IDENTIFICATION OF ACYLOXYACYL HYDROLASE, A LIPOPOLYSACCHARIDE-DETOXIFYING ENZYME, IN THE MURINE URINARY TRACT

APPROVED BY SUPERVISORY COMMITTEE

Robert S. Munford, M.D. _____

Leon Eidels, Ph.D. _____

Christopher Lu, M.D. _____

Kevin S. McIver, Ph.D. _____

Nicolai van Oers, Ph.D. _____

DEDICATION

Dedicated to my parents, Gene and Candace Feulner, my brother, Kenneth Feulner and Joseph Baur for their support, encouragement and love.

IDENTIFICATION OF ACYLOXYACYL HYDROLASE, A LIPOPOLYSACCHARIDE-DETOXIFYING ENZYME, IN THE MURINE URINARY TRACT

by

J. Amelia Feulner

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2003

IDENTIFICATION OF ACYLOXYACYL HYDROLASE, A LIPOPOLYSACCHARIDE-DETOXIFYING ENZYME, IN THE MURINE URINARY TRACT

Publication No. _____

J. Amelia Feulner, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2003

Supervising Professor: Robert S. Munford, M.D.

Acyloxyacyl hydrolase (AOAH) is a lipase that removes the secondary fatty acyl chains that are substituted to the hydroxyl groups of glucosamine-linked 3-hydroxyacyl residues in lipid A, the bioactive center of Gram-negative bacterial lipopolysaccharides (LPS). Such limited deacylation has been shown to attenuate cytokine and chemokine responses to LPS, suggesting a role for AOAH in modulating (downregulating) inflammatory responses to invading Gram-negative bacteria. Prior to the experiments described in this report, AOAH had only been found in myeloid lineage cells (monocyte-macrophages, neutrophils and dendritic cells). In the work presented here, AOAH was found in murine renal proximal tubule cells and in human renal

cortex. Proximal tubule cells are known targets for invading Gram-negative uropathogens and we hypothesize that possessing AOAH may help them degrade the LPS contained within these bacteria. I further found that AOAH is secreted from proximal tubules *in vitro* and that it can be detected in murine urine, where it is able to deacylate purified LPS. AOAH may also associate with downstream bladder epithelial cells (which do not express AOAH) and be processed by them to its more enzymatically active, mature form. Bladder cells that have taken up AOAH *in vitro* are able to deacylate LPS.

To determine the *in vivo* role of AOAH, I induced ascending urinary tract infections (UTIs) in wild type and AOAH null mice. To my surprise, AOAH null mice were able to clear bacteria from their urine faster than did wild type mice. An analysis of the immune response by histological analysis of bladder tissue and enumeration of neutrophils in the urine did not show a significant difference between wild type and AOAH null mice at any of the time points examined. Although I do not yet understand the mechanism for such increased clearance in AOAH null animals, we hypothesize that, due to their inability to deacylate LPS, they might have a more effective immune response to invading Gram-negative bacteria. A more detailed analysis of such responses to invading Gram-negative uropathogens will be important for understanding the *in vivo* role of AOAH in the urinary tract.

PRIOR PUBLICATIONS

J. A. Feulner, M. Lu, J. Shelton, J. Richardson, R.S. Munford, Identification of acyloxyacyl hydrolase, a lipopolysaccharide-detoxifying enzyme, in the murine urinary tract. Manuscript in submission, July 2003.

A.C. Walsh, **J. A. Feulner**, A. Reilly. Evidence for functionally significant polymorphism of human glutamate cysteine ligase catalytic subunit: association with glutathione levels and drug resistance in the National Cancer Institute tumor cell line panel. *Toxicol Sci*. 2001 Jun;61(2):218-23.

T.J. Sellati, D. A. Bouis, M.J. Caimano, **J. A. Feulner**, C.Ayers, E. Lien, and J. D. Radolf. Activation of Human Monocytic Cells by *Borrelia burgdorferi* and *Treponema pallidum* is Facilitated by CD14 and Correlates with Surface Exposure of Spirochetal Lipoproteins. *The Journal of Immunology*, 1999**,** 163: 2049-2056.

Table of Contents

List of Figures

List of Tables

Chapter One

Literature Review

Lipopolysaccharide (LPS)

Lipopolysaccharide History

Fever was one of the first recorded physical findings in medicine. Early investigators hypothesized that the inducer(s) of fever were physical entities and named them pyrogens, stemming from the Greek root pyr, meaning fire. Debates then arose as to whether fever was a manifestation of disease or a host defense against developing illness. Albrecht von Haller, a pioneer in the field of lipopolysachharide (endotoxin), showed that putrid (decomposing) tissue could induce fever in animals when re-injected intravenously [1]. In 1892, Richard Pfeiffer, a prized student of Koch, published that *Vibrio cholerae* had a toxin "closely attached to, and probably an integral part of, the bacterial body" [1]. This came at a time when most scientists believed pyrogens to be secreted proteins like the other known bacterial toxins. Pfeiffer is credited with coining the term endotoxin (although he never published it), which is still used today [2].

Endotoxin was first purified (crudely) around 1932 by Andre Boivin and Lydia Mesrobeanu using a trichloroacetic acid (TCA)-based method. Soon after, Walter T. J. Morgan and Walther F. Goebel used organic solvents and water to purify endotoxin. Both groups found

endotoxin to be composed of lipid and polysaccharide with very little if any associated protein [1]. While this crude preparation was a huge advance in our understanding of LPS, it was believed that alternative methods of purification would lead to a more highly purified product and a better understanding of the molecule. It was Otto Westphal and Otto Luderitz who succeeded in the landmark purification of endotoxin. Using a hot phenol-water extraction, they were able to obtain highly purified, biologically active endotoxin from a variety of Gram-negative bacteria. Their product lacked protein and was composed of just carbohydrate, fatty acids, and phosphorus [3]. It was they who first used the term lipopolysaccharide (LPS) to describe endotoxin, although the term was not immediately accepted by the scientific community of their day. Further studies by Westphal and Luderitz demonstrated that LPS was present on both pathogenic and non-pathogenic Gram-negative bacteria. It is now known that LPS is embedded in the outer leaflet of the outer membrane of Gram-negative bacteria (Figure 1.1) and that mutants with defects in the early stages of LPS biosynthesis are non-viable [4].

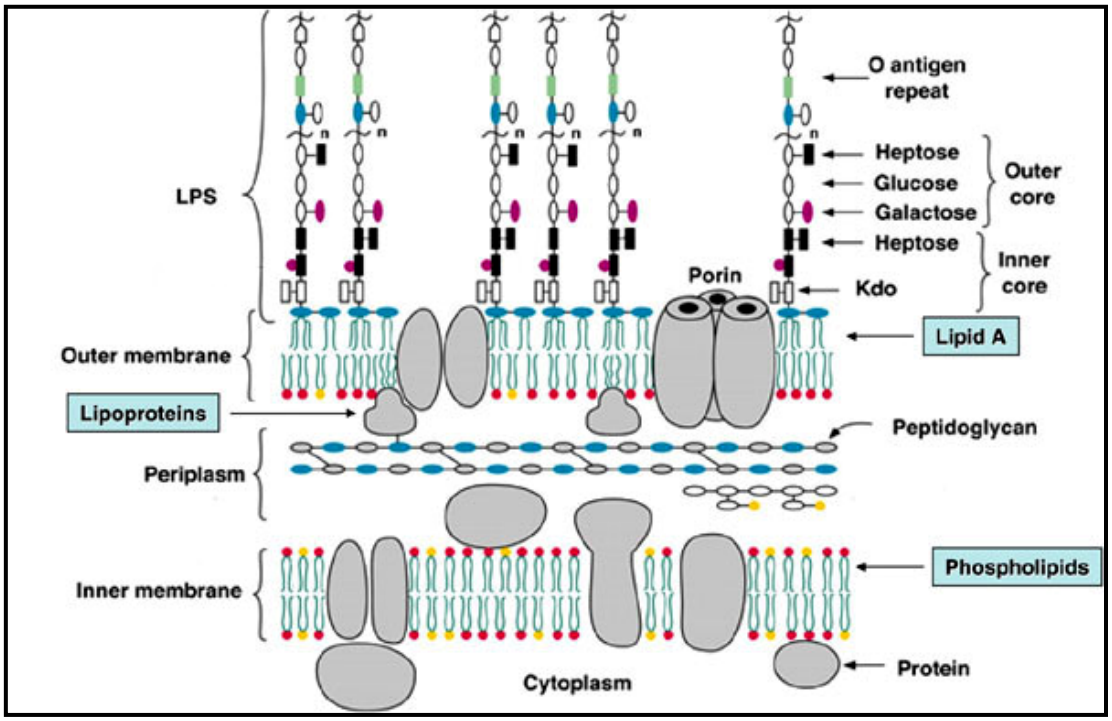


Figure 1.1 – *The composition of a Gram-negative bacterial membrane* [4]. *The inner or cytoplasmic membrane surrounds the bacterial cell. The periplasm, which contains peptidoglycan, is surrounded by the outer membrane. Lipopolysaccharide (LPS) is embedded in the outer leaflet of the outer membrane and is composed of three distinct components; lipid A, oligosaccharide core, and O-antigen. The oligosaccharide core contains a unique sugar, 2-keto-2-deoxyoctonate (KDO).*

Basic Structure of LPS

The availability of purified LPS made studies of the individual components of LPS possible [1]. The first bacterial LPS to be chemically characterized were those of the *Enterobacteriaceae* family (pathogenic and non-pathogenic bacteria located in the gut), such as *Salmonella* and *Escherichia*. When grown on solid agar, the colony morphology of all wild type strains of enteric bacteria look similar; it is complete and smooth (as opposed to some mutants, which have rough, irregular colony edges). After studying hundreds of enteric LPSs, Luderitz

proposed that all endotoxins are composed of three general components; the O-specific side chain, the oligosaccharide core, and lipid A (Figure 1.1 and 1.2) [1;5].

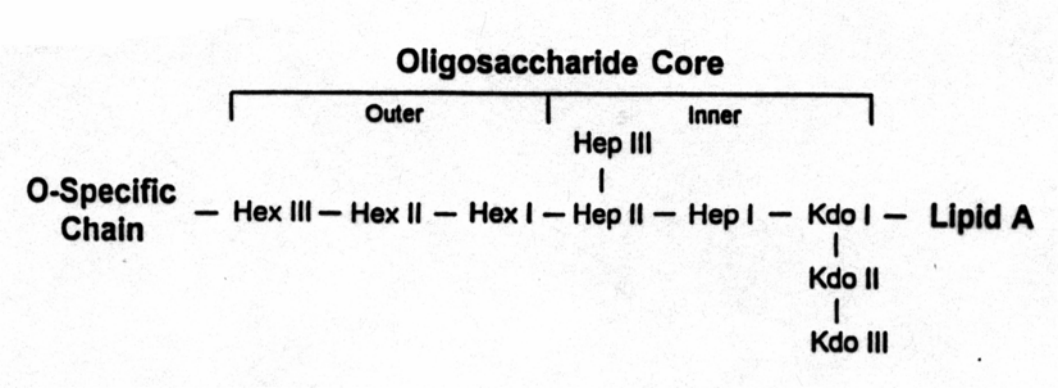


Figure 1.2 *General structure of smooth LPS showing the O-specific chain, inner and outer core, and lipid A. (KDO =2-keto-2-deoxyoctonate; Hep = heptose; Hex = hexose)*

O-antigen (O specific chain)

Studies in several different laboratories determined that the O-antigen was a complex polysaccharide, composed of repeating units of five to eight monosaccharides (galactose, rhamnose, mannose, and abequose in *S. typhimurium*). Different species of *Salmonella* had structurally different O-antigens, and antisera raised against one species did not cross-react with other species. This important understanding allowed scientists to classify bacteria into serotypes, a schema developed by Kauffmann, Luderitz and Westphal and published in 1960 [1]. The O-antigen has several biological activities, serving as receptors for bacteriophage, modulating the

activation of the alternative complement pathway, and inhibiting the attachment of the membrane attack complex to the bacterial outer membrane [4].

Core Oligosaccharide

It was later discovered that not all Gram-negative bacteria possess an O-antigen. Such bacteria are termed rough (R) because they form rigid, incomplete colonies on solid agar and autoagglutinate in saline. Studies of the core oligosaccharide were facilitated by the characterization of *Salmonella minnesota* and *Salmonella typhimurium* mutants. Several rough (R)-mutants were shown to have truncated polysaccharide cores due to defects in genes that code for glycosyl or phosphoryl transferases. Rough mutants synthesizing the entire core (but lacking O-antigen) were termed Ra. Those lacking the terminal sugar were named Rb, and the mutant possessing the shortest core was named Re [6]. Luderitz, Westphal, and colleagues proposed the structure of the *S. minnesota* core in 1967. Although core regions differ among bacterial species, all core regions contain an unusual sugar, 2-keto-3-deoxyoctonate (KDO). Other known residues are heptose, glucose, galactose, and N-acetylglucosamine [reviewed in [7]]. The minimum requirement for cell viability is a single molecule of KDO, as is present in Re *Salmonella* and *E. coli* LPS [8]. Based on the most severe mutant (Re), one can deduce that KDO must be directly attached to lipid A, the toxic moiety of LPS. Not much is known about the biological activities of the outer core region of LPS, but it is believed that both the outer and inner core carry epitopes for antibodies [4].

Lipid A

Westphal and Luderitz are credited with coining the term lipid A. As described in a 1954 review, they isolated a chloroform- and pyridine- soluble lipid structure from intact LPS by a thirty-minute treatment with HCl at elevated temperature [1;9]. Determining the structure of the extracted lipid was considerably more difficult than structuring either the core or O-antigen and it wasn't until 1983 that Takayama and colleagues published the complete and correct structure of lipid A [10]. It is now known that lipid A consists of a unique diglucosamine backbone (D-GlcN) that is β(1'-6) interlinked. Two phosphate groups are attached to the backbone at the 1 and 4' positions. These phosphates are sometimes modified with polar groups such as 4-amino-4-deoxy-L-arabinose (Ara4N) and/or ethanolamine, both of which are removed by the mild acid treatment used to purify and study lipid A. The fatty acid composition of lipid A was first described in *E. coli*. A total of six fatty acids chains are attached to the lipid A backbone, two via amide linkages and four via ester linkages. The amino-linked fatty acids were exclusively 3-hydroxymyristate, while the ester-linked fatty acids were of varying length (myristate, laurate, or palmitate). It was later discovered that two of the four ester-linked fatty acids were not directly attached to the lipid A backbone, but were actually bound to the 3-hydroxyl groups of one amide- and one ester-linked fatty acid [11]. Munford *et al.* termed these piggybacked, acyloxyacyl linked, fatty acids secondary, while those attached directly to the lipid A backbone were termed "primary" [10-12]. The ester- or amide-linked primary fatty acids are all 3-hydroxymyristates [14:0(3-OH)]. In independent studies, the structure of *S. typhimurium* lipid A was determined to be essentially identical to that of *E. coli*. Around 1985 investigators chemically synthesized *E. coli* lipid A [13]. This compound, called LA-15-PP (506), was later shown to be indistinguishable from lipid A purified from natural bacterial sources in a variety of biological assays [4;14;15].

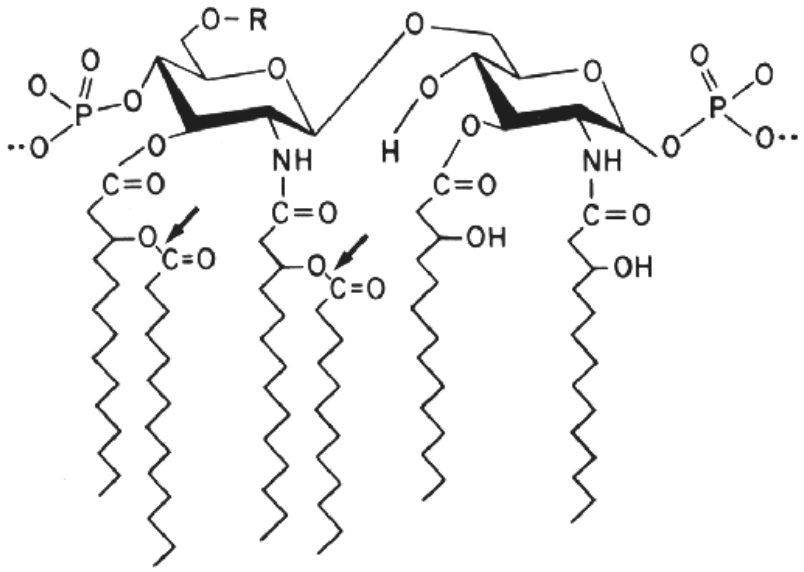
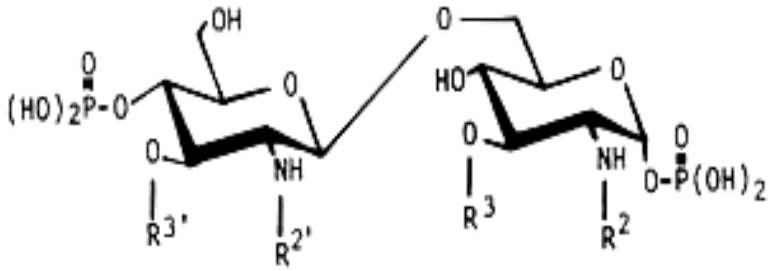


Figure 1.3 *Structure of S. typhimurium/E. coli Lipid A. The backbone of lipid A consists of a diglucosamine carbohydrate backbone in a beta 1-6 linkage. Primary fatty acyl chains are attached directly to the carbohydrate backbone by either ester- or amide-linkages. Secondary fatty acyl chains are attached to the 3-OH group of some of the primary fatty acyl chains. Arrows show sites of AOAH cleavage and the secondary fatty acyl chains.*

Structure-Function Relationships of Lipid A

Before the structure of LPS was completely solved, it was nearly impossible to determine which moiety was responsible for the bioactivities of LPS. Early studies of mutant *Salmonella* pointed to lipid A as the toxic principle, but because a complete core mutant was not viable, it was impossible to discount KDO or the combination of KDO and lipid A as the toxic moiety. Convincing evidence came from the analyses of LPS from a *Salmonella* mutant (Re) with chemically modified KDO. The mutant retained pyrogenicity and gave further evidence for the original hypothesis that lipid A was the toxic moiety of LPS [16]. The availability of a number of chemically synthesized lipid A structures allowed for the study of structure-function

relationships of lipid A. Table 1.0 describes the various properties of several of the chemically synthesized lipid A molecules. It should be noted that there are other names for these various compounds, but for simplicity I will continue to refer to only those compounds listed in Table 1.0. The complete chemical synthesis of *E. coli* lipid A (LA-15-PP) and the subsequent biological analyses firmly established the role of lipid A as the active component of LPS [15;17].



Compound	$R^{3'}$	$R^{2'}$	R^3	R^2	Remarks	**	***
LA-16-PP	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{16})	*S. minnesota*	7	3
LA-15-PP	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	*E. coli*	6	2
LA-22-PP	C_{14}-OH	C_{14}-O-(C_{14})	C_{14}-OH	C_{14}-O-(C_{14})	*C. violaceum*	6	2
LA-20-PP	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-O-(C_{16})	Precursor- Ib(IV_b)	5	1
LA-21-PP	C_{14}-OH	C_{14}-O-(C_{16})	C_{14}-OH	C_{14}-OH	Isomer of Ib	5	1
LA-14-PP	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	Precursor Ia (IV_a)	4	0

Table 1.0 *– Synthetic lipid A and disaccharide-type lipid A precursors [16]. Secondary fatty acyl chains are attached to the carbohydrate backbone at the $R^{3'}$, $R^{2'}$ (non-reducing sugar) R^3 and R^2 (reducing sugar).* ***** = total acyl groups per molecule; *** = total 3-acyloxyacyl groups (secondary fatty acyl chains) per molecule****.*

Chemically Modified LPS and Synthetic Lipid A Derivatives

Utilizing chemically modified Re LPS and synthetic lipid A derivatives, the critical components of lipid A needed to induce the biological activities of LPS were sought. Although it was demonstrated that a disaccharide backbone was more potent than a monosaccharide and that both phosphates play important roles [15;18;19], the fatty acid portion of lipid A was found to be the most important determinant in initiating the bioactivities of LPS. Using a variety of biological assays (pyrogenicity, lethal toxicity, and induction of the dermal Shwartzman reaction), it was demonstrated that natural, highly purified lipid A molecules from *E. coli* and *Salmonella* and the structurally equivalent synthetic lipid A (LA-15-PP or LA-16-PP, respectively) were far more potent than analogs with altered fatty acids [20]. The analysis of synthetic lipid A analogs with and without acyloxyacyl groups showed the vital importance of these ester-linked secondary fatty acids in nearly all of the biological assays tested. Compound LA-15-PP, which has two secondary fatty acids (comparable to wild type lipid A of *E. coli*), was the most potent, followed by compounds with one (LA-20-PP and LA-21-PP) and then three (LA-16-PP) acyloxyacyl groups, as determined in chick embryo lethality, pyrogenicity, and local Shwartzman assays. Compounds with no secondary fatty acids (LA-14-PP) were 100 times less potent than natural lipid A (Table 1.1) [16;21].

Endotoxic Activities of Synthetic Lipid A and Disaccharide-Type Lipid A Precursors

Compound	Lethal toxicity		Pyrogenicity (minimum effective dose, μg/kg)	Local Shwartzman reaction (preparatory activity)
	Chick embryos	Galactosamine-loaded mice		
LA-16-PP	–	+ + +	0.1–0.4	+
LA-15-PP	+ + +	+ + +	0.001–0.01	+ + +
LA-20-PP	ND	ND	0.01	+ +
LA-21-PP	ND	ND	0.05	+ +
LA-14-PP	–	+ + +	0.4–3.0	–

Table 1.1– *A comparison of the bioactivities of several synthetic lipid A and disaccharide-type lipid A precursors* [16]. *ND, not determined.*

In addition to the bioactivities of LPS mentioned above (pyrogenicity, tissue toxicity, and the dermal Shwartzman reaction), LPS is known to modulate many immune functions. The use of various chemically synthesized lipid A molecules (listed in Table 1.0) in a variety of *in vivo* and *in vitro* immunoassays yielded some interesting results. Compound LA-14-PP, which lacks secondary (acyloxyacyl) groups, retained nearly all of its *in vivo* immunostimulatory ability (Table 1.2) [16]. For example, compound LA-14-PP was only deficient in activating the human complement pathway and stimulating the production and secretion of human IL-1 [16;21].

Bioactivity (test animal)	LA-15-PP (2)**	LA-14-PP (0)***
Assays *in vivo*		
Adjuvant activity	+ + +	+ + +
Antibody response (mouse)	+ + +	+ +
DTH response (GP)	+ + +	+ +
Antitumor (mouse)	+ + +	+ + +
Analgesic (mouse)	+ + +	+ +
IFN induction (mouse)	+ + +	+ + +
TNF induction (mouse)	+ + +	+ +
Assays *in vitro*		
Mitogenicity (mouse)	+ + +	+ +
Macrophage-PGE_2 (mouse)	+ + +	+ +
Macrophage-IL-1 (mouse)	+ + +	+ +
Macrophage-O_2- (GP)	+ + +	+ +
Macrophage-GlcN (GP)	+ + +	+
Macrophage-IL-1 (human)	+ + +	-
Complement (human)	+ + +	-

Table 1.2 – *A comparison of immunostimulatory activities of various chemically synthesized lipid A molecules* [16]. *The absence of secondary fatty acyl chains (LA-14-PP) did not alter the mouse or guinea pig immune response in several assays tested. However, LA-14-PP was unable to stimulate the production or secretion of human IL-1 or to activate human complement.* ***(2)**, 2 acyloxyacyl groups; (0)***, zero acyloxyacyl groups (dLPS); GP, guinea pig; ND, not detected.***

It has been suggested that, upon ingestion by phagocytes, Gram-negative bacteria are rapidly killed. LPS, on the other hand, can be detected for days to months later [22]. Munford hypothesized that LPS might be catabolized within the cell and he began searching for enzymes (lipases) that might remove fatty acyl chains from lipid A. At this time, the correct structure of lipid A had not yet been elucidated and secondary fatty acyl chains were not known to exist. In order to begin his studies, he obtained a *Salmonella typhimurium* mutant, PR122 (from Paul Rick at USUHS). This strain lacks glucosamine deaminase and is therefore unable to metabolize glucosamine to glucose, so it incorporates radiolabeled glucosamine (N-Ac-^{14}C-glucosamine) into the carbohydrate backbone of LPS. Other bacterial components might also be labeled in this process, but are removed during LPS purification. In addition to labeling the carbohydrate backbone of lipid A, the fatty acyl chains were biosynthetically labeled with ^{3}H by adding ^{3}H-acetate to the bacterial growth medium. The resulting purified LPS (double-labeled LPS substrate) has a ^{14}C-labeled carbohydrate backbone and tritiated fatty acyl chains. It was only after the demonstration that neutrophils released the non-hydroxylated fatty acyl chains from the double-labeled LPS substrate that Wollenweber *et al*. reported the existence of acyloxyacyl linkages in lipid A [23].

