the TLR4-mediated MyD88 independent pathway. Studies by Yamamoto et al showed that the NF-κB and MAPK response to LPS in TRIF deficient mice was comparable to that seen in wildtype mice, yet TRIF and MyD88 double knockout mice had severely impaired NF-κB and MAPK activation. Furthermore, TRIF expression was crucial in activation of IRF3 and induction of IFN-β by LPS (36). TRIF is recruited to the TLR4 TIR domain by the adapter molecule TRAM and subsequently interacts with receptor-interacting protein 1 (RIP1), a serine/threonine kinase that is necessary for NF-κB activation (37, 38). However RIP1 is not involved in LPS induced IRF3 activation (39). TRIF activates IRF3 by recruiting TRAF3, a protein essential for induction of type I interferon's (40). TRAF3 associates with TRAF family member associated NF-kB activator (TANK), TANK binding kinase I (TBK1) and IKK to mediate downstream signaling events. The latter two proteins are critical for phosphorylation and activation of IRF3. IRF3 then binds to the interferon-sensitive response element (ISRE) to induce transcription of target genes including type I interferons (35).

6. Structure-bioactivity relationships of LPS

The lipid A moiety of LPS is responsible for its endotoxic properties (41, 42). The classical lipid A structure is that of *E.coli*. It has a 1,4 bisphosphorylated β-(1-6) linked D-glucosamine disaccharide backbone (Fig.3A). The backbone is substituted with six fatty acyl chains, four of which are in primary ester or amide linkage to the

disaccharide backbone, and two are in 3-acyl-oxyacyl linkage to the primary fatty acyl chains . Studies using synthetic analogues of lipid A demonstrated that the phosphorylation pattern, and the number and chain lengths of the fatty acyl chains are critical to maintaining the toxicity of LPS(43). Removal of one of the phosphate groups or saccharides reduces bioactivity by one-hundred and 10^7 fold respectively (9). Furthermore removal of one acyl group (to yield a pentaacyl lipid A) or addition of an acyl group (to yield heptaacyl lipid A) reduced bioactivity by one hundred fold. Kotani et al showed that compound 506 (or LA-15-PP), a synthetic lipid A which is structurally indistinguishable from *E.coli* lipid A had comparable bioactivity to the reference E.coli lipid A as evidenced by its potency in the rabbit pyrogenicity assay, the local Shwartzman reaction, and lethal toxicity assays (44). In addition, compound 406 (or LA-14-PP) which lacks the two secondary fatty acyl chains and is identical to lipid IV_A , a precursor of lipid A biosynthesis, was completely inactive in human cells (43, 45). These studies emphasize the importance of the secondary fatty acyl chains in the endotoxic activity of LPS.

Differences in lipid A structure and bioactivity have also been observed in nature. The numbers and types of Lipid A found in a single bacterial population can be regulated by environmental conditions. For example *Salmonella typhimurium* grown in low Mg^{2+} conditions activates its PhoP-PhoQ two component system leading to production of a mutant LPS called $PhoP^c$. The $PhoP^c$ mutant has an added 4-amino-arabinose and 2-hydroxymyristate on its lipid A (Fig. 5) (46). These modifications decrease the capacity of *S.typhimurium* LPS to stimulate TNF-α secretion from human monocytes (47). Similarly, *Yersinia pestis* undergoes lipid A modifications at different

temperatures. At 27°C (flea temperature), *Y.pestis* makes hexaacylated lipid A. At 37°C (mammalian host temperature), in contrast, the hexaacylated species is absent while tri- and tetraacylated species are predominant (48). The tetraacylated species had a significant reduction in ability to activate human cells as compared to its hexaacylated counterpart (48, 49). Production of tetraacyl LPS may serve as a mechanism for bacteria to evade host recognition (50).

The endotoxic action of LPS also depends on differences in host species. Whereas murine cells are able to recognize and initiate responses to lipid IV_A, human cells are unable to do so. The differential response has been attributed to both the TLR4 and MD-2 molecules (51).

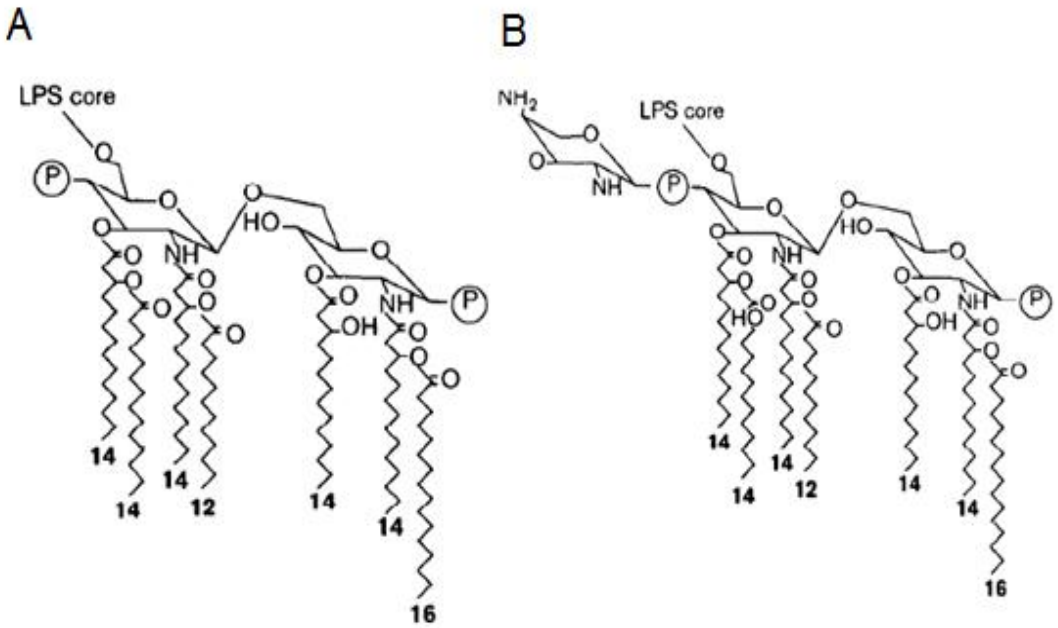


Figure 5: *Salmonella typhimurium* lipid A grown in (A) Mg^{2+} replete and low Mg^{2+} media.

Structure of LPS from (A) wildtype *S.typhimurium*. Growth in media with low Mg^{2+} causes modifications in the lipid A structure identical to $PhoP^c$ *S. typhimurium*

mutant (B). The mutant lipid A has a 4-amino-arabinose added to the 4’ phosphate and hydroxylation at the 2 position of the myristate (52) (Modified from Darveau P et al *Curr Opin Microbiol* 1:36-42)

7. Host inactivation of LPS

Recognition of LPS by the host is followed by activation of an immune response and microbial immunity. However uncontrolled inflammatory responses can be detrimental. Several mechanisms exist in the host to neutralize LPS thereby preventing harmful reactions to it.

Molecules that bind LPS and prevent it from engaging the MD-2—TLR4 complex

The O-antigen of LPS can be recognized by antibodies and lectins. Mannose-binding lectin (MBL) binds sugars that have C3-OH and C4-OH groups in an equatorial position on their microbial surfaces (53). MBL neutralizes LPS harboring these structures by preventing it from binding the MD-2—TLR4 complex, by binding calreticuin/CD91 C1q receptor complexes on macrophages, or by activating the classical complement pathway (54). Natural IgM and IgG antibodies have low affinity for LPS but binding promotes clearance of the LPS from circulation.

Cationic antimicrobial proteins (CAMPs) are concentrated in neutrophil granules and are released during phagocytosis. The positively charged domains of these molecules bind to negatively charged lipid A. Examples include bactericidal permeability-increasing protein (BPI), lactoferrin, lysozyme and cathelicidins. Of these, BPI has been most extensively studied. It is a 57kDa protein and a member of the BPI protein family, which also includes LBP (55). BPI outcompetes LBP for binding to lipid

A and prevents engagement of MD-2/TLR4 (56). CAP18 and CAP11 are cathelicidins derived from humans and guinea pigs respectively. Both have been shown to bind to LPS and to suppress LPS-induced TNF-α expression by macrophages (57).

LPS in the circulating blood can also be inactivated by host lipoproteins such as phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), and LBP. PLTP and LBP bind to LPS and transfer it to high density lipoprotein particles (HDL) (58). LPS that is bound to HDL is unable to activate the LPS signaling complex. Interestingly LBP has a concentration dependent dual role. At low concentrations, it binds to LPS aggregates and facilitates MD-2/TLR4 activation. On the other hand, high concentrations of LBP, such as those seen during the acute phase, inhibit LPS activity (59). LPS that is bound to the surface of monocytes may also be transferred to lipoproteins by soluble CD14 (60).

Inactivation of LPS after uptake by the liver and spleen

In rodents, approximately 70-80% of an intravenous does of LPS is quickly removed from the bloodstream by the liver (61). The LPS is taken up by Kupffer cells and subsequently moves into hepatocytes (62). Gram –negative bacteria are also removed from circulation by the liver and spleen (63). Disruption of macrophage function *in vivo* increases endotoxin-induced lethality in animals, suggesting that hepatic uptake and detoxification are crucial for preventing systemic reactions to LPS (64).

Enzymatic degradation of lipid A

Enzymatic degradation of LPS prevents full recognition by animal cells. Alkaline phosphatase and acyloxyacyl hydrolase (AOAH) are the two host enzymes known to mediate LPS catabolism. Exogenous alkaline phosphatase inactivates LPS by removing the phosphate groups and protects mice from *E.coli* induce sepsis (65, 66). AOAH selectively removes the secondary fatty acyl chains from lipid A and prevents prolonged responses to LPS.

B. ACYLOXYACYL HYDROLASE (AOAH)

1. AOAH deacylates LPS

LPS deacylation was first reported in Dictyostelium discoideum (slime mold), a primitive phagocyte that uses bacteria as a food source (67, 68). Dictyostelium has enzymes that can remove both nonhydroxylated and hydroxylated fatty acids from lipid A (68). In 1983, Hall and Munford reported enzymatic release of nonhydroxylated fatty acids from Salmonella LPS by human neutrophils (69). The enzyme responsible for the observed deacylation was called acyloxyacyl hydrolase (AOAH) and is the only mammalian enzyme known to deacylate LPS. It is interesting that AOAH cleaves only the secondary fatty acid chains (nonhydroxylated laurate and myristate) from *E.coli* lipid A leaving the primary fatty acids (3-hydroxymyristate) attached to the glucosamine backbone (Fig. 6A). The reaction products are thus fatty acids (usually myristate and laurate) and a tetraacyl LPS. AOAH was partially purified from HL-60 cells (human

promyelocytes) and used to deacylate LPS in vitro. As noted, the lipid A moiety of enzymatically deacylated LPS (dLPS) structurally resembles lipid IV_A (Fig. 6B), a tetraacyl intermediate in the biosynthesis pathway, and the chemically synthesized compound 406 (45, 70). As would be predicted from the structure-bioactivity studies discussed above, dLPS is much less potent than intact LPS.

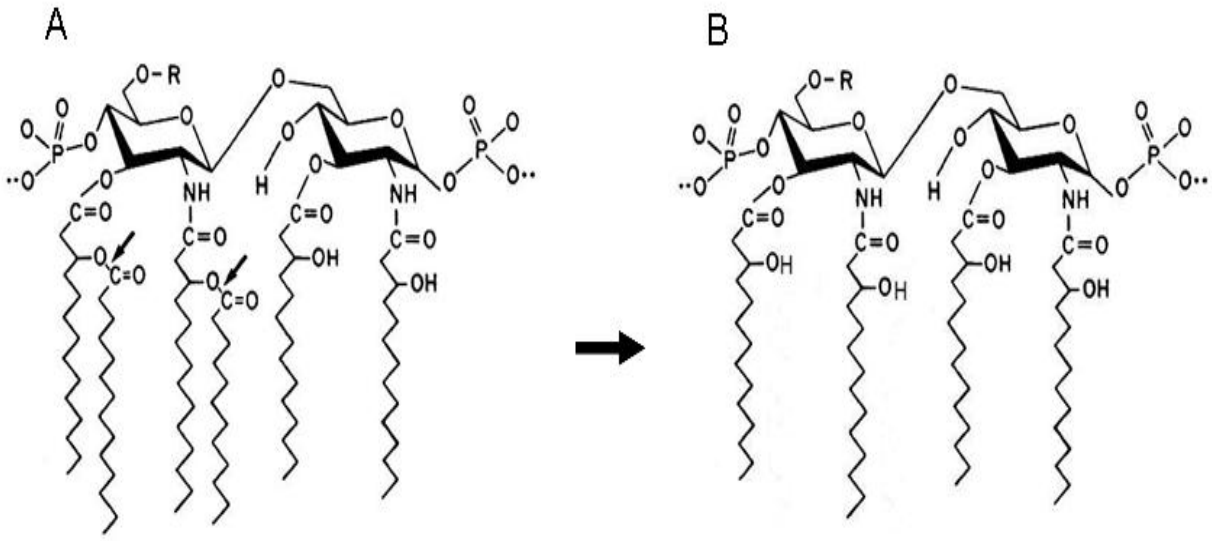


Figure 6: AOAH selectively cleaves secondary (piggyback) fatty acyl chains from lipid A moiety of LPS.
(A) Fully acylated *E.coli* lipid A with arrows showing AOAH cleavage sites. (B) The lipid A moiety of enzymatically deacylated LPS is structurally similar to lipid IV_A and compound 406 (11) (Modified from Erwin et al *J Biol Chem* 265:16444-16449).

Although the structure of lipid A is highly conserved, variations such as the extent of phosphorylation or the nature, length and attachment site of the acyloxyacyl groups exist in LPS derived from different bacteria. Interestingly, AOAH can act on

diverse LPS substrates. Erwin et al tested the ability of AOAH to deacylate LPS derived from *E.coli, Pseudomonas aeruginosa, Haemophilus influenzae, Neisseria meningitidis,* and *Neisseria gonorrhoeae in vitro* (11). In all cases the secondary fatty acids were removed regardless of their chain length or position. The primary fatty acids always remained attached to the backbone. AOAH appeared to have a preference for shorter, saturated non-hydroxylated secondary fatty acids.

The ability of AOAH to deacylate LPS in a physiological setting has been investigated. Both the cellular and extracellular components of an inflammatory exudate, elicited in a rabbit peritoneal cavity, participated in LPS deacylation in an AOAH like fashion (71). Among the components of the cellular extract, the mononuclear phagocytes were responsible for the majority of the deacylation activity observed. Neutrophils played a much less significant role. In addition, AOAH is capable of deacylating LPS in whole bacteria (72). Both mononuclear cells and neutrophils deacylated *E.coli* LPS in an inflammatory exudate though deacylation occurred relatively slowly.

Taken together these studies demonstrate that AOAH is a phagocytic-cell enzyme that deacylates LPS from a variety of bacteria that infect animals.

2. Deacylated LPS has reduced bioactivity

LPS that has been deacylated by AOAH is markedly less potent than intact LPS. The tissue toxicity of dLPS was measured by the dermal Shwartzman reaction and found to be 100-fold less than that of intact LPS (73). dLPS is also able to antagonize the ability of LPS to stimulate human cell types including neutrophils, THP-1 monocyte-macrophages and endothelial cells (74-77). dLPS can antagonize the LPS signaling

pathway by competing with LPS for binding to LBP, CD14 and MD-2 (76, 78). Therefore AOAH deacylation might reduce toxic reactions to Gram negative bacteria by detoxifying bacterial LPS and producing an LPS antagonist. However the antagonistic activity of dLPS varies among species and cell types. For example in a murine B-cell mitogenicity assay, the potency of dLPS was only reduced by a factor of ~30 relative to that of mock-treated LPS (73). In addition, whereas dLPS was able to antagonize responses to LPS in the human THP1 monocyte macrophage cell line, it did not block secretion of TNF-α in human whole blood cultures (79).

3. AOAH structure

Studies using recombinant AOAH have helped in deciphering the molecular structure of the enzyme. AOAH is a lipase encoded by a single RNA. It has been highly conserved during evolution (80, 81). AOAH is synthesized in BHK 570 cells as a 70k-Da precursor protein. It subsequently gets secreted or goes through cleavage in lysosomes to yield the ~60k-Da mature AOAH (Fig. 7) (82). Proteolytic cleavage increases the LPS deacylation activity of the enzyme by 10-20 fold (82). Mature AOAH consists of two disulfide linked subunits which is a unique property as very few lipases have more than one polypeptide chain. The large subunit contains the Gly-X-Ser-X-Gly sequence found at the active site of most lipases (83). Furthermore AOAH resembles members of the GDSL family of lipases which have a conserved Ser-Asp-His catalytic triad. Deletion of the Ser^{263} in the active site resulted in greater than 99% reduction in enzymatic activity toward LPS (82). The AOAH small subunit shares sequence similarity with saposins (sphingolipid activator proteins), small glycoproteins that

enhance the hydrolysis of glycosphingolipids by specific lysosomal hyrolases (84). Like most other saposins, the small subunit of AOAH has an N-linked glycosylation site and six cysteine residues, however the function of the glycosylation site remains unknown. The small subunit is involved in LPS recognition, activity, and intracellular localization of the enzyme.

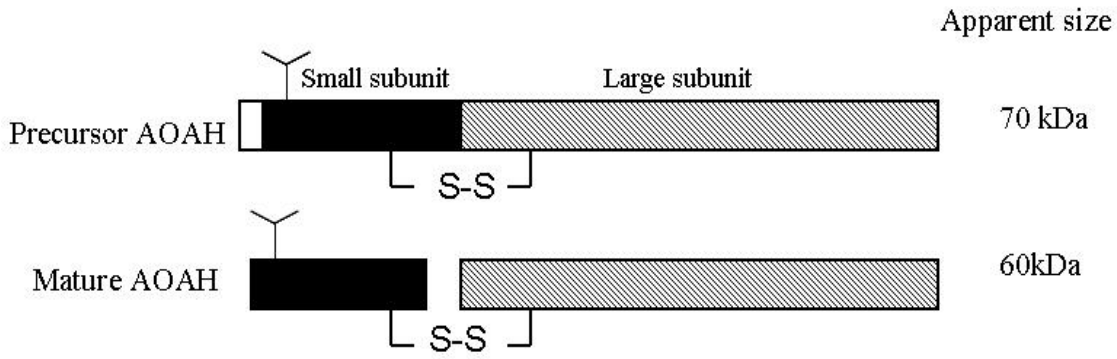


Figure 7: Schematic representation of AOAH biosynthesis showing conversion from precursor AOAH to mature AOAH

The branched lines indicate the glycosylation site in the small subunit. The apparent sizes of precursor and mature AOAH when run on non-reducing SDS-polyacrylamide gel are shown. (Modified from Staab et al *Jour Biol Chem* 269: 23736-23742).

4. Localization of AOAH

AOAH is expressed in cells of myeloid lineage (neutrophils, monocyte-macrophages, dendritic cells) and renal cortical epithelial cells (71, 85-87). Due to its low abundance, immunolocalization in primary cells has been unsuccessful. Studies

using indirect immunofluorescence on BHK cells transfected with an AOAH-expressing plasmid localized the enzyme to cytoplasmic vacuoles (82). The observation that ammonium chloride significantly decreased LPS deacylation led to the conclusion that AOAH is contained in an acidic compartment in human neutrophils (85).

5. AOAH is a phospholipase

Besides deacylation of LPS, AOAH is active towards other substrates. Munford and Hunter demonstrated that AOAH is a phospholipase A1/2 that can act on diverse glycerophospholipids and diglycerides (88). Interestingly it is the only known phospholipase that can cleave both palmitates from sn-1,2-dipalmitoylphosphatidyl-choline suggesting that it may be involved in the inactivation of surfactant lecithin. AOAH has a preference for cleaving shorter saturated acyl chains from the carbohydrate backbone of both glycerolipid and LPS substrates. It also has acyltransferase activities and was able to transfer acyl chains from LPS to acceptor lipids in vitro (88). The transfer of acyl chains from LPS to various lipids may be important in the reutilization of LPS derived fatty by animal cells. The presence of AOAH activity in phagocytic cells suggests it may participate in phospholipid turnover that accompanies phagocytosis or metabolic activation of phagocytes (88).

C. MACROPHAGE HETEROGENEITY

1. Origin of macrophages

Macrophages are professional phagocytic cells that play indispensible roles in maintaining tissue homeostasis, phagocytosing necrotic and foreign material, and

presenting and processing antigens (89). They derive from the myeloid lineage and are the end state of differentiation of blood monocytes. The phenotypic heterogeneity of macrophages results from their cellular differentiation, widespread tissue distribution, and their responsiveness to many endogenous and exogenous stimuli (90).

Differentiation and growth of macrophages are dependent on interactions with stroma of hematopoietic organs and lineage-determining cytokines like macrophage stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (90) . Transcription factors (PU.1) and other cytokines including IL-3 and TNF-family members are also involved in macrophage determination. Newly synthesized monocytes enter the bloodstream where they circulate for 1-3 days before entering tissues (91). Monocyte migration from the blood and through the endothelia, the interstitium and epithelia is mediated by adhesion molecules including integrins, selectins and epidermal growth factor seven-transmembrane spanning (EGF-TM7)-type receptors

(Fig 8) (90).

Macrophages migrate to all tissue compartments of the body and are assigned names based on their location. For example macrophages lining the vascular endothelial cells of the liver are called Kupffer cells, those found in the central nervous system are called microglial cells, osteoclasts are macrophages in bone, and alveolar macrophages reside in pulmonary airways. Although most tissue-resident macrophages of an adult animal are derived from circulating monocytes, there is evidence suggesting that local proliferation also plays a role in maintenance and renewal of many of these macrophage populations (92). Inflammatory insults like infection and trauma also lead to increased

recruitment of blood monocytes to inflamed tissue to aid in repopulation of tissue macrophages (89).

2. Macrophage activation

Macrophages exhibit another level of heterogeneity when they are activated by various endogenous and exogenous stimuli. They play key roles in innate and acquired immunity and their activation can be either pro-inflammatory or anti-inflammatory. Four distinct activation states have been described: classical, innate, alternative activation and deactivation (89).

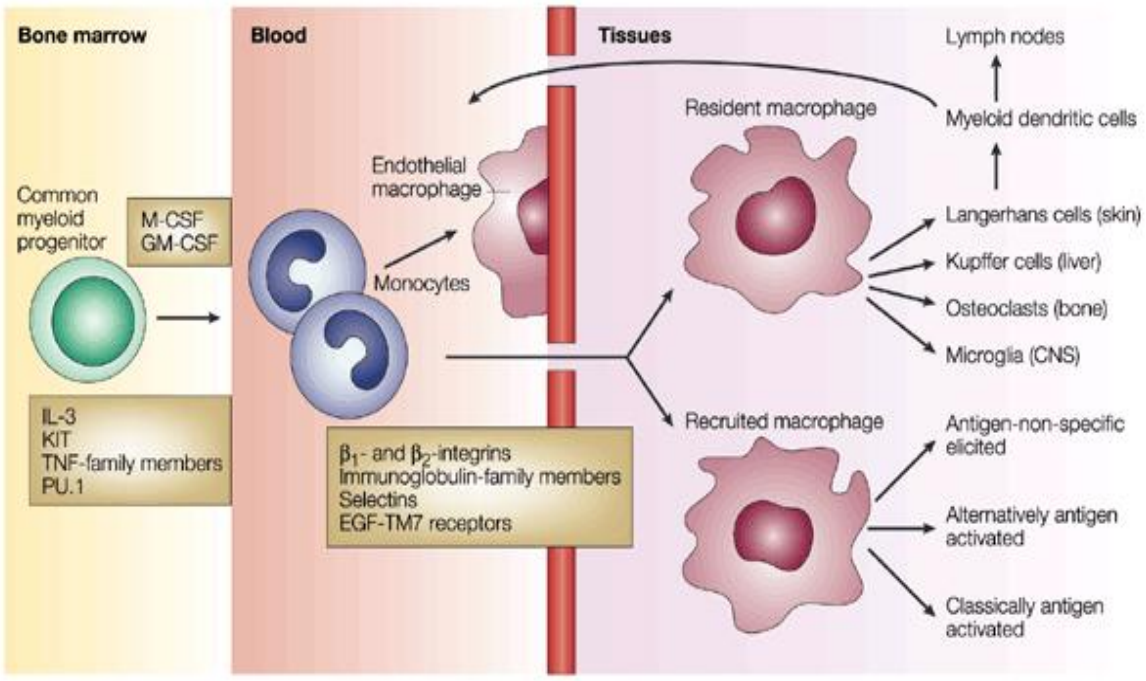


Figure 8: Differentiation and distribution of macrophages *in vivo*

Macrophages originate in the bone marrow from common myeloid progenitor cells which become committed to cells of monocytic lineage via multiple differentiation signals. Blood monocytes migrate to different tissues or are recruited in response to inflammatory stimuli (89). See text for details. (Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Immunol* Gordon et al 3:23-35 copyright 2005).

Classical activation. Classically activated macrophages (also known as M1 macrophages) develop in response to IFN-γ and LPS stimulation (93). They secrete large amounts of pro-inflammatory cytokines (e.g. IL-1β, IL-18, TNF-α, and IL-12) and chamomiles. They also have increased expression of MHC class II and co stimulatory molecules (CD80/CD86) leading to enhanced antigen presentation. M1 macrophages are associated with Th1 lymphocyte responses and are important in cell mediated immunity to intracellular pathogens (94). Their elevated microbicidal activity is mediated by mechanisms including restriction of iron from microorganisms, release of nitric oxide, acidification of the phagosome and synthesis of reactive oxygen intermediates (95).

Innate activation. Innate activation of macrophages involves direct recognition of microbial products (e.g. LPS) by pattern-recognition receptors such as Toll-like receptors and Nucleotide Oligomerization Domain (NOD)-like receptors (96, 97). Similar to classical activation, innate activation is characterized by secretion of pro-inflammatory cytokines and upregulation of costimulatory molecules.