Acyloxyacyl Hydrolase (AOAH)

In 1983, Hall and Munford identified an enzyme from neutrophils that partially deacylated *Salmonella typhimurium* LPS [12]. This enzyme, acyloxyacyl hydrolase (AOAH), was present in the granule fraction of human neutrophils and could remove the secondary

(nonhydroxylated laurate, myristate, and palmitate) fatty acids from the lipid A backbone of LPS. The primary fatty acids (3-OH-14:0) remained linked to the diglucosamine backbone [12].

In subsequent studies, partially purified acyloxyacyl hydrolase isolated from HL-60 cells (human promyelocytes) was used to deacylate LPS. The partially deacylated LPS product is termed deacylated LPS (dLPS); its lipid A moiety structurally resembles compound LA-14-PP (also called 406, precursor 1b, and lipid IVA). The dermal Shwartzman reaction, an assay for tissue toxicity, was utilized to test the potency of normal and AOAH-deacylated LPS (dLPS). In this assay, New Zealand White rabbits were injected intradermally with either enzyme-treated or control LPS, followed by an intravenous dose of LPS 20-24 hours later. Rabbits that were given dLPS had no hemorrhagic necrosis at the site of the intradermal injection, while the sites that were injected with intact LPS had lesions of 3 mm or greater. A dose response analysis revealed that dLPS was more than 100- fold less toxic than intact LPS [24]. These results were in keeping with data previously generated from chemically synthesized tetracyl lipid As [16;21].

As mentioned earlier, chemically synthesized lipid A molecules lacking acyloxyacyl-linked fatty acyl chains (compound LA-14-PP) retain their immunostimulatory ability, including the ability to stimulate mouse splenocyte mitogenesis (Table 1.2). In order to verify these results using AOAH-treated LPS (dLPS), Hall and Munford tested the ability of LPS and dLPS to stimulate murine splenocyte division. The results were similar to those obtained with compound LA-14-PP; when splenocytes were incubated with dLPS, their rate of division was reduced by a factor of 6 to 20 as compared to splenocytes incubated with intact LPS [24]. While there was thus an effect on mitogenesis, it was not as significant as the dramatic effect seen between LPS and dLPS in tissue toxicity assays (dermal Shwartzman reaction). Combined, these experiments gave validation to the studies of chemically synthesized lipid A molecules, for the enzymatically

deacylated lipid A was still attached to the core and O-antigen, which was not the case with the chemical derivatives. This work showed the importance of the secondary acyl chains in the bioactivity of LPSs, thus proving that the polysaccharide chain clearly plays a secondary role in the toxicity of LPS.

Since the elucidation of the correct lipid A structure, it has been shown that tetraacyl lipid A analogs, including AOAH-treated LPS (dLPS) and compound LA-14-PP, are able to antagonize LPS in human cells [25]. Deacylated LPS has been shown to inhibit neutrophil adherence to LPS stimulated endothelial cells [26], and also to inhibit prostaglandin E_2 production by neutrophils *in vitro* [27]. These compounds have also been shown to inhibit TNFα release from LPS-stimulated whole blood *ex vivo* [28] and abrogate the ability of LPS to stimulate endothelial cells *in vitro* [29]. Combined, these data clearly show the importance of acyloxyacyl groups in the bioactivities of LPS.

Deacylation of Diverse Lipopolysaccharides by AOAH

Although the general structure of lipid A is highly conserved, LPSs isolated from different bacteria can differ in many ways. The extent of phosphorylation, the polar group modifications of these phosphate groups, and the extent and type of acyloxyacyl groups attached to the lipid A backbone can vary in bacterial species. To study the specificity of AOAH for secondary (non-hydroxylated) fatty acids, the LPSs of *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* were treated with AOAH. Despite the structural differences in lipid A structure (namely the location and nature of the acyloxyacyl groups), AOAH was able to deacylate all LPSs to the same degree (~30% of the

total, or all of the secondary fatty acyl chains were removed). AOAH removed only the secondary fatty acyl chains, regardless of fatty acid chain length or the placement of the secondary fatty acyl chains on the reducing or non-reducing glucosamine. In each case, the primary fatty acyl chains (hydroxylated) remained attached to the lipid A backbone [30].

Purification of Acyloxyacyl Hydrolase (AOAH)

In 1989, Hall and Munford described the purification of acyloxyacyl hydrolase (AOAH). AOAH, when purified from HL60 cells, had the same specificity for secondary fatty acyl chains, a similar pH optimum (4.5), and the same Km (~0.55 μM) as did the original enzyme(s) isolated from the granule fraction of neutrophils [12;31]. The purified enzyme had an apparent size of 52 to 60 kDa and was composed of two disulfide-linked, glycosylated subunits. The AOAH cDNA was cloned in 1991 and the recombinant protein stably expressed in BHK570 cells [32]. Recombinant AOAH retained all of the characteristics of purified AOAH and is used in many of our studies [32].

Basic Structure of AOAH

Studies of recombinant AOAH protein produced a better understanding of the structure of AOAH and some of its supposed functions. Purified AOAH runs as a single band of approximately 60 kDa on a non-reducing SDS-polyacrylamide gel. Reduction of AOAH results in a separation of two subunits, of 50 kDa (large subunit) and ~10-14 kDa (small subunit). The sequence of AOAH reveals five potential (N-glycosylation) sites, one in the small subunit and four in the large subunit [32]. While the roles of such N-glycosylation sites are unknown, it has since been shown that the small subunit glycosylation site is not essential for either enzymatic

deacylation or intracellular localization [33]. The roles of the large subunit glycosylation sites are, at this time, unknown. In an attempt to reduce the complexity of the enzymes structure so that AOAH could be crystallized, several N-glycosylation mutants were constructed. While some glycosylation site mutants retained activity, others did not. Due to the lack of an appropriate antibody for detection by Western blot, it was never determined if the non-functional proteins were actually produced, since removing glycosylation sites may make proteins more susceptible to degradation.

AOAH appears to undergo two proteolytic processing events during maturation. The first is the removal of the leader sequence and an 11 amino acid propeptide just prior to the N-terminus of the small subunit, and the second is a cleavage between the small and large subunits (Figure 1.4) [32].

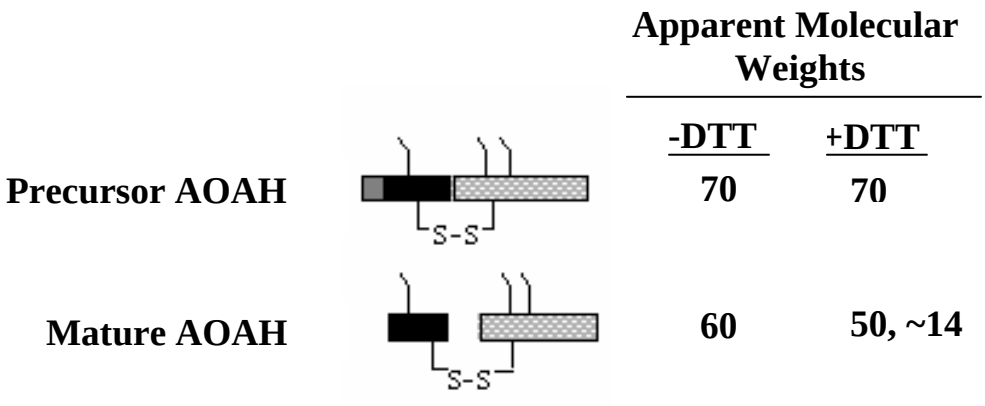


Figure 1.4 *Diagram of AOAH biosynthesis, showing the conversion of the precursor (pro-AOAH) into mature AOAH. Proteolysis removes the leader and pro-peptides and cleaves the precursor into a disulfide-linked heterodimer.*

Large Subunit

The large subunit of AOAH contains the sequence Gly-*X*-Ser-*X*-Gly, which is found at the active sites of many lipases. In fact, AOAH closely resembles members of the GDSL family of lipases, which have a conserved Ser-Asp-His catalytic triad [34] that is thought to be the active site for their various activities. Replacement of AOAH's serine (Ser^{263}) with Leu reduced its activity toward LPS by about 99% [33]. As mentioned earlier, the large subunit of AOAH is glycosylated although the importance of these sites is currently unknown. The large subunit of AOAH is highly conserved as shown (in part) below (Figure 1. 5 A-C).

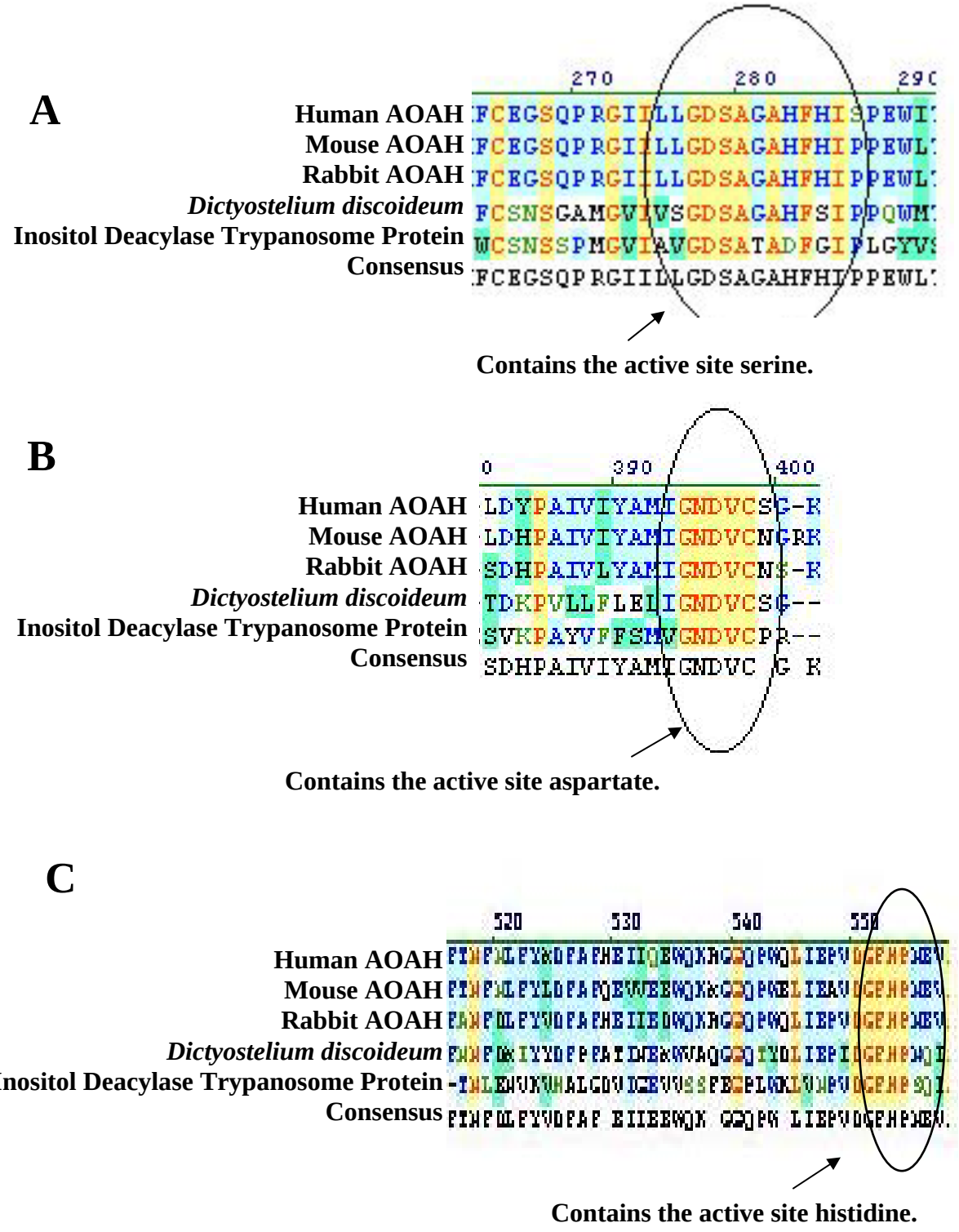


Figure 1.5 -- *Alignments of several stretches of amino acids from the large subunit of AOAH, derived from cDNAs from human, rabbit, mouse, Dictyostelium discoideum, and Trypanosome cDNA. The ovals represent areas of conservation including the serine (A), aspartate (B), and histidine (C) motifs present in all GDSL lipase family members* [34].

Small Subunit

The small subunit of AOAH shares sequence identity with a family of proteins called saposins. All saposin-like proteins (SAPLIPs) have six common cysteine residues and a N-linked glycosylation site that have been hypothesized to form a secondary structure of four disulfide-linked amphipathic helical bundles [35]. All members of the family interact with lipids, but seem to have diverse functions *in vivo*. The small subunit of AOAH is essential for the intracellular location and stability of AOAH and may also be involved in substrate recognition. The evidence for this comes from experiments where only the large subunit of AOAH was expressed in BHK 570 cells. In these cells, AOAH was less stable, less enzymatically active, and did not localize to the same intracellular compartment as did the full-length protein. In addition, a deletion of 32 amino acids within the small subunit of AOAH (including two of the six cysteines) resulted in an unstable protein that had approximately 40% of its native activity [33;35]. As is the case with other SAPLIPs, the function of the N-linked glycosylation is unclear. Deletion of the small subunit glycosylation site by site-directed mutagenesis did not alter the protein's stability, intracellular location, or its secretion. It did, however, increase its activity toward LPS by about 3-fold [33].

Precursor vs. Mature AOAH

Studies such as those described above were extremely helpful in understanding the structure-function relationships of AOAH, but they were not complete. When stably expressed in BHK 570 cells, recombinant AOAH is secreted into the culture supernatant as a approximately 70 kDa propeptide or precursor protein. The cell lysate fraction contains both the precursor and the previously described mature form of AOAH (~60 kDa). It was hypothesized

that, during maturation, the propeptide underwent intracellular proteolytic cleavage. In order to test this hypothesis, partially purified recombinant precursor was treated with trypsin or chymotrypsin, followed by analysis by a reducing or non-reducing SDS-PAGE. Both trypsin and chymotrypsin mimicked natural proteolytic cleavage, causing AOAH to separate into its large and small subunits on reducing SDS-PAGE. Maturation of AOAH (either naturally or via chymotrypsin treatment) increases its ability to deacylate LPS by 10- to 20- fold. Although AOAH is able to remove fatty acids from glycerophosphatidylcholine (GPC) *in vitro* [36], its activity toward this substrate is not altered by maturation [37].

Mannose 6 Phosphate Residues and Receptors

Newly synthesized proteins that contain an Asn-X-Ser/Thr motifs are covalently modified in the trans-Golgi network (TGN) by the addition of Asn-linked sugar chains that often contain mannose-6-phosphate (M6P) residues. Such proteins are recognized by mannose-6-phosphate receptors in the TGN and are either targeted to endosomes/lysosomes or are secreted from the cell [38]. Two mannose-6-phosphate receptors have been described. The first, an integral membrane glycoprotein with an apparent molecular weight of 215,000, binds M6P containing proteins independent of divalent cations. This receptor also binds insulin-like growth factor II (IGFII) and thus the receptor has been termed the CI-M6P/IGFII receptor. The second M6P receptor is also an integral membrane glycoprotein with an apparent molecular weight of 46,000. Because of its enhanced ligand binding affinity in the presence of divalent cations it has been termed the cation dependent (CD)-MPR [39;40]. As shown in Chapter Two, the secreted form of AOAH (pro-AOAH) uses M6P receptors on the plasma membrane to gain entry into cells.

Other Known Activities of AOAH

In addition to its role in deacylating LPS, the enzyme has been shown to have several other activities *in vitro*. AOAH preferentially cleaves saturated fatty acids from glycerophospholipids, lysophospholipids, and diacylglycerol with little to no preference for position (sn-1 vs sn-2, the names given to the two fatty acyl chains present in the above compounds). These studies were done by incubating either native or recombinant AOAH with glycerophospholipid substrates that had either saturated or unsaturated fatty acid chains in the sn-2 position and a saturated fatty acid at sn-1. When the sn-2 fatty acid was unsaturated, AOAH released only the saturated fatty acid from sn-1. When both sn-1 and sn-2 were occupied by saturated fatty acids, AOAH was able to release both saturated fatty acids [36]. These results indicated that fatty acid structure, and not position on the carbohydrate backbone, determined the enzyme's specificity. It was also shown that AOAH was able to transfer acyl chains to several lipid acceptors, and that the presence of free fatty acids in the reaction mixture did not inhibit such transfer. This suggests that AOAH is transferring fatty acyl chains from donor to acceptor rather than non-specifically associating with any available, free, fatty acyl chains [36]. These findings raise the possibility that AOAH may have a function(s) other than deacylating (detoxifying) LPS *in vivo*.

Localization of AOAH

As described earlier, AOAH was first detected in the granule fraction of human neutrophils and later purified from the HL-60 human promyelocyte cell line. Since its purification, AOAH has been detected in human and mouse myeloid lineage cells such as monocytes, macrophages, and, more recently, dendritic cells. My dissertation work will describe

the detection of AOAH in renal proximal tubule cells. This is the first description of AOAH in a non-myeloid cell. Such a finding encouraged me to study the role of AOAH in the urinary tract and the following paragraphs will discuss the current literature on such infections and some general properties of renal proximal tubule cells.

Kidney Architecture and Renal Proximal Tubule Cells

Our entire blood volume is filtered through the kidneys about 65 times each day. In doing so, the kidney(s) regulate our water and electrolyte balance and eliminate many metabolic waste products. The functional unit of the kidney, the nephron, is responsible for such functions and is composed of many cell types. Renal proximal tubule cells function within the kidney to regulate the water, ion, and small molecule concentrations in the blood. They are the first cell type in the nephron to actively reabsorb and secrete such molecules, which are filtered from the blood in the glomerulus. In doing so, they help to maintain the osmotic pressure and ionic composition of the fluids of the body [41].

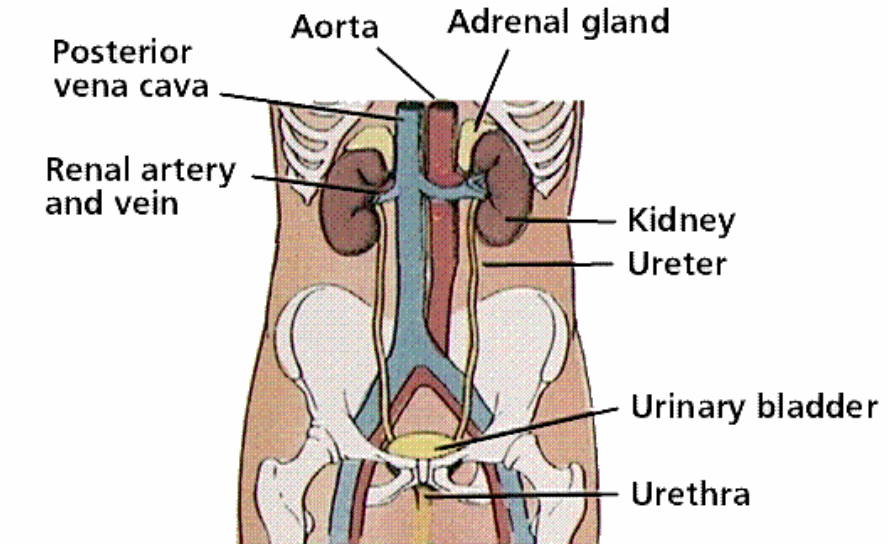


Figure 1.6 – *A diagram of the human urinary tract. Bacteria normally enter through the urethra and may ascend into the bladder, ureter(s), and kidneys(s). This figure was obtained from the website:* ***mcdb.colorado.edu/courses/ 3280/class08.html****.*

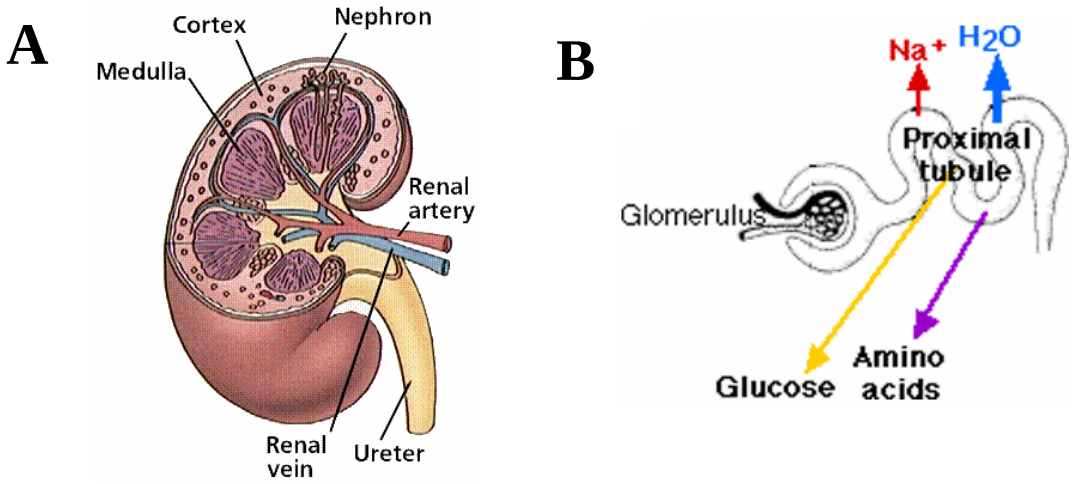


Figure 1.7 – ***Panel A****: a diagram of the kidney, showing the cortex, medulla, and placement of the nephron.* ***Panel B****: a schematic of the glomerulus and proximal tubule. One of the functions of the proximal tubule is regulate water and electrolyte balances in body fluids, and to do so, actively secrete and reabsorb water and other essential molecules from the glomerular filtrate. These pictures were obtained from the following website:* ***mcdb.colorado.edu/courses/ 3280/class08.html****.*

Urinary Tract Infections

Urinary tract infections (UTIs) are a significant cause of morbidity in the developed world and are one of the most common reasons for clinical visits to primary care, hospital, and extended-care facilities [42]. Cystitis, or bladder infection, is the most common manifestation of urinary tract infection. Symptoms include frequent or urgent voiding and suprapubic pain. Pyelonephritis, or infection of the kidney, is a more serious complication of urinary tract infection because of the destruction of kidney cells and the potential of the bacteria to enter the bloodstream. Symptoms of pyelonephritis include all of those described for cystitis plus flank pain, nausea, vomiting, fever, sweats, and malaise [43]. Pyelonephritis sometimes leads to bacteraemia [44]. UTIs affect women more frequently than men, probably due to the anatomy of the female urinary system as compared to that of the male (the female urethra is shorter and in closer proximity to areas of bacterial colonization such as the colon). It is estimated that one-third of American women will have a UTI before the age of 65 and that, of those women, 25 to 30% will have one or more recurrences within 3 to 6 months of their initial infection [45].

Uncomplicated urinary tract infections (which account for the majority of infections in adult women) are defined as those that occur in otherwise healthy individuals with normal immune status, respond well to antibiotic treatment, and in which recurrences are due to re-infections with strains other than the initial pathogen [44]. Complicated UTIs normally occur in individuals with urinary tract abnormalities and/or immune system functions such as diabetes, AIDS, and liver insufficiency. Complicated UTIs do not respond well to antibiotic therapy and recurrences are often due to relapse with the same pathogen [44;46]. Complicated UTIs will not be discussed in greater detail in this dissertation.

Etiology of Urinary Tract Infections

Approximately 80% of uncomplicated, community-acquired urinary tract infections are caused by uropathogenic *E. coli* (UPEC), which are facultative anaerobic Gram-negative rods. Other Gram-negative bacteria such as *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are also known to cause UTIs, but mostly in individuals with abnormalities in their urinary system or underlying immune dysfunction (complicated UTI). *Staphylococcus saprophyticus*, a facultative anaerobic, Gram-positive coccus, accounts for approximately 10 to 15% of UTIs [44;47]. Because of the prevalence of UPEC as the causative agent of UTIs and the existence of well-established murine models of infection, we chose to focus our studies on UPEC-induced urinary tract infections in the mouse.

The majority of bacteria that enter the urinary system do so via an ascending route; very few reach the kidneys via the bloodstream [44]. They must first gain access to the urethra and, if they survive the battery of host defenses that aim to eliminate them, they may travel to the bladder, ureters, and kidneys (Figure 1.6 and 1.7 A).

Virulence Factors Associated with Uropathogenic *E. Coli*

Adhesins (pili, fimbriae)

E. coli, like many enteric bacteria, is a heterogeneous species with members that differ widely in their ability to cause disease. With that said, strains that are able to colonize the bladders and/or kidneys during urinary tract infections typically have several common features. Arguably the most important virulence factors are the adhesins (also called pili or fimbriae),

which mediate bacterial binding and entry into bladder or kidney epithelium and result in the initiation of cellular inflammatory responses. Without these proteinaceous bacterial appendages, bacteria would be unable to gain a foothold on the host epithelium and would likely not cause disease. Martinez *et. al*. have demonstrated that type I pili are necessary to mediate not only adherence, but also invasion of bacteria into bladder epithelial cells [48]. Several adhesins have been described in the literature, these include type I, P, S, F1C, and Dr fimbriae, Afimbrial adhesin I (AFA I) and III (AFA III), Non-fimbrial adhesin 1 (Nfa-1), M and G-adhesin, and Curli [44]. Here I will describe only the type I and P fimbriae, the two most relevant to my experimental system.

Type I fimbriae

Because of their common occurrence on UPEC, type I fimbriae have been well studied. Although different pili bind specific cellular targets, their structures are strikingly similar. Therefore, the following description applies to both type I and P pili (as well as other adhesins). Genes that encode the structural and non-structural components of pili are located on large operons that usually consist of 9-12 genes. These genes include a structural subunit (Fim A; Pap A), accessory proteins (Fim I, C, D, F, and G; Pap H, C, D, E, and F), regulatory proteins (Fim B and E; Pap I and B), and the adhesin (Fim H; Pap G). The biogenesis of the pilus will not be discussed here, but like that of other adhesins, it involves a chaperone-usher pathway. Adherence and invasion by type I piliated bacteria is mediated by Fim H binding to mannosylated glycoproteins such as CD48, collagens, laminin, and fibronectin which are found a variety of host tissues [43;49]. It has recently been shown that type I pili bind UP1a, an integral membrane glycoprotein located on the lumenal surface of the bladder. In so doing, type I pili

induce exfoliation of the bladder epithelium via an apoptosis-like mechanism [50]. While binding to the bladder seems beneficial for the uropathogen, exfoliation is thought to be an effective innate host defense mechanism, clearing many bacteria from the urinary tract.

P pili

While type I pili can often be isolated from both pathogenic and non-pathogenic bacteria, p pili are rarely isolated from non-uropathogens and are the most commonly isolated fimbriae type from UPEC [51;52]. The adhesin, Pap G, mediates binding to glycolipid receptors (alpha-Gal-beta-(1-4)-Gal moieties), which are found on uroepithelial cells, renal proximal tubules, and renal vascular endothelium [44]. P blood group antigens, which are found on erythrocytes and uroepithelial cells, have also been shown to bind p pili. In fact, women with p-positive erythrocytes are more likely to get UTIs than are women who do not express such antigens. It is believed that p pili bind to the p blood group antigens expressed on uroepithelial cells [44]. Like type I pili, p pili also utilize a chaperone-usher pathway for pilus biogenesis. Studies in cynomogus monkeys have shown the vital importance of the p pilus in colonizing the kidneys. In these studies, p pilus negative strains of bacteria were able to colonize the bladder, but were unable to adhere to or cause pyelonephritis [53]. Likewise, studies in human volunteers have shown that p pili enhance the ability of bacteria to colonize the urinary tract [54]. In contrast, it has been shown that both p piliated and non p piliated strains of bacteria were able to bind to and invade proximal tubule cell *in vitro* [55;56].

Lipopolysaccharide

Particular O-antigens of LPS are often associated with uropathogenicity. The most common UTI- associated O-groups are O1, 2, 4, 6, 7, 8, 16, 18, 25, 50, and 75. In comparison to fecal isolates, UTI isolates are less diverse in their O serotypes (*ie.* similar O-groups predominate in UTI urine cultures). Women with vaginal colonization of serotypes 2, 4, 6, and 75 often develop UTI with these same serotypes. In contrast, women with other vaginal serotypes do not normally experience UTIs [49]. It is currently unknown what is unique about such serotypes. In addition to the O-antigen, the lipid A moiety of LPS is known to play a role in the virulence of type I piliated UPEC. Strains of bacteria that lack functional lipid A moieties are unable to stimulate appropriate inflammatory responses in bladder and kidney epithelial cells *in vitro* [57]. Mice that are unable to recognize LPS due to a mutation in TLR4 do not recruit neutrophils to the urine or bladder tissue and subsequently fail to clear UTIs [42;58-61]. In contrast, despite having a dysfunctional lipid A, bacteria that express p pili are still able to stimulate appropriate cytokine and chemokine responses *in vitro* and in murine models of UTI [57;62]. These data will be discussed in greater detail in Chapter 3 and in the discussion.

Toxins

Most UPEC produce toxins such as alpha-haemolysin (~50%) and cytotoxic necrotizing factor 1 (cnf1) [49]. Alpha-haemolysin is a heat-labile exotoxin that is encoded by genes *hly A, B, C, D,* and *tolC*, which are located on chromosomal pathogenicity islands or on transmissible plasmids [63]. Alpha-haemolysin is a pore-forming cytolysin that lyses erythrocytes by disrupting transmembrane ion gradients, raising intracellular osmotic pressure, and eventually lysing the cell. In addition to lysing red blood cells, alpha-hemolysin is thought to be able to lyse other

mammalian cells. *In vitro* data suggest that it may be able to lyse monocytes and granulocytes, but that it has little activity against lymphocytes [49;64]. Cnf 1, also a common toxin of UPEC, is associated with the O4 and O6 serotypes [65;66]. Cnf 1 affects the host cell cytoskeleton by post-translationally modifying the Rho GTP-binding protein responsible for formation of the cellular microfilament network [67]. In addition, *in vitro* work has suggested that Cnf 1 may increase the phagocytic behavior of epithelial cells, allowing the bacteria more efficient entry into cells [44].

Other virulence factors of UPEC

Uropathogens, like all *E. coli*, require iron for survival and thus encode the siderophores aerobactin and enterobactin, which help them sequester iron from their host. The mechanism by which aerobactin, which is predominately found on enteric pathogens, sequesters iron is well studied. Once secreted, the small siderophore is able to extract Fe^{3+} from host iron-binding proteins and channel it into the bacteria via an outer membrane receptor complex [68]. Enterobactin is expressed by both pathogenic and non-pathogenic bacteria and will not be discussed here [44]. In addition to iron acquisition systems, uropathogens also utilize polysaccharide capsules in their quest for host colonization. UPEC are known to produce type K1, 2, 5, 6, 12, 13, 29, and 51 capsules and to use these polysaccharides to evade host immune recognition and to inhibit opsonization [69]. Small percentages of UPEC, but a high percentage of *Proteus mirabilis*, produce urease. Urease is associated with an increased susceptibility to stone formation and pyelonephritis and acts by hydrolysing urea to ammonia and carbamate. Urease is further able to hydrolyse carbamate to ammonia and carbonic acid, thus increasing the pH of the urine and precipitating previously soluble polyvalent ions [44].

Known Host Defenses to Invading Uropathogens

Although they have been less well studied, several host responses to invading uropathogens are worth noting. UPEC are able to grow in urine despite the low pH, the force of flow during urination, and the high osmolarity. The host produces several inhibitors that are secreted into the urine. Tamm-Horsfall protein (THP), one such inhibitor, is a glycoprotein that binds to S fimbriae leading to the elimination of S-fimbriated strains from the urinary tract [70]. Other constitutive secretory components of normal urine are defensins, secretory IgA, uromucoid, and urea. As previously noted, binding of type I pili to bladder epithelial cells initiates exfoliation of the superficial cells that line the bladder, which is considered an innate host defense mechanism [71]. One of the most important host defenses toward invading uropathogens are neutrophils and macrophages which flux to site(s) of bacterial colonization and contribute greatly to bacterial clearance [43].

Ascending Urinary Tract Infections (UTIs)

In order to study the *in vivo* role of AOAH, I chose to induce unobstructed, ascending urinary tract infections in mice. Mice were the most suitable animal due to existing experimental protocols, the availability of AOAH null animals, and low cost. The method of Hagberg ***et. al***. was chosen and will be described in detail in the methods section of Chapter Three. Briefly, six to ten week old female mice are anesthetized and given a 50 µl injection of UPEC (suspended in PBS) via a soft polyethylene catheter into the bladder [72]. This is by far the most commonly

utilized model of UTI and while other models exist, they may not accurately mimic natural ascending UTI.

The role of toll-like receptor 4 (TLR4) in UTI

Even before the discovery of TLRs, it was known that, unlike C3H/HeN mice, C3H/HeJ mice were hyporesponsive to LPS. It is now known that C3H/HeJ mice have a point mutation in the toll-like receptor 4 (TLR4) gene which renders them unresponsive to LPS stimulation [73;73;74]. When subjected to ascending experimental UTI with UPEC, C3H/HeJ mice are unable to mount appropriate inflammatory responses (ie. neutrophils in urine and bladder and IL-6 in the urine) and fail to clear bacteria as efficiently as do C3H/HeN controls [42;58;59;61]. Recent data have shown that type I piliated bacteria not only invade bladder epithelium but are able to replicate and persist within bladder cells for months [75]. It has been suggested that the failure to recognize LPS contributes to the prolonged bladder colonization seen in infected C3H/HeJ mice [61]. Although bacteria have been detected in bladder cells up to six weeks after infection, I have not seen any data to suggest a difference between C3H/HeN and HeJ mice during such prolonged infection. Therefore, the ability to recognize LPS might mediate early or immediate immune responses to invading uropathogens but play a smaller role in more chronic infections. More recent data have suggested that mice that are transgenic for a mutant form of TLR4 in either bladder epithelial or hematopoietic stem cells are unable to mount appropriate inflammatory responses to UPEC and do not clear bacteria from their urinary tracts as efficiently as wild type controls [76]. In this study, $TLR4^{+}$ hematopoietic cells alone were not sufficient to activate appropriate immune responses or clear the bacteria [76]. Combined, these results suggest that TLR4 expression in the urinary tract and on immune cells plays a vital role in the recognition

and clearance of Gram-negative uropathogens. Without LPS recognition, mice are unable to mount appropriate innate immune responses that are necessary for bacterial clearance.

The role of lipid A in experimental ascending UTI

Although little *in vivo* work has addressed the role of lipid A in the establishment or persistence of UTI, several investigators have addressed its role *in vitro*. Polymyxin B (an antibiotic that inhibits the biological activities of LPS), bactericidal permeability-increasing protein (BPI)(a protein known to bind to and inhibit the bioactivities of LPS), and detoxified LPS (derived from a msbB *E. coli* mutant) were all able to reduce the IL-6 and IL-8 response of A498 kidney and 5637 bladder epithelial cells to type I piliated UPEC infection [57;75]. In contrast, the presence or absence of stimulatory LPS made no difference in infections with p piliated strains of bacteria [62]. Mutational inactivation of the *msbB* gene, which encodes an acyltransferase responsible for adding myristate (a secondary fatty acid) onto lipid A precursors, renders LPS non-toxic. Compared to wild type UPEC, *msbB* mutants were unable to elicit characteristic IL-6 or IL-8 inflammatory responses when they were used to activate epithelial cells. As was previously shown with polymyxin B, BPI, and detoxified LPS, the phenotype was seen only in type I piliated bacteria, not with p piliated strains [57;62;71]. This work suggests that type I pili and LPS work in concert to stimulate the epithelial cell inflammatory response to UPEC, but that p piliated strains function in an LPS-independent manner.

The *in vivo* role of LPS in UTI was examined by Frendeus *et al*. in C3H/HeJ and C3H/HeN mice. In this study, it was determined that a *msbB* mutation had no effect on the ability of p piliated UPEC to stimulate neutrophil recruitment into the urine after experimental ascending infection in C3H/HeN mice. C3H/HeJ mice were unable to recruit neutrophils to sites

of infection, regardless of the bacterial *msbB* genotype [62]. The authors did not use a type I piliated strain of bacteria in their studies. They concluded that p pili function *in vivo* in an LPS-independent manner to stimulate the characteristic inflammatory response to UPEC and that, surprisingly, TLR4 is essential for this response.

It is obvious that LPS plays a vital role in the modulation of Gram-negative UTI, since a failure to recognize LPS places mice at an increased risk of prolonged bacterial colonization. AOAH's previously described roles in modulating the bioactivities of LPS and its expression in the urinary tract make UTIs an interesting model to study. Throughout this dissertation I describe my efforts to understand the role of AOAH in the murine urinary tract.

CHAPTER TWO

Identification of Acyloxyacyl Hydrolase, a Lipopolysaccharide-detoxifying Enzyme, in the Murine Urinary Tract

Introduction

Gram-negative bacterial lipopolysaccharide (LPS) is a potent inducer of local and systemic inflammatory responses. Within the urinary tract, members of the receptor complex that initiates inflammatory responses to LPS (CD14 and TLR4) have been detected in uroepithelial cells in the bladder both *in vivo* [77] and in the T24, J82, and 5637 human bladder cell lines [71;77;78]. Murine renal proximal tubule cells, which possess several toll like receptors (TLR1, 2, 3, 4, and 6), CD14 and MD-2, are also invaded by uropathogenic bacteria during ascending urinary tract infections [53;79]. In addition to possessing such pattern recognition receptors, both bladder and proximal tubule epithelial cells are known to secrete IL-6 and IL-8 in response to bacterial and/or purified LPS stimulation [71]. Therefore, epithelial cells in the urinary tract are able to recognize and initiate innate immune responses to invading Gram-negative bacteria.

Acyloxyacyl hydrolase (AOAH) is a lipase that removes secondary fatty acyl chains (lauroyl, myristoyl, palmitoyl) that are substituted to the hydroxyl groups of glucosamine-linked 3-hydroxyacyl residues in lipid A, the bioactive center of LPSs [12]. As discussed in Chapter one, such limited deacylation has been shown to attenuate cytokine and chemokine responses to LPS, in keeping with the important role that acyloxyacyl linkages play in lipid A bioactivity [26;29;80] and

in the ability of Gram-negative bacteria to stimulate inflammation [29;81]. Prior to the experiments described in this report, AOAH had been found in myeloid-lineage cells (*ie.* neutrophils, monocyte-macrophages and dendritic cells) which can deacylate both purified LPSs and the LPS contained in intact Gram-negative bacteria [82-84]. I report here the unexpected finding that AOAH is also produced by renal cortical tubule epithelial cells (probably proximal tubules), which secrete it into the urine, where it can act on LPS. I also present evidence that the proximal tubule-derived enzyme can be taken up by downstream cells within the urinary tract and used by them to deacylate LPS. These observations raise the possibility that LPS deacylation plays a role in limiting inflammatory reactions to Gram-negative bacteria that enter the urinary tract.

Results

AOAH is produced in the kidney

I first determined the abundance of AOAH mRNA in different tissues by using a 1 kb, ^{32}P-labeled fragment of AOAH cDNA to probe a commercially-prepared membrane (BD Biosciences Clontech) that contained polyadenylated RNAs extracted from 8 tissues. We found intense hybridization to kidney mRNA (Figure 2.0a). An identical result was obtained using a different (non-overlapping) 350 bp ^{32}P-labeled fragment of AOAH cDNA as a probe (Figure 2.0b). In a survey of tissue lysates, several wild type murine tissues were homogenized in 0.2% Triton X-100 and assayed for AOAH activity. My results indicate that AOAH activity was also greatest in kidney (Figure 2.1). An analysis of the ethanol-soluble ^{3}H-lipids (from the tissue activity experiment) using thin-layer chromatography confirmed that only secondary acyl chains

were released from radiolabeled LPS, consistent with the known specificity of AOAH [12] (Figure 2.2).

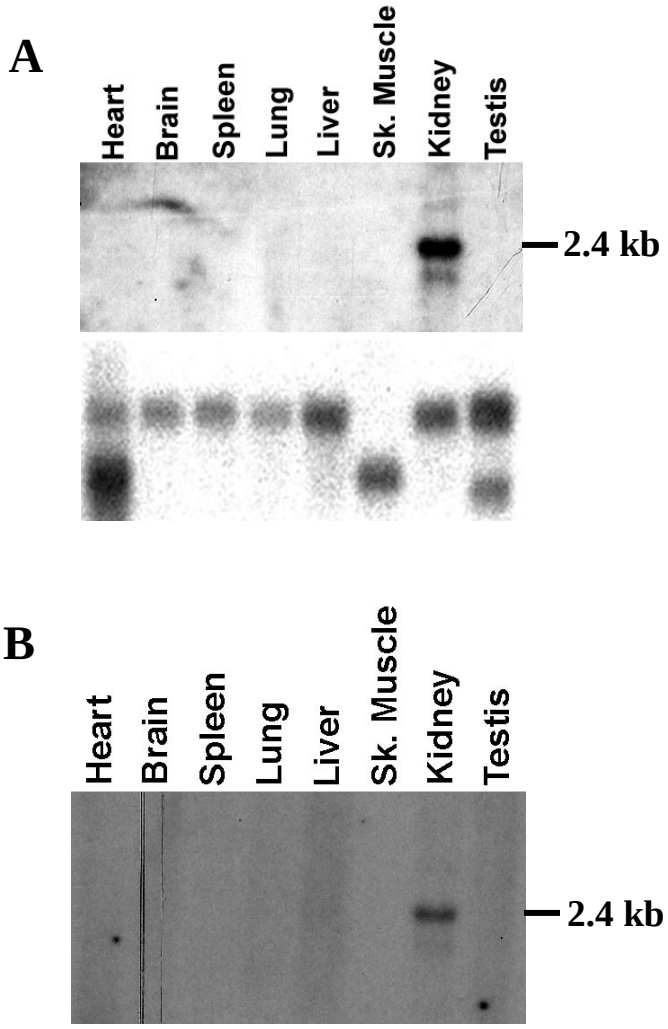


Figure 2.0. A *–AOAH expression in murine tissues. Northern analysis of multiple tissue RNAs (Clontech) using a 1 kb* ^{32}*P-labeled cDNA probe from the 5' coding region of AOAH (top). The blot was stripped and re-probed to detect murine β-actin (bottom).*
B *– Northern analysis of multiple tissue RNAs (Clontech) using a labeled 350 bp cDNA probe from the 3' non-coding region of mouse AOAH.*

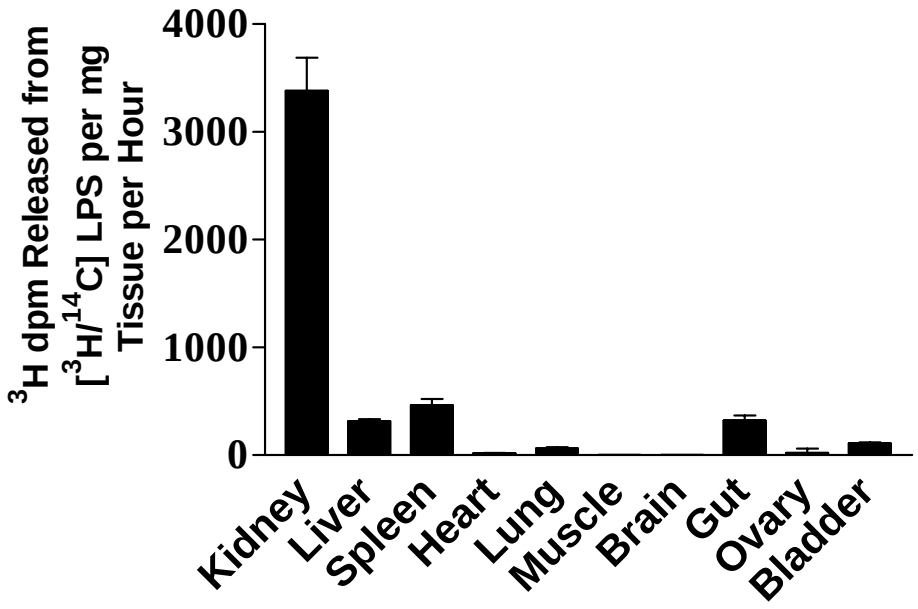


Figure 2.1 - *AOAH activity in lysates of freshly harvested C57Bl/6 mouse tissues. Measurements were performed in duplicate. The bars show standard deviations of data combined from 3 independent experiments.*

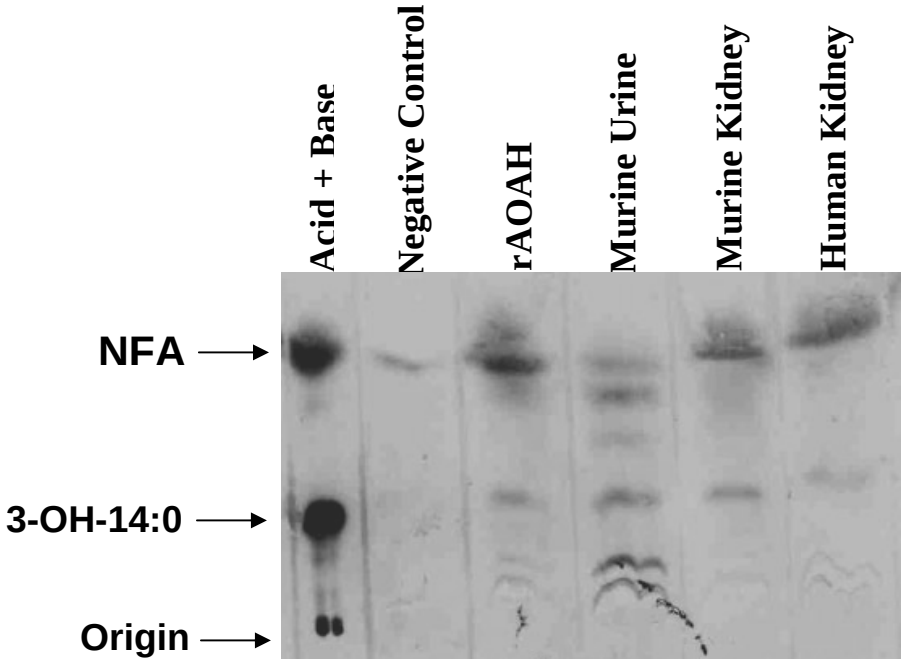


Figure 2.2 – *TLC analysis of the deacylation of LPS, showing that only secondary, non-hydroxylated (NFA) fatty acyl chains are released by purified recombinant AOAH (+ control), murine urine, and murine and human kidney. The negative control contains only PBS, no AOAH source. Acid and base treatment release both primary and secondary fatty acyl chains and serve as a reference.*

By *in situ* hybridization, using an antisense AOAH probe, the mRNA was localized to the renal cortex (Figure 2.3, Panel A and C). No hybridization was apparent in sections from kidneys of AOAH -/- mice [85] (Figure 2.4) or when sense AOAH probes were used (Figure 2.3, Panel B and D). Higher-power views revealed that the silver grains overlay proximal tubule cells and not glomeruli (Figure 2.3, Panel C). These data suggest that AOAH is produced within proximal tubules.