Alternative activation. Alternatively activated macrophages, also known as M2 cells, result from IL-4 and IL-13 stimulation (90). Both cytokines are produced by Th2 cells, mast cells and basophils and have anti-inflammatory effects on macrophages (90). Like classically activated macrophages, M2 cells also have elevated expression of MHC class II and costimulatory molecules. However, they have distinctive upregulation of arginase and lectin-like receptors (e.g. the mannose receptor) (98-100). Arginase is involved in tissue repair and the mannose receptor promotes the phagocytosis and endocytosis of host and exogenous ligands (95). M2 cells are also associated with allergy, anti-parasite responses, humoral immunity and fibrosis (94).

Macrophage deactivation. Deactivation of macrophages is induced by IL-10, TGF-β, glucocorticoids and other regulatory mechanisms (90). Deactivated macrophages are associated with anti-inflammatory cytokine production and reduced MHC class II expression. Scavenging activity is also upregulated (95).

3. Macrosialin/CD68 expression in macrophages

Membrane antigens serve as markers for macrophages both *in vivo* and *in vitro*. F4/80 and sialoadhesin are examples of plasma membrane antigens with macrophage restricted expression. In 1991 Rabinowitz et al used FA/11, a rat monoclonal antibody to identify a sialoglycoprotein in murine macrophages. The protein was named macrosialin (101).

Macrosialin is a heavily glycosylated 87-115kDa transmembrane protein highly expressed by macrophages and to a lesser extent, by dendritic cells (102). The

extracellular domain has two distinct regions: a membrane-distal mucin-like domain, and a membrane proximal domain homologous to the lysosomal associated glycoprotein (lamp/lgp) family of proteins. Members of the lamp family are ubiquitously expressed and found in lysosomal membranes. Lamp family members have a high degree of homology across different species. Accordingly, macrosialin has 72% identity and 80.6% similarity with CD68, its human homologue (102). Macrosialin and CD68 both have macrophage restricted expression, similar molecular masses and comparable glycosylation patterns. Both are located intracellularly in endosomes or lysosomes and a small amount is found on the cell surface (102, 103).

Macrosialin is differentially glycosylated in response to inflammatory stimuli. Whereas resident peritoneal macrophages express low levels of a non-lectin binding glycoform, the presence of inflammatory stimuli leads to a 17-fold increase in macrosialin expression and lectin binding capabilities (102). Both glycoforms contain N-linked glycans and O-linked sugar structures which account for 21kDa and 30-40% respectively of the molecular mass of macrosialin. (101). The O-linked sugars and sialic acid prevent proteolysis and protect cell membranes from complement and degradative enzymes (104, 105). These properties would be useful in lysosomes which are rich in acid, proteolytic enzymes and complement. Although macrosialin has been shown to bind oxidized low density lipoprotein (OxLDL), its function remains unknown (106).

D. NON-ALCOHOLIC FATTY LIVER DISEASE

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the presence of predominantly macrovesicular hepatic steatosis that is not associated with alcohol consumption. It occurs as a histological spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Whereas simple steatosis is fat in the liver without hepatocellular damage, NASH is fat in the liver accompanied by inflammation and hepatocyte damage (107). NAFLD affects 70 million adults in the United States (108). Most patients are asymptomatic except for occasional fatigue and malaise (109). NAFLD can be detected in humans by magnetic resonance spectroscopy and liver biopsy. Liver enzymes are usually not elevated and are not a reliable method of detection (110). Depending on the underlying pathogenesis, NAFLD can be categorized as primary or secondary (108). Secondary NAFLD results from drugs, viral infections and toxins. Primary NAFLD is the more common of the two. It is associated with obesity, diabetes, insulin resistance and the so-called “metabolic syndrome.”

2. Pathogenesis

The risk factors for NAFLD and NASH include central or visceral abdominal obesity, insulin resistance, hyperglycemia and hypertriglyceridemia (111). Insulin resistance plays a key role in development and progression of NAFLD but a cause and effect relationship between the two has not been determined. Increase in visceral adipose tissue (VAT) mass is very common in NASH and contributes to pathogenesis by increasing blood levels of free fatty acids and pro-inflammatory cytokines (112).

The liver plays an important role in whole-body lipid and carbohydrate metabolism. NAFLD develops when there is a disruption in the normal mechanisms for synthesis, transport and removal of free fatty acids and triglycerides (113). When the rate of import or synthesis of fatty acids by hepatocytes exceeds that of export or catabolism, steatosis occurs. The physiological balance that prevents lipid accumulation in the liver can be disrupted in four major ways (Fig. 9) (113).

1) Increased delivery and uptake of free fatty acids into hepatocytes

Adipose tissue secretes several molecules that are involved in metabolism (114). These include mediators of carbohydrate metabolism (leptin, resistin and adiponectin), lipid metabolism (lipoprotein lipase and apolipoprotein E) and adipocytokines (TNF-α, TGF-β and IL-6). Adipose TNF-α is upregulated in obese animals and contributes to insulin resistance. It activates IκB kinase β (Iκκβ) in an autocrine and paracrine fashion, thereby inhibiting phosphorylation of insulin receptor substrates (IRS-1 and IRS-2) (113). Insulin-mediated suppression of hormone-sensitive lipase (HSL) is also disrupted leading to an increased flux of free fatty acids from VAT to the portal circulation. Studies in mice have shown that targeted disruption of the Iκκβ gene ameliorates insulin resistance (115).

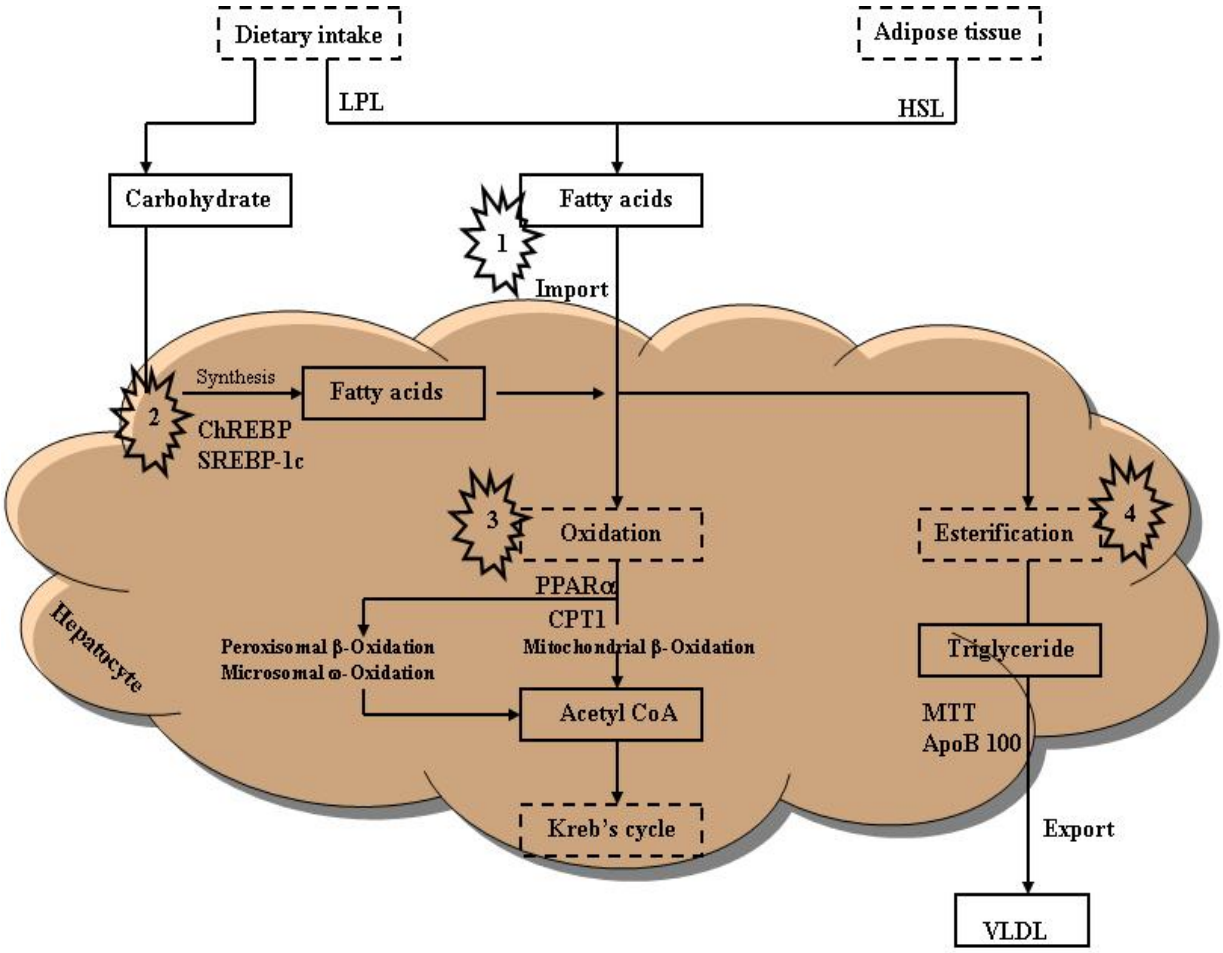


Fig 9: Lipid metabolism and development of steatosis in the liver

In the postprandial state, triglycerides obtained from the diet are transported in the blood as chylomicrons. LPL hydrolyzes lipids in the chylomicrons and releases free fatty acids. In the fasting state, insulin levels decrease allowing the HSL-mediated release of free fatty acids from adipose tissue. The liver absorbs circulating free fatty acids and also synthesizes them via SREBP-1c and ChREBP. Clearance of fatty acids from the liver is followed by β-oxidation in the mitochondria, peroxisomes and microsomes leading to acetyl CoA generation. Excess fatty acids are re-esterified to triglycerides and exported into the blood as VLDL. A disruption in any of the points numbered in the figure can lead to NAFLD. LPL. Lipoprotein lipase; HSL, hormone sensitive lipase; ChREBP, carbohydrate response element-binding protein; CPT1, carnitine palmonitoyl transferase 1; SREP-1c, sterol regulatory element-binding protein 1c; VLDL, very low density

protein; PPARα, peroxisome proliferator –activated receptor a. (Modified from Anstee Q et al *Int J Exp Pathol* 87: 1-16)

2) Increased hepatic de novo free fatty acid and triglyceride synthesis. Oleic acid is a product of *de novo fatty* acid synthesis and excess amounts have been demonstrated in human and murine models of NAFLD (116). After a meal, high plasma glucose and insulin levels activate rapid synthesis of free fatty acids within hepatocytes. Carbohydrate response element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c) are transcription factors that mediate de novo lipogenesis by increasing transcription of lipogenic enzymes (117). CHREBP is activated by glucose and binds to carbohydrate response elements in genes encoding pyruvate kinase, acetyl CoA carboxylase and fatty acid synthetase. SREBP-1c promotes insulin mediated hepatic lipid synthesis by increasing expression of acetyl CoA synthetase, which in turn increases production of acetyl CoA. It also increases hepatic triglyceride content by inhibiting microsomal triglyceride transfer (MTT) protein expression, leading to a decrease in very low-density lipoprotein formation (VLDL).

3) Decreased triglyceride export and synthesis of VLDL. Free fatty acids can be cleared from the liver by oxidation or by esterifcation to triglyceride and export as VLDL. Transfer of lipid to apolipoprotein B100 by MTT is the rate-limiting step in VLDL lipoprotein assembly. Individuals with a mutation in MTT develop abetalipoproteinaemia which is characterized by severe steatosis (118).

4) Decreased elimination of free fatty acids due to impaired hepatic mitochondrial β-oxidation. Oxidation of fatty acids takes place primarily in the mitochondria (119). Carnitine palmitoyl transferase 1 (CPT-1) mediates the esterification and import of fatty acids into the mitochondrial matrix. CPT-1 is very sensitive to the effects of upregulated hepatic fatty acids and can be inhibited by malonyl CoA, the first intermediate of fatty acid synthesis. Alternative oxidation pathways such as peroxisomal β-oxidation and microsomal β-oxidation are utilized when CPT-1 and mitochondrial β-oxidation become overwhelmed. Peroxisome proliferator activated receptor alpha (PPAR-α) also plays a key role in mitochondrial β-oxidation of fatty acids (117). Mice deficient in PPAR-α develop extensive hepatic steatosis after short-term fasting due to their diminished hepatic oxidation capacity (120).

3. Progression from NAFLD to NASH: The "two hit-hypothesis"

The "two-hit hypothesis" has been used to describe the mechanisms that promote progression of steatosis (NAFLD) to steatohepatitis (NASH) (121). The "first hit" refers to hepatic steatosis caused by obesity and insulin resistance. Steatosis then predisposes the liver to additional damaging factors including lipopolysaccharide (LPS). The "second hit" leads to steatohepatitis and is caused by oxidative stress.

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the cell (113). Mitochondrial dysfunction and large amounts of hepatic fatty acids lead to increased production of ROS. Oxidative stress causes cellular damage and release of pro-inflammatory cytokines.

4. Animal models of NAFLD and NASH

Several animal models that exhibit hepatic steatosis have been described in the literature yet none of them displays the entire NASH phenotype observed in clinical practice. A true animal model of NASH should have the pathological pattern of liver injury which constitutes fatty change with an inflammatory infiltrate, and the metabolic abnormalities seen in NASH such as obesity, insulin resistance and dyslipidemia (111). Models that meet most of these criteria are the most useful in studying the impact of metabolic abnormalities on the pathogenesis of NASH. Below is a description of some of the models that have been useful in the study of NASH.

1) Genetic models of NASH

Hepatic steatosis develops when fatty acid delivery to the liver exceeds oxidative requirements. CD36 (fatty acid translocase) is a fatty acid transporter expressed in peripheral tissues (113). Mice deficient in CD36 have increased levels of circulating free fatty acids and triglycerides and hepatic insulin resistance due to dysfunctional fatty acid storage (122). The mice develop hepatic steatosis and are unable to suppress gluconeogenesis in the liver. Another genetic model of steatosis associated with insulin resistance is the SREBP-1c transgenic mouse. SREBP-1c is a lipogenic transcription factor and mice overexpressing the protein in adipose tissue develop diabetes, severe insulin resistance and hepatic lipid accumulation. Ballooned hepatocytes and Mallory bodies which are histological features of NASH are also present.

Leptin, a hormone produced by adipose tissue, plays an important role in food intake and energy expenditure. Mice deficient in leptin (*ob/ob*) overeat when given access to standard chow. They develop insulin resistance, hepatic steatosis and type 2 diabetes. Although leptin deficient mice exhibit the metabolic abnormalities associated with NAFLD, they do not spontaneously progress from steatosis to steatohepatitis (111). A low dose of LPS or a diet deficient in methionine and choline are often used as a second insult to produce steatohepatitis (111).

2) Models of NASH caused by overnutrition

High fat and high carbohydrate diets have both been used to trigger steatosis in mouse models. Both lead to increased hepatic fatty acid uptake and *de novo* lipogenesis. A diet rich in carbohydrate (65% sucrose) led to the development of insulin resistance and macrovesicular steatosis in C57Bl/6 mice after 8 weeks of feeding (123). Similarly mice on a high-fat diet developed steatohepatitis after 10 weeks of feeding (124). They also had increased levels of plasma insulin, total cholesterol and hepatic triglycerides. By 50 weeks they developed marked hepatic steatosis and inflammation. Steatohepatitis induced by a high fat diet is associated with increased portal endotoxin levels in some mouse strains (125).

5. Hypothesis: AOAH prevents NASH

The histological features of NASH are similar to those of alcoholic liver disease, suggesting that both diseases have common pathogenic mechanisms (126, 127). Studies in rodents revealed that gut-derived bacteria and endotoxin modulate alcohol-induced

liver damage. Chronic alcohol administration increases the intestinal permeability to endotoxin which is then delivered by the portal venous system to the liver. The increased endotoxin in the liver activates Kupffer cells to secrete cytotoxic products via the MD-2—TLR4 pathway resulting in liver injury (128-130).

In recent years, human and animal studies indicate that gut microbiota may also play a role in the development of NASH. Surgical procedures that trigger intestinal stasis and bacterial overgrowth hasten the progression of steatosis in obese patients (131). Furthermore, in comparison with healthy controls, patients with NASH had a higher prevalence of small intestinal bacterial overgrowth as measured by a 14C-D-xylose and lactulose breath test (132). The NASH patients also had significantly higher levels of serum TNF-α. Interestingly Cope et al demonstrated that in the absence of ethanol ingestion, genetically obese mice had increased production of gastrointestinal ethanol that was significantly decreased by antibiotic treatment (133). The obesity-related increases in ethanol production by intestinal bacteria were thought to alter the intestinal barrier and promote translocation of endotoxin to the portal circulation. Accordingly, ob/ob mice have been shown to have increased intestinal permeability and portal endotoxemia (134). Consequently elevated endotoxin levels activate liver cells to release ROS and inflammatory cytokines including TNF-α. TNF-α interferes with insulin signaling, promoting insulin resistance. This in turn alters carbohydrate and lipid metabolism resulting in hepatocyte lipid accumulation (135, 136).

AOAH detoxifies Gram-negative bacterial LPS and is mainly produced by Kupffer cells in the liver (61). LPS deacylation by AOAH prevents prolonged inflammatory responses to LPS in the liver and spleen. *Aoah*$^{-/-}$ mice are unable to

deacylate LPS and develop significant hepatic inflammation when given small doses of LPS (61). Given that high-fat diet feeding alters intestinal flora and increases plasma endotoxin levels in mice, it stands to reason that AOAH may prevent diet –induced steatohepatitis (137). Demonstrating that AOAH can protect mice from NASH would further verify the role of endotoxin in the development of NASH, and provide the groundwork for investigating a link between patterns of AOAH expression in humans and susceptibility to NASH.

CHAPTER TWO: REGULATION OF AOAH EXPRESSION

A. INTRODUCTION

AOAH is a highly conserved LPS-deacylating enzyme found in neutrophils, macrophages and dendritic cells. It selectively removes the secondary (acyloxyacyl-linked) fatty acyl chains which are required for full recognition of LPS by the vertebrate MD-2—TLR4 complex. Inactivation of LPS by AOAH is crucial to preventing prolonged responses to LPS. Therefore studies that delineate the expression and regulation of the enzyme are important.

AOAH expression and activity can be regulated by LPS both *in vivo* and *in vitro*. Lu et al demonstrated that treatment of immature dendritic cells with anti-inflammatory cytokines and an agonistic CD40 antibody decreased their AOAH activity whereas LPS treatment increased it (138). Similarly, Cody et. al found that treating explanted murine macrophages with 100ng/ml LPS induced a 10-fold increase in AOAH mRNA abundance as early as two hours after stimulation. mRNA abundance peaked at six hours with a 20-fold increase above baseline and remained elevated for 72 hours (139). As little as 0.01ng/ml of LPS was sufficient to induce AOAH expression and the increase in AOAH mRNA coincided with a sustained increase in enzymatic activity. Induction of AOAH by LPS required de novo protein synthesis since treatment with cyclohexamide, a protein synthesis inhibitor, significantly decreased AOAH mRNA levels. IFN-γ also increased AOAH mRNA expression in macrophages though to a lesser extent than LPS. The anti-inflammatory cytokine IL-10 did not inhibit LPS induced AOAH mRNA expression which is unlike other LPS inducible genes. This suggested that AOAH might

work in concert with IL-10 and other anti-inflammatory mediators to limit inflammatory responses to LPS.

Although AOAH activity is minimally expressed or absent in mouse plasma, it is present in normal rabbit plasma and increases markedly after LPS challenge. Rabbits challenged intravenously with LPS had a 16-fold increase in plasma AOAH activity as early as 90 minutes after challenge. Induction of AOAH mRNA expression by LPS has also been reported in both the liver and the lung. Since approximately 70-80% of an intravenous dose of LPS ends up in the liver, we wanted to explore how AOAH activity is regulated by LPS in this organ. We observed that LPS induces AOAH enzymatic activity that remains elevated for several days.

B. MATERIALS AND METHODS

1. Mouse Strains

C57BL/6 (B6) mice were housed in a specific pathogen-free barrier facility at UT Southwestern and fed a standard chow diet.

2. Preparation of Rough LPS (*E. coli* O14)

LPS from *E.coli* O14 bacteria were extracted and purified using the Galanos procedure for isolating rough LPS (140). Bacteria were pelleted by centrifugation then washed twice with water, once with 80% and 95% ethanol, once with acetone and three times with ether. Cells were then allowed to dry in a desiccator. The dry bacteria were then extracted for 15 minutes one ice with PCP (90% (w/w) phenol: chloroform: petroleum ether in a volume ratio of 2:5:8) centrifuging at 5000rpm for 10 minutes to pellet the cells. The supernatants were pooled and bubbled with argon to remove the chloroform and petroleum ether. LPS was carefully precipitated with water (10μl at a time), pelleted by centrifugation, then washed twice with 80% phenol and three times with anhydrous ether. The pellet was resuspended in water and extracted twice with ether to eliminate unwanted lipids. After adding 20mM ammonium acetate, 2 vol 95% ethanol were added to precipitate the LPS at 4^{o}C. The purified LPS was washed with 95% ethanol and anhydrous ether, and allowed to dry in a dessicator.

Purity of the LPS was confirmed by silver stained SDS PAGE (Fig. 10) and by its failure to stimulate IL-6 release from *Tlr4*$^{-/-}$ macrophages in vitro. Whereas wildtype macrophages secreted approximately 0.3ng/mg of IL-6 during a 4 hour incubation with 100ng/ml PS, no IL-6 was detected in media overlying Tlr4$^{-/-}$ macrophages incubated

with 10µg of LPS. LPS was resuspended in 0.1% (v/v) triethylamine, 10mM Tris HCl by vigorous vortexing or sonication and stored at -20C.

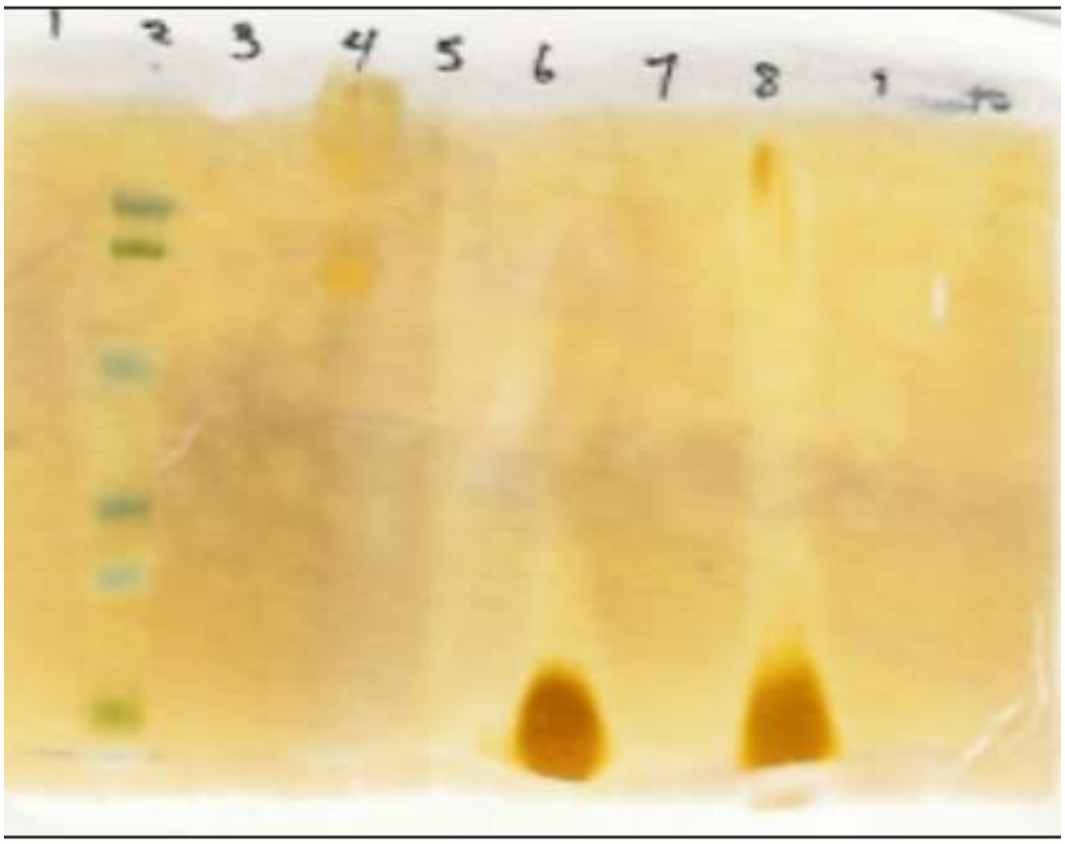


Figure 10: Silver stained SDS PAGE gel of purified O14 LPS

Lane 1, low molecular weight standard; lane 4, 10µl of BSA; lane 6, 10µg of LPS: lane 8, 10µg of LPS treated with proteinase K.

3. LPS Challenge

E.coli O14 LPS was diluted in phosphate buffered saline (PBS) and vortexed vigorously before use. B6 mice were injected with either 10μg intravenously (i.v.) or 25 μg intraperitoneally (i.p.) in a 250 μl volume. The mice were euthanized at different time points after LPS challenge and livers were harvested and stored at -20^{o}C.

4. AOAH Activity Assays

Approximately 150 mg of liver tissue were transferred to a 1.5ml microphage tube with 600μl of cold lysis buffer (0.1% Triton X-100, 2mM phenylmethylsulfonyl fluoride, PBS pH 7.2) and homogenized using a sonicator (Branson Sonifier 450, VWR, West Chester PA). The lysates were diluted 1:10 in sterile PBS and 20μl were assayed for AOAH activity as described previously (141). In brief, lysates were incubated at 37^{o}C overnight in reaction mixture (20mM sodium acetate pH 6, 0.9% NaCl, 1mg/ml BSA, 0.1% Triton X-100) with double-radiolabeled *Salmonella typhimurim* LPS (^{3}H-acyl fatty chains and ^{14}C glucosamine backbone) as the substrate. One ml 100% ethanol was added to precipitate undeacylated LPS and BSA by centrifugation at 4^{o}C. The ethanol supernatant containing free ^{3}H labeled fatty acids was added to 200 μl SDS-EDTA (2% SDS, 5 mM EDTA) and 3 ml scintillation cocktail (BudgetSolve, Research Products International Corp. IL) and dpm were counted using a scintillation counter (Packard Tricarb 2100 TR) with external quench correction.