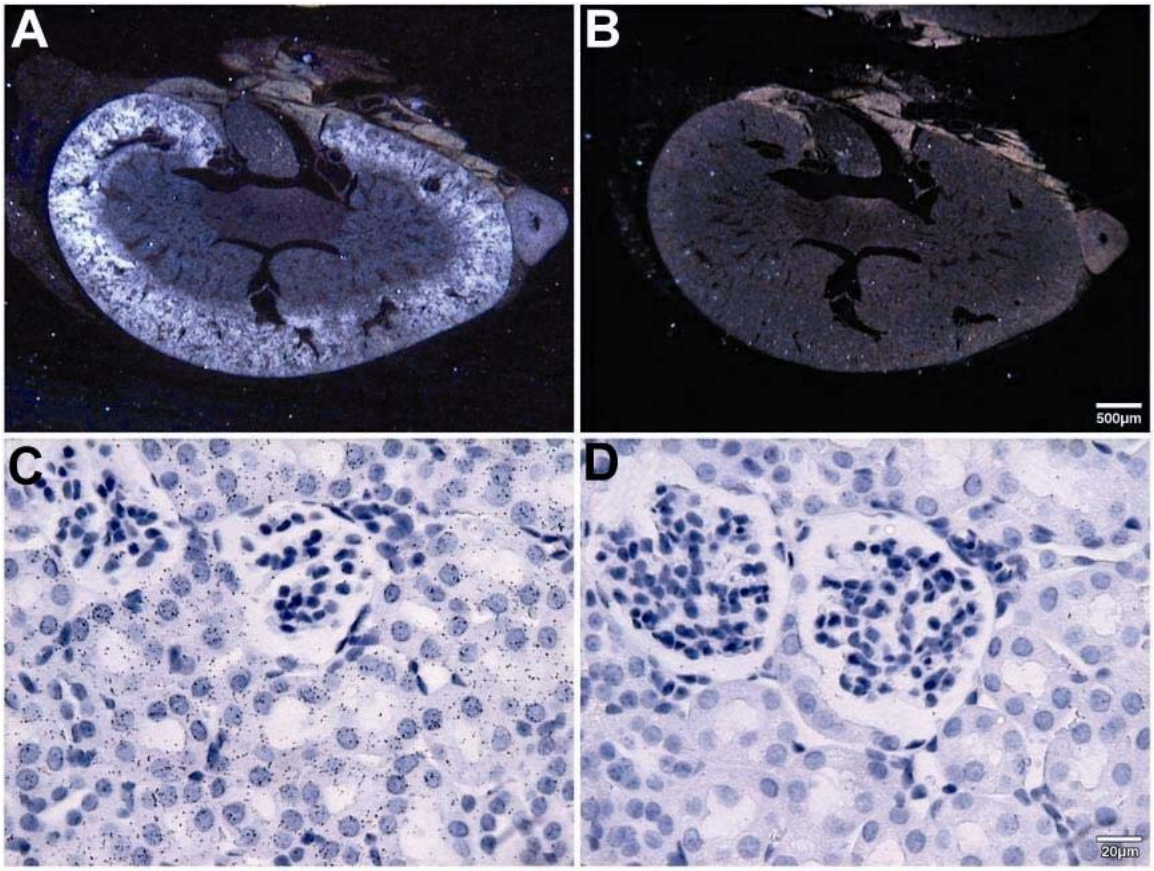


Figure 2.3 - *Localization of AOAH mRNA in murine kidney by in situ hybridization. A and C, antisense probe. B and D, sense probe. The antisense probe hybridized to the renal cortex (panel A) and was found over cortical tubules (panel C). The bars indicate 500 µm (A and B) or 20 µm (C and D). This experiment was repeated 3 times; each experiment used kidney from a different mouse.*

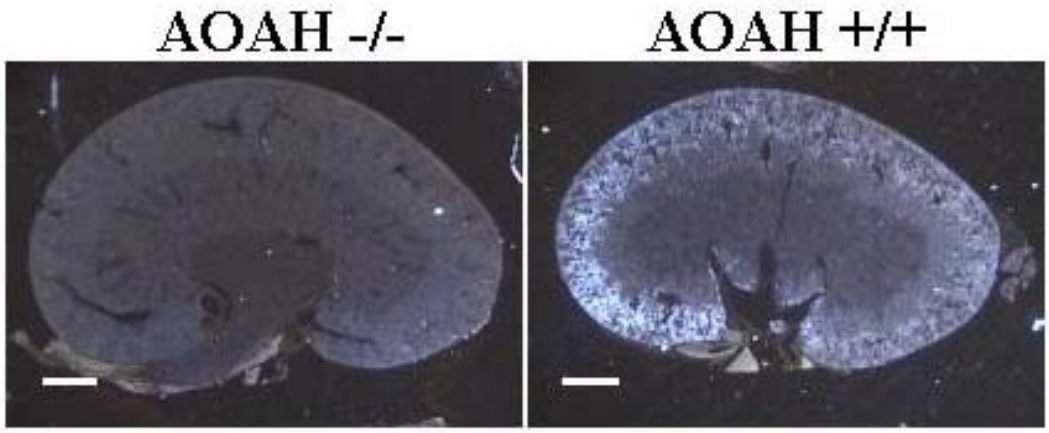


Figure 2.4 – *Localization of AOAH mRNA in wild type and AOAH null murine kidneys by in situ hybridization. The antisense AOAH probe hybridized to the renal cortex of the wild type mouse only. The bars indicate 1mm.*

I next assayed freshly-isolated renal cortex, renal medulla, and urinary bladder for AOAH activity and for the presence of AOAH mRNA. Whereas all of these tissues had AOAH activity (Figure 2.5), AOAH mRNA was detected by real-time PCR exclusively in the renal cortex (Figure 2.6). The lack of AOAH mRNA in the bladder and the presence of the protein in washed sample (no urine) suggests that bladder cells might associate with AOAH secreted from another cell in the urinary tract. However, the presence of AOAH activity in the medulla might be explained by the AOAH that was present in the urine.

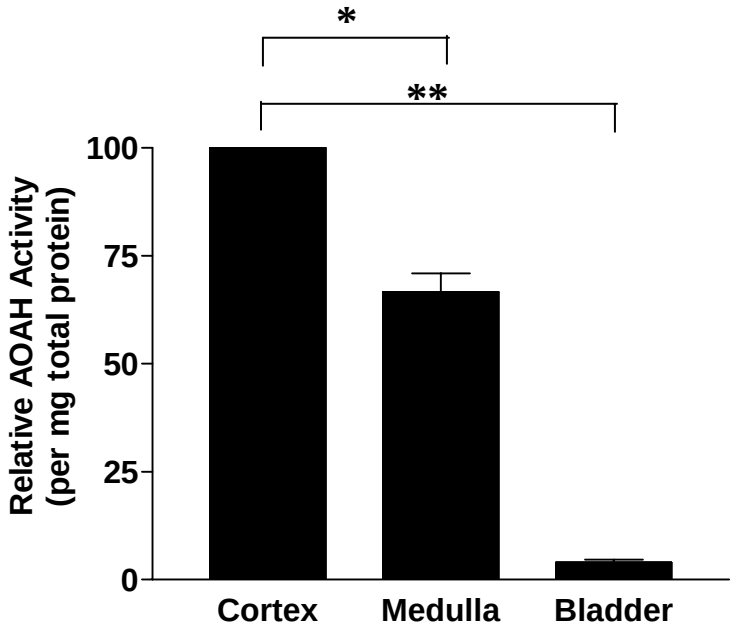


Figure 2.5 - *AOAH activity in lysates of renal cortex, medulla, and washed bladder. Each bar shows the mean and SE of 3 or more measurements. The mean activity per mg protein in 7 bladder samples was 2.5% of the activity in total kidney lysates. Statistics were performed with GraphPad software using a paired, two-tailed, t test. * p=0.159, ** p=0.0002.*

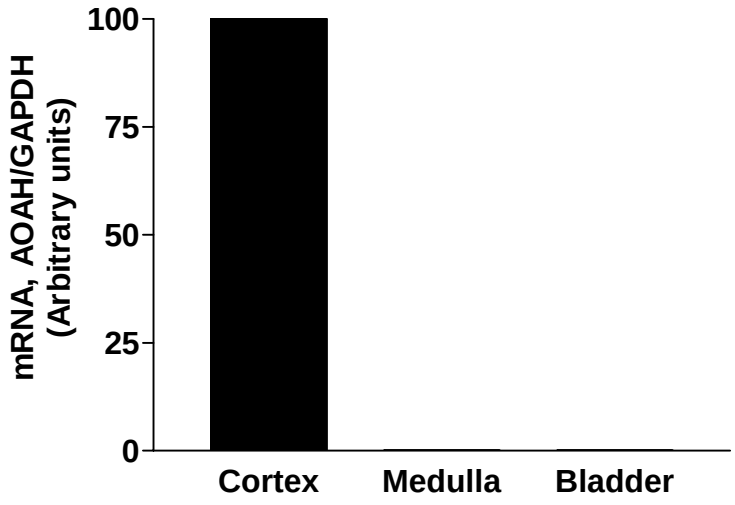


Figure 2.6 - *Real-time PCR analysis of AOAH and GAPDH mRNA in renal cortex, medulla, and bladder. GAPDH mRNA was used as the reference control. The experiment was performed in duplicate and repeated twice, using tissues from different mice, with similar results.*

AOAH is present in human kidney

In order to confirm the presence of AOAH in human kidney, we isolated normal human medulla and cortex from patients undergoing radical nephrectomy for renal cell carcinoma. As expected from the murine data, both cortex and medulla fractions had considerable AOAH activity (Figure 2.7). Thin layer chromatography confirmed that only the secondary fatty acyl chains were being removed by human kidney cell lysates (Figure 2.2), confirming that AOAH is responsible for the deacylation of LPS seen in Figure 2.7.

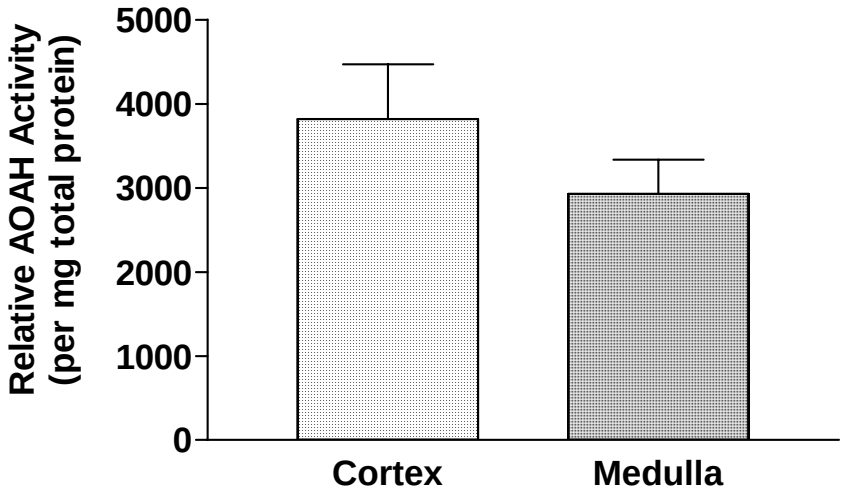


Figure 2.7 – *LPS deacylation by human kidney. Sections of human cortex and medulla were excised from the normal tissue surrounding a renal tumor. Samples were homogenized, diluted in PBS, and the supernatants were incubated with [$^{3}H/^{14}C$]LPS (1ug) at 37ºC for 18 hours before adding ethanol and further steps described in Methods. Background deacylation (no enzyme) has been subtracted.*

When probed with a 786 bp ^{32}-P labeled section of AOAH cDNA, a commercially-prepared (MTN, BD Biosciences Clontech) membrane containing 12 different polyadenylated human RNAs showed no hybridization to the kidney (Figure 2.8). AOAH was detected in the thymus, spleen, liver, placenta, lung, and peripheral blood leukocytes. Because the human kidney is such a large organ, it is possible that none or only a small fraction of the total isolated RNA was derived from the cortex, where we would expect all of the message to be. Comparing the Northern blots of human and murine tissues also suggests that AOAH is more abundantly expressed in myeloid cells in man than in mouse.

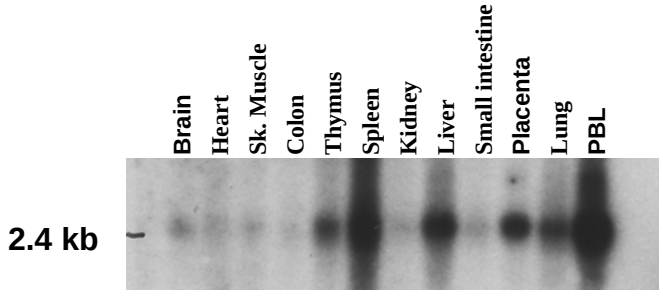


Figure 2.8 – *AOAH mRNA expression in human tissues. A human 12-lane Multiple Tissue Northern Blot (MTN) (Clontech) was probed with a 786 bp ^{32}-P labeled cDNA fragment from the 5' coding region of AOAH. The blot was exposed to Kodak film for 4 days at -70℃ before processing. The blot was probed with beta-actin by Simon Daefler's group and showed equal loading of all lanes (data not shown). The blot was exposed to a phosphoimager screen overnight prior to the experiment and was completely blank.*

Proximal tubule cells secrete pro-AOAH

I next used an *in vitro* system to ask if proximal tubule cells secrete AOAH. In cultured fibroblasts, recombinant human AOAH is synthesized as a precursor (pro-AOAH, apparent Mr =

70,000) that is proteolytically processed to form the mature enzyme, a heterodimer in which large and small subunit peptides are disulfide-linked (Figure 2.9.A). Treatment with dithiothreitol (DTT) cleaves the two subunits, which then migrate at apparent molecular Mr of ~50,000 and ~14,000 when analyzed by SDS-PAGE [33]. Previous studies found that the mature enzyme is 10- to 20-fold more active in deacylating LPS *in vitro* than is pro-AOAH [33]. To study the biosynthesis of AOAH by renal cortical tubule cells, we used a porcine proximal tubule cell line, LLC-PK1 [86]. AOAH was successfully immunoprecipitated from both lysates and culture medium of LLC-PK1 cells that had been allowed to incorporate ^{35}S-methionine and ^{35}S-cysteine for 5 hours. The cell lysates contained both precursor (pro-AOAH, apparent Mr = 70,000) and mature (apparent Mr = 60,000) enzyme, while only the precursor was found in the medium (Figure 2.9.B). As expected, treatment with DTT did not change the size of the precursor but it decreased the apparent Mr of mature AOAH to ~50,000 (only the large subunit is shown). Similar results were obtained by immunoprecipitation and Western blot analysis of media and lysate fractions of LLC-PK1 cells (Figure 2.9.C). Proximal tubule cells thus can release AOAH precursor (pro-AOAH) into their growth medium *in vitro*, suggesting that they may also do so *in vivo*.

Before I found that LLC-PK1 cells make AOAH, we tested several other proximal tubule cell lines for AOAH activity. Human (A498), opposum (OKP), mouse (MCT), and rat (NRK-52E) proximal tubule cells were all AOAH negative. This result suggests that AOAH might be lost as cells differentiate in long-term cell culture. Indeed, I followed the method of Triffilis *et. al* [87] for isolating primary proximal tubules from both human and mouse kidney and found that AOAH activity rapidly decreased as I passed the cells *in vitro*. Investigators wishing to study epithelial responses to LPS or the LPS- induced inflammatory response in such cells should be

aware of the presence (or likely absence) of AOAH, a LPS-deacylating enzyme, in their cell lines.

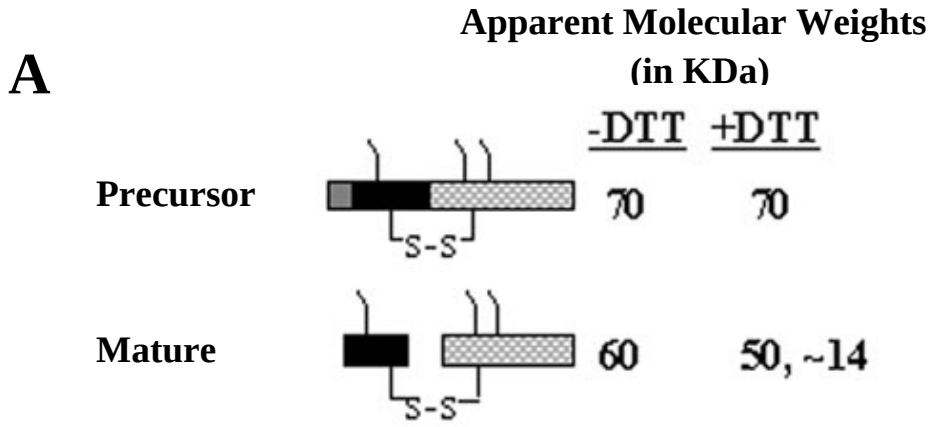


Figure 2.9.A – *Diagram of AOAH biosynthesis, showing the conversion of the precursor (pro-AOAH) into mature AOAH. Proteolysis removes the leader and pro-peptides and cleaves the precursor into a disulfide-linked heterodimer.*

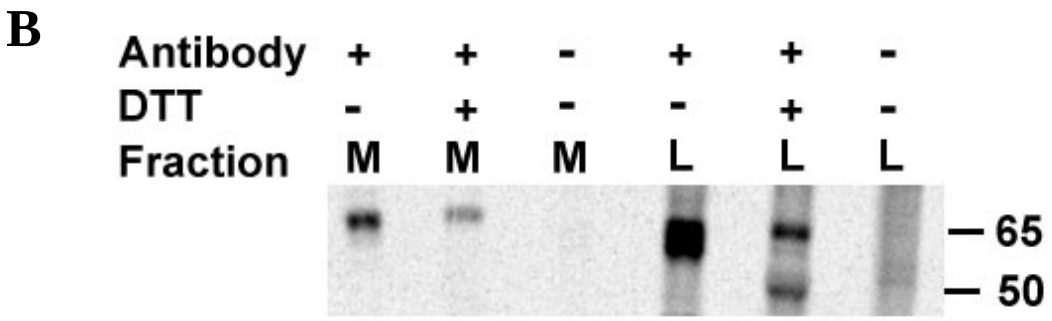


Figure 2.9. B – *Production of ^{35}S-AOAH by porcine proximal tubule cells in vitro. Labeled AOAH was immunoprecipitated with either anti-murine AOAH monoclonal antibody 2F3-2A4 (+), or control IgG (-) as described in Methods and studied by SDS-PAGE (+/- DTT) and autoradiography. M = media, L = lysate.*

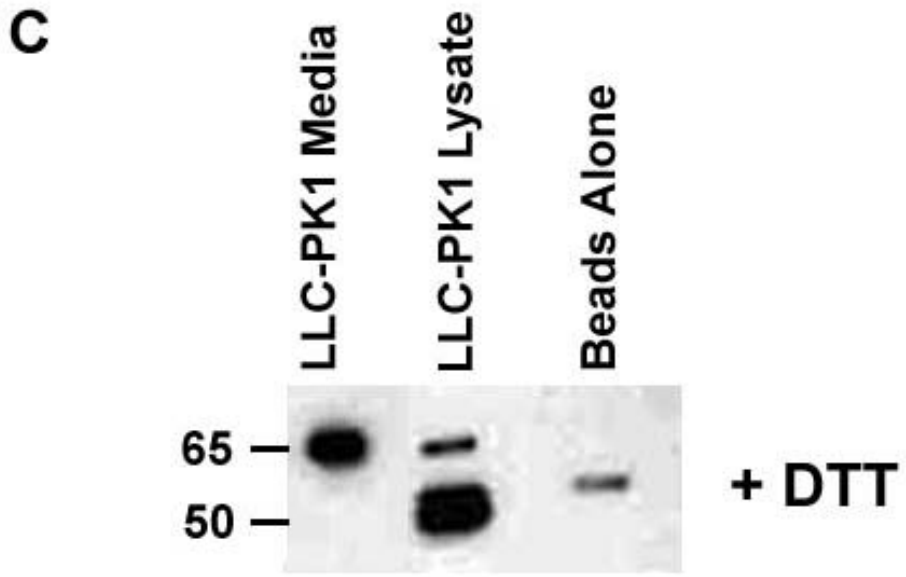


Figure 2.9.C – *Western blot analysis of AOAH production by LLC-PK1 cells in vitro. Media and lysate fractions were immunopreciptated with antibody 2F3-2A4, run on SDS-PAGE in the presence of DTT, and analyzed by Western blot as described in Methods. The ~55 kDa band seen in the lysate and beads alone lane is the heavy chain of the IgG used to immunoprecipitate AOAH. The LLC-PK1 Media lane is underexposed and the cross-reaction (antibody) is not detected.*

AOAH is found in voided urine.

Consistent with secretion of AOAH by cortical tubule cells *in vivo*, AOAH activity was present in freshly voided murine urine (Figure 2.10, 2.11). Urine from AOAH null mice was not active (Figure 2.10), indicating that the deacylating activity detected in wild-type urine is due to AOAH. Thin layer chromatographic analysis of the above reaction revealed that only the secondary, non-hydroxylated fatty acids were removed by wild type urine, in keeping with AOAH being responsible for the deacylation of LPS (Figure 2.2). The reaction mixture used in

AOAH activity assays contains Triton-X, a detergent that is not present in the urinary system. In order to simulate *in vivo* conditions better, I assayed freshly voided murine urine for its ability to deacylate ^{3}H-[LPS] substrate in the absence of detergent. My results indicate that urine is able to deacylate radiolabeled LPS substrate under these conditions (Figure 2.11), suggesting that soluble AOAH may act on extracellular LPS within the urine.

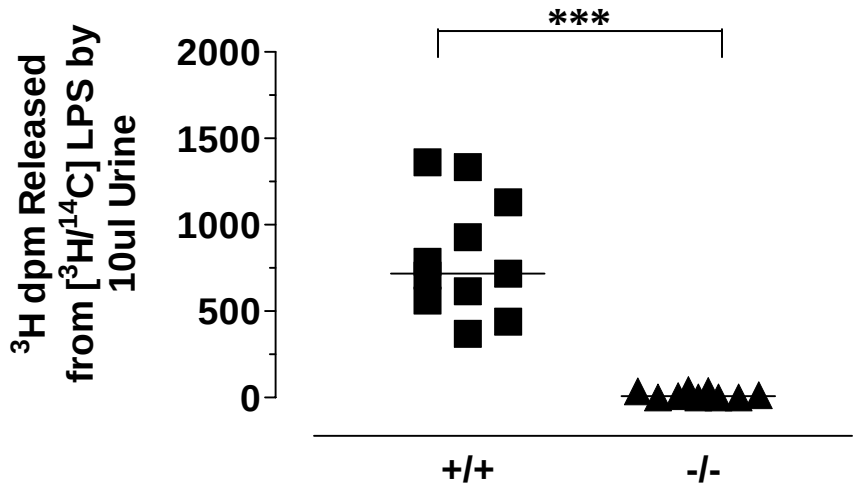


Figure 2.10 – *LPS deacylation by urine from AOAH +/+ and -/- mice. Urine (10 µl) was added to 490 µl AOAH reaction mixture containing 1 µg [^{3}H/^{14}C]LPS and incubated at 37℃ for 18 hrs before adding ethanol and further steps as described in Methods. Statistics were performed with GraphPad software using a two-tailed, paired t test (*** p<0.001).*

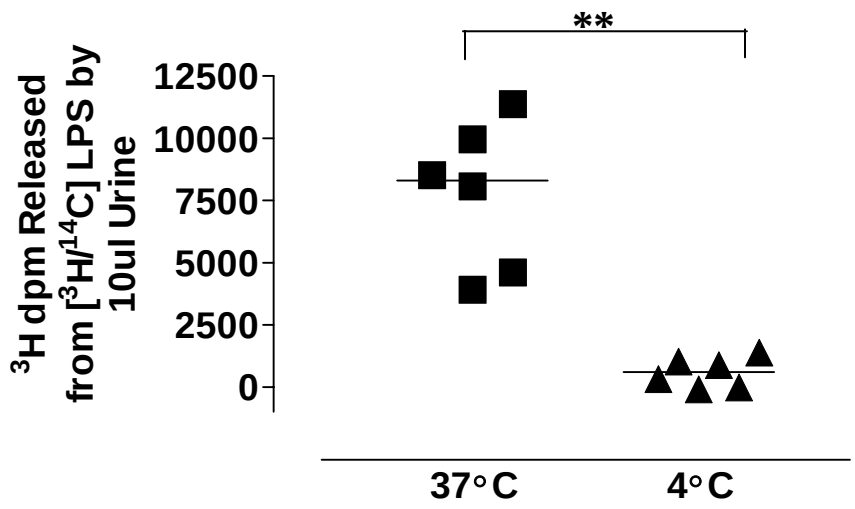


Figure 2.11- *LPS deacylation by murine urine. Fresh urine (10µl) was incubated with [^{3}H/^{14}C]LPS (0.5µg) at 4℃ (control) or 37℃ for 18 hrs. AOAH reaction mixture was then added to provide protein for co-precipitation of intact LPS, followed by ethanol. The remaining steps are described in Methods. Statistics were performed with GraphPad software using a two-tailed, paired t test (*** p= 0.0017).*

To determine if the AOAH in the urine is the precursor or the mature form, I collected urine from both wild type and AOAH null mice, precipitated AOAH with an anti-mAOAH monoclonal antibody, and performed a Western blot as described in Methods. Urine AOAH was found to be in the mature (Mr~60,000, reduced 50,000) form (Figure 2.12). Although the low pH of the urine may create an environment that favors proteolytic cleavage of pro-AOAH as it descends through the urinary tract, I was unable to show that urine from AOAH -/- mice can cleave pro-AOAH *in vitro* (data not shown). Alternatively, epithelial cells might process the precursor and return the mature enzyme to the urine, or mature AOAH may be secreted by

proximal tubules *in vivo*. Some of these experiments will be discussed in more detail later in this chapter.