5. Protein Assays

Protein concentration in liver lysates were measured by using the Biorad Protein Assay (Biorad, Hercules, CA). The protein assay reagent was diluted 1:5 and 200µl were combined with 5µl of sample in a 96 well plate. The plate was incubated for 10 minutes at room temperature and the absorbance measured at 595nm using an MRX Microplate Reader (Dynex Technologies Inc. Chantilly, VA).

6. Quantitative PCR

Total RNA was isolated from snap-frozen livers without thawing (RNeasy mini kit; Qiagen). RNA was reverse- transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad laboratories, USA) and stored at -20^{0}C. SYBR Green PCR Master Mix (Applied Biosystems) was combined with the cDNA template and primers in a 96 well optical reaction plate. Real-time PCR was performed in an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). The following primers were used for AOAH mRNA amplification: 5-'GCC ATG ATT GGA AAC GAT GTT-3 and 5'-GTT GGC GTA CAT TTG TTC AGG A-3'. The PMF612 and pGTeasy18srRNA plasmids containing known amounts of murine AOAH and 18s rRNA cDNA respectively were used to produce dose-response curves. 18srRNA amplification was achieved using the following primers: 5'-ACC GCA GCT AGG AAT AAT GG AA' and 5'-CCG TCC CTC TTA ATC ATG GC-3'. The reaction conditions used were 50^{o}C for 2min; 95^{o}C for 10min; and 95^{o}C for 15sec, 60^{o}C for 1min for 40 cycles. All sample and standard reactions were done in duplicate.

C. RESULTS

1. LPS augments hepatic AOAH mRNA expression

Previous studies showed that LPS induces AOAH mRNA expression in the liver. To determine how long induction lasts and if there is a corresponding increase in activity, B6 mice were challenged i.v. with 10μg of *E.coli* 014 LPS. At 1, 3, 5, and 7 days after challenge, the mice were euthanized and their livers were tested for AOAH activity and mRNA expression. There was a 5 fold increase in AOAH mRNA expression by day 3 that lasted through day 5. (Fig. 11A) AOAH mRNA returned to just above baseline levels by day 7. Enzymatic activity of AOAH correlated with mRNA expression with a 3 fold peak by day 5 post challenge (Fig 11B). Hepatic AOAH activity remained significantly above baseline levels through day 7.

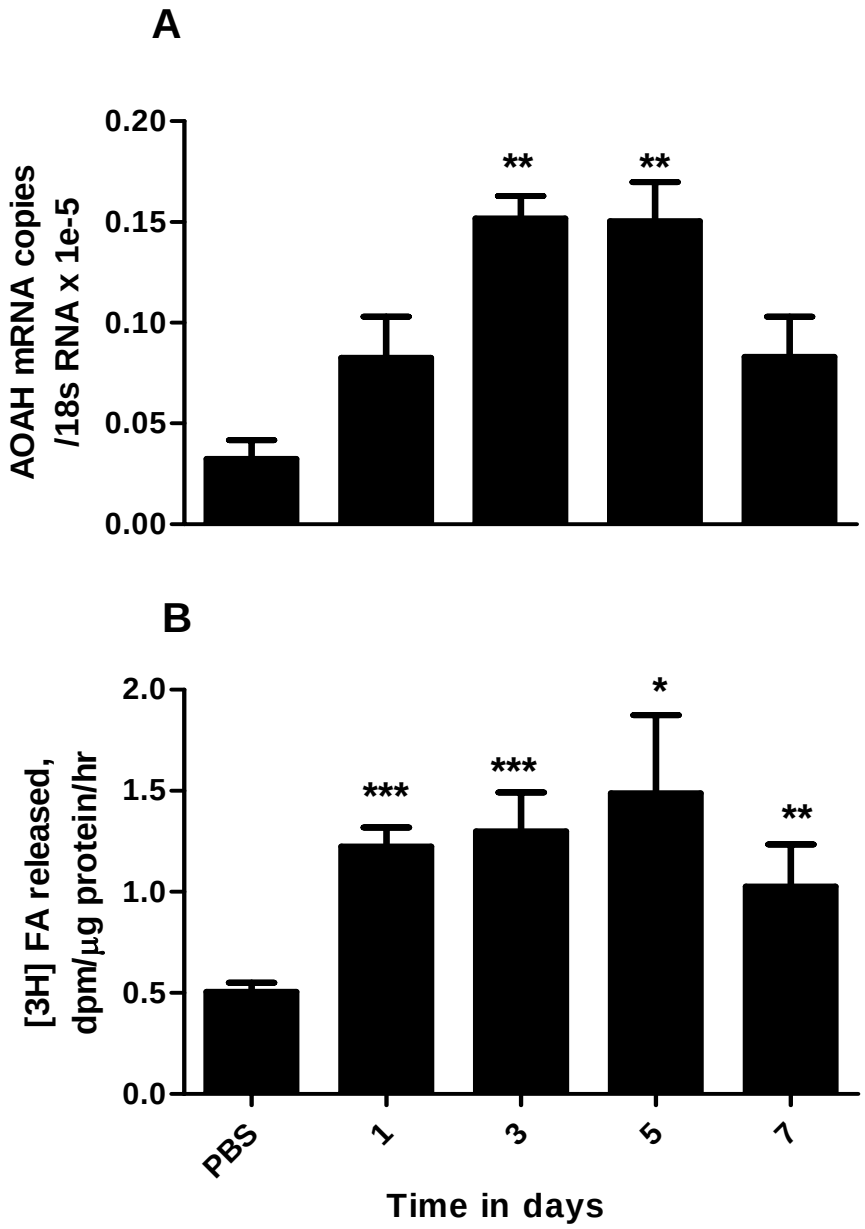


Figure 11: LPS causes transient upregulation of hepatic AOAH mRNA andactivity. B6 mice were i.v. injected with either 10μg *E.coli* O14 LPS or PBS. Livers were harvested at the indicated times after challenge. AOAH mRNA (A) and activity (B) were measured as described in *Methods*. Data are expressed as mean ± SD, n=3. *P<0.05; **P<0.01

2. Serum AOAH activity is induced by LPS

Very little AOAH can be detected in normal mouse serum. We asked whether LPS challenge was sufficient to increase AOAH activity in the serum. B6 mice were i.p. injected with 25μg of LPS and bled through the retroorbital vein at 1, 3, 5, 7 and 9 days post challenge. Serum AOAH activity increased slightly by day 1, peaked at day 3 with a 1.5 fold increase and returned to baseline levels by day 7 (Fig. 12A). A concurrent increase in hepatic AOAH activity was also observed (Fig. 12B).

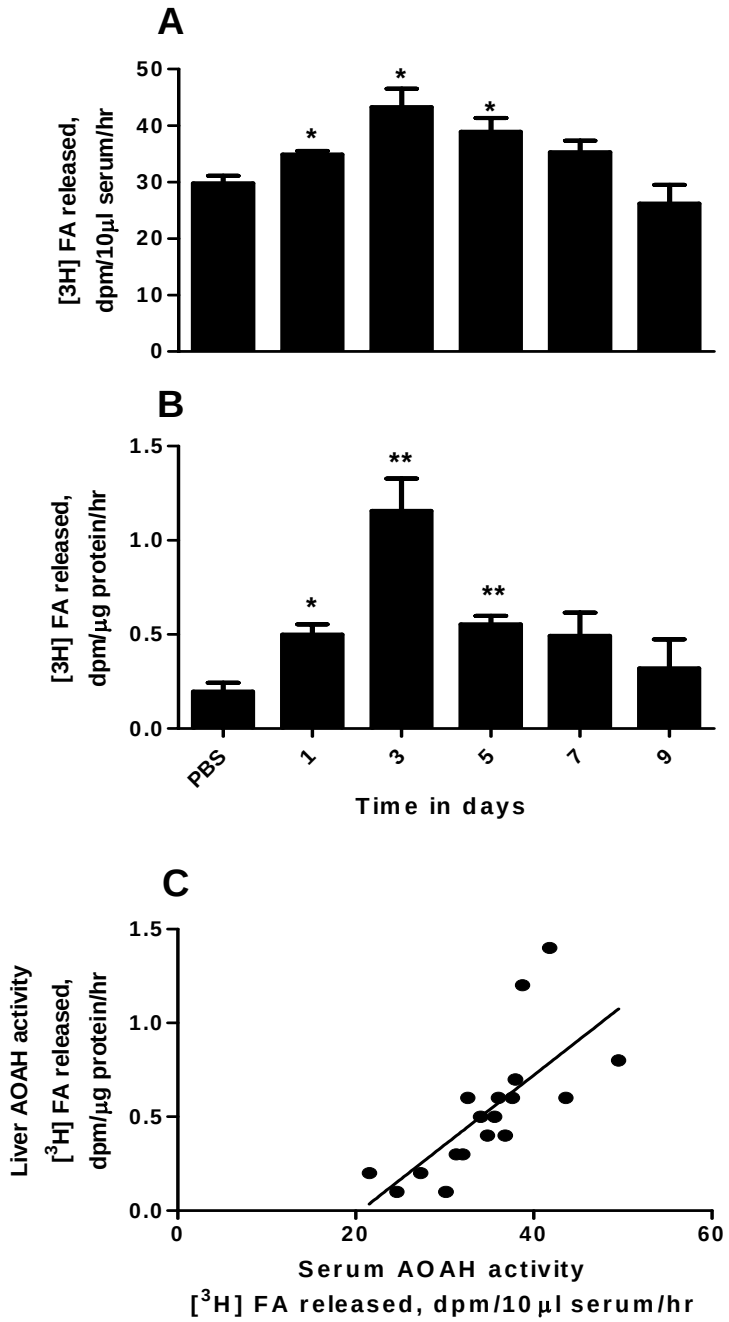


Fig. 12: Serum AOAH activity is induced by LPS

B6 mice were i.p. injected with either PBS or 25µg of LPS. **(A)** Livers were harvested at the times indicated after LPS challenge and AOAH activity was measured. (B) AOAH activity in 10µl of serum obtained from mice by retroorbital bleeds. (C) Correlation between liver and serum AOAH activity after LPS challenge. R^2=0.5, p<0.001

D. DISCUSSION

Prior to these studies, the induction of murine AOAH by LPS *in vivo* had not been explored extensively. Cody et al reported that AOAH mRNA in the liver continued to increase within an 8 hour period following LPS challenge however the duration of induction was not studied (139). The experiments described in the present study demonstrate that a single dose of LPS is sufficient to cause a small yet significant increase in hepatic AOAH mRNA that lasts for several days.

AOAH is the only mammalian enzyme known to deacylate LPS. The deacylated LPS is markedly attenuated in toxicity. Therefore upregulation of AOAH may be one of the mechanisms which animal cells use to limit inflammatory responses to this highly potent stimulus. IFN-γ, like LPS, is able to activate macrophages through the classical pathway leading to enhanced endocytic functions and ability to kill intracellular pathogens. It is also capable of inducing AOAH mRNA expression in macrophages (139). However, unlike LPS mediated induction, AOAH mRNA induced by IFN-γ accumulates slowly and declines rapidly.

The liver plays a crucial role in removing LPS from the circulating blood; thus hepatic LPS detoxification may be important in recovery from Gram-negative bacteremia. We observed a 5-fold increase in hepatic AOAH mRNA 3 days following an i.v. LPS challenge. The observed increase in AOAH mRNA was sustained till day 5 post challenge and was accompanied by an increase in AOAH enzymatic activity (Fig. 11). On day 7, hepatic AOAH activity was still significantly above baseline levels.

The sustained increase in AOAH expression and activity are quite unusual as most LPS inducible genes return to baseline levels shortly after induction. This unique

property may be attributed to the slow kinetics of the enzyme (72). LPS in circulation is quickly cleared up by the liver where Kupffer cells deacylate it over many hours (61). Accordingly, the observed increase in AOAH roughly paralleled the time-course of hepatic LPS deacylation.

Administration of LPS via the peritoneal route also induced AOAH enzymatic activity. Moreover a concurrent increase in serum activity was observed suggesting that the enzyme may be exported into the circulation by the liver (Fig. 12). Serum AOAH activity has also been found in mice infected with a recombinant adenovirus carrying the AOAH gene and in transgenic mice overexpressing AOAH (142) (Chapter Three). Increased hepatic AOAH activity might confer protection from some of the long-term reactions to Gram negative bacterial infection and might be beneficial in a clinical setting.

CHAPTER THREE: CHARACTERIZATION OF CD68p-AOAH TRANSGENIC MICE

A. INTRODUCTION

Monocyte-macrophages play a key role in the innate immune response to Gram-negative bacteria. They sense minute quantities of LPS and initiate inflammatory responses that provide effective host defense. The LPS molecule has a common architecture which consists of the O-antigen, the core region and lipid A. The lipid A moiety is the most conserved region of LPS and is responsible for most of its biological activity. In general, lipid A is a bisphosphorylated glucosamine disaccharide with hydroxyacyl chains directly linked at positions 2, 2', 3 and 3' (10). The hydroxyl groups of some the hydroxyacyl resides may be substituted with secondary acyl chains (laurate, myristate or palmitate) to form an acyloxyacyl linkage. Once inside the host, the lipid A portion of LPS binds to LPS-binding protein (LBP) which facilitates the binding of LPS to CD14. This enables transfer of LPS to the MD-2—TLR4 complex found mainly on the surface of monocytes. Activation of TLR4 by LPS results in the recruitment of MyD88, activation of NFκB and secretion of pro-inflammatory cytokines like TNF-α, IL-6 and IL-12 (35).

Although cytokine production is important for resolution and clearance of infection, overproduction can be harmful to the host and can lead to multiple organ failure and mortality. The host has developed several mechanisms to limit responses to LPS. These include proteins that bind directly to LPS and prevent it from interacting

with the MD-2—TLR4 complex, inactivation of LPS by its rapid removal from circulation, and diminution of cellular pathways that have been activated by LPS (64).

Another important mechanism by which animals can limit their responses to LPS is via enzymatic degradation of lipid A. Dephosphorylation and deacylation are the two known inactivating enzymatic reactions. Exogenously administered alkaline phosphatase has been shown to reduce the toxicity of LPS *in vivo (64, 143, 144)*. However the site(s) of lipid A that undergo dephosphorylation and the ability of the enzyme to dephosphorylate LPS from diverse Gram-negative bacteria are still undetermined (64). The second inactivating reaction is carried out by AOAH, a highly conserved host lipase that selectively removes the secondary acyl chains from lipid A. Deacylated (tetraacylated) LPS has a marked reduction in bioactivity and acts in vitro as an antagonist competing with LPS for binding to the MD-2—TLR4 complex (76, 145). AOAH is expressed by monocyte-macrophages, neutrophils, and as well as by renal cortical epithelial cells which secrete it into the urine. A host defense role for AOAH was suggested in studies showing that AOAH deficient mice develop long-lasting hepatomegaly, prolonged immunosuppression, and have exaggerated polyclonal antibody production in response to small amounts of LPS or Gram-negative bacteria (35, 61, 138, 146). Deacylation of LPS by AOAH may thus be a necessary mechanism for animals to recover from infection by Gram-negative bacteria that produce hexaacylated LPS.

Based on these findings, we hypothesized that increased constitutive expression of AOAH in macrophages will hasten recovery from LPS exposure in vivo. Promoter fragments of c-fms, CD11b and scavenger receptor A (SR-A) have been used by others

to drive transgene expression to macrophages, however none of these have been ideal because they are either expressed in only a subset of macrophages or they give rise to expression in non-macrophage cell types (147). We generated transgenic mice using human CD68 promoter sequences to drive high levels of AOAH expression. CD68 and its murine homologue macrosialin, are transmembrane proteins expressed in the endosomal compartment of all cells of mononuclear phagocyte lineage including monocytes, macrophages, microglia, osteoclasts, and to a lesser extent, dendritic cells (102). We found that human CD68 sequences can drive constitutive overexpression of murine AOAH in its normal cell environment-specifically in macrophages and dendritic cells. The resulting CD68p-AOAH transgenic mice deacylated LPS more efficiently than their wildtype littermates and were protected from LPS induced hepatomegaly.

A. MATERIALS AND METHODS

1. Construction of CD68p-AOAH Transgenic mice

Murine AOAH cDNA sequence was amplified from pSF451, a plasmid containing mouse cDNA, by PCR. The primers 5'-TCT AGA CAC CAT GAA GTT TCC CTG GAA AGT CTT CAA GAC C-3 and 5'-CTA CTT ATC GTC GTC ATC CTT GTA ATC GTG TCC TCC TTG GTC TC-3'were used in the amplification and the reaction conditions were 95^{o}C for 3min; and 95^{o}C for 40sec, 60^{o}C for 40sec, 72^{o}C for 1.5min for 35 cycles. The resulting PCR product had an added 5' XbaI site and a 3' Flag epitope (underlined bases). The PCR product was cloned into pCR 2.1 to introduce a second XbaI site downstream of the Flag sequence. The mAOAH-Flag fragment was released from the plasmid by XbaI digestion and inserted into pcDNA3 vector containing 2940 bp of the human CD68 promoter and 83bp of the first intron of the human CD68 gene (Fig. 13). The construct was isolated from this plasmid (pCDNA3-hCD68p-IVS-mAOAHFlag) using KpnI and PsiI and purified. Injection into C576BL/6 eggs and insertion into pseudopregnant foster mothers was carried out by the UT-Southwestern transgenic mouse core. Two founder lines (7802 and 7803) were confirmed by PCR analysis of genomic DNA extracted from the tails of mice; the primers amplified a 548bp region from the distal portion of the CD68 promoter to the proximal portion of the AOAH gene: 5'-GAA GCA GGG CCA ACA GTC CCC T-3' and 5'-CTC TCC ATC GCC ACC TGG ACT-3'.

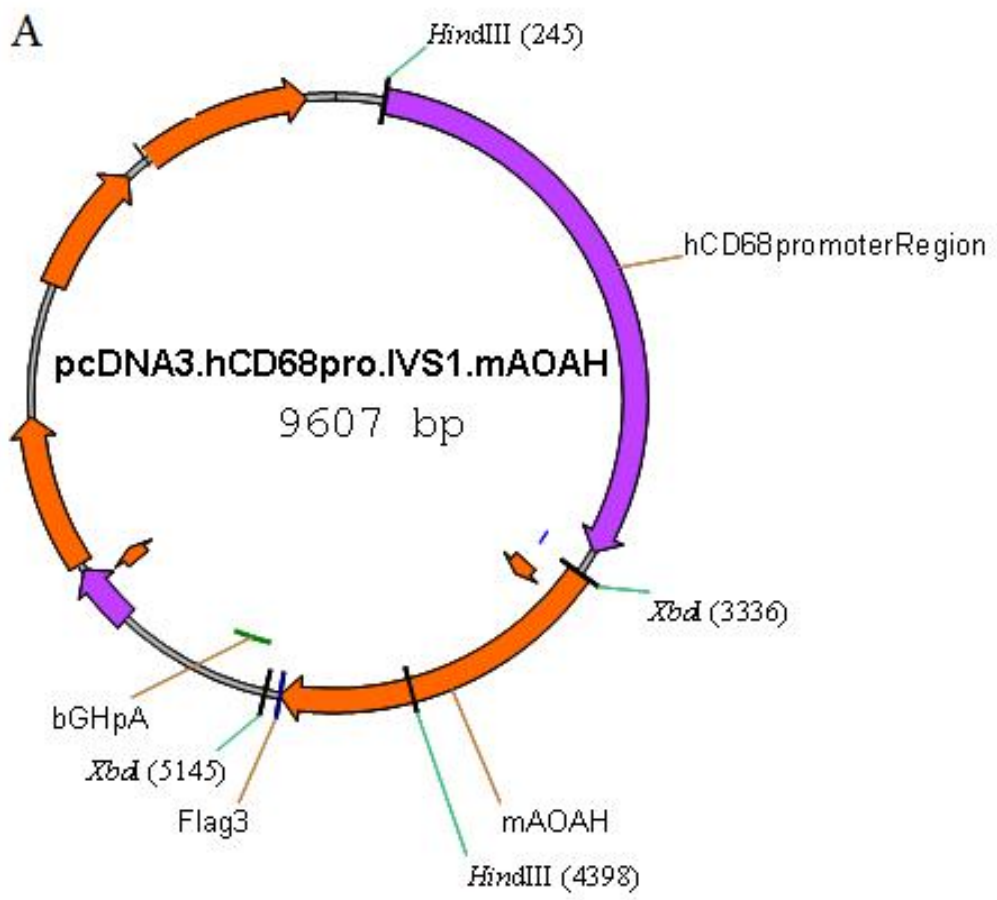


Figure 13: Schematic of pcDNA3.hCD68pro.IVS1.mAOAH plasmid

pcDNA3 vector with FLAG-tagged murine AOAH cDNA cloned downstream of human CD68 promoter sequence. AOAH cDNA was cloned into the vector at the XbaI sites. (bGHpA, bovine growth hormone polyadenylation sequence).

2. LPS Preparations

Double-radiolabeled Rc *Salmonella typhimurium* PR122 LPS that had 175,000 ^{3}H dpm in the fatty acyl chains and 10,000 ^{14}C dpm in the glucosamine backbone per microgram was prepared as previously described (141). Unlabeled LPS from *E. coli* O14 was purified by phenol-chloroform-petroleum ether extraction as described above (140). Prior to injection LPS was diluted in sterile PBS by vortexing vigorously.

3. AOAH activity assays

Mice were deeply anesthetized with isofluorane before the liver, kidney, spleen, lung, small intestine and heart were harvested. The tissues were rinsed in sterile PBS, weighed and sonicated in 600µl lysis buffer (0.1% Triton X-100 in PBS pH 7.2). AOAH activity in the tissues was measured as described previously using double radio-labeled *Salmonella typhimurium* LPS (141). Serum (10µl) collected from mice by tail vein bleeds, dendritic cells and macrophages were also tested for AOAH activity.

4. Bone marrow derived macrophages and dendritic cells

Tibias and fibulas were obtained from mice and immersed in 70% ethanol for 20 minutes then washed with cold complete RPMI (RPMI 1640, 10% inactivated FBS (Hyclone), 100µM non-essential amino acids (GIBCO BRL), 10µM sodium pyruvate, 25mM Hepes, 2mM L-glutamine (GIBCO BRL), 50µM 2-mercaptoethanol, 100U/ml penicillin and 0.1 mg/ml streptomycin). Both ends of the bones were cut with a razor and marrow was flushed out with 10ml of cRPMI and a 25G needle. Cell clumps were broken by passing the cells through a 22G needle and 10 ml syringe five times in a

culture dish. The cells were subsequently transferred to a 50ml conical tube and centrifuged at 800g for 10 minutes. The supernatant was discarded and cells were treated with 3ml of red blood cell lysis buffer for 5 minutes at room temperature. Cells were then underlaid with 5ml of heat inactivated fetal calf serum and washed twice.

Dendritic cells were obtained by resuspending cells at 2 X 10^6 cells/ml in cRPMI containing 10ng/ml rmGMCSF and seeding them in 6 well plates (3ml/well). The cells were incubated at 37^{o}C with 5% CO_2. On day 3 of culture, non-adherent cells were carefully removed and 2ml of fresh medium containing rmGMCSF were added. An additional 2ml of fresh medium were added on day 5. On day 7 the media containing non- adherent dendritic cells were harvested and purified using anti-CD11c monoclonal antibody N418 coupled to magnetic beads according to manufacturer's directions (Miltenyi Biotech Inc., Auburn CA). Flow cytometric analysis on a FACScalibur (Becton Dickinson) revealed that more than 94% of the sorted cells were CD11c+

Macrophages were obtained by resuspending marrow in DMEM medium supplemented with 15% FCS, 50% L cell supernatant, 100U/ml penicillin and 0.1 mg/ml streptomycin. The cells were incubated for 6 days at 37^{o}C with 5% CO_2. The cells were lifted by incubating them with 10mM EDTA in PBS and incubating at 37^{o}C for 5 minutes. Approximately 98% of the lifted cells were F4/80+ as determined by flow cytometric analysis.

5. Protein assays

Protein concentration in samples were measured by using the Biorad Protein Assay (Biorad, Hercules, CA) according to manufacturer's directions.

6. Uptake and deacylation of LPS by peritoneal macrophages

Peritoneal macrophages were obtained by injecting mice with 2ml of aged Brewer's thioglycollate (Difco laboratories Detroit, MI). The mice were sacrificed 4 days later and their peritoneal cavities flushed with RPMI. 1.5 x 10^6 cells/ml were cultured in 6 well plates in complete RPMI medium. Non-adherent cells were washed off after 3 hours and fresh cRPMI medium was added. One μg /ml of double labeled *Salmonella typhimurium* LPS was added to the cells in a total volume of 1ml cRPMI. At 6 and 12 hours, the media were collected, and cells were washed with fresh RPMI and harvested with 1ml of lysis buffer (0.1% Triton X-100 in PBS pH 7.2). The dpm in 100μl of the cell lysate were counted to estimate the cell associated LPS. To determine the amount of ^{3}H dpm fatty acid released into the cells and media, 500μl of both the media and cell lysates were precipitated with 1ml ethanol and incubated at -20^{o}C for 30 minutes. The ^{3}H dpm in the ethanol supernatant were measured and the dpm in the wildtype and transgenic cells were corrected for non-specific deacylation by subtracting the dpm released by the *Aoah*$^{-/-}$ cells. The percent deacylation for each time point was estimated using the formula: [ethanol soluble ^{3}H dpm released in cells + supernatant]/[^{3}H dpm in cells + supernatant] x 100%.