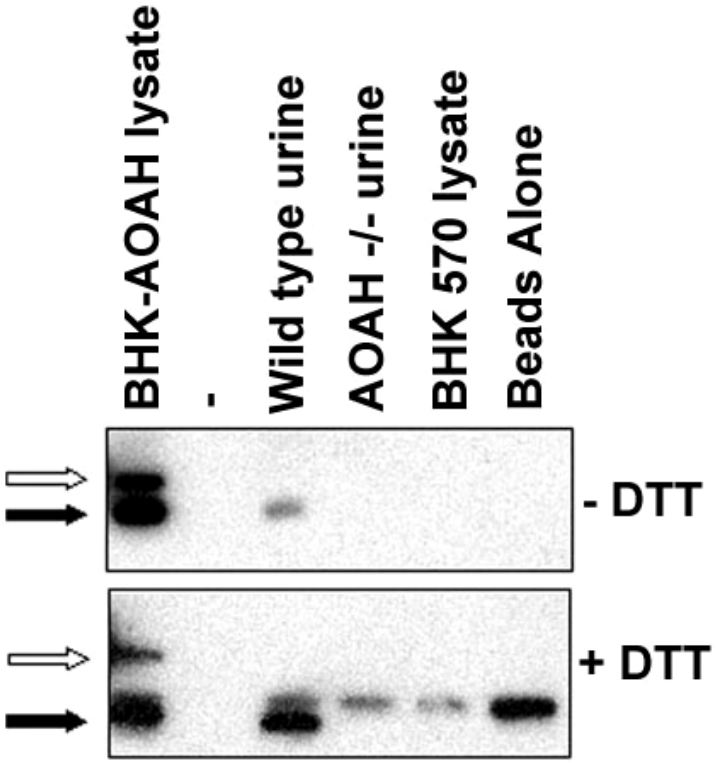


Figure 2.12. *Mature AOAH is found in urine. Equal volumes of wild type and AOAH null urine were immunoprecipitated with an anti-murine AOAH monoclonal antibody and assayed by Western blot as described in Methods. Lysates of BHK cells transfected with AOAH were used as the positive control. The results are representative of 3 experiments with similar results. Note in the upper panel that the BHK-AOAH cell lysate contains both pro-AOAH (open arrow) and mature AOAH (solid arrow). Wild type urine only has mature AOAH. After treatment with DTT (lower panel), mature AOAH migrates with apparent Mr = 50,000 (solid arrow). The band at apparent Mr = 55,000 in the lower panel is the heavy chain of the murine mAb used for immunoprecipitation.*

Bladder cells take up pro-AOAH

In order to test the hypothesis that secreted AOAH could be used by non-expressing bladder cells, I added medium containing pro-AOAH (from confluent AOAH-transfected BHK 570 cells) to cultures of T24 human bladder cells. Binding of AOAH to T24 bladder cells was readily detected, and it could be blocked by adding mannose-6-phosphate (M6P) or ammonium chloride but not glucose-6-phosphate (G6P) or mannose (Figure 2.13. Panels A and B). These results strongly suggest that the uptake of AOAH, a heavily N-glycosylated protein [32;33], is mannose-6-phosphate receptor-dependent. Since the specific activity of the enzyme (^{3}H-fatty acids released from [^{3}H/^{14}C]LPS per μg AOAH protein) was ~50-fold higher in the T24 cell lysates than in the BHK medium (Figure 2.13, panel C), it is likely that pro-AOAH is processed to mature AOAH by the T24 cells [33].

Previous work by Staab and colleagues found that the maturation of AOAH is dependent upon low pH [33]. Her data, obtained by analyzing the cell lysate fractions of AOAH-transfected BHK 570 cells before and after treatment with 10 mM ammonium chloride (NH_4Cl) (an agent known to raise the intracellular pH), showed that ammonium chloride treatment blocked the expression of mature AOAH. In apparent disagreement with these data, I found that ammonium chloride was able to block the association of AOAH with T24 cells, but had no effect on the maturation of the enzyme (specific activity, Figures 2.13. Panel C). Since AOAH uptake is mediated by the M6P receptor and expression of the M6P receptor on the cell surface may be disrupted by ammonium chloride [88;89] ammonium chloride probably prevents the internalization of AOAH, but does not interfere with maturation by proteolytic leavage. This interpretation suggests that AOAH maturation may not be acid-dependent.

We also found that other cell lines ($IMCD_3$ mouse collecting duct, 5637 human bladder, and CHO-CD14 chinese hamster ovary cells) are able to take up pro-AOAH (from either LLC-PK1 or AOAH transfected BHK 570 cells) in a M6P-dependent fashion (Figure 2.14).

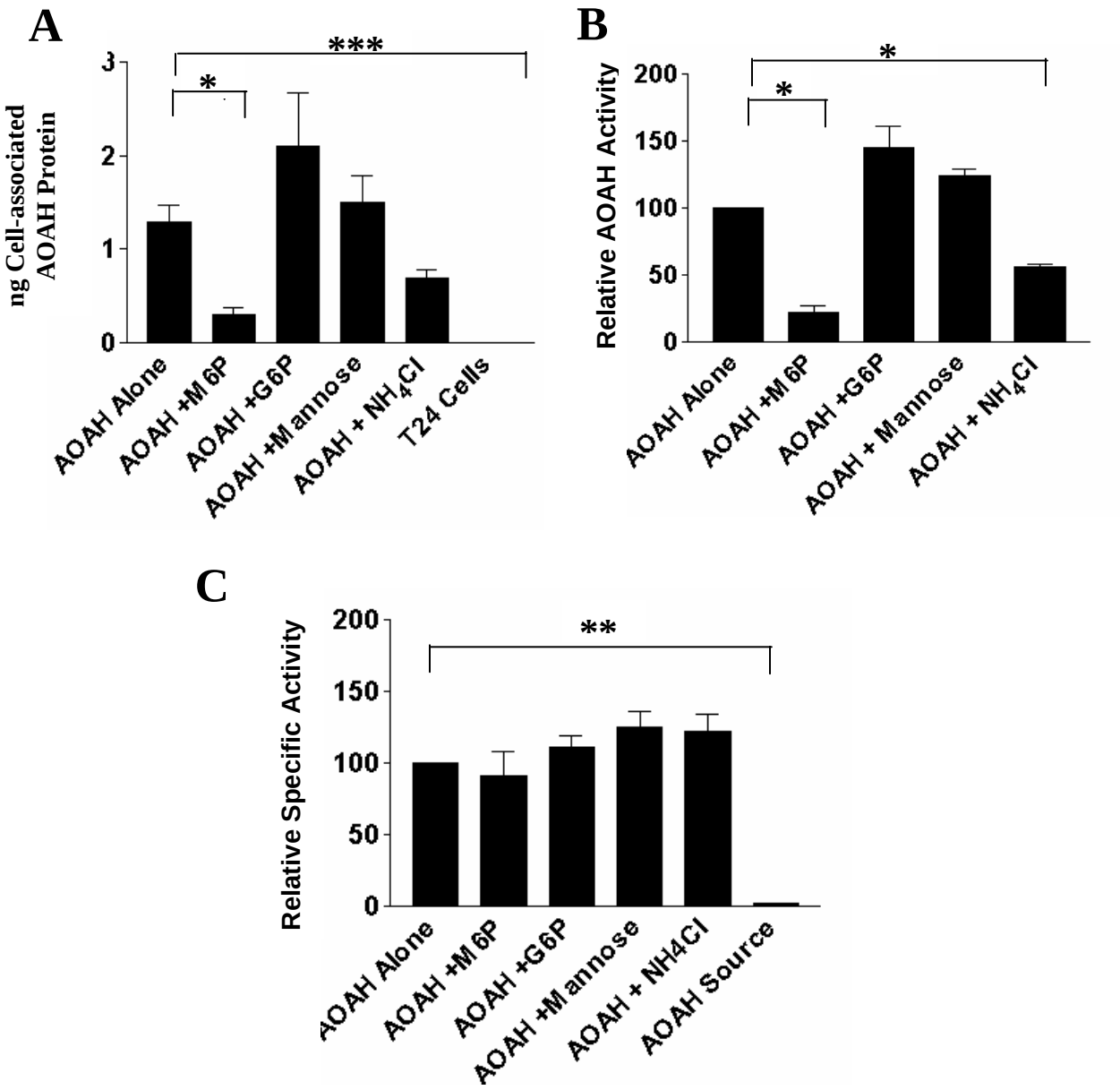


Figure 2.13 – **A**. *Uptake of pro-AOAH by T24 bladder cells after incubation with AOAH-containing medium for 5 hrs in the presence or absence of 10 mM M6P, G6P, NH_4Cl or mannose. Washed cells were lysed and AOAH protein was assayed by ELISA.* **B**. *AOAH activity in T24 lysates, expressed relative to the activity observed in cells that took up AOAH in the absence of inhibitor. Each bar shows the mean and SE of data from 4 independent experiments.* **C**. *AOAH specific activity (activity/ng protein) in T24 cell lysates. Compared with the AOAH added in the medium, cell-associated AOAH had much greater specific activity, reflecting its activation by the T24 cells. M6P inhibited AOAH binding (A) but did not prevent activation of the cell-associated AOAH (C). Statistics were performed with GraphPad software using a two-tailed, paired t test (*p< 0.1, **p< 0.001, ***p< 0.0001).*

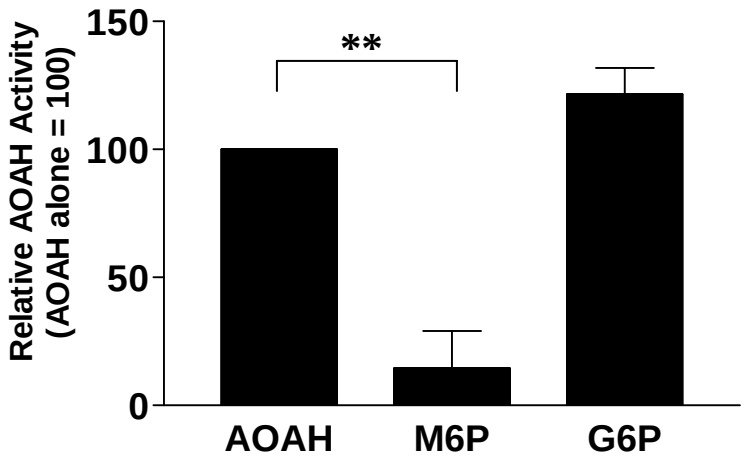


Figure 2.14– *Uptake of LLC-PK1 AOAH by 5637 human bladder cells. Media from confluent LLC-PK1 cells was overlaid onto 5637 bladder cells in the presence or absence of 10 mM M6P or G6P. The ability of the washed cell lysate fraction to remove ^{3}H fatty acids from double-labeled substrate was analyzed at 2 and 5 hours after incubation with AOAH. The bars show standard errors of data combined from three separate experiments. Statistics were performed with GraphPad software using a two-tailed, paired t test (**p=0.002).*

Bladder cells do not re-secrete mature AOAH.

In order to test the hypothesis that epithelial cells within the urinary tract might process precursor AOAH and return the mature form to the urine, the following experiment was performed. Media from confluent AOAH-transfected or untransfected BHK 570 cells was overlaid onto washed T24 bladder cells for 8 or 24 hours. Pre- and post-incubation media were assayed for both activity (ability to remove fatty acyl chains from [$^{3}H/^{14}C$]LPS substrate) and total ng of AOAH (via ELISA) in order to determine their specific activities. If the bladder cells are able to secrete mature AOAH into their culture medium, post-incubation media should have an increased specific activity (activity/ng protein). My results show that the specific activity of the media did not change over time (Figure 2.15), but, as was shown previously, once associated

with the T24 cells, AOAH had a great increase in specific activity. These data suggest that while bladder cells are able to mature AOAH, they do not return it to their medium (or that the culture conditions do not allow such release). These data do not rule out the possibility that another cell type in the urinary tract is responsible for maturing and re-secreting AOAH or that bladder cells may re-secrete AOAH *in vivo*.

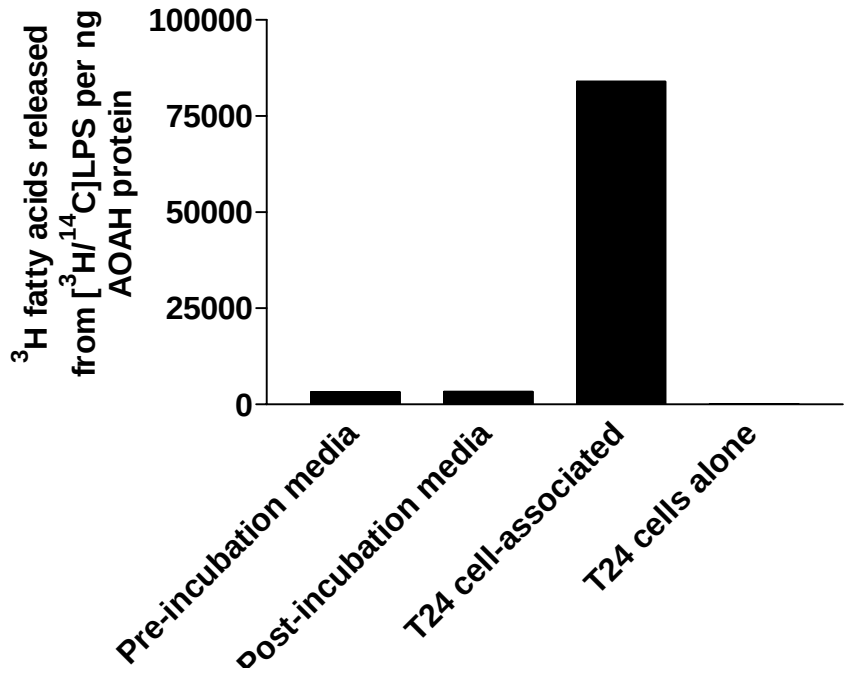


Figure 2.15 – *Bladder cells do not secrete mature AOAH into their medium in vitro. T24 bladder cells were washed with PBS and incubated with AOAH-transfected or untransfected BHK 570 cell media for 8 or 24 hours at 37ºC. Media and lysates were assayed for AOAH activity and total AOAH protein (ELISA) as described in Methods. The experiment was done in duplicate and repeated twice with similar results.*

Bladder cells that have taken up AOAH can deacylate LPS.

I next asked if bladder cells that take up AOAH can use the enzyme to deacylate LPS. T24 cells were incubated with confluent medium from AOAH-transfected BHK 570 cells or medium from confluent, untransfected BHK 570 cells for 5 hours, washed, and reincubated in medium that contained 125 ng/ml [^{3}H]LPS . At 24 and 48 hours, cells were washed and then lysed to measure the cell-associated ^{3}H radioactivity and the fraction of the ^{3}H that was ethanol-soluble (i.e., released from the LPS backbone). As shown in Figure 2.16, acquisition of AOAH allowed the cells to deacylate a significant fraction of the LPS that became cell-associated over time, whereas control cells were unable to deacylate cell-associated LPS.

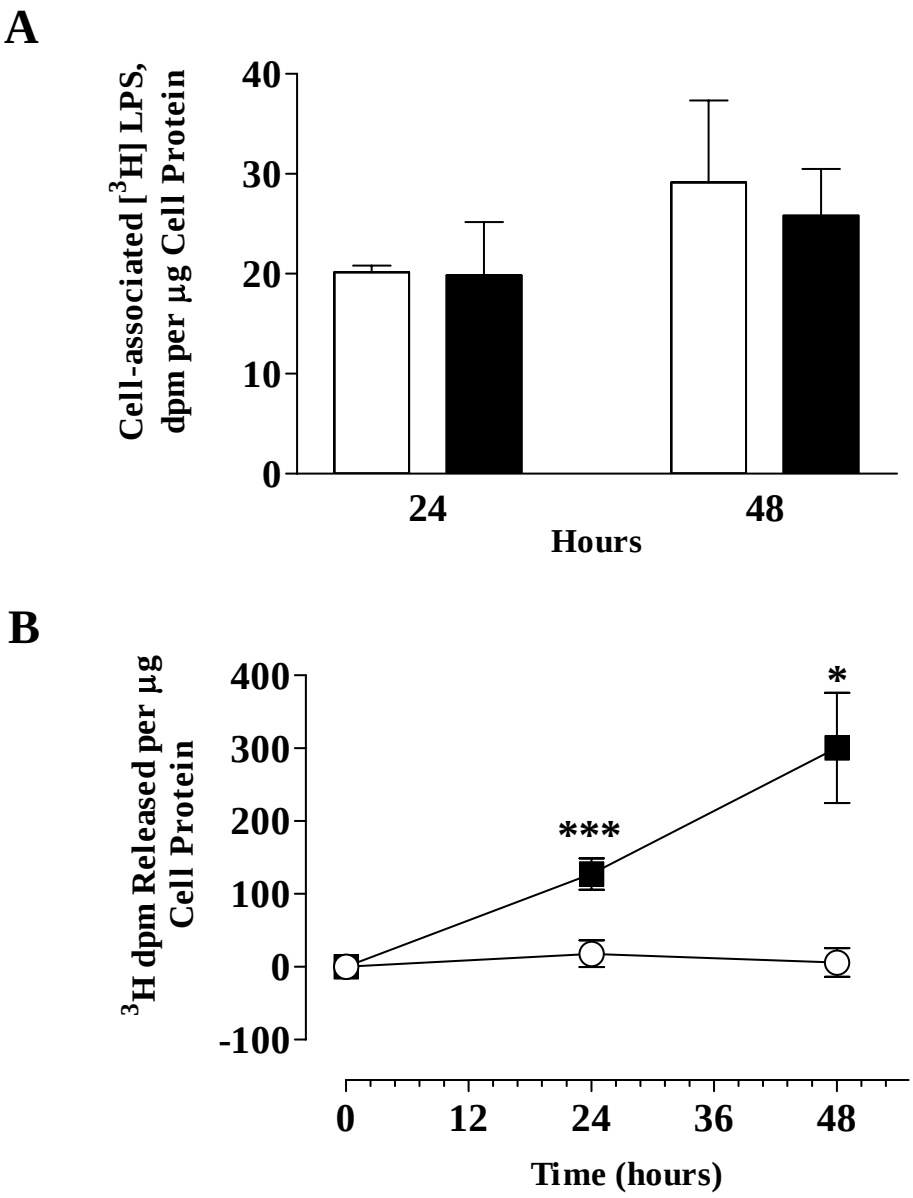


Figure 2.16 – *AOAH confers LPS-deacylating activity to bladder cells. T24 cells were allowed to take up AOAH for 5hrs, washed, and then incubated with ^{3}H-LPS (125 ng/ml) for the times indicated. Control cells were incubated with medium that did not contain AOAH. Whereas control and AOAH-containing cells took up similar amounts of ^{3}H-LPS (A), only the AOAH-containing cells removed ^{3}H-fatty acids from the LPS backbone (B). The data represent combined results of three separate experiments; the error bars represent 1 SEM. Solid squares and bars, T24 cells with AOAH. Open circles and bars, control T24 cells. Statistics were performed with GraphPad software using a two-tailed, paired t test (***p = 0.0009, *p = 0.0147).*

METHODS

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Chemical Co, St. Louis, MO.

Mouse strains. Specific pathogen-free mice were housed in the UT Southwestern Animal Resource Center and fed a standard diet. ICR (Harlan, Indianapolis, IN), C57Bl/6 (Harlan) or 129S6/SvEvTac (Taconic, Germantown, NY) mice were used. AOAH null 129 and C57Bl/6 mice were produced as described by Lu *et al* [85].

Northern Analysis. A Mouse Multiple Tissue Northern Blot (BD Biosciences Clontech) was probed with a ^{32}P-radiolabeled 1 kb (*Asp*718 to *Hind*III) cDNA fragment of the 5' coding region of mouse AOAH cDNA (Genebank # AF018172). The blot was stripped and re-probed with a 350 bp cDNA probe from the 3' (*Ahd*I to *Xba*I) non-coding region of mouse AOAH as above. The blot was then stripped and reprobed with a 500 bp *EcoR*I to *EcoR*I fragment of the murine β-actin cDNA (a kind gift from I. Shimomura) [90]. A Human Multiple Tissue Northern Blot (BD Biosciences Clontech) was probed with a 786 bp ^{32}P- labeled cDNA fragment from the 5' coding region of human AOAH.

AOAH Activity Assays. Three month-old female C57Bl/6 mice were anesthetized (ketamine-acepromazine), sacrificed by cervical dislocation, then selected tissues were rinsed with ice-cold saline and weighed. Each tissue was transferred to a microfuge tube that contained 500 µl lysis buffer (0.2% triton X-100 in PBS, pH 7.2, with 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin, and 2.5

mM EDTA) on ice, and sonicated (Branson Sonifier 450, VWR, West Chester, PA) to complete disruption (approximately 30 sec). The lysates were diluted 1:10 in sterile PBS and 10 µl was assayed for AOAH activity at 37°C as previously described [91], using a double-labeled [^{3}H-acyl chains/^{14}C-glucosamine backbone] *S. typhimurium* LPS as substrate. Mouse urine was assayed using the same reaction mixture [91] or by adding 0.5 µg double-labeled *S. typhimurium* LPS substrate and sodium acetate, pH 5 (final concentration = 3 mM) to 10 µl urine (final volume = 15µl) and incubating at either 4°C or 37°C for 18 hrs.

Thin-layer chromatography. Twenty µls murine urine and 100 µls human or murine kidney lysate (sonicated briefly in 0.1% Triton X-100 in PBS) was incubated in 400 µls AOAH reaction mix plus approximately 1µg purified [^{3} H]LCD25-O9 LPS and incubated overnight at 37°C. A chloroform/methanol extraction was performed to isolate the soluble fatty acyl chains. Briefly, 450 µl of the overnight reaction was placed in a glass tube to which 3.75x volume chloroform:methanol 1:2 (v:v) was added. Samples were vortexed, incubated at RT for 10 min., and extracted in 1.25 volume chloroform, 1 volume water, and 1/100th volume glacial acetic acid. The chloroform layer was removed, washed three times and dried under argon. Samples were resuspended in 50 µl chloroform:methanol 1:1 (v:v) and loaded onto a silica gel G TLC plate. Primary and secondary fatty acids (for reference) were obtained by hydrolyzing LPS sequentially with 4 M HCl and 4 M NaOH. The hydrolyzed fatty acids were extracted into chloroform, dried under argon, resuspended in 50µl chloroform:methanol 1:1 (v:v) and loaded onto a silica G plate. The TLC was run in petroleum ether:diethyl ether:acetic acid; 70:30:1. The plate was sprayed with ^{3}H-enhance, incubated at -70°C for one week and visualized by autoradiography using Kodak film.

Generation of AOAH null mice. C3H/HeN and C57Bl/6 AOAH knockout mice were generated as follows. The murine AOAH gene was disruption in 129/SvEvTac ES cells by inserting a neomycin resistance gene into the first exon of AOAH, eliminating a 705 bp region that encodes untranslated mRNA, the translation start site, the leader and pro-peptide sequences, and 41 amino acids of the small subunit of AOAH. Mouse DNA was screened by Southern blot, using an *EcoR*I-*BamH*I probe derived from the 5' genomic sequence upstream of the long arm of the targeting vector. 129/SvEvTac heterozygous mice were crossbred to the F_2 generation at which time they were crossbred with wild type C57Bl/6 and C3H/HeN mice. Heterozygous mice from each backcross were bred through 8 generations, so that only 0.39% of the genes were of 129/SvEvTac origin. Progeny of F_8 homozygote knockouts and wild type littermates were used in all experiments presented in this thesis.

***In situ* hybridization and riboprobes.** A 1 kb fragment of the 5' coding region of AOAH cDNA (5'-*Asp*718 to 3'-*Hind*III) was inserted into pBlueScript KS+ (Stratagene, La Jolla, CA) at the *Asp*718 and *Hind*III sites. The plasmid was linearized with *Bgl*II and a 650 bp antisense riboprobe was generated by *in vitro* transcripton from the T7 promoter using the Ambion Maxiscript kit (Ambion, Austin, TX). A 517 bp sense riboprobe was similarly generated using the T3 promoter according to the manufacturer's instructions. Briefly, 300 ng of template DNA was transcribed with T7 (antisense) or T3 (sense) polymerase in the presence of 200 μCi of [α -^{35}S]-UTP (800 Ci/mmol) (Amersham, Piscataway, NJ) for 1 hr at 37ºC. DNAse I was added for 15 min at 37ºC and all enzymatic activity was then terminated with 1 μl of 0.5M EDTA. The volume was brought to 75 μl with DEPC water and 50 μl was run through a Rnase-free G-50

column (Roche, Basel Switzerland). The probes were stored at -80ºC and used within 2 days of preparation.

Female ICR mice (Harlan) were anesthetized (ketamine-acepromazine) and tissues were isolated following transcardial perfusion with cold heparinized DEPC-saline and then with chilled 4% formaldehyde/DEPC-PBS, pH 7.4 (freshly prepared from paraformaldehyde). Samples were incubated in 4% formaldehyde for 16 hrs and then transferred to sterile DEPC-saline. Kidneys were dehydrated and paraffin-embedded, and 4 μm sections were placed onto microscope slides treated with Vectabond (Vector Laboratories, Burlingame, CA). Slides were stored desiccated at 4ºC until use. *In situ* hybridization was preformed as previously described [92], using the riboprobes described above. The *in situ* hybridization using wild type and AOAH null mice were performed as above using 129 female mice.

Real-time PCR. Total RNA was isolated from bladders and from pooled renal cortex and medulla fractions obtained from wild type mice (RNAqueous Kit, Ambion, Austin, TX). A region of the AOAH cDNA was amplified using primers Seq_mAOAH-ex12F (CCAACTCTCTGGTGTAACTGGATTT) and Seq_mAOAH-ex12R (TCTCAAACGATGGTAAATGGATTTT). TaqMan® MGB probe (FAM™ dye-labeled) ACGAGTGGAATTGAAG and primers were designed and synthesized by Applied Biosystems (Foster City, CA). Plasmid pMF612, which contains murine AOAH cDNA, was used as a reference molecule for the standard curve calculation. TaqMan® Rodent GAPDH Control Reagents were used to measure GAPDH gene expression. All real-time PCR reactions were performed by M. Lu in a 25 μl mixture with Taqman one step RT-PCR Master Mix Reagents Kit on the ABI PRISM® 7700 Sequence Detection System.

Cell culture. LLC-PK1 porcine proximal tubule cells (ATCC, CL-101) were cultured in low-glucose DMEM (Invitrogen, Carlsbad, CA), 5% heat-inactivated FCS (Hyclone, Logan, UT), penicillin, streptomycin, glutamine (PSG, Invitrogen) at 37ºC in an atmosphere of 5% CO_2. T24 human bladder cells (ATCC, HTB-4) were cultured in Vitacell McCoy's 5a Medium (ATCC) supplemented with 10% FCS and 2% PSG at 37ºC, 5%CO_2. AOAH-transfected BHK 570 cells [33] and untransfected BHK cells were cultured in DMEM with glutamine and 4.5 g/L glucose (Fisher Scientific), 10% FCS, and 2% PSG as above. 5637 human bladder cells (HTB-9) and CHO K1 (CCL-61) were obtained from ATCC and $IMCD_3$ cells were a kind gift of Dr. Christopher Lu (UT Southwestern).

Antibodies. Rabbit anti-human AOAH polyclonal antibodies (E553) bind porcine, but not murine, AOAH. The available murine monoclonal antibodies to human AOAH also do not bind murine AOAH. After numerous unsuccessful attempts at producing antibodies to murine AOAH in rabbits, we (W. Lai, Dept. Pathology) immunized AOAH -/- mice thrice, at monthly intervals, with 100 μg of a plasmid that expressed the murine AOAH cDNA from a CMV promoter. We then administered approximately 10^9 plaque-forming units of an adenoviral vector that produces murine AOAH, prepared as previously described [93]. When the mice still did not have high anti-AOAH antibody titers, we (Mei Zhang) expressed the large subunit of murine AOAH in *E. coli* using the pET-30 LIC vector (Novagen); inclusion bodies were subjected to preparative SDS-PAGE and bands containing recombinant AOAH were cut out and injected, emulsified in complete or incomplete Freund adjuvant, at monthly intervals (3 injections). Although the antibody titers remained low, we fused spleens from immunized mice with SP 2/0-

IL-6 myeloma cells and screened the resulting hybridomas to isolate anti-murine AOAH IgG antibodies. Approximately one liter of hybridoma supernatant was purified on a protein A Sepharose column and concentrated using an Amicon Centricon YM-100 (Millipore, Bedford, MA). The purified antibody, 2F3-2A4, is a mouse anti-mouse AOAH monoclonal antibody of the IgG2a, kappa isotype.

Immunoprecipitation of biosynthetically radiolabeled AOAH. One million LLC-PK1 cells were seeded into Costar brand 75 cm^2 tissue culture-treated flasks (Corning, Acton, MA). Two days later, the cells were washed twice with pre-warmed, sterile PBS, pH 7.2, and incubated with methionine- and cysteine-free DMEM (Sigma) at 37ºC, 5% CO_2 for 20 to 30 min. 1 mCi ^{35}S-labeled methionine and cysteine (1175 Ci/mmol) (ICN, Costa Mesa, CA) was then added to 5 ml pre-warmed, methionine- and cysteine-free DMEM with 50 µl dialyzed heat-inactivated FCS (Hyclone, Logan, UT) and overlaid onto washed LLC-PK1 cells. Incubation was continued at 37°C in 5% CO_2 for 5 hrs. The flasks were then placed on ice, the medium was removed and saved at 4°C, and the monolayers were washed thrice with PBS, pH 7.2. I then added 0.6 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 8.0, 10 mM PMSF and 2.5 mM EDTA) on ice, rocking occasionally for 20 min. The cells were pelleted by centrifugation (5 min, at 4°C) and the lysate supernatants were transferred to fresh microfuge tubes. The media and lysates were pre-cleared with 50 µl of a 50% slurry of Pansorbin (CalBiochem, La Jolla, CA) that had been allowed to bind non-immune rabbit IgG. Specific immunoprecipitation was then performed using rabbit anti-human AOAH IgG bound to protein A Sepharose CL-4B (Amersham Biosciences, Uppsla, Sweden).

Immunoprecipitation and western blot of murine urine. C57Bl/6 mouse urine was stored at 4°C overnight after adding protease inhibitors (1 mM PMSF and 2 μg/ml leupeptin). Protein A Sepharose beads were washed 5 times with IP wash buffer (0.1% Triton X-100, 50 mM Tris-Cl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and 1 mM PMSF, 2 μg/ml leupeptin), and resuspended in 1 ml wash buffer on ice. The pH of the urine was adjusted to 7.6 with 1 M Tris, pH 8.0, and brought to a final concentration of 0.1% SDS, 0.1% Triton X-100, and 1 mM PMSF and 10 μM leupeptin. Three ml urine was pre-cleared at 4°C for 1 h by adding 150 μl of a 50% slurry of protein A Sepharose in IP wash buffer and gentle rotation. The samples were spun and the supernatants were poured into fresh 15 ml conical tubes to which 300 μl of protein A Sepharose (pre-bound with 20 μg mouse anti-murine AOAH mA2F3-2A4 in a 50% slurry) and 30 μg BSA were added. After gentle rotation for 1 hr at 4°C, the beads were washed 4 times with IP wash buffer and once with PBS. Twenty μl of sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added and the samples were immediately heated (100°C, 5 min) and loaded onto a 4-20% gradient gel (BioRad, Hercules, CA). The gel was run at 100V for approximately 2 hours and then transferred to a Immobilon^{TM}-P membrane (Millipore, Bedford, MA) at 4°C overnight. The blot was blocked in 4% dry milk/TTBS (2 mM Tris-HCl, pH 7.5, 15 mM NaCl, 0.05% Tween 20) for one hr at room temperature and probed with primary antibody 2F3-2A4 (4.5 μg/ml) for one hour at RT. Following 5 washes in TTBS, the blot was incubated with peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham) for 1 hr at RT. The membrane was washed five times as above, treated with SuperSignal West Pico ECL reagent (Pierce, Rockford, IL), and exposed to Kodak film to visualize the bands.

Uptake and deacylation of LPS by bladder cells. T24 cells were seeded into 6 well culture dishes (2 x 10^5 cells per well). Two days later, media from confluent AOAH- transfected BHK cells and untransfected BHK 570 cells were cleared by centrifugation and added to washed T24 cells in the presence or absence of 10 mM mannose-6-phosphate (M6P), 10 mM glucose-6-phosphate (G6P), 10 mM D(+) mannose, or 10mM ammonium chloride. After 5 hr at 37°C, the cells were washed 3 times with PBS and re-incubated with 0.8 ml of T24 cell media that contained 100 ng *E. coli* LCD25 [^{3}H]LPS, specific activity = 4200 dpm/ng [94]. The cells in two wells were immediately lifted and placed at 4°C. The remaining cells were incubated at 37°C, 5% CO_2 for 1 hr, washed twice with PBS, and re-incubated with fresh T24 cell media for 24 and 48 hrs. Pelleted cells were washed once with PBS and lysed in 0.6 ml PBS, 0.1% Triton X-100. 0.25 ml was removed to a fresh microfuge tube and the insoluble [3H]LPS was removed by centrifugation after adding 0.625% Triton X-100, 1.25 mg/ml BSA, and 1 ml 100% ethanol and incubation at –20°C for 20 min. The radioactivity in the ethanol-soluble fraction and the original lysate was counted (Packard Tri-carb 2100TR, PerkinElmer, Boston, MA) and analyzed to obtain the percentage of the ^{3}H dpm that was released from the [^{3}H]LPS over time. Protein assays were done on cell lysates by using the Bradford method (BioRad).

AOAH ELISA. Ninety-six well, square-bottom, NUNC brand Maxisorb ELISA plates (Fisher Scientific) were coated with 0.5 µg/ml 3C5 (8F8) monoclonal mouse anti-human AOAH antibody in 0.1M $NaHCO_3$ overnight at 4°C. Sample wells were washed 3 times in PBST (0.005% Tween 20 in PBS, pH 7.2) and blocked with 1% milk/PBST for 1 hr at 37°C. Samples and standards were diluted in 1% milk/PBST and incubated at 4°C overnight or at 37°C for 2 hrs. After washing, secondary antibody (E553, purified polyclonal rabbit anti-human AOAH,

final concentration 10 μg/ml in 1% milk/PBST) was added for one hour at 37°C. After washing 5 times, the tertiary antibody (biotin goat anti-rabbit (Zymed, San Francisco, CA) was then added (0.075 μg/ml) for 1 hr at 37°C. 2 μg/ml alkaline phosphatase-conjugated streptavidin (Jackson Laboratories) in 1% milk/PBST was added to each well and incubated for 30 min at 37°C. The plate was washed as above and developed with alkaline phosphatase substrate (5 mM Sigma 104 and 0.1M Sigma Alkaline buffer 221 in water). Optical densities were read at 405 nm and analyzed on an Elisa Plate Reader (MRX Revelation, Dynex Technologies, Chantilly, Virginia).

DISCUSSION

Prior to these studies, AOAH had been found only in the myeloid cells (neutrophils, monocyte-macrophages, and dendritic cells) that comprise the front-line innate defense against invading bacteria and fungi. In the work presented here, AOAH mRNA was localized to renal cortical tubule cells of the murine kidney. It was further shown that AOAH is secreted from proximal tubule cells *in vitro* and present in freshly voided murine urine. The presence of AOAH protein in the washed urinary bladder, combined with the absence of mRNA in this tissue, suggests that the enzyme may be produced and secreted by cortical tubule cells *in vivo* and "shared" with downstream cells in the urinary tract. In support of this hypothesis, the secreted form of AOAH (pro-AOAH) was able to associate with bladder epithelial cells *in vitro* and was used by these cells to deacylate cell-associated LPS. I believe this to be the first description of enzyme sharing in the urinary tract. Although the urinary bladder has AOAH

activity, I did not attempt to immunolocalize AOAH within this tissue due to my inability to localize AOAH in the kidney (where I expect AOAH to be the most concentrated). As discussed in Chapter 4, once appropriate antibodies and/or immunhistochemical techniques are optimized, immunolocalization experiments should be performed on the urinary bladder.

As expected, only the precursor form of AOAH was detected in the culture medium of LLC-PK1, porcine proximal tubule cells. I therefore hypothesized that freshly voided murine urine would contain the precursor form of AOAH. To my surprise, I found that voided murine urine contains the mature, highly active form of AOAH. It is possible that the low pH of the urine may create an environment that favors proteolytic cleavage of pro-AOAH as it descends through the urinary tract. Alternatively, proximal tubules may secrete the mature form *in vivo* or epithelial cells that line the urinary tract might process the precursor and return the mature enzyme to the urine. Western blot analysis of pro-AOAH before and after incubation with AOAH null urine suggested that urine alone is unable to mature AOAH, for pro-AOAH did not mature in the presence of urine. T24 human bladder cells were able to mature pro-AOAH, but only that which was cell-associated, for the culture medium did not accumulate any mature AOAH over time. While this suggests that bladder cells are unable to release mature AOAH *in vitro*, it does not rule out the possibility that bladder or other cells in the urinary tract mature AOAH *in vivo*. I believe more experiments are necessary to determine the mechanism by which mature AOAH is produced in urine.

In the original descriptions of AOAH, Staab ***et. al.*** reported a dependence on pH for the maturation of AOAH. In her studies, the addition of 10 mM ammonium chloride to AOAH transfected BHK570 cells completely ablated the presence of mature AOAH in cell lysate fractions (mature AOAH has never been seen in the cell supernatant) [33]. In apparent opposition,

my studies have shown that ammonium chloride treatment (either pre-treatment or given concurrent with the AOAH source) does not prevent the maturation of the AOAH that has associated with T24 bladder cells, although it does block association. The addition of ammonium chloride disrupts the intracellular location of M6P receptors, sequestering them in distinct endosomal compartments where they are not usually present. Not only does this effectively remove M6P receptors from the cell surface but it also affects the intracellular trafficking of newly synthesized proteins with M6P moieties [95]. The data presented by Staab *et. al* might now be interpreted differently. Newly synthesized and precursor AOAH that had been secreted from the cell would be unable to traffic to their appropriate intracellular locations, possibly inhibiting their maturation. These data do not address whether AOAH must first be secreted from the cell in order to then re-enter and become mature, or whether some pro-AOAH might be targeted to endosomes for maturation before secretion. It should also be noted that my system for detecting maturation (activity assays and ELISA) is much more sensitive (based on my experiments with IP/Western blot vs. ELISA) than the Staab *et. al* method (pulse chase of radiolabeled [^{35}S]methionine and [^{35}S]cysteine) and therefore it is possible that there is some maturation of AOAH present in the Staab *et. al* method that has gone undetected. Removing M6P receptors from the cell surface would be expected to inhibit association of pro-AOAH with T24 bladder cells, but should have no effect on the maturation of any AOAH that did become cell-associated.

The data presented here describe a potential mechanism of enzyme sharing in the urinary tract. AOAH, an enzyme well described for its role in detoxifying LPS, is produced and secreted by renal proximal tubule cells and detected in voided murine urine as a mature, highly active protein. The mere presence of AOAH in the urine, combined with its ability to deacylate

LPS in the absence of detergent (Figure 2.13), suggests a possible role in defense against invading uropathogens. In addition to its potential role in the detoxification of LPS on bacteria that gain entry into the fluid-filled urinary system, AOAH may also play a role in modulating the host response to cell-associated bacteria in the bladder and kidney. *In vitro*, AOAH is able to associate with bladder cells and once associated, is used by these cells to deacylate LPS. I believe that this may be beneficial to the bladder cells, for the inability to deacylate the LPS on resident, replicating bacteria might lead to an overstimulation of the immune response and possible immune damage. The same is true of the proximal tubule cells themselves. It is well documented that p piliated (pyelonephritic) bacteria have the ability to gain entry into the kidney and once there, bind to renal proximal tubule cells. The presence of AOAH in these cells may help downregulate prolonged LPS induced inflammatory responses in the kidney. Based on the observations presented here, future studies will focus on the role, if any, of AOAH in Gram-negative urinary tract infections.

CHAPTER THREE

The In Vivo Role of Acyloxyacyl Hydrolase (AOAH)

INTRODUCTION

Gram-negative bacterial urinary tract infections (UTIs) affect millions of people worldwide. UTIs affect women more frequently than men, probably due to the anatomy of the female urinary system as compared to that of the male. Symptomatic infections arise when bacteria adhere to epithelial cells that line the urethra, bladder and, in some cases, the kidneys. The invading bacteria must overcome a battery of host defenses that can kill or neutralize them. Known host defenses include the low pH, rapid flow, and high osmolarity of the urine, and constituents of normal urine such as defensins, secretory IgA, Tamm-Horsfall protein (THP), uromucoid, and urea [43;96]. Bacterial multiplication is also limited by exfoliation of the superficial cells that line the bladder [43;50;96]. Exfoliation is an apoptotic event that occurs between 2 and 6 hours post-infection and considered an effective innate immune response to invading uropathogens.

Uropathogenic *E. coli* (UPEC), the etiologic agents of nearly 80% of UTIs, possess so-called "virulence" factors that favor their survival and multiplication within the urinary tract. Recent studies have revealed that bacterial adherence and colonization of bladder epithelium are maximal when bacteria express type I pili [48;75]. Host production of cytokines and chemokines, most notably IL-6 and IL-8, precedes the influx of inflammatory cells to the local site of infection [57;71]. This innate immune response to invading *E. coli* is triggered in the bladder by type I pili in a LPS-dependent manner,

since polymyxin B or detoxified LPS inhibit IL-6 production in cultured bladder epithelial cells that have been invaded by Gram-negative uropathogens [71]. Moreover, the cytokine response of cultured epithelial cells was abrogated after infection with a *msbB* strain (lacking secondary fatty acyl chains) [57].

As mentioned previously, AOAH is a host lipase that cleaves the non-hydroxylated (secondary) fatty acyl chains from the lipid A portion of LPS. In human cells, this event leads to the detoxification of LPS, as reflected by its diminished ability to induce cellular inflammatory responses. *In vitro* data have suggested that deacylated LPS may function differently in mouse cells. In one experiment, the difference in the ability of dLPS and fully acylated LPS to stimulate murine splenocyte mitogenesis was much smaller (~6-20 fold reduction with dLPS) than would be expected for human splenocytes (~100 fold reduction with dLPS) [97]. It is now appreciated that there are subtle differences in the structures of murine and human TLR4s that might account for this species-specific difference [98;99]. For example, murine TLR4 might recognize a LPS structure independent of secondary fatty acyl chains, such that deacylating LPS (with AOAH) does not completely inhibit TLR4's ability to signal. On the other hand, human TLR4 probably recognizes LPS in a way that is dependent on the secondary fatty acyl chains. Removing them (dLPS) abrogates signaling through TLR4. These data suggest that dLPS may not be as strong an antagonist in murine cells, but it probably does not exclude a role for AOAH in the detoxification of LPS in the mouse. As described in Chapter two of this thesis, AOAH is highly expressed in renal proximal tubules of both mice and humans. It is secreted from these cells and is easily detected in the urine and urinary bladder of wild type mice. *In vitro* data suggest that secreted AOAH may associate with bladder cells

and be used by them to deacylate associated LPS. This chapter will describe my efforts to determine the *in vivo* role of AOAH in the urinary tract during Gram-negative urinary tract infection.

Results

Wild type and AOAH null mice differ in rate of bacterial clearance.

In order to examine the *in vivo* role of AOAH, we challenged AOAH null and wild type C57Bl/6 mice with the uropathogenic *E. coli* strain f11 CNF 1^+, originally isolated from a patient with cystitis [100] and provided to us by C. Virginia Lockatell (V.A. Medical Center, Baltimore Maryland). We chose this strain for several reasons. Firstly, it has been well characterized by Warren *et. al* [100] and shown to cause urinary tract infection when injected into the urethra of mice. Secondly, cystitis strains have been shown to cause a more severe (duration and intensity) bladder infection in mice as compared to pyelonephritic strains. As mentioned in the literature review, p piliated (pyelonephritic) bacteria are the most common UPEC isolated from human patients with kidney infection and therefore one might assume that p pili are necessary for kidney infection. This is not the case, for Warren *et. al* have shown that of the several cystitis and pyelonephritic strains tested, cystitis strains (including f11) caused a more severe kidney infection [100]. Thirdly, strain f11 CNF 1^+ expresses type I pili that allows for its identification by a simple agglutination test (mannose inhibited agglutination of 1% yeast, as described in Methods). A fourth consideration is the fact that type I pili

stimulate the host immune system via an LPS-dependent mechanism, whereas p pili initiate responses independently of LPS.

In my experiments, six to ten week-old female wild type and AOAH null C57Bl/6 mice were injected intra-urethrally with (1×10^9 CFU/ml [5×10^7 total]) bacteria in PBS. Urine was collected at 6, 12, 24, 48, and 72 hours post-infection and assayed for bacterial growth (colony counts on MacConkey agar) and neutrophils (enumeration using a hemocytometer). Our results indicate that, at early time points, both wild type and AOAH null mice have similar bacterial loads in their urine, but that by 48 and 72 hours post-infection, the AOAH null mice have significantly fewer bacteria in their urine than do wild type mice (Figure 3.0 and 3.1 A-E).