7. Measurement of *in vivo* LPS deacylation

Radiolabled LPS (5 μg) was suspended in 200 μl PBS and injected into the lateral tail vein. Mice were euthanized 4 hours later and their livers were harvested.

Approximately 100 mg of tissue were sonicated in lysis buffer (0.1% Triton X-100 in PBS pH 7.2). The radioactivity in the lysates was measured by scintillation counting and the ratio of ^{3}H to ^{14}C dpm in the lysates was calculated. Removal of ^{3}H labeled fatty acids by AOAH decreases the $^{3}H/^{14}C$ ratio of the injected LPS. Since some of the ^{3}H counts in the tissue may be in LPS-derived fatty acids that are either free or incorporated into other molecules, we also precipitated the undeacylated LPS in the lysates by adding 2 volumes of 100% ethanol. The ^{3}H dpm in the ethanol supernatant were subtracted from the ^{3}H dpm in the tissue lysate to give the LPS-associated ^{3}H dpm. Percent deacylation was calculated using the formula: (1-[final ratio]/[starting ratio]) X 100.

8. Quantitative PCR

RNA was isolated from cells or snap frozen tissues (RNeasy mini kit; Qiagen), DNAseI treated (Boehringer Mannheim), and reverse- transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad laboratories, USA). SYBR Green PCR Master Mix (Applied Biosystems) was combined with the cDNA template and primers in a 96 well optical reaction plate. Real-time PCR was performed and the following primers were used for AOAH mRNA amplification: 5-'GCC ATG ATT GGA AAC GAT GTT-3 and 5'-GTT GGC GTA CAT TTG TTC AGG A-3'. Transgene mRNA was quantitated by amplifying the FLAG region using the primers 5'-ACC AAG GAG GAC ACG ATT ACA AG-3' and 5'-CCG CCA GTG TGA TGG ATA TCT-3.' The PMF612, pGTeasy18srRNA, and pcDNA3-CD68IVS1mAOAH plasmids containing known amounts AOAH, 18s rRNA and FLAG (transgene derived AOAH) cDNA respectively were used to produce dose-response curves. The reaction conditions used

were 50^{o}C for 2min; 95^{o}C for 10min; and 95^{o}C for 15sec, 60^{o}C for 1min for 40 cycles. All sample and standard reactions were done in duplicate.

9. Preparation of *E.coli* O14 bacteria

E.coli O14 bacteria were grown in LB media till mid-log phase (approximately 4 hours). The bacteria were washed and resuspended in PBS to a concentration of $2.5x10^{8}$ colony forming units (cfu). This is equal to an absorbance of 0.8 at 600nm.

10. *In vivo* LPS Challenge

To evaluate the impact of AOAH on LPS-induced cytokine release, wildtype and transgenic mice were injected with 5μg of *E.coli* 014 i.p. They were bled 1 and 4 hours after challenge and serum levels of TNF-α and IL-6 were measured using ELISA (BD Biosciences). To assess clinical responses to LPS, we injected mice with 10 mg/kg *E.coli* O14 LPS i.p. They were weighed daily and observed for signs of clinical distress by an observer who had no knowledge of the treatment groups. A clinical score was assigned according to the following criteria: *Appearance*:- 0, normal, 1, lack of grooming, 2 , coat rough, possible nasal or ocular discharge, 3,coat very rough, abnormal posture, eyes sunken and glazed; *Clinical signs*:- 0, normal, 1, diarrhea, 2, respiratory rate and or depth altered, 3, cyanotic extremities, labored breathing; *Unprovoked behavior*:- 0, normal, 1, minor changes, 2, abnormal behavior, less mobile, less alert, inactive when activity expected, 3, paralysis, inability to remain upright, shivering, convulsion. Scores for each of the three categories were summed so that the maximum score per mouse each day was 9.

11. Estimation of hepatomegaly

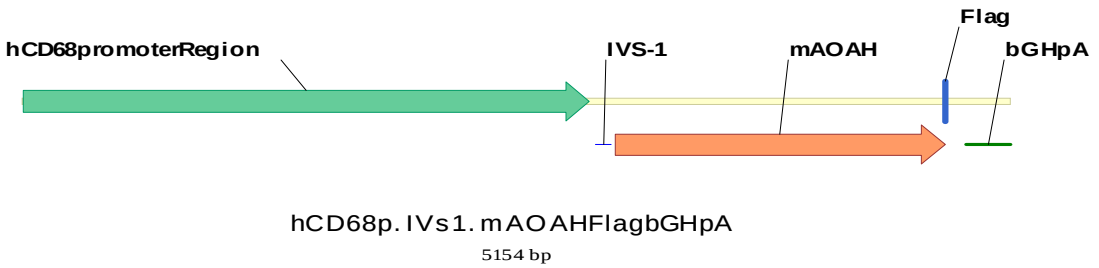
Sex and age matched wildtype and CD68p-AOAH Tg mice were challenged with 30μg of *E.coli* 014 LPS or bacteria (1.5-3 x 10^8 cfu) i.v. or i.p. Mice were euthanized 7-9 days after challenge and their livers were harvested and weighed. The liver weight/body weight fraction (%) was calculated.

C. RESULTS

1. Generation of CD68p-AOAH transgenic mice and detection of AOAH activity in serum.

In order to generate transgenic mice that overexpress AOAH in macrophages, FLAG- tagged murine AOAH cDNA sequence was inserted into an expression vector downstream of human CD68 promoter sequences, the 83bp first intron of the CD68 gene (IVS-1), and a polyadenylation signal (Fig. 14A). The IVS-1 sequence has been shown by others to act as a macrophage specific enhancer when cloned downstream of human CD68 promoter sequence. The transgene construct was injected into male pronuclei of fertilized B6 oocytes and inserted into pseudopregnant foster mothers by the UT Southwestern transgenic core facility. The two transgenic founder lines (7802 and 7803) that were obtained appeared normal and produced viable offspring. AOAH activity was detected in serum obtained from CD68p-AOAH transgenic mice and was substantially higher than that of their B6 wildtype counterparts (Fig. 14B). Serum AOAH activity was subsequently used as a screening method for high expressing transgenic mice.

A



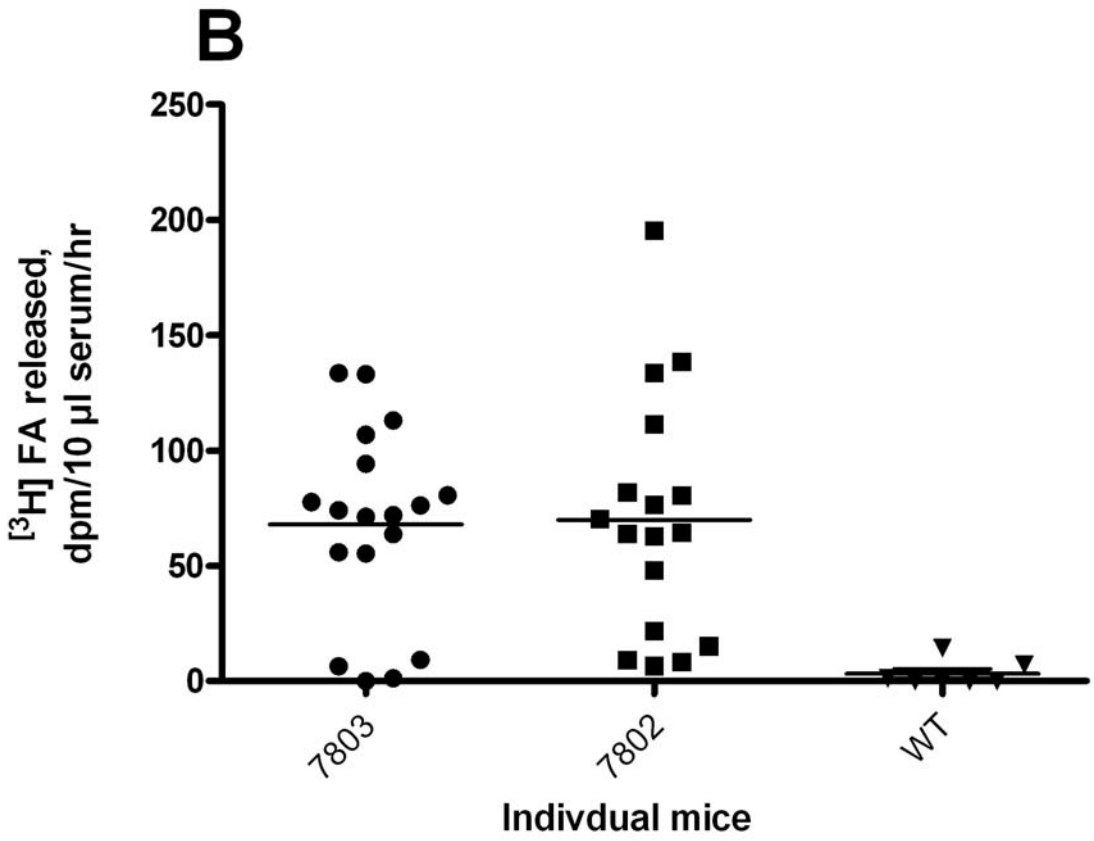


Figure 14: Constitutive AOAH expression in serum of CD68p-AOAH transgenic mice

(A) Schematic of transgene showing human CD68 promoter and 83bp of the first intron of the CD68 gene (IVS-1) driving expression of FLAG-tagged murine AOAH cDNA. (B) 10µl of serum obtained from mice were tested for AOAH activity as described in *Methods.*

2. CD68p-AOAH transgenic mice have constitutive high-level expression of AOAH in macrophages and dendritic cells

Transgene expression was assessed at the mRNA level by quantitative real-time PCR. AOAH mRNA expression in thioglycollate elicited peritoneal macrophages, bone marrow derived macrophages and dendritic cells was compared in wildtype and CD68p-AOAH transgenic mice. Dendritic cells and peritoneal macrophages from transgenic mice had approximately 16 fold and 25 fold higher expression of AOAH than did wildtype mice respectively, while bone marrow macrophages had 37 fold higher expression (fig 15A). This indicated that the majority of AOAH measured in transgenic mice was due to transgene expression. AOAH enzymatic activity in the cell lysates was also measured and found to be elevated in cells from transgenic mice (fig 15B). These results led to the conclusion that the transgene drives high-level constitutive expression of AOAH in macrophages and dendritic cells.

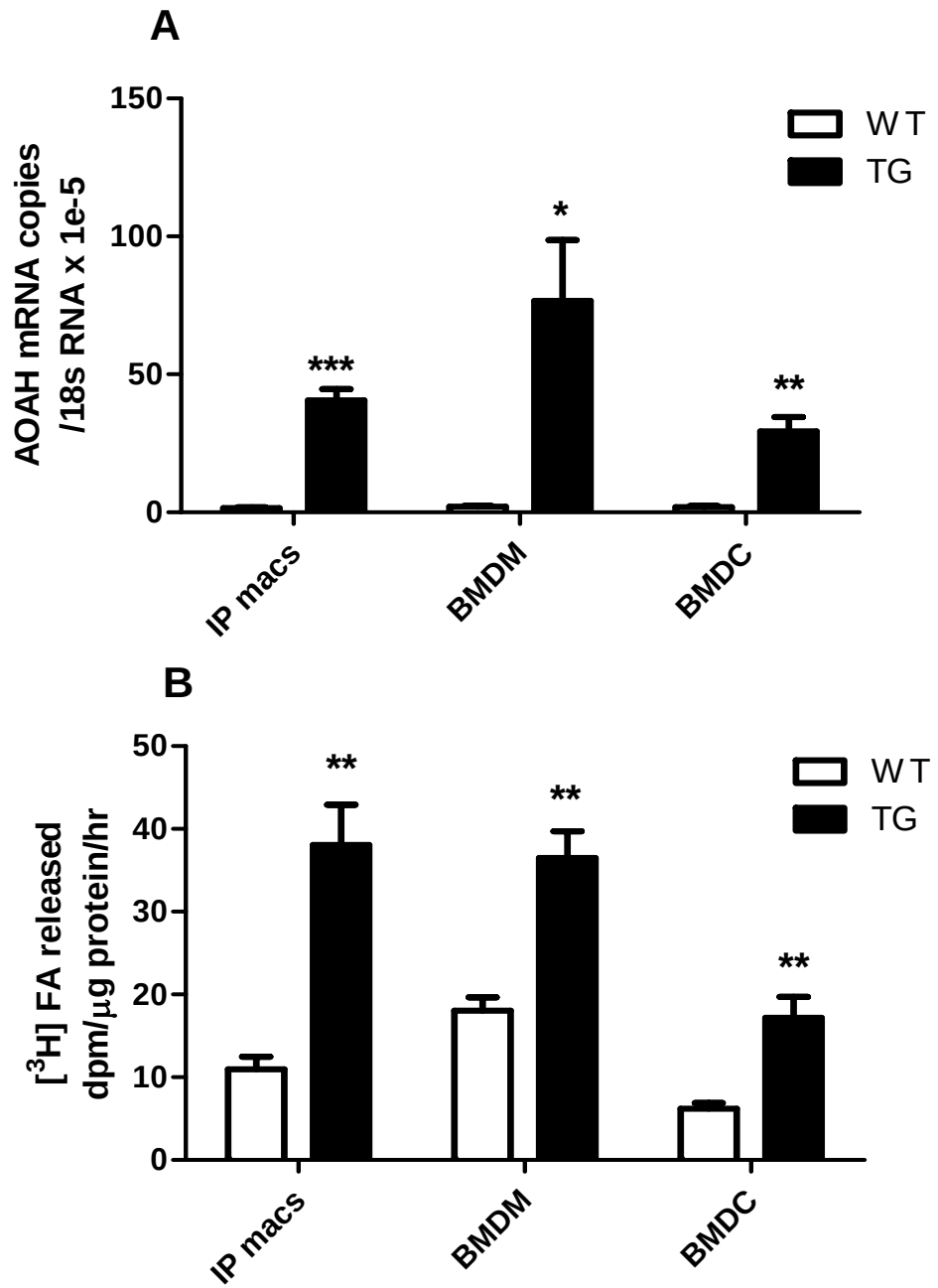


Figure 15: CD68p-AOAH transgenic mice have increased AOAH expression in macrophages and dendritic cells

RNA was extracted from peritoneal macrophages (IP macs), bone marrow derived macrophages (BMDM) and dendritic cells (BMDCs). AOAH mRNA was assessed by

real time PCR. (B) AOAH activity in the cells was estimated by incubating cell lysates with radiolabeled LPS and quantifying release of ^{3}H fatty acids.

3. Elevated AOAH expression in macrophage-rich tissues of CD68p-AOAH transgenic mice

The macrophage/dendritic cell-specific expression of the transgene led us to predict that phagocyte rich tissues would have elevated AOAH expression. AOAH mRNA was measured in liver, spleen lung and kidney. Tissues from transgenic mice expressed AOAH abundantly with highest expression observed in the spleen and lowest in the liver (Fig. 16A). Transgene specific FLAG mRNA expression was also assessed in these tissues. As expected, FLAG expression was absent in wildtype tissues (Fig. 16B). In transgenic mice, it was highly expressed in the spleen and to a lower extent, the liver. The transgene expression pattern was comparable to macrosialin expression observed by others and is consistent with macrophage-specific targeting.

AOAH is produced abundantly by renal cortical epithelial cells in wildtype mice (87). Accordingly, we found high-level AOAH mRNA expression in kidneys of both wildtype and transgenic mice (Fig 16A). FLAG mRNA was minimally expressed or absent in the kidneys of transgenic mice confirming that transgene expression occurs primarily in tissues rich in macrophages and dendritic cells.

Furthermore tissue AOAH enzymatic activity was compared in wildtype and transgenic mice and a substantial increase was found in the liver, spleen and lung of transgenic mice (Fig 16C). The liver had the highest AOAH activity while the lung had the lowest.

We also observed a strong correlation between liver and plasma AOAH activity in transgenic mice (Fig 16D).

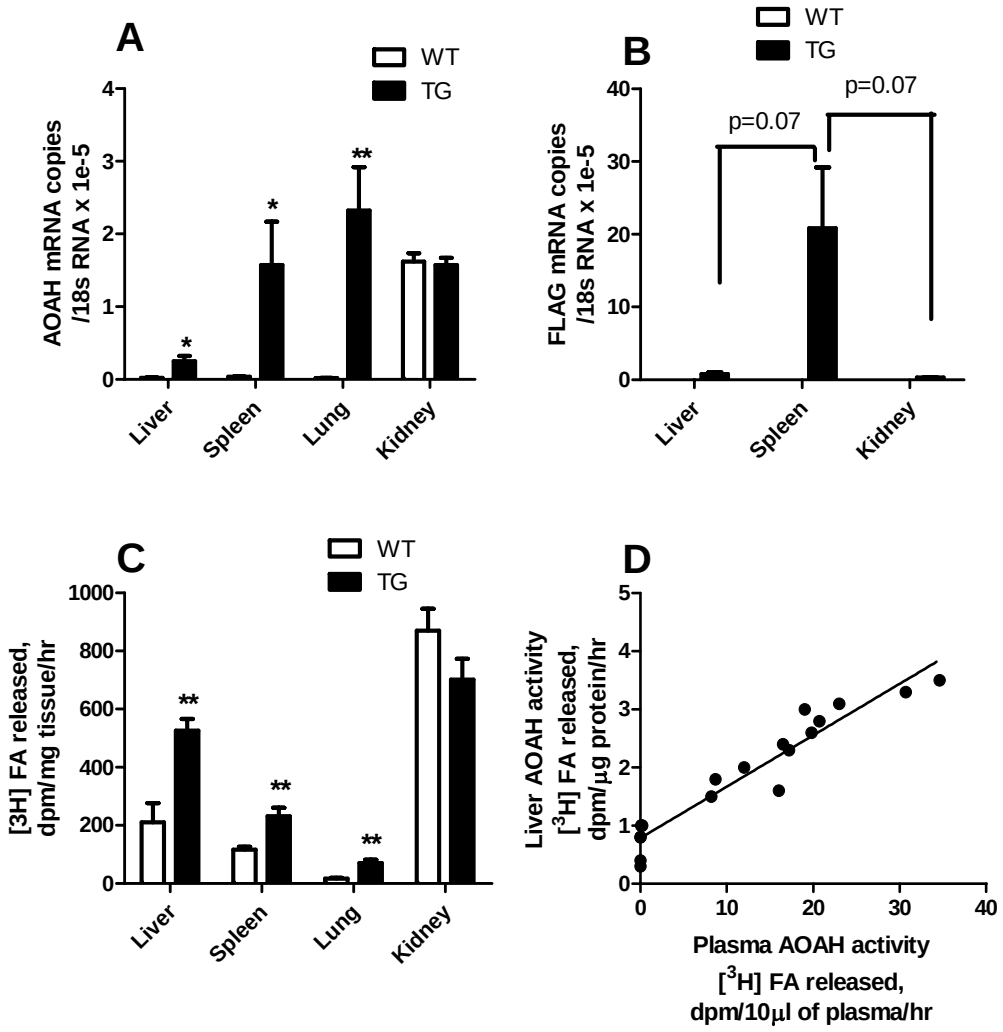


Figure 16: Increased AOAH mRNA expression and activity in macrophage-rich tissues

Total RNA was isolated from liver, spleen, lung and kidney. Expression of (A) AOAH mRNA and (B) transgene-specific FLAG mRNA was assessed by real time PCR. (C) Tissue AOAH activity was estimated by incubating tissue lysates with radiolabeled LPS substrate and measuring ethanol soluble ^{3}H fatty acids released. (D) Positive correlation between liver and plasma AOAH activity.

4. AOAH overexpression allows more rapid *in vitro* LPS deacylation

The rate of LPS deacylation by explanted macrophages was measured by incubating thioglycollate-elicited peritoneal macrophages with radiolabeled LPS for 6 or 12 hours. At the end of the incubation period the media were harvested and cells were washed twice and resuspended in lysis buffer. Whereas both transgenic and wildtype macrophages had comparable amounts of cell associated LPS (Fig. 17C), transgenic macrophages deacylated 6% of the LPS while wildtype macrophages deacylated only 2% at the end of 6 hours (Fig. 17A). Similarly, after incubation with LPS for 12 hours, transgenic macrophages deacylated twice as much LPS as did wildtype macrophages (Fig. 17B).

5. *In vivo* LPS deacylation occurs more rapidly in CD68p-AOAH transgenic mice

To determine if overproduction of AOAH also allows for a higher rate of LPS deacylation *in vivo*, we intravenously injected mice with 5 μg of radiolabeled LPS and harvested their livers 4 hours later. Deacylation of the LPS, which was rapidly taken up by the liver, was measured as described previously (141). By 4 hours, CD68p-AOAH transgenic mice had released approximately 16% of ^{3}H radioactivity from liver-associated LPS while the wildtypes had only released an average of 9% (Fig. 18). The fatty acid composition of the LPS recovered from the livers were analyzed. Non-hydroxylated fatty acids (laurate and myristate) were less abundant in LPS isolated from transgenic mice confirming more rapid deacylation with increased AOAH expression.

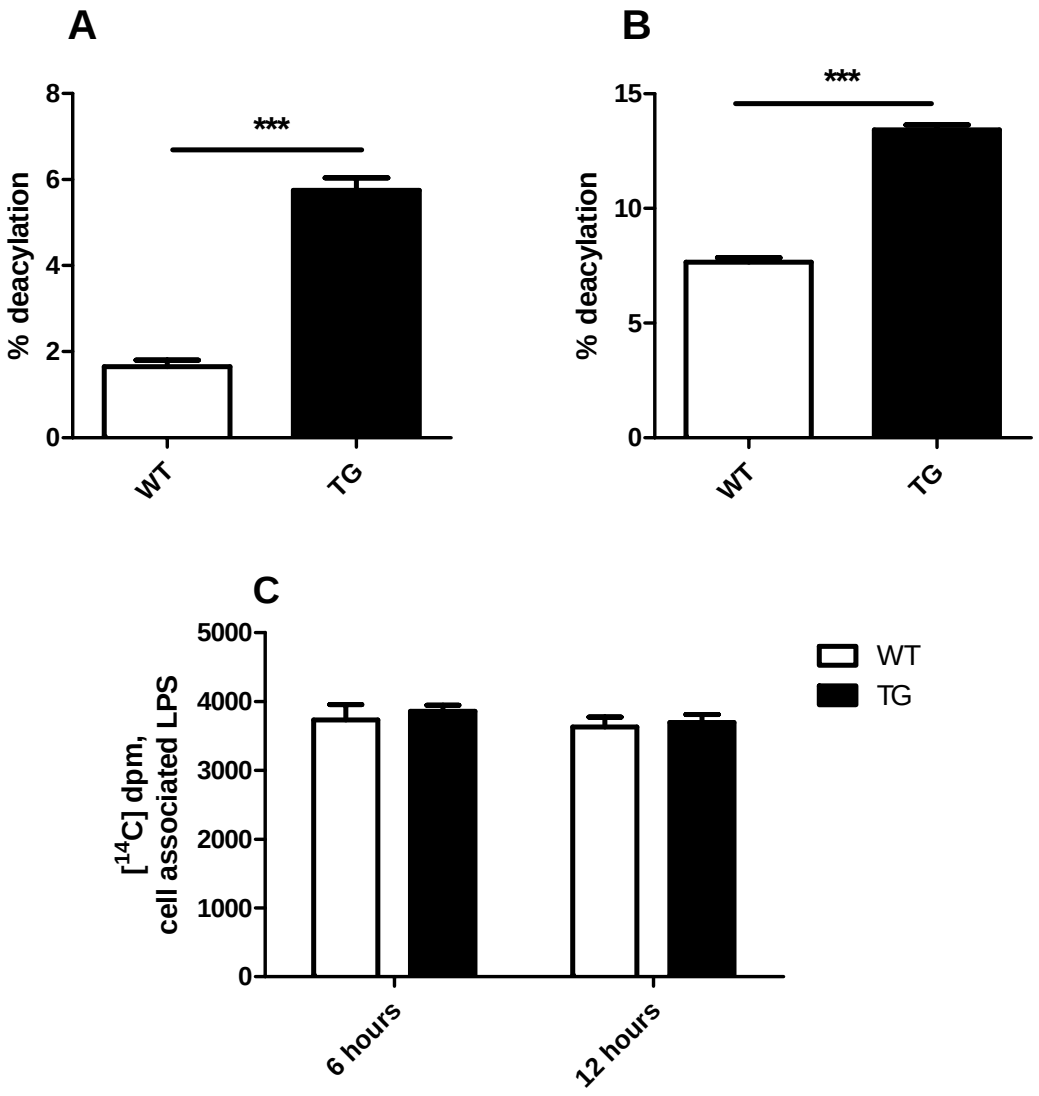


Figure 17: Rapid *in vitro* deacylation in transgenic macrophages

Thioglycollate elicited peritoneal macrophages were incubated with radiolabled LPS for either (A) 6 hours or (B) 12 hours. Deacylation was determined by calculating the ratio of ethanol soluble ^{3}H fatty acids to ^{3}H dpm associated with cells and supernatant. Maximum deacylation is 33% (release of two of six acyl chains). (C) Cell-associated LPS was estimated by measuring the ^{14}C counts in cell lysates.

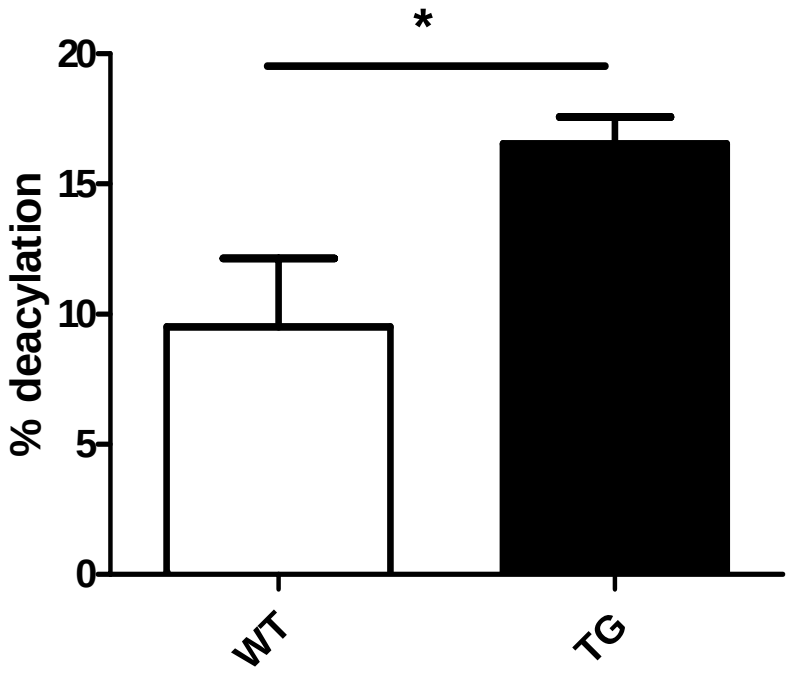


Figure 18: CD68p-AOAH transgenic mice deacylate LPS more rapidly *in vivo*
Mice were intravenously injected with 5μg of radiolabled LPS and their livers were harvested 4 hours after challenge. LPS deacylation in liver was determined by using the formula: [1-(final ratio)/(starting ratio)] X 100 where "starting ratio" is the ^{3}H dpm to ^{14}C dpm ratio in the LPS and "final ratio" is the ^{3}H dpm to ^{14}C dm in the tissue lysate.

6. Increased AOAH expression does not interfere with acute inflammatory responses to LPS.

The AOAH mediated conversion of fully acylated LPS to deacylated LPS takes place slowly, over a period of many hours (61). Therefore AOAH expression in mice is not likely to alter the rapid inflammatory response normally observed within minutes of exposure to LPS. Accordingly, transgenic mice overexpressing AOAH expressed

plasma levels of IL-6 and TNF-α that were similar to those of wildtype mice in response to an intraperitoneal dose of 10 μg of O14 *E.coli* LPS (Fig. 19 A & B).

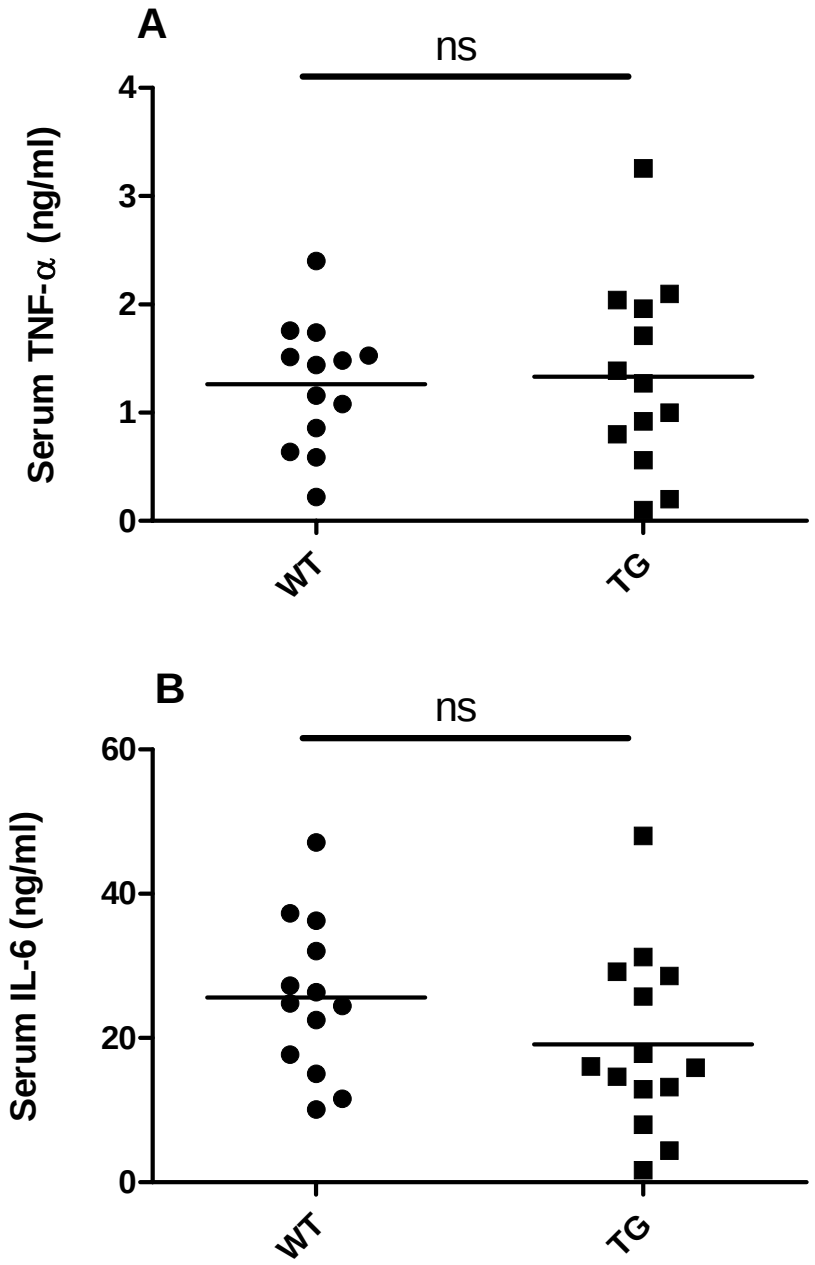


Figure 19: Increased AOAH expression does not interfere with early inflammatory responses to LPS.

10 μg of O14 *E.coli* LPS were injected into mice through the peritoneal route. Blood was collected (A) 1hour later to measure serum TNF-α and (B) 4 hours later to measure serum IL-6 by ELISA.

7. Increase in AOAH expression shortens recovery time from LPS challenge

In contrast with acute responses, AOAH has a protective effect from chronic and long term responses to LPS (35, 61, 146). We hypothesized that transgenic mice expressing large amounts of AOAH would recover more quickly from LPS challenge. Wildtype and CD68p-AOAH transgenic mice were intraperitoneally injected with 10mg/kg O14 LPS. They were weighed daily and a quantitative assessment of pain or distress (eg. coat appearance, ocular discharge, diarrhea and unprovoked behavior) was made by an observer who had no knowledge of the treatment groups. Both transgenic and wildtype mice showed significant weight loss one day after injection (Fig. 20A). However the transgenic mice began to regain weight more rapidly and reached their original weight faster than wild type mice. Though clinical score was higher in wildtype mice, it did not reach statistical significance (20B).

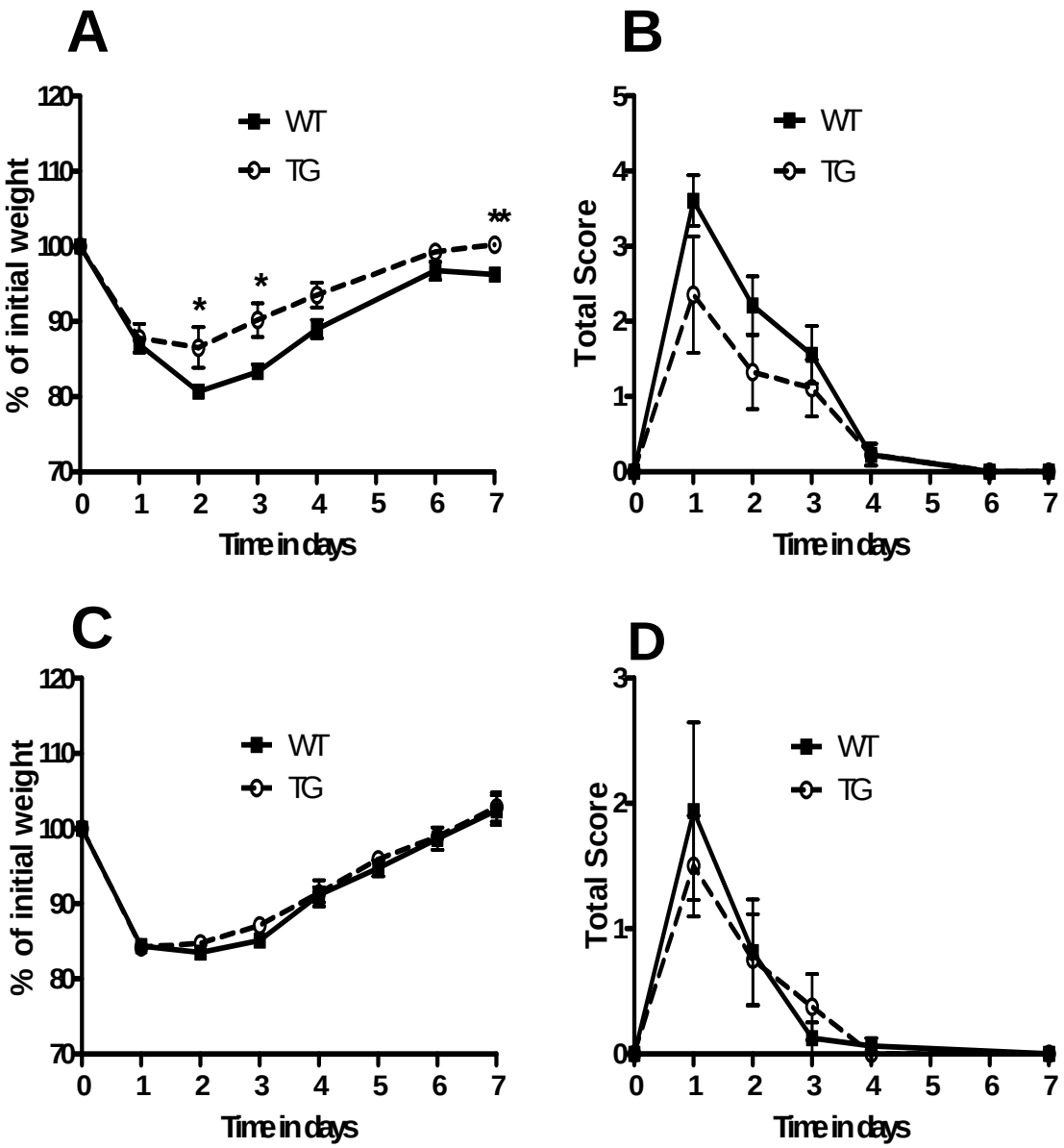


Figure 20: CD68p-AOAH transgenic mice recover more rapidly from LPS challenge

(A&B) *E.coli* O14 LPS (10mg/kg body weight) or 1mg/kg UT12 antibody (C&D) was i.p. injected into wildtype and transgenic mice. Weights (A&C) and clinical scores (B&D) were monitored daily for 7 days.

8. AOAH overexpression does not protect against MD-2—TLR4 activation by an agonistic monoclonal antibody

It is possible that the ability of transgenic mice to recover more quickly from LPS challenge is conferred by an activity of the enzyme that is unrelated to its ability to deacylate LPS. To explore this possibility, mice were injected intraperitoneally with 20 μg of UT12, an agonistic monoclonal antibody to the MD-2—TLR4 complex (148). UT12 induces stimulatory signals that are similar to those induced by LPS. The weights of mice and clinical scores were monitored after injection for seven days. Similar changes in weight and clinical scores were observed in wildtype and transgenic mice (Fig 20C &D). Overproduction of AOAH thus enables more rapid weight gain following LPS challenge but has no such effect when the LPS signaling pathway (MD-2—TLR4) is activated by a non-LPS agonist.

9. CD68p-AOAH transgenic mice are less susceptible to LPS induced hepatomegaly

LPS deacylation in the liver is carried out primarily by Kupffer cells (61) Mice deficient in AOAH were unable to deacylate LPS and developed long-lasting hepatomegaly after a single i.v. dose of 5 μg *E.coli* O14 LPS (0.25 mg/kg body weight) (61). However, much higher doses of LPS could induce hepatomegaly in wildtype mice, indicating that the increase in liver size is a dose-dependent response to LPS. We hypothesized that an increase in hepatic AOAH would prevent or reduce the development of hepatomegaly when LPS was administered intravenously. Transgenic and wildtype mice were challenged with PBS or 30 μg (1.5mg/kg) of *E. coli* O14 LPS

and their livers were weighed 7 days after injection. Livers from wildtype mice were approximately 15% larger than those from transgenic mice (Fig. 21A), and there was a negative correlation between hepatic AOAH activity and liver size in individual mice (Fig. 21B).

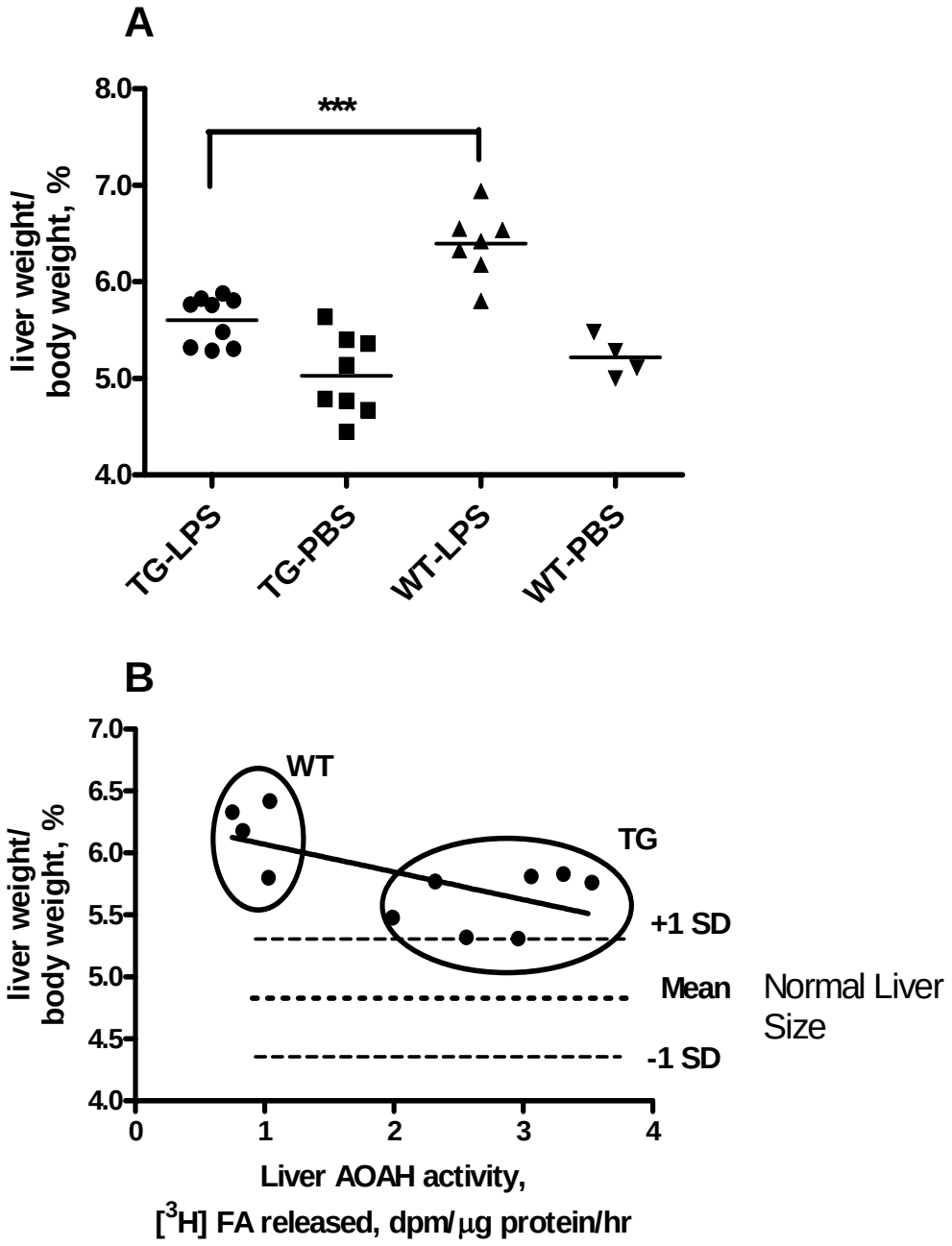


Figure 21: Mice overexpressing AOAH are less susceptible to LPS-induced hepatomegaly

Wildtype and transgenic mice were intravenously injected with either PBS or 30μg of LPS. (A) Livers were harvested 7 days later and weighed. The liver weight/body weight fraction (%) is plotted. (B) Correlation between liver weight/body weight % and hepatic AOAH activity in transgenic and wildtype mice after LPS challenge. The horizontal lines indicate the mean +/-1 S.D. for normal mice. R^2=0.4, $p<0.05$

10. CD68p-AOAH transgenic mice are less susceptible to hepatosplenomegaly induced by Gram negative bacteria

Given that animals are usually exposed to LPS in its natural setting, the Gram-negative bacterial cell wall, we wanted to find out if AOAH-overexpressing mice would be protected from a Gram-negative bacterial challenge. We intraperitoneally injected mice with an avirulent strain of *E.coli* (1.5-3 X 10^8 colony forming units) and weighed their livers and spleens 9 days later. Spleens from wildtype mice were approximately 70% larger than spleens from their transgenic counterparts (Fig. 22A). The livers from wildtype mice also tended to be larger than those from transgenic mice (p=0.055, Fig 22B).

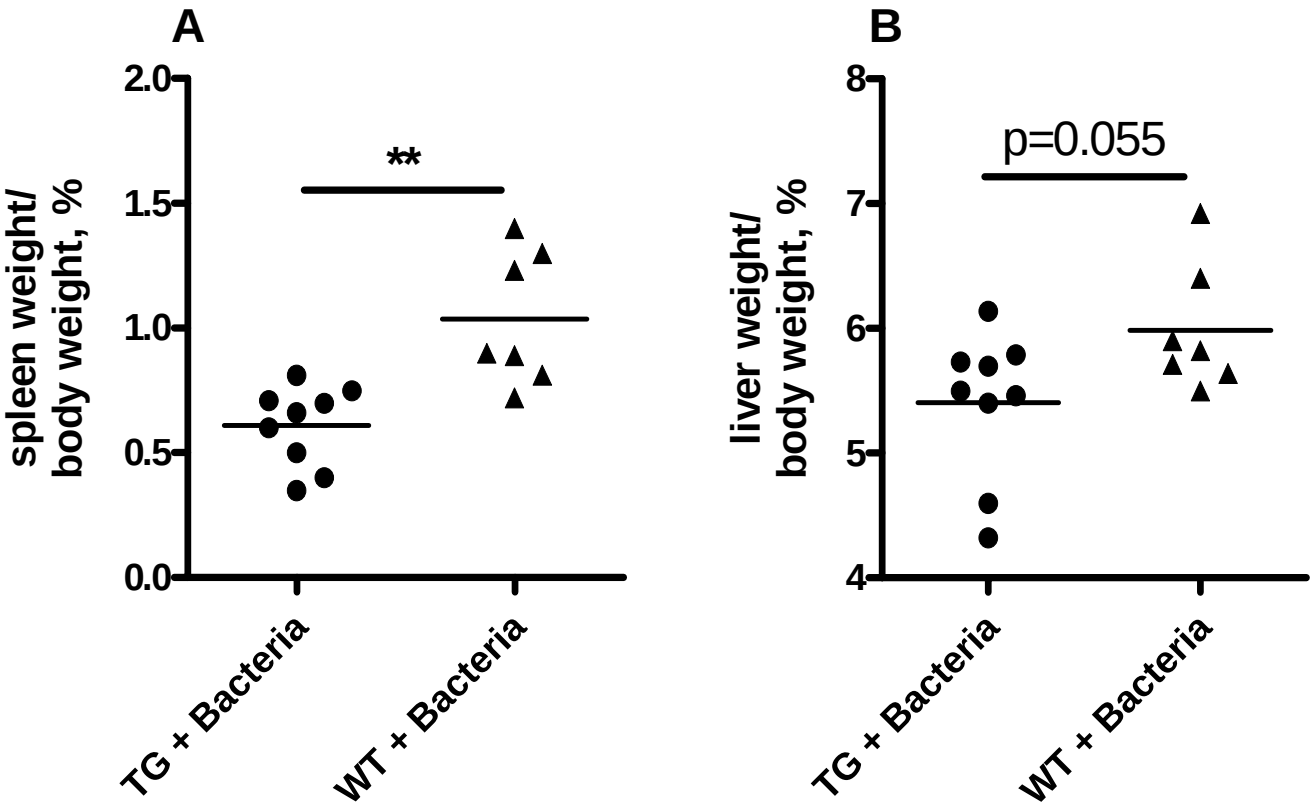


Figure 22: AOAH overexpression renders mice less susceptible to hepatosplenomegaly induced by Gram negative bacteria.

Mice were challenged with 1.5-3 x 10^8 cfu of *E.coli* O14 bacteria through the peritoneal route. Spleens (A) and livers (B) were harvested and weighed 9 days after challenge. ***p<0.005

D. DISCUSSION

Animals have developed mechanisms to protect them against potential microbial invaders. Most rely on germ-line encoded receptors known as pattern recognition receptors (PRRs) to recognize particular microbial molecules (96). The molecules or patterns recognized by PRRs are found on a variety of microbes and are key targets for detection of invading organisms by the innate immune system. Although they are termed pathogen-associated molecular patterns (PAMPs), these structures are not limited to pathogens. They are also found on commensals (normal flora) and microbes that occupy habitats besides the host. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins are two classes of PRRs in mammals. Both recognize different but specific motifs on microbes. For example MD-2—TLR4 best recognizes LPS molecules that are hexaacylated, Nod1 and Nod 2 recognize distinct motifs within peptidoglycans, TLR2/TLR1 heteromers recognize triacylated lipopeptides and TLR2/TLR6 heteromers recognize diacylated lipopeptides (81, 149, 150).

LPS is a major structure sensed by host defense cells during interaction with Gram-negative bacteria and it potently activates the innate immune system (81). Interestingly, vertebrates have a highly conserved enzyme that selectively removes the secondary acyl chains on lipid A required for full recognition by host cells (73). Much of what is known about the host defense role of AOAH stems from studies using mice deficient in AOAH (*Aoah* $^{-/-}$). Shao et al demonstrated that AOAH-producing Kupffer cells in the liver deacylate LPS. *Aoah* $^{-/-}$ mice failed to deacylate LPS and developed hepatomegaly that persisted for at least 3 weeks after a single intravenous dose (61). Furthermore administration of an AOAH-producing adenoviral vector to *Aoah*$^{-/-}$ mice

restored LPS deacylation and prevented LPS induced hepatomegaly. Since intravenously-adenovirus vectors largely produce their cargo transgenes in hepatocytes, we wanted to determine if increasing expression of AOAH in cells that normally make it would allow wildtype mice to resist LPS challenge (151). To this end, we utilized the CD68p-IVS-1 expression cassette which drives the expression of other transgenes in macrophages *in vivo* (147, 152, 153). The resulting CD68p-AOAH transgenic mice had high expression of AOAH in macrophages and dendritic cells and secreted the enzyme into the blood (Fig. 14 & 15). There was also abundant AOAH expression in macrophage-rich tissues (liver spleen and lung) (Fig. 16A & C). We observed an unexpected discrepancy between AOAH mRNA expression and activity. In comparison to the spleen and lung, the liver had the highest AOAH enzymatic activity however its AOAH mRNA abundance was the lowest. Though the reason for this discrepancy was not apparent, it suggests that post-transcriptional factor(s) may be involved in the regulation of transgene derived AOAH. High levels of AOAH activity that were not due to transgene expression were present in the kidneys of both wildtype and transgenic mice confirming that transgene expression occurs primarily in macrophage-rich tissues (Fig. 16C).

Constitutive overexpression of AOAH increased the rate of LPS deacylation both *in vitro* and *in vivo* (Fig. 17& 18). In contrast it did not interfere with the initial pro-inflammatory response to LPS, in keeping with previous observations that AOAH-mediated deacylation/inactivation occurs over many hours and does not diminish acute reactions to LPS *in vivo* (Fig. 19).

Two different test systems demonstrated that elevated constitutive AOAH expression protects mice from LPS challenge. First, after a 10mg/kg LPS challenge, transgenic mice returned to their pre-challenge weights more rapidly than did the wildtype mice (Fig. 20A). Importantly AOAH overexpression did not hasten recovery from weight loss induced by a potent non-LPS MD-2—TLR4 agonist, confirming that the enzyme's ability to deacylate LPS is required for its beneficial effect (Fig. 20C). Second, mice with high constitutive AOAH activity were protected from developing LPS and Gram-negative bacteria induced hepatosplenomegaly (Fig. 21 & 22).

Overexpression of AOAH had been previously achieved by intravenously administering an AOAH-producing adenovirus into wildtype mice (142). Although there was an increase in hepatic deacylation of LPS, the increase was transient and adenoviral infection induced hepatitis and splenomegaly. Here, we have generated healthy mice that constitutively express large amounts of AOAH in macrophages and dendritic cells, and are protected from long term reaction to LPS *in vivo*.

Our findings raise the possibility that overexpressing AOAH in macrophages and/or dendritic cells could hasten recovery from Gram-negative infections in other animals, including humans. In addition, they suggest that genetic variability in AOAH expression might affect recovery from diseases caused by Gram-negative bacteria that produce hexaacyl LPS (50).

CHAPTER FOUR: THE ROLE OF AOAH IN NON-ALCOHOLIC FATTY LIVER DISEASE AND NON-ALCOHOLIC STEATOHEPATITIS

A. INTRODUCTION

There is a high prevalence of non-alcoholic fatty liver disease (NAFLD) in Western countries due to epidemics of obesity and diabetes. NAFLD and non-alcoholic steatohepatitis (NASH) are often associated with an altered lipid profile and metabolic abnormalities collectively known as the metabolic syndrome. Although it is widely accepted that liver steatosis is associated with insulin resistance, the exact mechanism is still unclear. Clinical and experimental studies suggest that NAFLD is simply the hepatic manifestation of the metabolic syndrome (109).