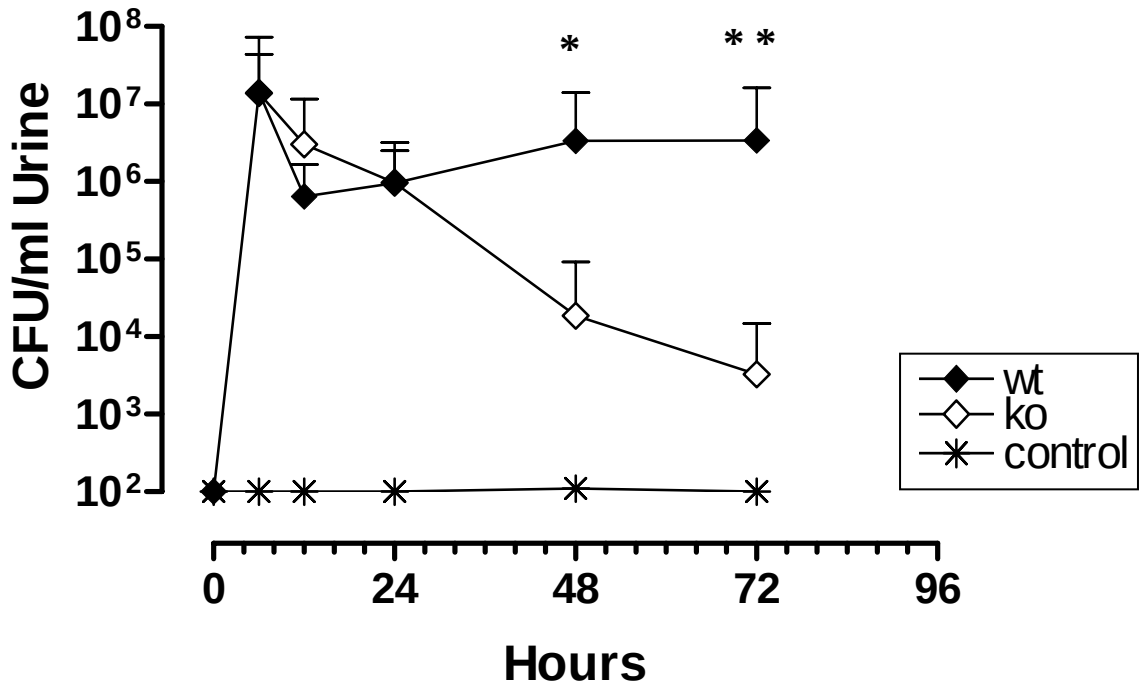


Figure 3.0 – *C57BL/6 AOAH null mice clear bacteria from their urine faster than do wild type mice. The data represent combined results of 5 independent experiments, (~30 mice per group per time point); the error bars represent 1 SD.* * ***p=0.0034, ** p=0.018***

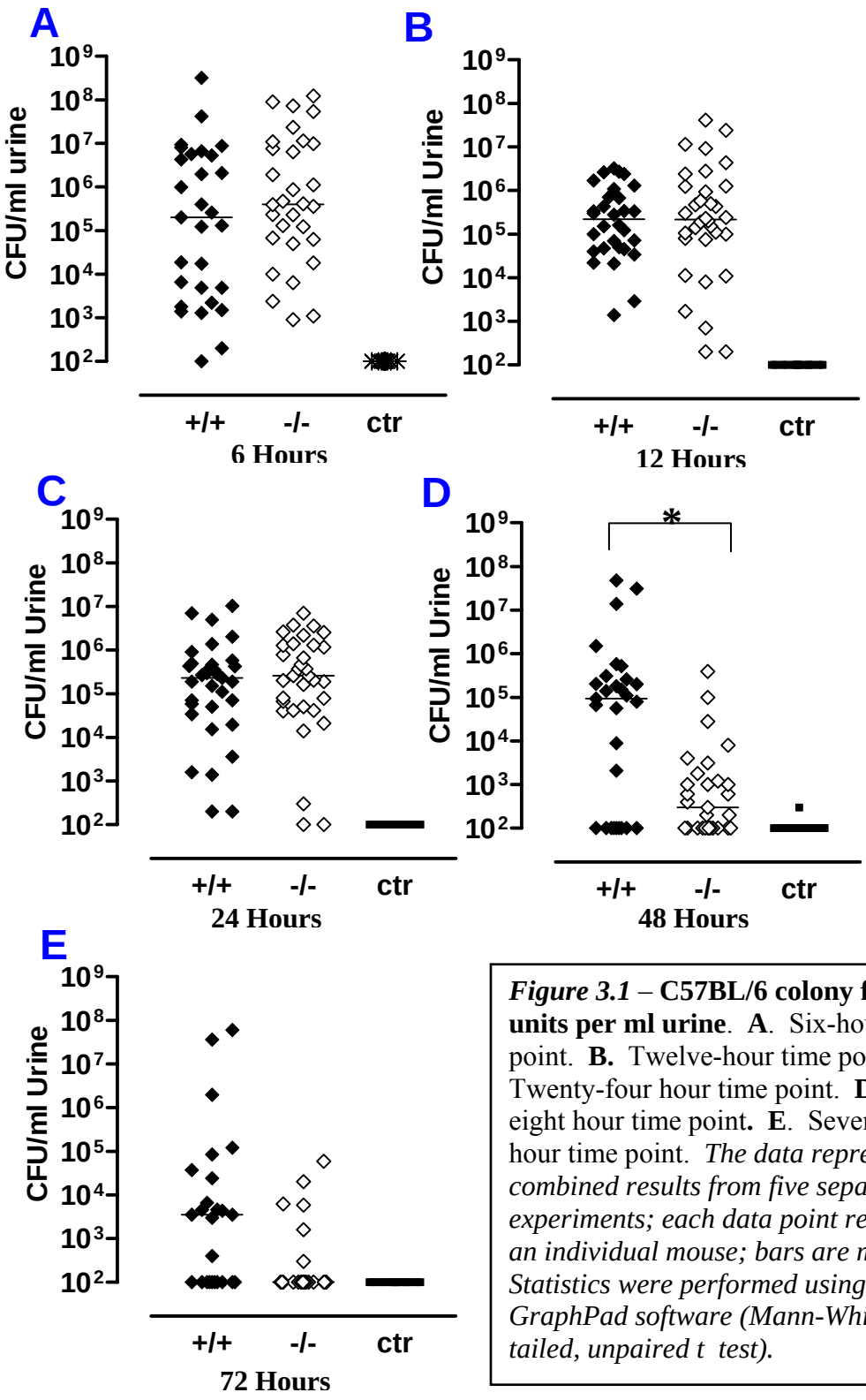


Figure 3.1 – **C57BL/6 colony forming units per ml urine**. **A**. Six-hour time point. **B.** Twelve-hour time point. **C**. Twenty-four hour time point. **D**. Forty-eight hour time point**. E**. Seventy-two hour time point. *The data represent combined results from five separate experiments; each data point represents an individual mouse; bars are medians. Statistics were performed using GraphPad software (Mann-Whitney, 2-tailed, unpaired t test).*

AOAH null and wild type mice have similar numbers of bacteria in their bladders at 72 hours.

Previously considered extracellular "pathogens", UPEC are now being re-examined in light of recent evidence that type I piliated bacteria are able to bind to, invade, and persist within bladder epithelial cells for weeks to months [75]. *In vitro*, 5637 bladder cells respond to piliated bacteria with an initial IL-6 response, peaking between two and twenty-four hours, and falling to baseline levels despite the persistence of intracellular bacteria [71]. In order to assess the number of bacteria present within the bladder tissue following experimental ascending UTI, C57BL/6 mice were sacrificed at 72 hours post-infection. This time point was chosen because it allowed me to follow the course of infection to its endpoint (an early collection time requires the mouse to be sacrificed before we gain essential data concerning the outcome of infection). To our surprise, both wild type and AOAH null mice had bacteria present in their bladders (at levels similar to reports from other investigators), but there was no significant difference in the bacterial loads between wild type and AOAH null mice at 72 hours (Figure 3.2).

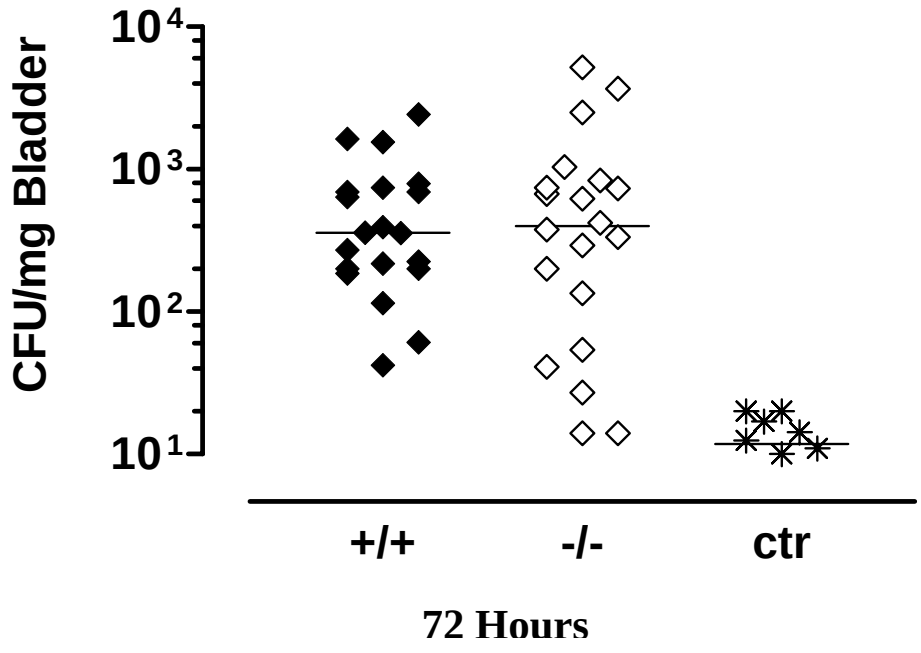


Figure 3.2 – *Wild type and AOAH null C57BL/6 colony forming units per mg bladder tissue at 72 hours post-infection with f11 CNF 1+ (cystitis strain UPEC). Urinary bladders were rinsed with sterile PBS, homogenized, serially diluted, and plated on MacConkey agar plates. Ten control mice were analyzed and had no bacteria present in their bladders. Each data point represents an individual mouse; the data represent combined results of 3 separate experiments; the bars are medians.*

Although we could detect bacteria in the kidneys of both wild type and AOAH null mice at early time points, virtually all bacteria are cleared from the kidneys by 72 hours post-infection (data not shown).

In order to assess the bacterial load in the bladder at an earlier time point, the experiment was repeated and four wild type and five AOAH null mice were sacrificed at 24 hours post-infection, while five wild type, five knock-out, and two controls were followed for 72 hours to ensure the experiment was consist with previous data. Although

the sample size is small, it appears that wild type and AOAH null mice have similar numbers of bacteria associated with their bladders at 24 hours post-infection (Figure 3.3).

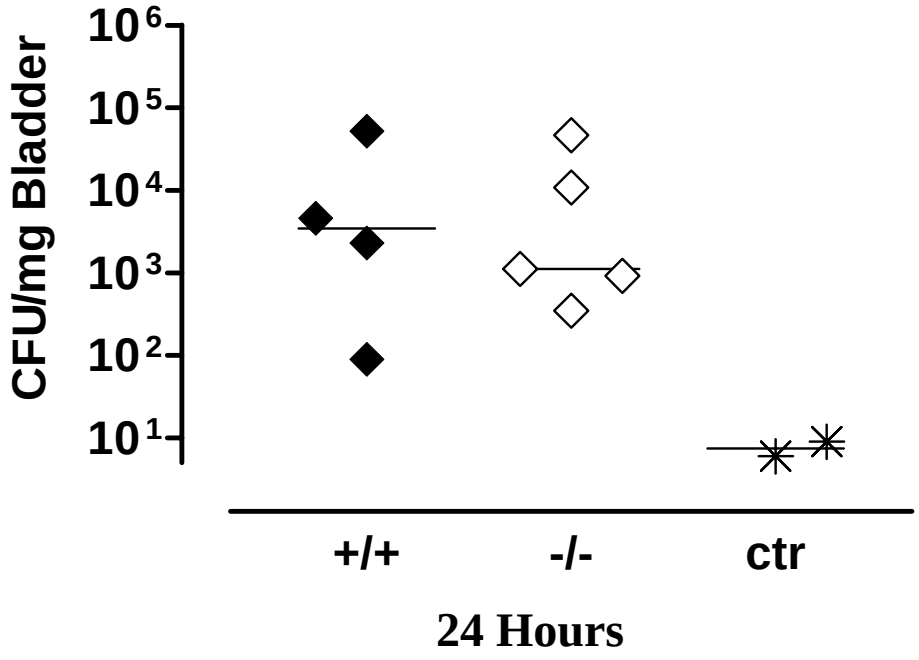


Figure 3.3—*Colony forming units per mg bladder tissue in C57BL/6 mice at 24 hours post-infection with f11 CNF 1+ (cystitis strain UPEC). Mice were sacrificed at 24 hours. The urinary bladders were rinsed with sterile saline, homogenized, serially diluted in sterile PBS, and plated on MacConkey agar for CFU determination. Control mice were negative. Each data point represents an individual mouse; bars are medians.*

The immune response to invading UPEC

The presence of neutrophils in the urine is a clinical marker of urinary tract infection that is commonly used in the initial diagnosis of UTI. We sought to determine the numbers of neutrophils in the urine throughout the duration of our experiment as an

indication of the immune response to the ascending infection. Immediately after collecting the urine, neutrophil numbers were assessed by diluting urine 1:1 in trypan blue and counting on a hemocytometer. We found that the numbers of neutrophils secreted into the urine were not significantly different at 6, 12, or 24 hours. Mirroring the number of bacteria in the urine, wild type mice had significantly more neutrophils in their urine at 48 and 72 hours than did AOAH null mice (Figure 3.4).

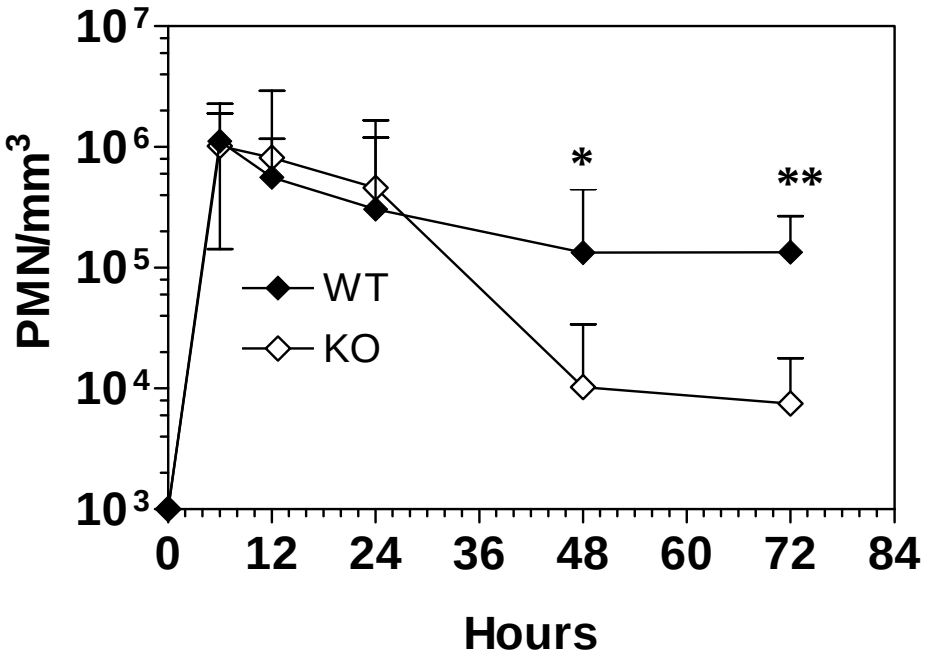


Figure 3.4 – *AOAH null and wild type C57BL/6 neutrophil response to ascending UTI. The data represent combined results from 5 separate experiments; ~ 30 mice per group except 72 hour time point where there are 9 mice per group (I did not always assay neutrophils on the day of sacrifice); bars represent 1 SD. * p=0.0067, ** p=0.0152*

Histological analysis of C57Bl/6 bladder and kidney samples.

In order to assess the local inflammatory response to ascending UTI, bladders were collected at the time of sacrifice (72h), bisected, and fixed in 4% formaldehyde as described in Methods. After two days in fixative, the bladders were paraffin-embedded and 4 μm sections were cut and stained with hematoxylin-and-eosin (H&E). Dr. Zhou, a renal pathologist, kindly scored each specimen. He was blinded to the experimental grouping of the mice and scored on a scale of 0-3 as described in Methods. Briefly, each section was scored for the extent of inflammatory cell influx into the tissue and interstitial edema. Our results indicate that, at 72 hours, there is no significant difference between wild type and AOAH null mice in their mean histological scores. Both wild type and AOAH null mice had a significant amount of inflammation as compared to the PBS injected control mice (Figure 3.5).

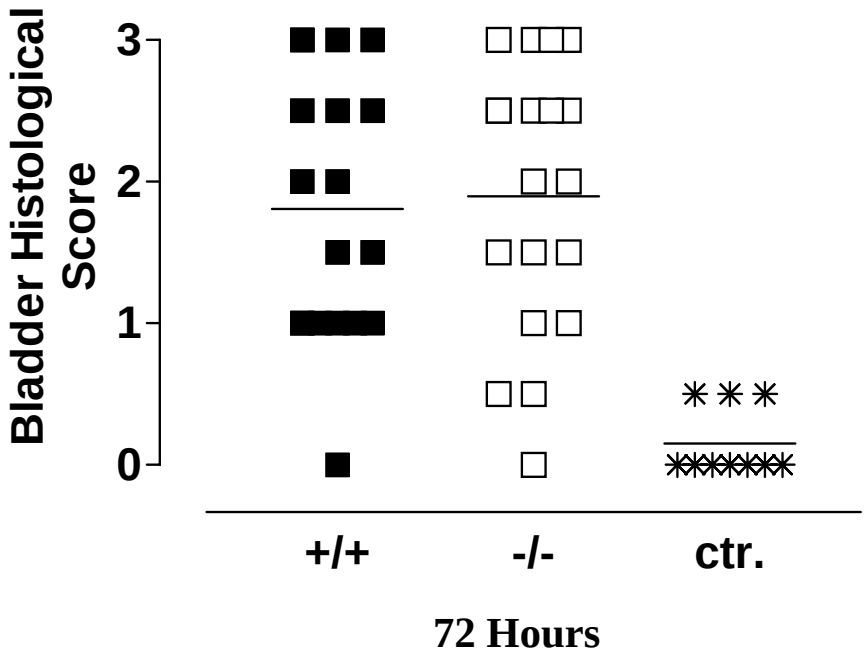


Figure 3.5—*C57BL/6 Bladder Histological Scoring. Wild type and AOAH null mice were sacrificed at 72 hours post-infection, and the excised bladder was rinsed, fixed, sectioned and stained with H&E. Analysis was preformed by Dr. Joseph Zhou, blinded for experimental group, on a scale of 0-3 (see Methods). Each point represents an individual mouse; bars are medians.*

At this 72-hour time point, there are significantly fewer bacteria and neutrophils in the urine of AOAH null animals (Figure 3.0 and 3.1E). With the clearance of bacteria, one might expect there to be a diminished inflammatory response in the bladder, but this does not seem to be the case. The sustained inflammatory response seen in the bladder tissue of AOAH null mice may, then, be a significant finding. I hypothesize that despite the clearance of bacteria, the inability to deacylate LPS might cause the AOAH null mice to sustain a prolonged inflammatory response, potentially proving to be detrimental to the bladder itself. On the other hand, since equal numbers of bacteria were detected in the

bladders at the time of sacrifice, one might expect to find a similar level of tissue inflammation.

In an attempt to localize bacteria within the bladder epithelium, unstained sections of bladder from infected and control wild type and AOAH null mice were Gram-stained and analyzed by Dr. Zhou, blinded to the experimental grouping of the mice. Unfortunately, no bacteria were detected in any of four wild type, four AOAH null, or two control animals. Hultgren and colleagues have shown by electron microscopy that internalized UPEC are not evenly dispersed throughout the bladder tissue; rather, they form focal lesions within the epithelium. In fact, Mulvey *et. al* have called such lesions "bacterial factories" [75]. A recent publication by Hultgren and colleagues has suggested that *E. coli* form biofilms within the bladder epithelial cells and has termed such bacterial focuses "biofilm-like pods". It is possible that the 4 μm sections examined in my studies were not representative of the entire bladder. Sections taken from other planes of the bladder might have contained bacteria (as would be expected from the CFU/mg bladder determinations). Alternatively, 72 hours may be too late to detect bacteria in the bladder tissue. An earlier time point, such as the 6 and 24 hour time point used by Mulvey and colleagues, may allow for better visualization due to the increased numbers of bacteria present.

An analysis of inflammation in the bladders of C57Bl/6 mice at 24 hours post-infection

I next wondered if the inflammatory response within the bladders of wild type and AOAH null mice might be different at 24 hours, just prior to the clearance of bacteria from AOAH null urine. To assess this, C57BL/6 mice were sacrificed at 24 hours post-

infection and bladders were processed as described in Methods. The results suggest that wild type and AOAH null mice do not have statistically different levels of inflammation at 24-hours post infection with f11CNF 1^+ (Figure 3.6).

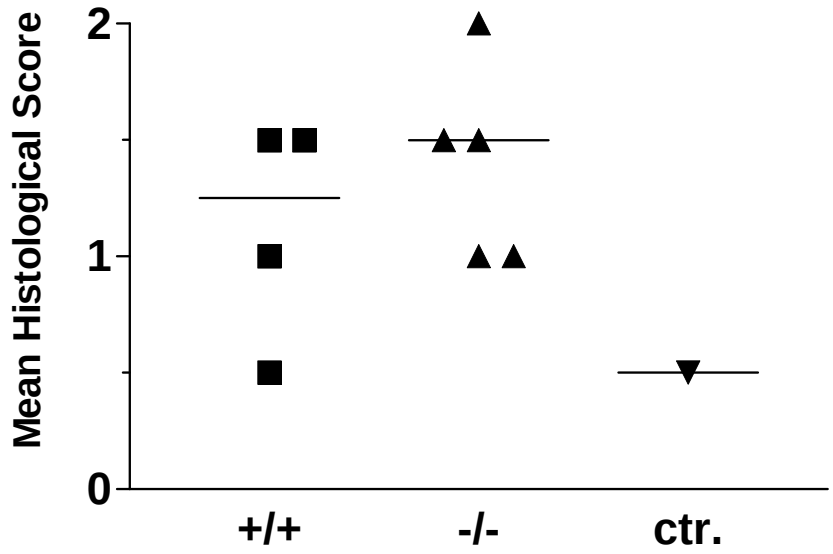


Figure 3.6 – *The mean histological score of C57BL/6 bladders at 24 hours post-infection with f11 CNF 1^+. Each data point represents an individual mouse; bars are medians.*

C3H/HeN mice are also susceptible to ascending UTI.

In order to verify the finding that AOAH null mice clear bacteria from their urine faster than do wild type animals, female C3H/HeN mice were subjected to experimental UTI. Like C57Bl/6 mice, both wild type and AOAH null C3H/HeN mice were susceptible to UTI, averaging approximately 10^6- 10^7 bacteria per ml of urine at the six though 24 hour time points (Figure 3.7). As was the case with C57Bl/6 mice, C3H/HeN

AOAH null mice had fewer numbers of bacteria in their urine by 48 hours than did wild type mice. Although not significant (probably due to the small number of animals in each group), and highly variable, AOAH null mice continued to have fewer bacteria in their urine throughout the duration of the experiment than did wild type animals (Figure 3.7).

To assess the immune response to the invading UPEC, urine was diluted in trypan blue and neutrophils were enumerated using a hemocytometer. Although the numbers remained high throughout the experiment, there was no significant difference in the number of neutrophils present in the urine of either wild type or AOAH null mice (Figure 3.8).

In order to determine the number of bacteria in the bladders and kidneys of wild type and AOAH null animals, four wild type and five AOAH null mice were sacrificed at 24 hours, and four wild type and four AOAH null mice were sacrificed at 10 days post-infection. Bladders and kidneys were aseptically removed, rinsed, serially diluted and plated on MacConkey agar. Although our results indicate that there is no significant difference in the number of bacteria in the bladders or kidneys of wild type and AOAH null C3H/HeN mice at 24-hours or 10-days post-infection (Figure 3.9 A and B), it appears that over time, bacteria are cleared faster from AOAH null animals.