Insulin resistance and obesity are also associated with low grade chronic systemic inflammation. In animal models of obesity, the adipose tissue produces increased amounts of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, all of which disrupt insulin signaling (112). TNF-α contributes to insulin resistance by increasing serine phosphorylation of IRS-1 leading to its inactivation. The resulting insulin resistance promotes excessive lipid storage in the liver and adipose tissue.

Recently, TLR4 has been shown to link the innate immune system and metabolism. Shi et al demonstrated that saturated free fatty acids (palmitate, myristate and oleate), often upregulated in obese patients, activate TLR4 signaling and stimulate cytokine expression in macrophages and adipocytes (154). TLR4 signaling leads to

activation of NF-κB, JNK and SOCS pathways which are all capable of inducing insulin resistance. Mice deficient in TLR4 had a reduction in hepatic steatosis and triglyceride content, and were protected from lipid induced insulin resistance (154-156).

Interestingly, several studies suggest that LPS from the gut microbiota is a source of low grade inflammation in obese animals. Antibiotic treatment of diabetes-prone rats modified their intestinal flora, decreased the incidence and delayed the onset of type I diabetes (157). Moreover, Cani et al showed that high fat feeding altered the composition of bacterial species in the gut and increased plasma endotoxin levels in mice (137). To causally link plasma endotoxemia caused by high-fat diet with metabolic disease, the authors mimicked low grade inflammation by infusing mice with LPS for one month. Chronic infusion of LPS induced liver insulin resistance and increased hepatic triglyceride levels. Mice deficient in CD14 were resistant to the metabolic features induced by the high fat diet and LPS infusion (137). Perhaps the most striking evidence for gut microbiota as a cofactor in metabolic dysfunction comes from studies using germ free mice. Colonization of germ free mice with normal microbiota from conventional mice led to increased liver triglycerides, a 60% increase in body fat content and insulin resistance despite a reduction in food intake (158).

Thus LPS produced in the gut by gram-negative bacteria is translocated into intestinal capillaries and may be transported to target tissues by lipoproteins where it activates the MD-2—TLR4 complex and triggers production of pro-inflammatory cytokines (137, 155, 159). These cytokines are deleterious to insulin signaling and lead to insulin resistance and hepatic fat accumulation.

Based on these studies, we hypothesized that failure to inactivate the increased levels of LPS caused by overnutrition would promote low grade inflammation and hepatic steatosis. AOAH inactivates LPS by selectively removing the secondary fatty acyl chains required for full recognition by vertebrate cells. *Aoah*$^{-/-}$ mice are unable to deacylate LPS and develop prolonged immunosuppression, hepatomegaly and polyclonal antibody production in response to LPS (35, 61, 146). We subjected *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ mice to a high-carbohydrate diet for 7-9 weeks and assessed their livers for fat accumulation. We also explored the role of AOAH in diet induced insulin resistance.

MATERIALS AND METHODS

1. Mice

Mice deficient in AOAH *(Aoah*$^{-/-}$) were produced as previously described (146). The *Aoah*$^{-/-}$ construct was backcrossed eight generations into C57Bl/6 background. Mice deficient in both AOAH and TLR4 were obtained by breeding *Aoah*$^{-/-}$ mice with C57BL/10ScN mice which have a TLR4 deletion (146) All mice used in experiments were male.

2. Mouse diet

Liquid AIN-76A purified diet was purchased from Dyets Inc. in powder form and stored at 4^{o}C. The diet contained 19.3%, protein, 11.9% fat and 68.8% carbohydrate (Table 1). 272.85g of the diet was added to 860ml of cold autoclaved water and blended for 30 seconds. More water was added to make a final volume of 1L. Liquid diet was stored at 4^{o}C for no more than 3 days.

3. Mouse feeding

Mice (2/cage) were fed 40ml of liquid AIN-76A diet in feeding cups daily. The mice were not given access to water or normal chow for the duration of the experiment. The volume of food consumed per cage was recorded daily. The feeding cups were washed every other day before adding liquid diet.

Ingredient	Grams/Liter of Diet
Casein (100 Mesh)	53.0
DL-Methionine	0.8
Maltose Dextrin	177.1
Cellulose	13.25
Corn Oil	13.25
Salt Mix #201200	9.27
Vitamin Mix #300050	2.65
Choline Bitartrate	0.53
Xanthan Gum	3.0

Table 1: Ingredients of AIN-76A diet. (Modified from Dyets Inc. www.dyets.com)

4. Intraperitoneal insulin sensitivity test (IPIST)

After 9 weeks of feeding, mice were fasted overnight. The following day, they were placed individually in fresh cages without food. Their weights were recorded and the volume of insulin working solution required for a 1U/kg dose of insulin was calculated. The AccuChek blood glucose meter was calibrated with the standard strip supplied by the manufacturer. Basal glucose concentration was measured at t= -15min in each mouse by removing the mouse from the cage, placing it on top of its cage and cutting the tip of the tail with a clean pair of scissors. A small blood sample (not the first drop) was applied directly onto the test strip placed in the blood glucose monitor. Pressure was applied to the tail to allow the blood to clot and the mouse was returned to its cage. After

the basal glucose concentrations were taken in all mice, 1U/kg of insulin was intraperitoneally injected (t=0min) into each mouse at 60 second intervals between mice. At t=15min, blood glucose was measured again starting with the first mouse that was injected with insulin. The clot was removed from the tail to restart bleeding. The tail was massaged if blood flow was inadequate. Blood glucose was measured again at t=30, 45 and 60 min and every 30 minutes until 180 minutes after insulin injection. At the end of the experiment the mice were returned to their cages with food. Blood glucose levels were plotted versus time and statistical analysis was done using 2-sided ANOVA.

5. Harvesting mouse tissues

After 7-9 weeks on a high carbohydrate diet, mice were weighed, deeply anesthetized with isofluorane and bled through the retroorbital vein. The whole liver was harvested, weighed and small sections were taken for H&E and Oil-red-O staining. The rest of the liver was snap frozen in liquid nitrogen and stored at -80^{o}C.

6. Oil-red O staining

Livers were harvested from mice and small pieces (0.5-3 mm cubed) were fixed in 4% paraformaldehyde in PBS overnight at 4^{o}C. The livers were transferred to 5ml tubes containing 30% sucrose in PBS and incubated at 4^{o}C overnight or until tissues sank to the bottom of the tube. The tissues were embedded in OCT and the tissue block holder was frozen in isopentane and liquid nitrogen. Tissues were stored at -20^{o}C until ready for use. A stock solution of Oil-red O (0.3% in isopropanol) was prepared and allowed to stand for 2-3 days at room temperature. Working solution of Oil-red O was prepared

freshly by mixing 2 volumes of deionized water (dH_20) with 3 volumes of Oil-red O stock solution. The solution was thoroughly mixed, allowed to stand at room temperature for 10 minutes then filtered through Whatman #7 filter paper. Livers were cryosectioned and fixed in 10% buffered formalin for 10 minutes. They were rinsed in dH_20 three times for 5 minutes and stained in Oil-red O for 10 minutes. The liver slides were counterstained with haematoxylin and quickly placed in dH_20. They were rinsed in cold running tap water for 6 minutes, transferred to dH_20 and then dabbed dry. Beads of water left on the slide were aspirated. The slides were mounted with 32µl of Vectashield and a coverslip, and stored at 4^oC.

7. Hematoxylin and eosin H&E staining

Liver sections were fixed in 10% formalin for 48 hours at room temperature. The livers were transferred to tubes containing PBS and sent to the UT Southwestern Pathology core facility for H&E staining.

8. Measurement of serum lipids and ALT

Mice were bled from the retroorbital vein and blood was allowed to clot at room temperature. The serum was sent to the UT Southwestern mouse metabolic phenotyping core facility for measurement of serum ALT, triglycerides and cholesterol.

9. Enzymatic tissue cholesterol and triglyceride determination

Approximately 100 mg of frozen liver tissue were homogenized in a glass tube with 4ml of chloroform/methanol (2:1 v/v) for 30 seconds. 1ml of 50mM NaCl was added to the

homogenate which was then vortexed and centrifuged at 1500g for 30 minutes. The organic phase was carefully removed from under the cellular trash and placed into a new glass tube. It was washed twice by adding 1ml of 0.36M $CaCl_2$/methanol, vortexed and centrifuged at 1500g for 10 minutes. After the second wash, the organic phase was transferred to a volumetric flask, chloroform was added to a final volume of 5ml and it was incubated overnight at room temperature. To measure triglycerides, 50µl of lipid sample were added to a tube with 10µl of 50% Triton X-100. The samples were vortexed and dried under nitrogen until there was only triton left at the bottom of the tube. 800µl of triglyceride enzymatic reagent (Sigma) were added to the samples which were then vortexed for 4 minutes and incubated at 37^{o}C for 15 minutes. The absorbance of the samples was read on a spectrophotometer at 520nm.

For cholesterol determination 100µl of sample were added to 10µl Triton X-100 and dried under nitrogen. 800µl of cholesterol enzymatic reagent (Roche) were added to the tube and absorbance was read at 500nm.

10. Histological scoring for NASH

H&E-stained sections of livers were graded by a pathologist for steatosis and inflammation based on the following criteria:

Steatosis: 0=None, 1= < 5% of cells, 2 = 5-25% of cells, 3 = 25-50% of cells, 4 = 50-75% of cells, 5 = 75-100% of cells.

Inflammation: 0 = none, 1 = one or less focus per 100X field, 2 = >1 focus/10X field

C. RESULTS

1. *Aoah*$^{-/-}$ mice develop NASH in response to a high-carbohydrate diet

Sex- and age-matched AOAH wildtype and knockout mice were fed liquid AIN-76A diet, which is high in carbohydrate, for 7-9 weeks. The volume of food consumed was monitored daily and the mice were weighed every other day. Both groups of mice gained weight at the same rate and appeared healthy for the duration of the experiment. At the end of the feeding period, their livers were weighed and processed for histology. The body and liver weights were comparable in both groups of mice, yet we observed that livers from knockout mice had a marked increase in neutral lipids as observed by oil-red-o staining (Fig. 23 A & B). Haematoxylin and eosin (H&E) staining confirmed the presence of inflammation as well as patchy but prominent macrovesicular steatosis in the livers of *Aoah* $^{-/-}$ mice (Fig. 24 A-D). Little or steatohepatitis was observed in the livers of *Aoah*$^{+/+}$ mice. The livers were histologically graded for steatosis and inflammation by a pathologist who had no knowledge of the treatment groups. *Aoah*$^{-/-}$ mice had significantly higher liver pathology scores than did the *Aoah*$^{+/+}$ mice (Fig. 25). Ballooning degeneration, a marker for progressive NASH, (126) was also observed in *Aoah*$^{-/-}$ livers. Serum from wildtype and knockout mice tested negative for mouse hepatitis virus confirming that viral infection did not contribute to the observed pathology.

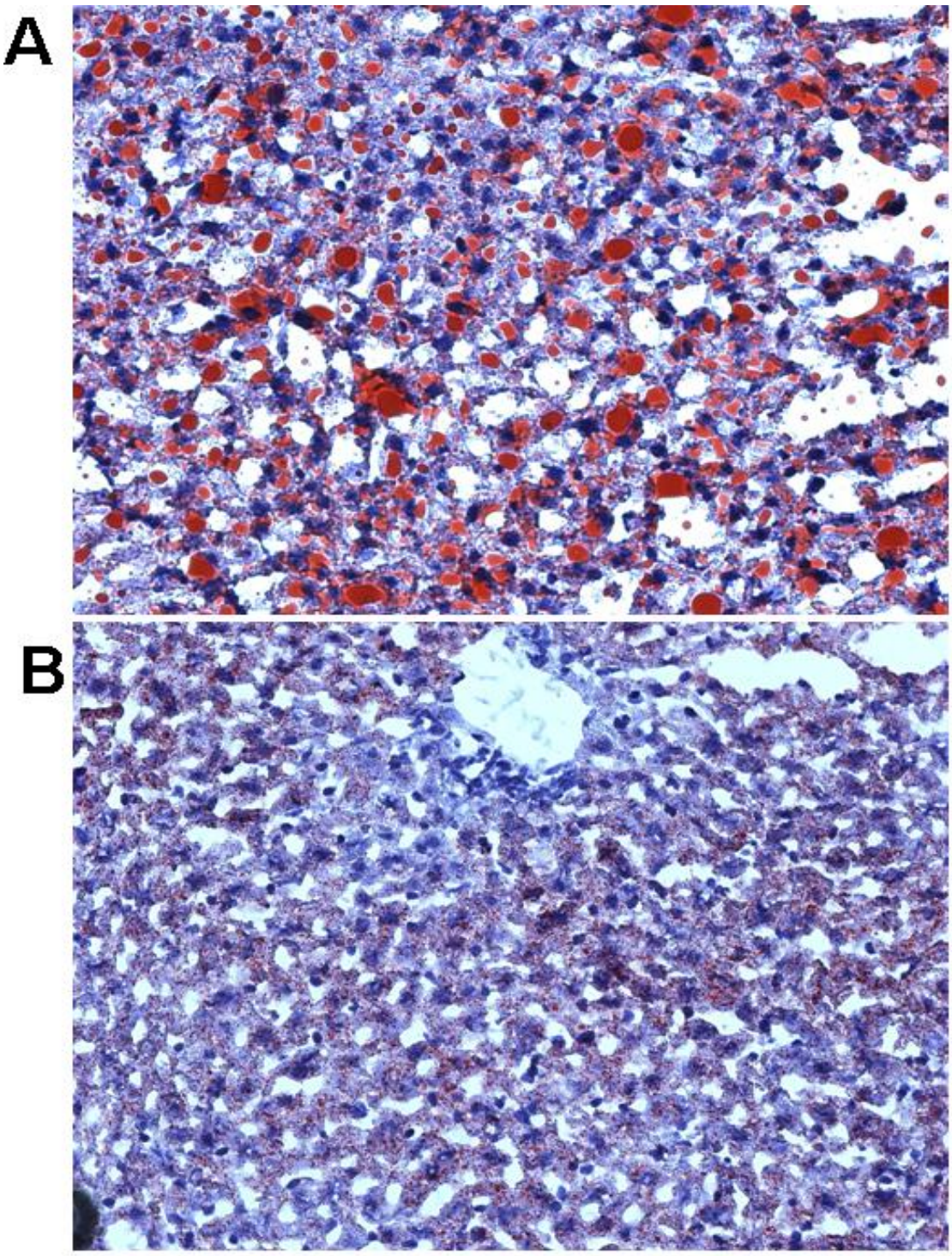


Fig 23: High-carbohydrate diet induces hepatic fat accumulation in *Aoah*$^{-/-}$ mice.
Oil-red O stains of paraformaldehyde fixed liver sections obtained from (A) *Aoah*$^{-/-}$ and (B) *Aoah*$^{+/+}$ mice after high-carbohydrate feeding for 9 weeks

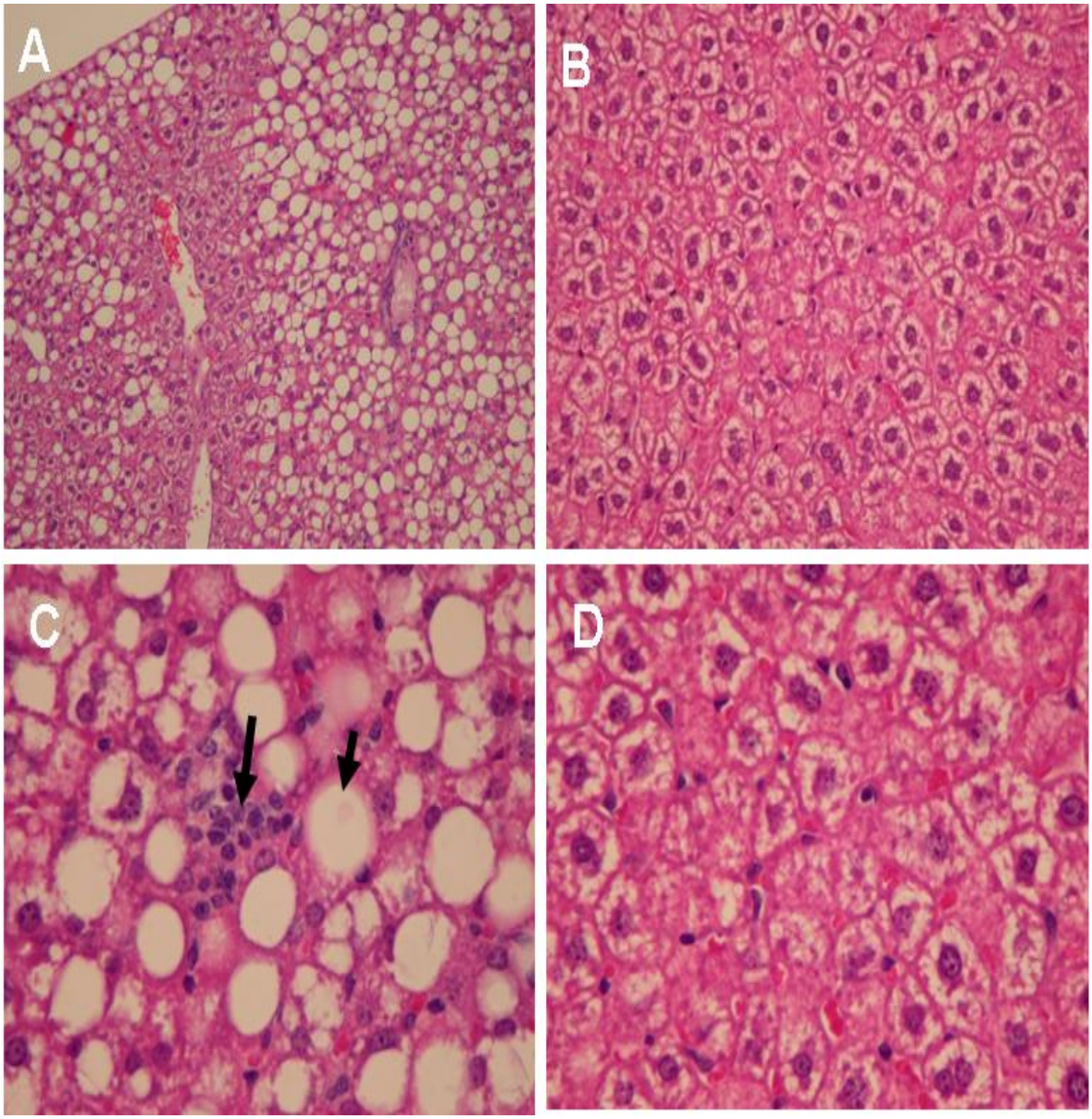


Fig 24: *Aoah*$^{-/-}$ mice develop patchy steatosis and inflammation in response to high-carbohydrate diet

H& E stains of livers from (A & C) *Aoah*$^{-/-}$ and (B &D) *Aoah*$^{+/+}$ mice. Arrows in C point to inflammation and fat droplet (steatosis). Magnification: 100X in A & B, 200X in C & D.

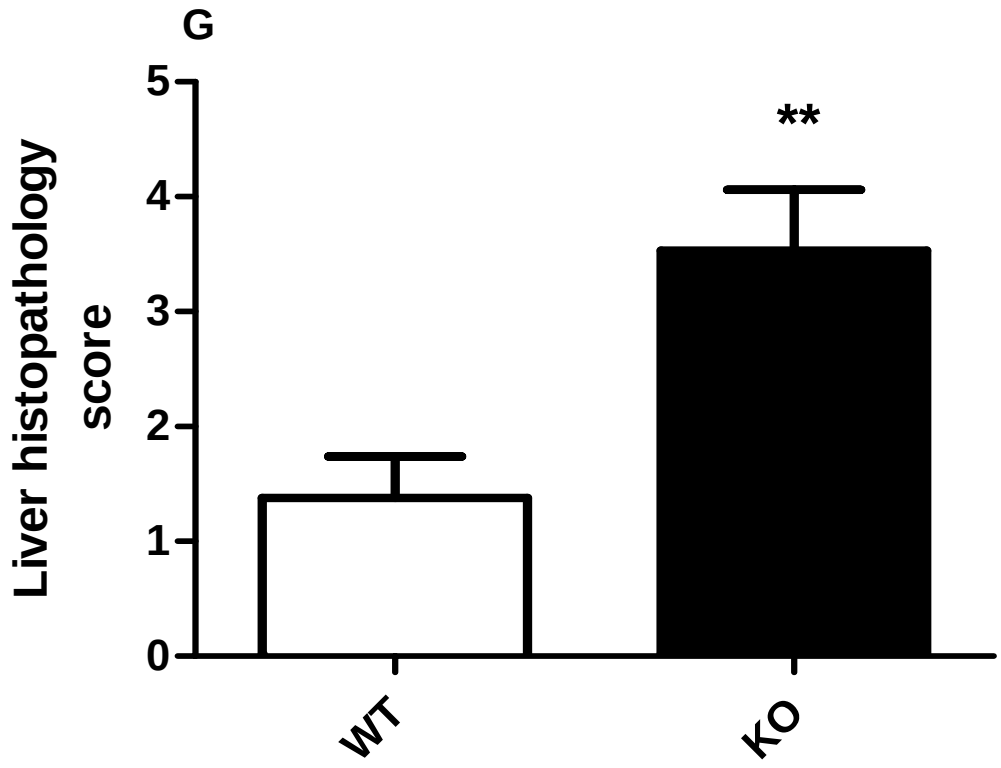


Fig. 25: *Aoah*$^{-/-}$ mice have increased histopathology in response to high-carbohydrate diet

H&E liver sections were scored for steatosis and inflammation as described in *Methods*.

2. Steatohepatitis in *Aoah*$^{-/-}$ mice is not due to triglycerides or cholesterol

We hypothesized that the steatosis observed in the liver histology of *Aoah*$^{-/-}$ mice was due to an increase in the esterification of hepatic free fatty acids to triglycerides, or increased levels of cholesterol. Livers were homogenized and the lipids were extracted with chloroform/methanol. Hepatic triglyceride and cholesterol content were determined as described in *Methods*. Surprisingly, triglyceride and cholesterol levels were similar in the livers of wildtype and knockout mice indicating that the steatosis observed was due to other lipids (Fig. 26A & B). We also observed similar levels of serum ALT between the two groups of mice (Fig 26C).

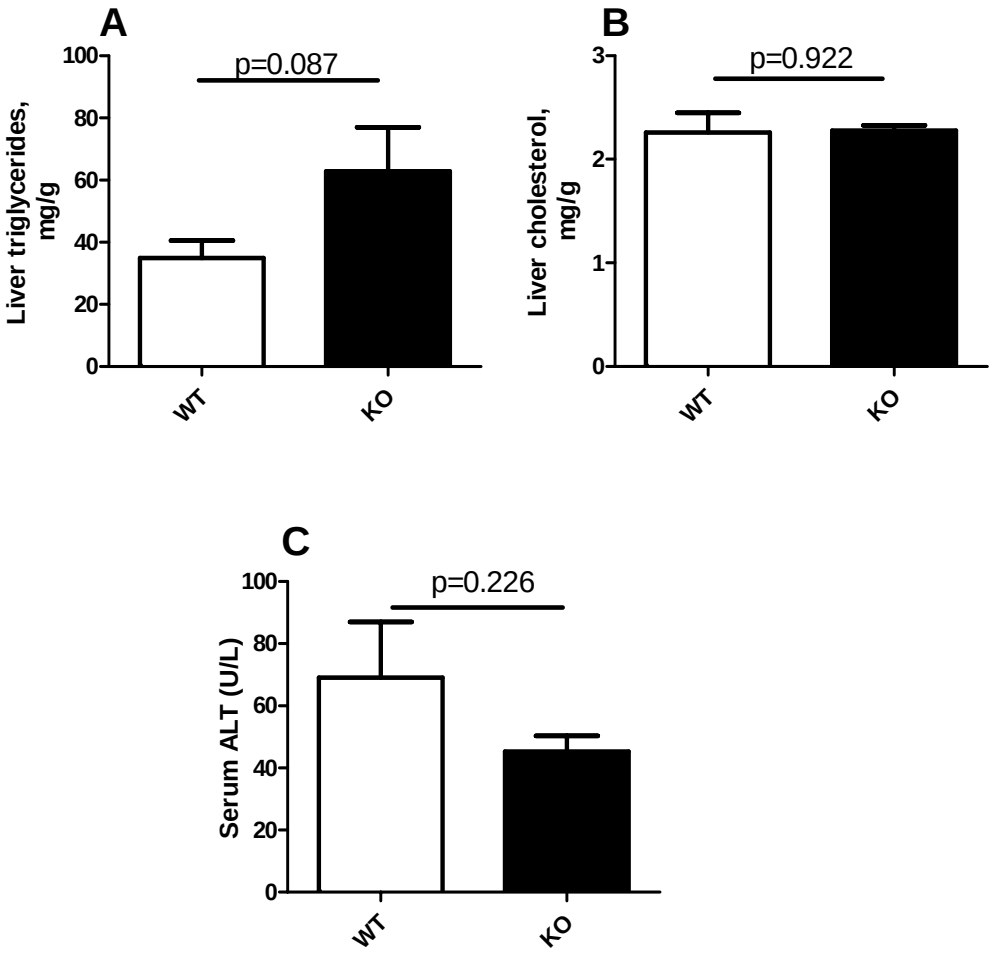


Figure 26: Diet-induced fatty liver in *Aoah* $^{-/-}$ mice is not due to hepatic triglycerides or cholesterol

Hepatic (A) triglyceride and (B) cholesterol content in the *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice. (C) Serum ALT levels.

3. *Aoah*$^{-/-}$ mice develop hyperlipidemia in response to high-carbohydrate diet

NAFLD and NASH are associated with the metabolic syndrome of which hyperlipidemia is a feature. We wanted to determine if the steatohepatitis observed in *Aoah*$^{-/-}$ mice was accompanied by hyperlipidemia. We measured triglyceride and cholesterol content in the serum and observed that *Aoah*$^{-/-}$ mice had significantly higher levels of both than did *Aoah*$^{+/+}$ mice (Fig 27A & B). This result suggests that high-carbohydrate feeding of *Aoah*$^{-/-}$ mice creates a model for NASH that is associated with metabolic abnormalities.

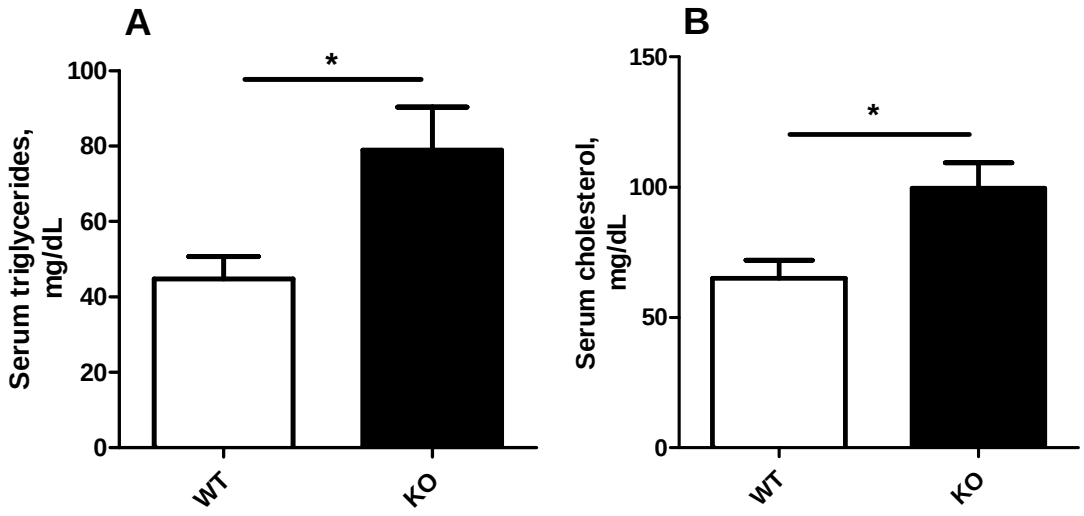


Figure 27: Hypertriglyceridemia and hypercholesterolemia in *Aoah*$^{-/-}$ mice on high-carbohydrate diet

Serum was obtained from AOAH wildtype and knockout mice through the retroorbital vein and tested for (A) triglyceride and (B) cholesterol content.

4. TLR4 deficiency alleviates diet induced steatohepatitis but not hyperlipidemia in *Aoah*$^{-/-}$ mice

The TLR4 receptor has been implicated in the crosstalk between inflammatory and metabolic signals. TLR4 deficient mice were protected from diet induced obesity, insulin resistance and hepatic steatosis (155, 156). We reasoned that steatohepatitis and hyperlipidemia observed in *Aoah*$^{-/-}$ mice would be abated if the mice also lacked TLR4. Mice that were deficient in both AOAH and TLR4 (DK) were fed the high-carbohydrate diet for 9 weeks. Despite similar food intake, DK mice gained weight at a slower rate than *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ mice for the first few weeks of feeding. All three groups of mice had identical weight gain percentage at the end of the feeding period and comparable levels of hepatic triglycerides and cholesterol (Fig. 28). As expected, livers from DK mice had little or no steatohepatitis and their histopathology scores were significantly lower than those from *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$mice (Fig. 29A). In contrast, serum triglyceride and cholesterol levels remained elevated in DK mice (Fig. 30A & B).

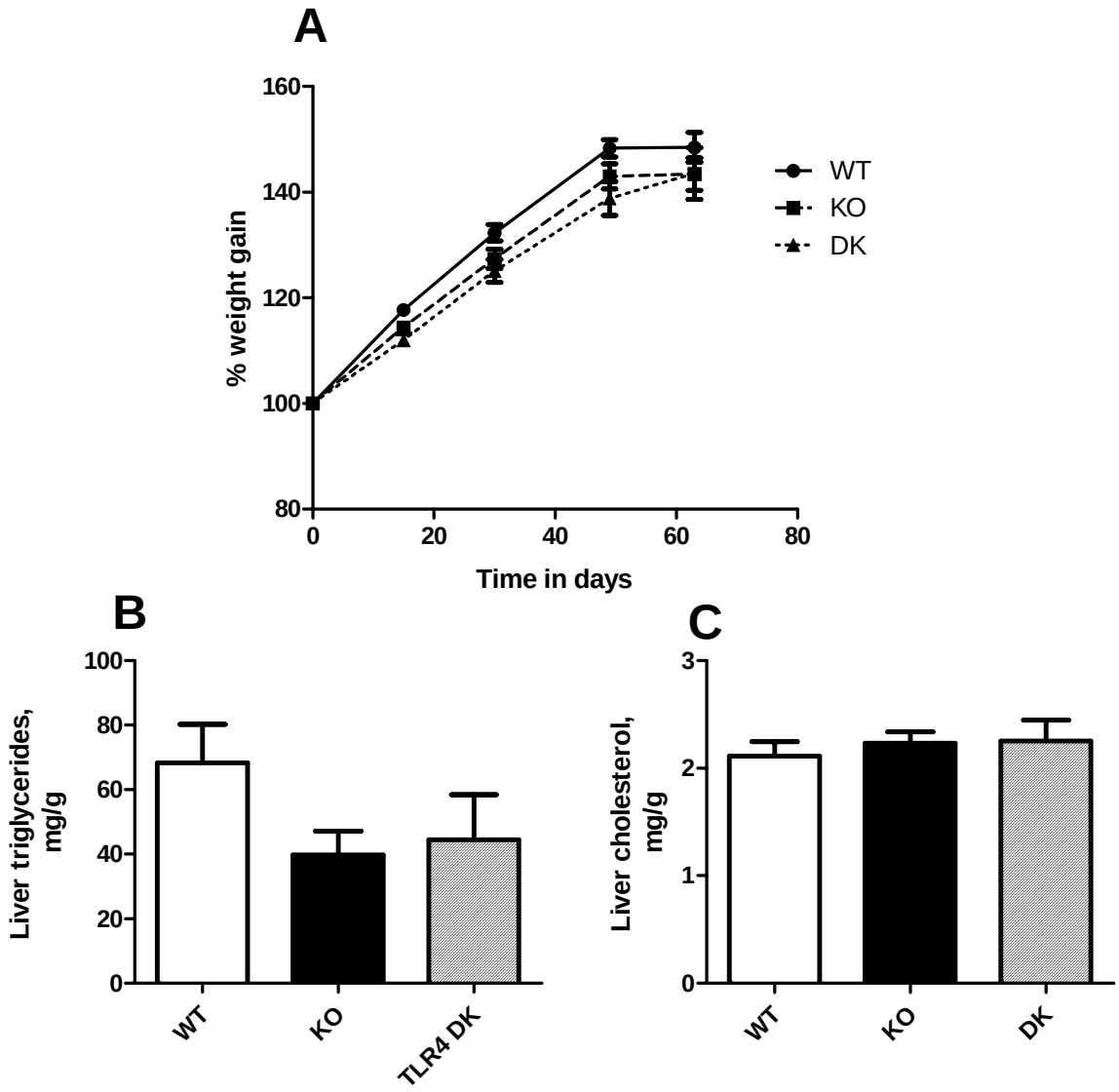


Figure 28: Growth curves and hepatic lipids of *Aoah*$^{-/-}$ (KO), *Aoah*$^{+/+}$ (WT) and *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ (DK) mice on high-carbohydrate diet.

(A) Mice were weighed periodically during 9 weeks of high carbohydrate diet feeding. Percent weight gain is plotted. Livers were harvested after feeding period and triglyceride (B) and cholesterol levels were determined.

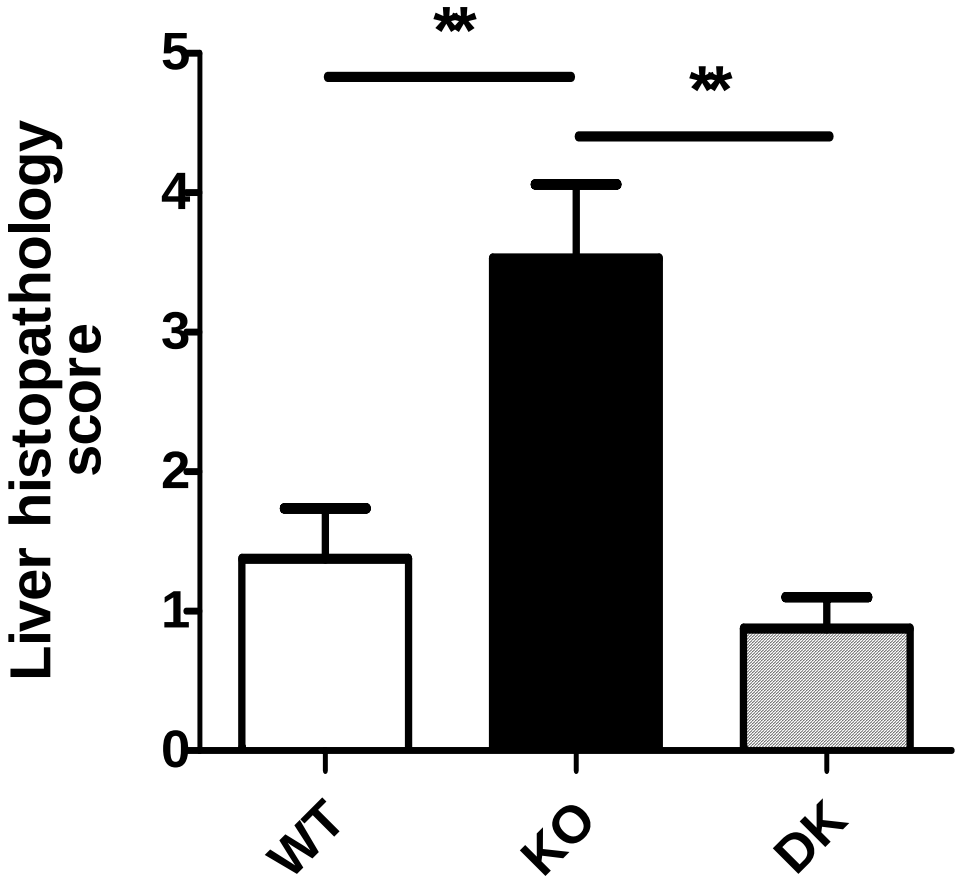


Figure 29: *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ (DK) mice are protected from diet-induced steatohepatitis. Formalin fixed liver sections were H&E stained and assessed for steatosis and inflammation

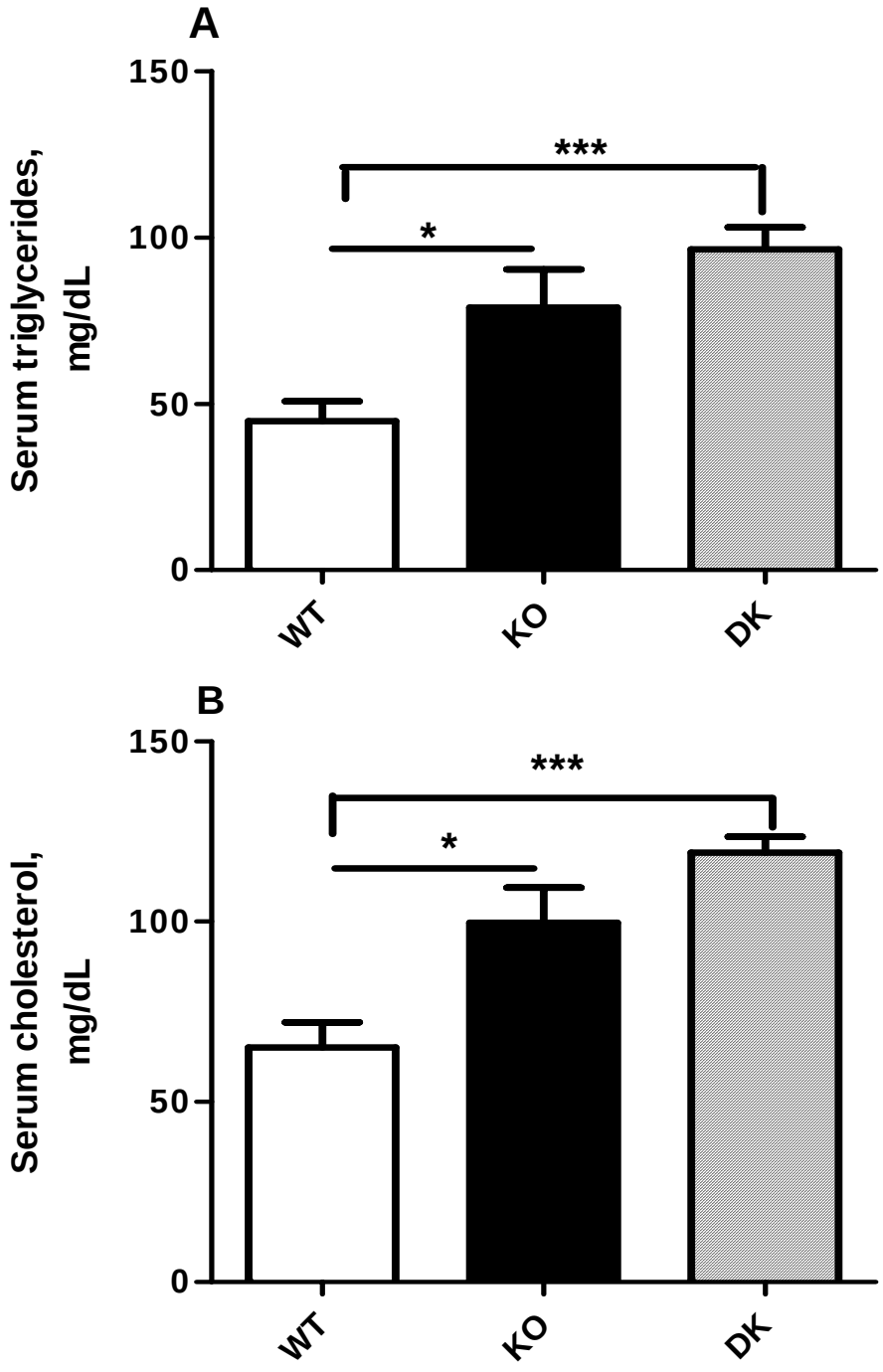


Figure 30: *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ (DK) mice are not protected from hyperlipidemia induced by a high-carbohydrate diet

Aoah$^{+/+}$ (WT), *Aoah*$^{-/-}$ (KO) and DK mice were bled through the retroorbital vein after 9 weeks on a high carbohydrate diet . Serum was obtained to determine (A) triglyceride and (B) cholesterol content.

5. ***Tlr4*$^{-/-}$*Aoah*$^{-/-}$ (DK) mice develop impaired insulin sensitivity in response to high-carbohydrate diet.**

In human patients, insulin resistance is a predictor of NAFLD and NASH (160). We wanted to find out if *Aoah*$^{-/-}$ mice had impaired insulin sensitivity in response to a high –carbohydrate diet and if TLR4 deficiency had a protective effect.

After 9 weeks on a high-carbohydrate diet, mice were fasted overnight and the intraperitoneal insulin sensitivity test (IPIST) was performed (see *Methods*). Surprisingly both *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ mice were more sensitive to the hypoglycemic effects of insulin than were DK mice. 60 minutes after insulin injection, *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice showed a 61% and 49% decrease respectively in blood glucose levels whereas DK mice had only a 35% reduction in glycemia (Fig. 31). The difference in insulin response was significant between WT and DK mice. Thus high-carbohydrate diet caused a slight decrease in insulin sensitivity in *Aoah*$^{-/-}$ mice which was more pronounced in mice that were deficient in both AOAH and TLR4.

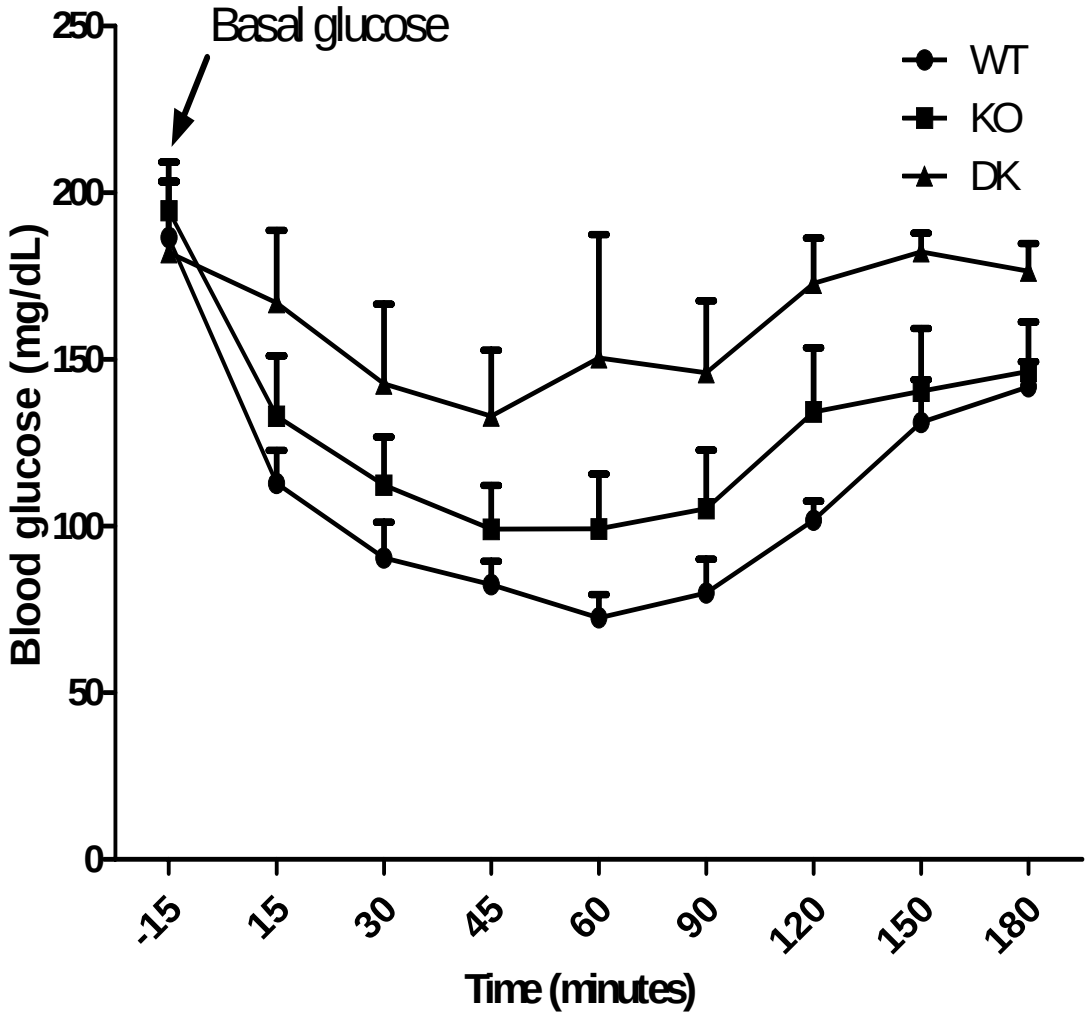


Figure 31: Impaired insulin sensitivity in *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ (DK) mice.

Intraperitoneal insulin sensitivity test (IPIST). After 9 weeks of a high-carbohydrate diet, the IPIST test was performed. Basal blood glucose levels were measured at t=-15 min. 1U/kg insulin was intraperitoneally injected into mice at t=0min. Blood samples were collected from the tip of mouse tails at the times indicated and blood glucose was determined. (P=0.02 between WT and DK mice, 2way ANOVA)

DISCUSSION

The results presented here demonstrate a new model for NAFLD. When *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$mice were fed a diet rich in carbohydrate for 7-9 weeks, they consumed comparable amounts of food and gained weight are the same rate. In spite of this, *Aoah*$^{-/-}$ mice developed steatohepatitis, hypertriglyceridemia and hypercholesterolemia that were minimal or absent in *Aoah*$^{+/+}$ mice. Whereas liver steatosis was confirmed by Oil-red O and H&E staining, neither triglyceride nor cholesterol content was elevated in livers of Aoah$^{-/-}$ mice. It is noteworthy that the steatosis observed was patchy hence the liver pieces used for triglyceride determination may not be representative of the triglyceride content in the whole liver. On the other hand, the observed steatosis may be due to other lipids. Puri el al have shown that in addition to triglycerides, diacylglycerols, phospholipids and lysophospholipids are also elevated in livers of NAFLD and NASH patients (161). The fact that AOAH is a phospholipase that can act on diverse phospholipids and diglycerides strengthens the possibility that steatosis in *Aoah*$^{-/-}$ mice may be due to other lipids besides triglycerides. Analysis of total hepatic lipid content is required to characterize the steatosis observed in these mice.

Normal serum ALT levels in *Aoah*$^{-/-}$ mice indicated that steatohepatitis was not severe enough to cause hepatocyte damage. Feeding for longer periods may be sufficient to raise serum ALT levels, however NAFLD and NASH are not always accompanied by elevated liver enzymes (110). Hypertriglyceridemia is associated with diets rich in carbohydrate and low in fats. Whether the hypertriglyceridemia observed in *Aoah*$^{-/-}$ is a cause or a consequence of liver steatosis is unknown at this time.

Given that LPS is the known substrate for AOAH, the presence of hepatic steatosis and hyperlipidemia in Aoah$^{-/-}$ mice suggests an important role for LPS in high carbohydrate induced NAFLD. Mice deficient in TLR4 cannot respond to LPS and are protected from diet-induced hepatic steatosis and insulin resistance (154-156). Similarly we found that *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ mice on a high-carbohydrate diet gained weight at a slower rate and were protected from steatohepatitis. In contrast they maintained hyperlipidemia and had decreased insulin sensitivity as measured by the intraperitoneal insulin sensitivity test. The absence of steatosis in hyperlipidemic, insulin resistant *Tlr4*$^{-/-}$ *Aoah*$^{-/-}$ mice suggests that fatty liver and insulin resistance may occur independently of each other. Steatosis in mice lacking AOAH may be due to their inability to deacylate LPS (since steatosis is abated in mice deficient in TLR4) but increased serum lipids and insulin resistance may be as a result of another property of AOAH, namely lipid metabolism.

A limitation of this study is the lack of *Tlr4*$^{-/-}$*Aoah*$^{+/+}$ controls. Based on the data presented, we would expect that these mice would be protected from hepatic steatosis but not hyperlipidemia and insulin resistance in response to a high-carbohydrate diet.

Our results correlate with others that show that inactivation of TLR4 blunts diet-induced liver steatosis. But they differ in that our TLR4 deficient mice had exacerbated insulin resistance. This distinction may simply be due to differences in diet (high-carbohydrate vs. high-fat), mouse strains (C57BL/10ScN; C57Bl/6 vs.C3H/HeJ) or to the lipid metabolism function(s) of AOAH. The third possibility deserves further investigation.

CONCLUSIONS AND FUTURE DIRECTIONS

1. Characterization of CD68p-AOAH transgenic mice

Prior to this study, *Aoah*$^{-/-}$ mice had been used to investigate the role of AOAH in host defense. Here, we used transgenic mice to show that AOAH prevents long-term responses to LPS. CD68p-AOAH transgenic mice overexpressing AOAH in macrophages deacylated LPS at a faster rate *in vivo*, recovered more rapidly from O14 LPS challenge and were protected from bacterial and LPS induced hepatosplenomegaly. Future directions for this project include immunolocalization of AOAH in macrophages. Low abundance of AOAH in primary cells has made immunolocalization unsuccessful. The CD68p-AOAH transgene has a FLAG sequence downstream of the AOAH cDNA sequence. An anti-FLAG antibody may prove useful in localizing transgene-derived AOAH expression in macrophages and may be suggestive as to where AOAH is expressed in other myeloid cells. Moreover a positive signal for FLAG exclusively in macrophages would further attest to the macrophage specific expression of the transgene.

Explanted macrophages from CD8p-AOAH transgenic mice were shown to deacylate rough LPS at a faster rate than wildtype macrophages. It would also be important to determine if transgenic macrophages can deacylate whole bacteria more rapidly. In addition, experiments to determine if transgenic mice are able to deacylate and recover from LPS obtained from a wide variety of bacteria would equally be vital.

1. The role of AOAH in NAFLD and NASH

The results presented here indicate that AOAH plays a role in diet-induced metabolic dysfunction. *Aoah*$^{-/-}$ mice had hyperlipidemia and fat accumulation in their livers in response to a diet rich in carbohydrates. Whereas hepatic steatosis was attenuated in *Tlr4*$^{-/-}$*Aoah*$^{-/-}$ mice, insulin resistance and hyperlipidemia persisted. Several experiments need to be performed in order to understand the mechanism of diet induced steatohepatitis in *Aoah*$^{-/-}$ mice.

The observed steatosis in livers of *Aoah*$^{-/-}$ mice was not due to increased triglyceride levels. A lipidomic analysis of the liver needs to be performed to identify the lipids observed in the histological sections. In addition, levels of ChREBP and SREBP-1c, both of which mediate *de novo* lipogenesis in response to glucose and insulin, need to be quantified. Pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) produced via activation of TLR4 disrupt insulin signaling leading to insulin resistance and excessive hepatic lipid storage. The livers, adipose tissue and serum of *Aoah*$^{-/-}$ mice on a high-carbohydrate diet need to be tested for increased levels of these cytokines.

To further investigate the role of AOAH and LPS derived from intestinal flora in diet-induced hepatic steatosis, mice on a high-calorie diet may be treated with antibiotics to reduce gut flora. *Aoah*$^{-/-}$ mice treated with antibiotics would be expected to have a decrease in hepatic steatosis.

As mentioned earlier, the high-carbohydrate feeding experiment needs to be repeated in *Tlr4*$^{+/+}$*Aoah*$^{-/-}$ mice. Inactivation of TLR4 in these mice should protect them from diet induced hepatic steatosis.

Finally to confirm the decrease in insulin sensitivity observed in mice that lack AOAH, insulin signaling needs to be studied in these mice. Levels of insulin in the blood may be measured and tissues examined for increased serine phosphorylation of IRS-1.

REFERENCES

1. Caroff, M., and D. Karibian. 2003. Structure of bacterial lipopolysaccharides. *Carbohydr Res* 338:2431-2447.
2. Dixon, D.R., and R.P. Darveau. 2005. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. *J Dent Res* 84:584-595.
3. Medzhitov, R., and C.A. Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296:298-300.
4. Beutler, B., and E.T. Rietschel. 2003. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* 3:169-176.
5. Rietschel, E.T., and J.M. Cavaillon. 2003. Richard Pfeiffer and Alexandre Besredka: creators of the concept of endotoxin and anti-endotoxin. *Microbes Infect* 5:1407-1414.
6. Caroff, M., D. Karibian, J.M. Cavaillon, and N. Haeffner-Cavaillon. 2002. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes Infect* 4:915-926.
7. Hitchcock, P.J., L. Leive, P.H. Makela, E.T. Rietschel, W. Strittmatter, and D.C. Morrison. 1986. Lipopolysaccharide nomenclature--past, present, and future. *J Bacteriol* 166:699-705.
8. 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.

9. Rietschel, E.T., T. Kirikae, F.U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A.J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, and et al. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J* 8:217-225.

10. Seydel, U., B. Lindner, H.W. Wollenweber, and E.T. Rietschel. 1984. Structural studies on the lipid A component of enterobacterial lipopolysaccharides by laser desorption mass spectrometry. Location of acyl groups at the lipid A backbone. *Eur J Biochem* 145:505-509.

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

12. Moran, A.P., M.M. Prendergast, and B.J. Appelmelk. 1996. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* 16:105-115.

13. Kahler, C.M., and D.S. Stephens. 1998. Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). *Crit Rev Microbiol* 24:281-334.

14. Mandrell, R.E., and M.A. Apicella. 1993. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. *Immunobiology* 187:382-402.

15. Estabrook, M.M., J.M. Griffiss, and G.A. Jarvis. 1997. Sialylation of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. *Infect Immun* 65:4436-4444.

16. Kulshin, V.A., U. Zahringer, B. Lindner, C.E. Frasch, C.M. Tsai, B.A. Dmitriev, and E.T. Rietschel. 1992. Structural characterization of the lipid A component of pathogenic Neisseria meningitidis. *J Bacteriol* 174:1793-1800.

17. Schletter, J., H. Heine, A.J. Ulmer, and E.T. Rietschel. 1995. Molecular mechanisms of endotoxin activity. *Arch Microbiol* 164:383-389.

18. Tobias, P.S., K. Soldau, and R.J. Ulevitch. 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J Exp Med* 164:777-793.

19. Hailman, E., H.S. Lichenstein, M.M. Wurfel, D.S. Miller, D.A. Johnson, M. Kelley, L.A. Busse, M.M. Zukowski, and S.D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med* 179:269-277.

20. Poltorak, A., X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, 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.

21. Qureshi, S.T., L. Lariviere, 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.

22. 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.

23. 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.

24. Visintin, A., E. Latz, B.G. Monks, T. Espevik, and D.T. Golenbock. 2003. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J Biol Chem* 278:48313-48320.

25. Byrd, C.A., W. Bornmann, H. Erdjument-Bromage, P. Tempst, N. Pavletich, N. Rosen, C.F. Nathan, and A. Ding. 1999. Heat shock protein 90 mediates macrophage activation by Taxol and bacterial lipopolysaccharide. *Proc Natl Acad Sci U S A* 96:5645-5650.

26. Triantafilou, K., M. Triantafilou, and R.L. Dedrick. 2001. A CD14-independent LPS receptor cluster. *Nat Immunol* 2:338-345.

27. Heine, H., V.T. El-Samalouti, C. Notzel, A. Pfeiffer, A. Lentschat, S. Kusumoto, G. Schmitz, L. Hamann, and A.J. Ulmer. 2003. CD55/decay accelerating factor is part of the lipopolysaccharide-induced receptor complex. *Eur J Immunol* 33:1399-1408.

28. O'Neill, L.A., and A.G. Bowie. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7:353-364.

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

30. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P.F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 167:5887-5894.

31. Li, S., A. Strelow, E.J. Fontana, and H. Wesche. 2002. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A* 99:5567-5572.

32. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D.V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature* 383:443-446.

33. Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, and S. Akira. 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* 6:1087-1095.

34. Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature* 410:37-40.

35. Lu, Y.C., W.C. Yeh, and P.S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. *Cytokine* 42:145-151.

36. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301:640-643.

37. Fitzgerald, K.A., D.C. Rowe, B.J. Barnes, D.R. Caffrey, A. Visintin, E. Latz, B. Monks, P.M. Pitha, and D.T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* 198:1043-1055.

38. Oshiumi, H., M. Sasai, K. Shida, T. Fujita, M. Matsumoto, and T. Seya. 2003. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem* 278:49751-49762.

39. Cusson-Hermance, N., S. Khurana, T.H. Lee, K.A. Fitzgerald, and M.A. Kelliher. 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. *J Biol Chem* 280:36560-36566.

40. Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L.C. Hsu, G.G. Wang, M.P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann, and M. Karin. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439:204-207.

41. Galanos, C. 1975. Physical state and biological activity of lipopolysaccharides. Toxicity and immunogenicity of the lipid A component. *Z Immunitatsforsch Exp Klin Immunol* 149:214-229.

42. Loppnow, H., I. Durrbaum, H. Brade, C.A. Dinarello, S. Kusumoto, E.T. Rietschel, and H.D. Flad. 1990. Lipid A, the immunostimulatory principle of lipopolysaccharides? *Adv Exp Med Biol* 256:561-566.

43. Loppnow, H., H. Brade, I. Durrbaum, C.A. Dinarello, S. Kusumoto, E.T. Rietschel, and H.D. Flad. 1989. IL-1 induction-capacity of defined lipopolysaccharide partial structures. *J Immunol* 142:3229-3238.

44. Kotani, S., H. Takada, M. Tsujimoto, T. Ogawa, I. Takahashi, T. Ikeda, K. Otsuka, H. Shimauchi, N. Kasai, J. Mashimo, and et al. 1985. Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from an Escherichia coli re-mutant. *Infect Immun* 49:225-237.

45. Ulmer, A.J., H. Heine, W. Feist, S. Kusumoto, T. Kusama, H. Brade, U. Schade, E.T. Rietschel, and H.D. Flad. 1992. Biological activity of synthetic phosphonooxyethyl analogs of lipid A and lipid A partial structures. *Infect Immun* 60:3309-3314.

46. Garcia Vescovi, E., F.C. Soncini, and E.A. Groisman. 1996. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. *Cell* 84:165-174.

47. Guo, L., K.B. Lim, J.S. Gunn, B. Bainbridge, R.P. Darveau, M. Hackett, and S.I. Miller. 1997. Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. *Science* 276:250-253.

48. Kawahara, K., H. Tsukano, H. Watanabe, B. Lindner, and M. Matsuura. 2002. Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature. *Infect Immun* 70:4092-4098.

49. Rebeil, R., R.K. Ernst, B.B. Gowen, S.I. Miller, and B.J. Hinnebusch. 2004. Variation in lipid A structure in the pathogenic yersiniae. *Mol Microbiol* 52:1363-1373.

50. Munford, R.S. 2008. Sensing gram-negative bacterial lipopolysaccharides: a human disease determinant? *Infect Immun* 76:454-465.

51. 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.

52. Darveau, R.P. 1998. Lipid A diversity and the innate host response to bacterial infection. *Curr Opin Microbiol* 1:36-42.

53. Hoffmann, J.A., F.C. Kafatos, C.A. Janeway, and R.A. Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313-1318.

54. van de Wetering, J.K., L.M. van Golde, and J.J. Batenburg. 2004. Collectins: players of the innate immune system. *Eur J Biochem* 271:1229-1249.

55. Wittmann, I., M. Schonefeld, D. Aichele, G. Groer, A. Gessner, and M. Schnare. 2008. Murine bactericidal/permeability-increasing protein inhibits the endotoxic activity of lipopolysaccharide and gram-negative bacteria. *J Immunol* 180:7546-7552.

56. Weiss, J. 2003. Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against Gram-negative bacteria. *Biochem Soc Trans* 31:785-790.

57. Nagaoka, I., S. Hirota, F. Niyonsaba, M. Hirata, Y. Adachi, H. Tamura, and D. Heumann. 2001. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-alpha by blocking the binding of LPS to CD14(+) cells. *J Immunol* 167:3329-3338.

58. Vesy, C.J., R.L. Kitchens, G. Wolfbauer, J.J. Albers, and R.S. Munford. 2000. Lipopolysaccharide-binding protein and phospholipid transfer protein release lipopolysaccharides from gram-negative bacterial membranes. *Infect Immun* 68:2410-2417.

59. Zweigner, J., H.J. Gramm, O.C. Singer, K. Wegscheider, and R.R. Schumann. 2001. High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. *Blood* 98:3800-3808.

60. 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 transferring cell-bound LPS to plasma lipoproteins. *J Clin Invest* 108:485-493.

61. Shao, B., M. Lu, S.C. Katz, A.W. Varley, J. Hardwick, T.E. Rogers, N. Ojogun, D.C. Rockey, R.P. Dematteo, and R.S. Munford. 2007. A host lipase detoxifies bacterial lipopolysaccharides in the liver and spleen. *J Biol Chem* 282:13726-13735.

62. Ge, Y., R.M. Ezzell, R.G. Tompkins, and H.S. Warren. 1994. Cellular distribution of endotoxin after injection of chemically purified lipopolysaccharide differs from that after injection of live bacteria. *J Infect Dis* 169:95-104.

63. Munford, R.S., C.L. Hall, J.M. Lipton, and J.M. Dietschy. 1982. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of Salmonella typhimurium lipopolysaccharides. *J Clin Invest* 70:877-888.

64. Munford, R.S. 2005. Detoxifying endotoxin: time, place and person. *J Endotoxin Res* 11:69-84.

65. Koyama, I., T. Matsunaga, T. Harada, S. Hokari, and T. Komoda. 2002. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clin Biochem* 35:455-461.

66. Verweij, W.R., H. Bentala, A. Huizinga-van der Vlag, A. Miek van Loenen-Weemaes, K. Kooi, D.K. Meijer, and K. Poelstra. 2004. Protection against an Escherichia coli-induced sepsis by alkaline phosphatase in mice. *Shock* 22:174-179.

67. Nigam, V.N., D. Malchow, E.T. Rietschel, O. Luderitz, and O. Westphal. 1970. [Enzymatic liberation of long-chain fatty acids from bacterial lipopolysaccharides with the aid of extracts from amoebae of Dictyostelium discoideum]. *Hoppe Seylers Z Physiol Chem* 351:1123-1132.

68. Rosner, M.R., R.C. Verret, and H.G. Khorana. 1979. The structure of lipopolysaccharide from an Escherichia coli heptose-less mutant. III. Two fatty acyl amidases from Dictyostelium discoideum and their action on lipopolysaccharide derivatives. *J Biol Chem* 254:5926-5933.

69. 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 U S A* 80:6671-6675.

70. Raetz, C.R., C.M. Reynolds, M.S. Trent, and R.E. Bishop. 2007. Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* 76:295-329.

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

72. 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 peritoneal inflammatory exudate. *J Biol Chem* 274:36579-36584.

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

74. 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.

75. 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.

76. 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.

77. 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.

78. Coats, S.R., T.T. Pham, B.W. Bainbridge, R.A. Reife, and R.P. Darveau. 2005. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. *J Immunol* 175:4490-4498.

79. Kovach, N.L., E. Yee, R.S. Munford, C.R. Raetz, and J.M. Harlan. 1990. Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human whole blood ex vivo. *J Exp Med* 172:77-84.

80. 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.

81. Munford, R.S., and A.W. Varley. 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. *PLoS Pathog* 2:e67.

82. 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.

83. Upton, C., and J.T. Buckley. 1995. A new family of lipolytic enzymes? *Trends Biochem Sci* 20:178-179.

84. 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.

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

86. 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.

87. Feulner, J.A., M. Lu, J.M. Shelton, M. Zhang, J.A. Richardson, and R.S. Munford. 2004. Identification of acyloxyacyl hydrolase, a lipopolysaccharide-detoxifying enzyme, in the murine urinary tract. *Infect Immun* 72:3171-3178.

88. 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.

89. Gordon, S., and P.R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5:953-964.

90. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

91. Taylor, P.R., and S. Gordon. 2003. Monocyte heterogeneity and innate immunity. *Immunity* 19:2-4.

92. Hume, D.A., I.L. Ross, S.R. Himes, R.T. Sasmono, C.A. Wells, and T. Ravasi. 2002. The mononuclear phagocyte system revisited. *J Leukoc Biol* 72:621-627.

93. Ma, J., T. Chen, J. Mandelin, A. Ceponis, N.E. Miller, M. Hukkanen, G.F. Ma, and Y.T. Konttinen. 2003. Regulation of macrophage activation. *Cell Mol Life Sci* 60:2334-2346.

94. Gordon, S. 2007. Macrophage heterogeneity and tissue lipids. *J Clin Invest* 117:89-93.

95. Martinez, F.O., A. Sica, A. Mantovani, and M. Locati. 2008. Macrophage activation and polarization. *Front Biosci* 13:453-461.

96. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197-216.

97. Franchi, L., J.H. Park, M.H. Shaw, N. Marina-Garcia, G. Chen, Y.G. Kim, and G. Nunez. 2008. Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell Microbiol* 10:1-8.

98. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 176:287-292.

99. Doyle, A.G., G. Herbein, L.J. Montaner, A.J. Minty, D. Caput, P. Ferrara, and S. Gordon. 1994. Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur J Immunol* 24:1441-1445.

100. Munder, M., K. Eichmann, J.M. Moran, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol* 163:3771-3777.

101. Rabinowitz, S.S., and S. Gordon. 1991. Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli. *J Exp Med* 174:827-836.

102. Holness, C.L., R.P. da Silva, J. Fawcett, S. Gordon, and D.L. Simmons. 1993. Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem* 268:9661-9666.

103. Saito, N., K.A. Pulford, J. Breton-Gorius, J.M. Masse, D.Y. Mason, and E.M. Cramer. 1991. Ultrastructural localization of the CD68 macrophage-associated antigen in human blood neutrophils and monocytes. *Am J Pathol* 139:1053-1059.

104. Kozarsky, K., D. Kingsley, and M. Krieger. 1988. Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc Natl Acad Sci U S A* 85:4335-4339.

105. Fearon, D.T. 1978. Regulation by membrane sialic acid of beta1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc Natl Acad Sci U S A* 75:1971-1975.

106. Ramprasad, M.P., W. Fischer, J.L. Witztum, G.R. Sambrano, O. Quehenberger, and D. Steinberg. 1995. The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. *Proc Natl Acad Sci U S A* 92:9580-9584.

107. Adams, L.A., and K.D. Lindor. 2007. Nonalcoholic fatty liver disease. *Ann Epidemiol* 17:863-869.

108. Zivkovic, A.M., J.B. German, and A.J. Sanyal. 2007. Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. *Am J Clin Nutr* 86:285-300.

109. Marchesini, G., R. Marzocchi, F. Agostini, and E. Bugianesi. 2005. Nonalcoholic fatty liver disease and the metabolic syndrome. *Curr Opin Lipidol* 16:421-427.

110. Browning, J.D., L.S. Szczepaniak, R. Dobbins, P. Nuremberg, J.D. Horton, J.C. Cohen, S.M. Grundy, and H.H. Hobbs. 2004. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 40:1387-1395.

111. Larter, C.Z., and M.M. Yeh. 2008. Animal models of NASH: Getting both pathology and metabolic context right. *J Gastroenterol Hepatol*

112. Diehl, A.M. 2005. Lessons from animal models of NASH. *Hepatol Res* 33:138-144.

113. Anstee, Q.M., and R.D. Goldin. 2006. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int J Exp Pathol* 87:1-16.

114. Brunt, E.M. 2004. Nonalcoholic steatohepatitis. *Semin Liver Dis* 24:3-20.

115. Yuan, M., N. Konstantopoulos, J. Lee, L. Hansen, Z.W. Li, M. Karin, and S.E. Shoelson. 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 293:1673-1677.

116. Araya, J., R. Rodrigo, L.A. Videla, L. Thielemann, M. Orellana, P. Pettinelli, and J. Poniachik. 2004. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)* 106:635-643.

117. Sanyal, A.J. 2005. Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2:46-53.

118. Wetterau, J.R., L.P. Aggerbeck, M.E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D.J. Rader, and R.E. Gregg. 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science* 258:999-1001.

119. Rao, M.S., and J.K. Reddy. 2001. Peroxisomal beta-oxidation and steatohepatitis. *Semin Liver Dis* 21:43-55.

120. Hashimoto, T., W.S. Cook, C. Qi, A.V. Yeldandi, J.K. Reddy, and M.S. Rao. 2000. Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem* 275:28918-28928.

121. Day, C.P., and O.F. James. 1998. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 114:842-845.

122. Coburn, C.T., F.F. Knapp, Jr., M. Febbraio, A.L. Beets, R.L. Silverstein, and N.A. Abumrad. 2000. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of CD36 knockout mice. *J Biol Chem* 275:32523-32529.

123. Feldstein, A.E., A. Canbay, M.E. Guicciardi, H. Higuchi, S.F. Bronk, and G.J. Gores. 2003. Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. *J Hepatol* 39:978-983.

124. Ito, M., J. Suzuki, S. Tsujioka, M. Sasaki, A. Gomori, T. Shirakura, H. Hirose, A. Ishihara, H. Iwaasa, and A. Kanatani. 2007. Longitudinal analysis of murine

steatohepatitis model induced by chronic exposure to high-fat diet. *Hepatol Res* 37:50-57.

125. Yoshimatsu, M., Y. Terasaki, N. Sakashita, E. Kiyota, H. Sato, L.J. van der Laan, and M. Takeya. 2004. Induction of macrophage scavenger receptor MARCO in nonalcoholic steatohepatitis indicates possible involvement of endotoxin in its pathogenic process. *Int J Exp Pathol* 85:335-343.

126. Matteoni, C.A., Z.M. Younossi, T. Gramlich, N. Boparai, Y.C. Liu, and A.J. McCullough. 1999. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 116:1413-1419.

127. Menon, K.V., G.J. Gores, and V.H. Shah. 2001. Pathogenesis, diagnosis, and treatment of alcoholic liver disease. *Mayo Clin Proc* 76:1021-1029.

128. Thurman, R.G. 1998. II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. *Am J Physiol* 275:G605-611.

129. Su, G.L. 2002. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol* 283:G256-265.

130. Wheeler, M.D. 2003. Endotoxin and Kupffer cell activation in alcoholic liver disease. *Alcohol Res Health* 27:300-306.

131. Haines, N.W., A.L. Baker, J.L. Boyer, S. Glagov, H. Schneir, J. Jaspan, and D.J. Ferguson. 1981. Prognostic indicators of hepatic injury following jejunoileal bypass performed for refractory obesity: a prospective study. *Hepatology* 1:161-167.

132. Wigg, A.J., I.C. Roberts-Thomson, R.B. Dymock, P.J. McCarthy, R.H. Grose, and A.G. Cummins. 2001. The role of small intestinal bacterial overgrowth,

intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 48:206-211.

133. Cope, K., T. Risby, and A.M. Diehl. 2000. Increased gastrointestinal ethanol production in obese mice: implications for fatty liver disease pathogenesis. *Gastroenterology* 119:1340-1347.

134. Brun, P., I. Castagliuolo, V. Di Leo, A. Buda, M. Pinzani, G. Palu, and D. Martines. 2007. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 292:G518-525.

135. Uysal, K.T., S.M. Wiesbrock, M.W. Marino, and G.S. Hotamisligil. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610-614.

136. Hotamisligil, G.S., P. Peraldi, A. Budavari, R. Ellis, M.F. White, and B.M. Spiegelman. 1996. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665-668.

137. Cani, P.D., J. Amar, M.A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A.M. Neyrinck, F. Fava, K.M. Tuohy, C. Chabo, A. Waget, E. Delmee, B. Cousin, T. Sulpice, B. Chamontin, J. Ferrieres, J.F. Tanti, G.R. Gibson, L. Casteilla, N.M. Delzenne, M.C. Alessi, and R. Burcelin. 2007. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56:1761-1772.

138. 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.

139. 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. *Journal of Endotoxin Research* 4:371-379.

140. Galanos, C., O. Luderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur J Biochem* 9:245-249.

141. Munford, R.S., and A.L. Erwin. 1992. Eukaryotic lipopolysaccharide deacylating enzyme. *Methods Enzymol* 209:485-492.

142. Coulthard, M.G., J. Swindle, R.S. Munford, R.D. Gerard, and R.S. Meidell. 1996. Adenovirus-mediated transfer of a gene encoding acyloxyacyl hydrolase (AOAH) into mice increases tissue and plasma AOAH activity. *Infect Immun* 64:1510-1515.

143. Poelstra, K., W.W. Bakker, P.A. Klok, M.J. Hardonk, and D.K. Meijer. 1997. A physiologic function for alkaline phosphatase: endotoxin detoxification. *Lab Invest* 76:319-327.

144. Beumer, C., M. Wulferink, W. Raaben, D. Fiechter, R. Brands, and W. Seinen. 2003. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther* 307:737-744.

145. Teghanemt, A., D. Zhang, E.N. Levis, J.P. Weiss, and T.L. Gioannini. 2005. Molecular basis of reduced potency of underacylated endotoxins. *J Immunol* 175:4669-4676.

146. Lu, M., M. Zhang, A. Takashima, J. Weiss, M.A. Apicella, X.H. Li, D. Yuan, and R.S. Munford. 2005. Lipopolysaccharide deacylation by an endogenous lipase controls innate antibody responses to Gram-negative bacteria. *Nat Immunol* 6:989-994.

147. Lang, R., R.L. Rutschman, D.R. Greaves, and P.J. Murray. 2002. Autocrine deactivation of macrophages in transgenic mice constitutively overexpressing IL-10 under control of the human CD68 promoter. *J Immunol* 168:3402-3411.

148. Ohta, S., U. Bahrun, R. Shimazu, H. Matsushita, K. Fukudome, and M. Kimoto. 2006. Induction of long-term lipopolysaccharide tolerance by an agonistic monoclonal antibody to the toll-like receptor 4/MD-2 complex. *Clin Vaccine Immunol* 13:1131-1136.

149. Buwitt-Beckmann, U., H. Heine, K.H. Wiesmuller, G. Jung, R. Brock, S. Akira, and A.J. Ulmer. 2006. TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J Biol Chem* 281:9049-9057.

150. Philpott, D.J., and S.E. Girardin. 2004. The role of Toll-like receptors and Nod proteins in bacterial infection. *Mol Immunol* 41:1099-1108.

151. Kopfler, W.P., M. Willard, T. Betz, J.E. Willard, R.D. Gerard, and R.S. Meidell. 1994. Adenovirus-mediated transfer of a gene encoding human apolipoprotein A-I into normal mice increases circulating high-density lipoprotein cholesterol. *Circulation* 90:1319-1327.

152. Shi, C., M. Sakuma, T. Mooroka, A. Liscoe, H. Gao, K.J. Croce, A. Sharma, D. Kaplan, D.R. Greaves, Y. Wang, and D.I. Simon. 2008. Downregulation of the forkhead transcription factor Foxp1 is required for monocyte differentiation and macrophage function. *Blood*

153. Gough, P.J., S. Gordon, and D.R. Greaves. 2001. The use of human CD68 transcriptional regulatory sequences to direct high-level expression of class A scavenger receptor in macrophages in vitro and in vivo. *Immunology* 103:351-361.

154. Shi, H., M.V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J.S. Flier. 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 116:3015-3025.

155. Poggi, M., D. Bastelica, P. Gual, M.A. Iglesias, T. Gremeaux, C. Knauf, F. Peiretti, M. Verdier, I. Juhan-Vague, J.F. Tanti, R. Burcelin, and M.C. Alessi. 2007. C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. *Diabetologia* 50:1267-1276.

156. Tsukumo, D.M., M.A. Carvalho-Filho, J.B. Carvalheira, P.O. Prada, S.M. Hirabara, A.A. Schenka, E.P. Araujo, J. Vassallo, R. Curi, L.A. Velloso, and M.J. Saad. 2007. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 56:1986-1998.

157. Brugman, S., F.A. Klatter, J.T. Visser, A.C. Wildeboer-Veloo, H.J. Harmsen, J. Rozing, and N.A. Bos. 2006. Antibiotic treatment partially protects against type

1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? *Diabetologia* 49:2105-2108.

158. Backhed, F., H. Ding, T. Wang, L.V. Hooper, G.Y. Koh, A. Nagy, C.F. Semenkovich, and J.I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* 101:15718-15723.

159. Vreugdenhil, A.C., C.H. Rousseau, T. Hartung, J.W. Greve, C. van 't Veer, and W.A. Buurman. 2003. Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons. *J Immunol* 170:1399-1405.

160. Angelico, F., M. Del Ben, R. Conti, S. Francioso, K. Feole, S. Fiorello, M.G. Cavallo, B. Zalunardo, F. Lirussi, C. Alessandri, and F. Violi. 2005. Insulin resistance, the metabolic syndrome, and nonalcoholic fatty liver disease. *J Clin Endocrinol Metab* 90:1578-1582.

161. Puri, P., R.A. Baillie, M.M. Wiest, F. Mirshahi, J. Choudhury, O. Cheung, C. Sargeant, M.J. Contos, and A.J. Sanyal. 2007. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 46:1081-1090.