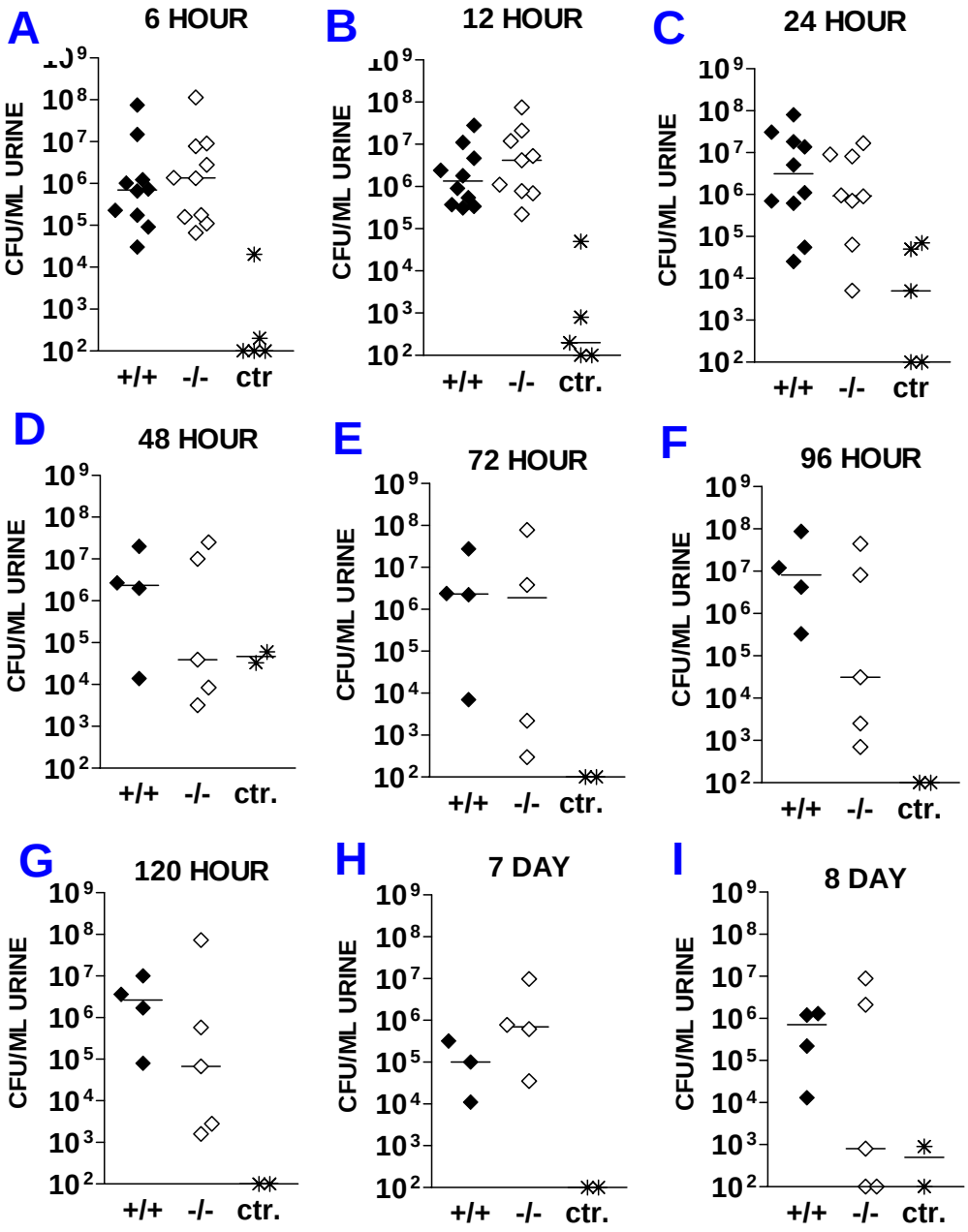


Figure 3.7 *– C3H/HeN colony forming units per ml urine over time. Female wild type (+/+) and AOAH null (-/-) mice were given 1 x 10^9 CFU/ml strain f11 CNF 1+ . Urine was assayed for bacterial growth over time. Each point represents an individual mouse; bars are medians.*

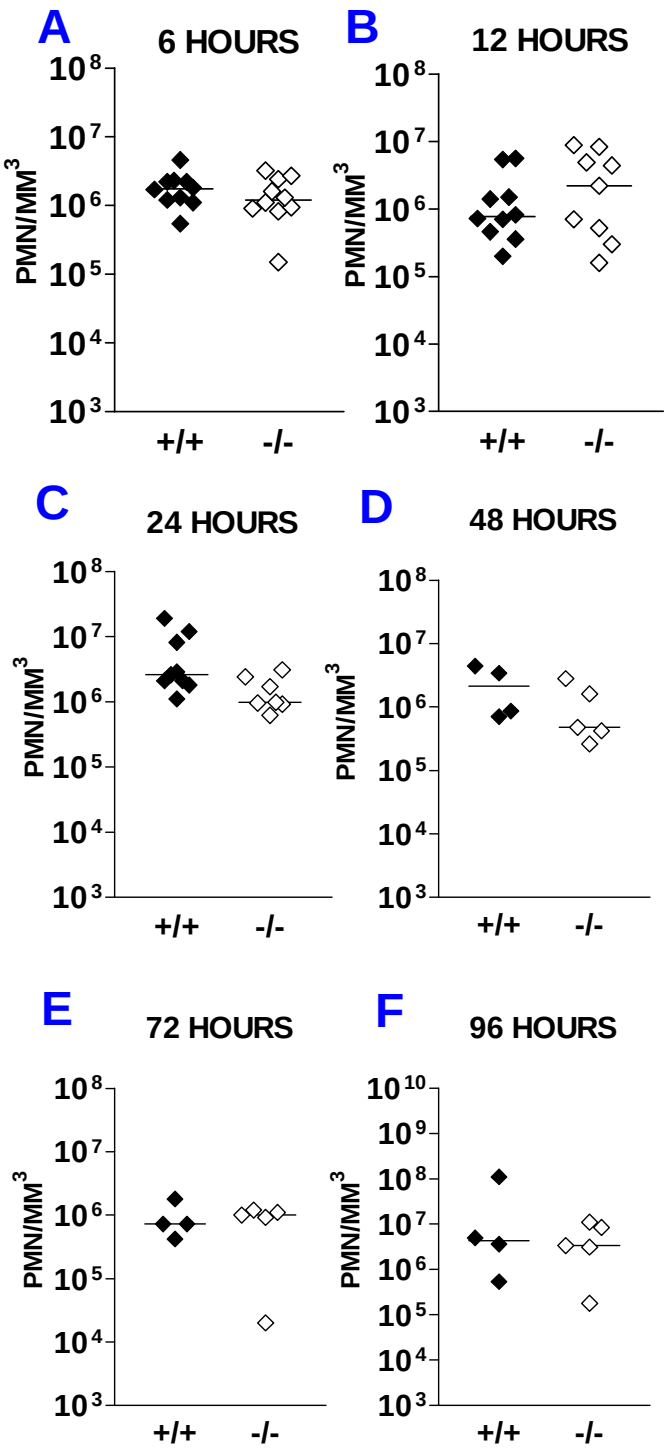


Figure 3.8 – *Number of neutrophils in the urine of +/+ and -/- C3H/HeN mice after infection with f11 UPEC. Urine was collected as described in methods, diluted 1:1 in 1% trypan blue, and placed under a hemocytometer for enumeration. Each point represents an individual mouse; bars are medians.*

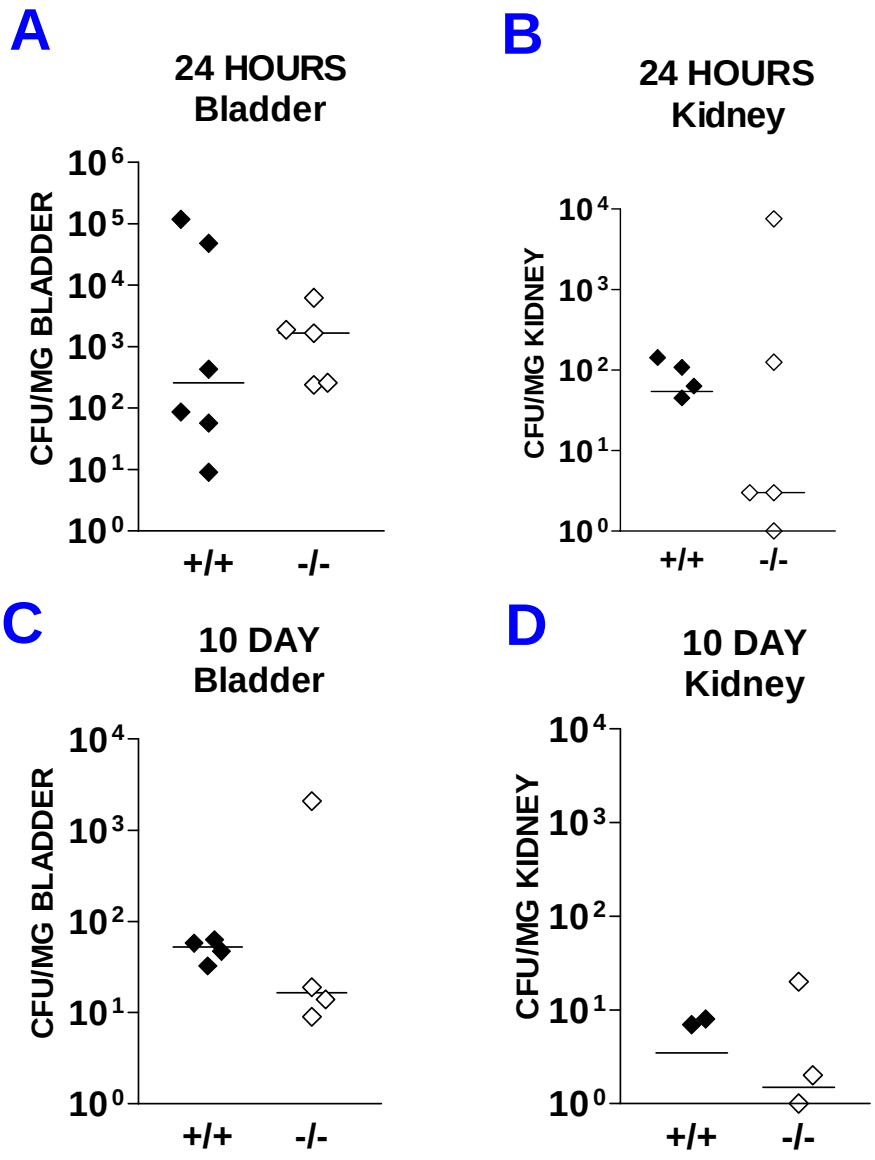


Figure 3.9 – *Colony-forming units per mg bladder or kidney tissue in wild type and AOAH null C3H/HeN mice at 24-hour and 10-day post-infection. Control mice were negative at all time points and in all tissues.*

Mice do not have detectable levels of anti-f11 LPS in their urine.

I next wondered if AOAH null mice might have an abundance of anti-LPS antibodies in their urine that could explain their ability to clear uropathogens faster than wild type mice. Although it is unlikely that the mice have seen the particular strain of bacteria (f11 CNF 1+) used to give them UTIs, it's possible that they possess anti-LPS antibodies that might cross-react with the LPS present in the f11 strain. I used an ELISA to detect anti-f11 LPS in the urine of both wild type and AOAH null C57Bl/6 and C3H/HeN mice. To begin these studies, pre-immune serum was collected from five AOAH null and four wild type C57Bl/6 mice that had previously been given UTIs with the f11 strain. Mice were immunized by injecting a sub-cutaneous dose of heat-killed f11 CNF 1+ bacteria as described in the Methods. Two weeks later, serum was collected and assayed for anti-f11 LPS IgG, IgM, and IgA antibodies. AOAH null mice had significantly more anti-f11 IgG, IgM, and IgA f11 LPS antibodies in their post-infection serum than did wild type mice (Figure 3.10 A-C). Wild type and AOAH null mice had similar levels of anti-f11 LPS IgG, IgM, and IgA antibodies in their pre-immune serum (Figure 3.10 B-E) Neither wild type or AOAH null (C57Bl/6 or C3H/HeN) mice had any anti-f11 LPS IgG, IgM, or IgA in their urine (data not shown).

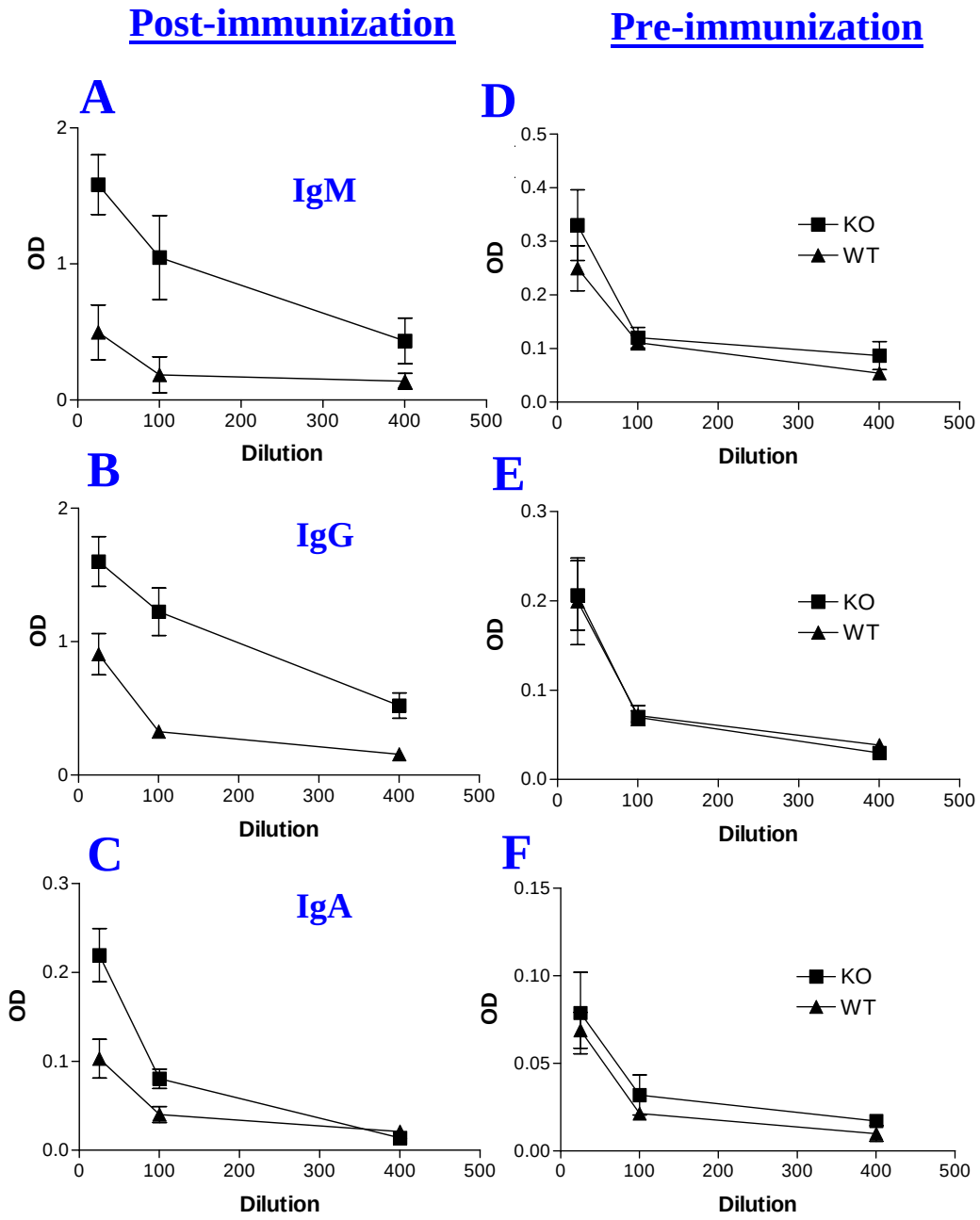


Figure 3.10 – *Anti-f11 LPS antibody titers in serum from C57Bl/6 mice. Mice were immunized with heat-killed f11 CNF 1+ as described in Methods.* ***A****. Post-immune serum, anti-f11 LPS IgM* ***B.*** *Post-immune serum, anti-f11 LPS IgG.* ***C****. Post-immune serum, anti-f11 LPS IgA.* ***D****. Pre-immune serum, anti-f11 LPS IgM.* ***E.*** *Pre-immune serum, anti-f11 LPS IgG.* ***F****. Pre-immune serum, anti-f11 LPS IgA.* **(A-C):** *Each point represents four wild type and five AOAH null mice, assayed in duplicate.* ***(D-F):*** *Each point represents three wild type and three AOAH null mice, assayed in duplicate. The error bars represent SEM.*

Methods

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Chemical Co, St. Louis, MO.

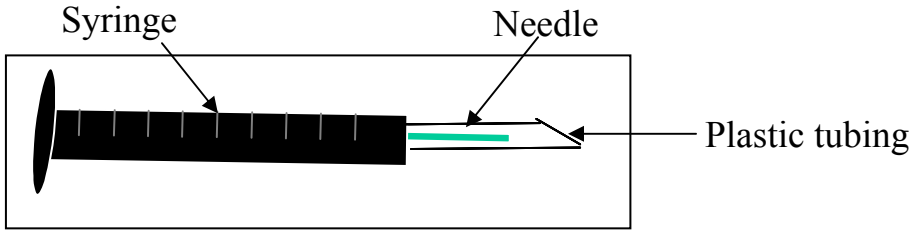
Mouse strains. Specific pathogen-free mice were housed in the UT Southwestern Animal Resource Center and fed a standard diet. ICR (Harlan, Indianapolis, IN), C57Bl/6, or C3H/HeN, wild type and AOAH null [85] mice were used.

Bacterial Strains. *E. coli* strain f11 CNF 1^+ (f11) was obtained from C. Virginia Lockatell, V.A. Medical Center, Baltimore Maryland, and is described in [100]. Strain f11 expresses a type I pilus which can be verified by a simple agglutination test. Agglutination was assessed before and during every experiment by adding 50-100µls bacterial suspension (or one colony) to 50-100 µls of 1% yeast (Fleischmann's® active dry yeast, bought at the grocery store) +/- an equal volume of 2.5 mM D-mannose on a sterile petri dish and gently mixing. Agglutination is evident within one minute. *E. coli* strain FN414 is a previously described non-pathogenic strain originally isolated from the stool of a healthy individual. It was also obtained from C. Virginia Lockatell, V.A. Medical Center, Baltimore Maryland, and is referenced in [100]. It does not express a type I pilus and therefore does not agglutinate a 1% yeast solution. Approximately one week before each experiment, bacteria were streaked onto Trypticase Soy Agar (TSA) plates, incubated at 37°C overnight, and stored at 4°C for a maximum of one week before use. Three days prior to the experiment, one to five colonies were inoculated into 5 mls LB medium and grown overnight at 37°C with shaking. The five ml culture was transferred to 50 mls LB medium and allowed to grow at 37°C with shaking for 2 days. On the

morning of the experiment, bacteria were spun at 5000 rpm in 50 ml conical tubes at 4°C for 5 minutes and washed one time in sterile 1x PBS (pH 7.4). Bacteria were resuspended in 20 mls of sterile 1x PBS and diluted to an OD_{600} = 1.1, (Spectronic® 20 Genesys™, Spectronic Instruments, Rochester, NY) which is approximately equal to 1 x 10^9 CFU/ml, as pre-determined by growth curve analysis. The bacteria were then kept on ice until inoculation.

AOAH Activity Assays. All mouse urine was pre-tested for AOAH activity by assaying 5-10 µls at 37°C as previously described [91], using a double-labeled [^{3}H-acyl chains/^{14}C-glucosamine backbone] *S. typhimurium* LPS as substrate and incubating at 37ºC overnight.

Catheters. Prior to the experiment, catheters were prepared by placing a piece of sterile intramedic PE-10 tubing (Fisher Scientific, cat # 22204008) over a 30-gauge needle (in a sterile hood) with forceps. The tubing was cut at an angle with the bevel of the needle approximately one centimeter from the tip of the needle (Figure 3.10). Catheters were incubated under UV-light for 30 minutes and stored in closed and taped petri dishes until use. Approximately five mice can be injected with the same catheter.



* Diagram of a catheter attached to a syringe. The PE-10 tubing is placed over a 30-gauge needle and cut at an angle.

Urine Collection. The perineal skin of all mice was sprayed with 70% EtOH and the mice were returned to their own clean cage and allowed to dry. Urine was collected onto sterile petri dishes (Fisher Scientific) after picking up the mice and sometimes gently pushing on their bladders. Urine that came into contact with feces or other areas of the perineum was discarded and not analyzed.

Experimental Ascending Urinary Tract Infections. Just prior to an experiment, time zero urine was collected from all mice and stored on ice. Six to ten week-old wild type and AOAH null, female, C57Bl/6 or C3H/HeN mice were anesthetized IP with 0.05 mls ketamine-acepromazine (2.5 mls ketamine (Ketaset, Fort Dodge Animal Health, Burns Veterinary Supply, Rockville Centre, NY), 0.5 mls acepromazine (Aceproject, Vetus Animal Health, Burns Veterinary Supply, Rockville Centre, NY), and 7.0 mls sterile water) and placed in sterile, clean cages. Once asleep, the mice were secured, lying supine, onto a piece of Styrofoam with two large rubber bands over each hind leg. Bacteria (1×10^9 CFU/ml) were drawn into a 1 ml tuberculin syringe and a pre-made and sterilized catheter was attached. The catheter was gently inserted into the urethra of the mouse, first entering perpendicular to the mouse and then slowly angling to become more parallel. The catheter was never forced; when it is in the correct place, it will glide into the bladder and one will know it is in the correct place. Once in place, 0.05 mls of bacteria were gently and slowly expulsed into the bladder. The catheter was slowly removed and no more manipulations were made. The mice were placed in clean cages,

which had been placed on an electric heating-pad (high setting)(Walgreen's) and allowed to awaken (~20-30 minutes). Control mice (at least two per group) were injected as above with 0.05 mls sterile PBS (pH 7.2). The mice remained in the lab throughout the duration of the experiment so as not to infect the mouse colony and for ease of urine collection. All cages were placed on heating pads (low setting) and were covered at night to ensure complete darkness.

Colony-forming Unit (CFU) Determination. MacConkey agar (Voigt Global Distribution LLC) plates were prepared weeks in advance of the experiment by autoclaving media and pouring 15 mls per plate into sterile petri dishes. Plates were cooled overnight at room temperature, dried overnight at 37ºC, and stored, sealed in original packaging at 4ºC until use. Four (or more) hours before use, plates were removed from their packaging and placed at 37ºC to warm-up. Urine, bladder or kidney lysates were serially diluted (Table 3.0) into sterile PBS (pH 7.2) and 100 µls were plated. Bacteria were spread by either the traditional glass rod method or by placing 3 to 5 glass beads (3 mM in diameter) (Fisher Scientific) onto each plate and shaking for approximately 30 seconds. The glass bead method is much faster and works better than the traditional glass rod method. Plates were incubated at 37ºC overnight and stored at 4ºC until colonies could be counted. If possible, two dilutions were counted (between 50 and 500 colonies) and the average was recorded. The presence of type I pili was confirmed by mannose-sensitive agglutination of 1% yeast at least twice during the experiment.

Samples (Infected mice)	Time (hours)	Dilution(s)
Urine	0	1:10
Urine	6-24	1:10-1:10^5
Urine	48-end of experiment	1:10-1:10^4
Bladder	0-48	1:10-1:10^4
Bladder	48-end of experiment	1:10-1:10^3
Kidney	0-48	1:10- 1:10^3
Kidney	48-end of experiment	1:10

Table 3.0 – *Dilution guidelines for ascending urinary tract infection. Note, all samples from control mice should be diluted 1:10 at all time points.*

Neutrophil determination. Five µl of fresh, mixed, murine urine was added to an equal volume of 1% trypan blue and placed under a hemocytometer. Neutrophils were counted in at least two large squares and averaged. To calculate the number of neutrophils/mm^3, this value was multiplied by the dilution factor and 10,000.

Bladder and Kidney CFU and Histology. Mice were anesthetized at 24 or 48 hours post-infection with ketamine/acepromazine and sacrificed by cervical dislocation. The abdomens were sprayed with 70% ETOH and the bladder and both kidneys were aseptically removed into weigh boats. Bladders were bisected [in the first three experiments this was done along the wrong axis (↔), but the last two were bisected along

the correct, sagittal axis (↑)] and rinsed with sterile saline to remove any residual urine. Half of the bladder and one kidney was weighed, placed into a 5 ml polystyrene FACs tube (Falcon #2054), pre-filled with 0.025% Triton X-100 in PBS on ice, for future colony-forming unit determination. One half of the bladder and the other kidney were immersed in 4% formaldehyde (freshly prepared from paraformaldehyde in 1x PBS) and stored on ice. Tissues in formaldehyde were rocked for two days at 4ºC at which time the formaldehyde was replaced with PBS. Tissues were dehydrated, paraffin-embedded, and 4 μm sections were placed onto microscope slides and stained for hematoxylin-and-eosin (H&E) [92].

Tissues in 0.025% Triton X-100 were homogenized to complete disruption with a clean (washed in 70% ETOH, and two sterile water washes) hand-held Tissue Tearor (Biospec Products, Inc. Model 985-370) for approximately 30 seconds. Disrupted tissues were serially diluted in sterile PBS and 100 μl were plated onto MacConkey agar plates as per Table 3.0.

Histological Scoring Index. Bladders were scored, blinded to experimental group, on a scale of 0-3 as previously described [100]. In brief, a score of zero (no inflammation); one, mild (infiltration of low numbers of neutrophils in the lamina propria, little or no interstitial edema, and the absence of regenerative hyperplasia in the luminal epithelium); two, moderate (moderate numbers of neutrophils in the lamina propria, moderate interstitial edema, and moderate generalized hyperplasia of the lumina epithelium); and three, severe (diffuse infiltration of large numbers of neutrophils in the lamina propria, severe interstitial edema, and severe generalized hyperplasia of the luminal epithelium).

Preparation of LPS. Strain f11 CNF 1+ (RM 945) was streaked onto a Trypticase Soy Agar (TSA) plate from a glycerol stock and grown overnight at 37ºC. Several colonies were transferred to 500 mls of LB and grown for 48 hours at 37ºC with shaking. Bacteria were spun at 1500 rpm (Beckman Coulter, Fullerton, CA) for 10 minutes at 4ºC, washed once in 20 mls of sterile water, and resuspended in 21 mls of water. Twenty mls of phenol (27g of phenol resuspended in 3 mls water) was added to the 20 ml bacterial suspension in a 68ºC water bath with continuous mixing. The mixture was kept at 68ºC (with mixing) for approximately 20 minutes (or until one phase is clearly present). In order to separate into two phases, the mixture was placed in an ice/water bath for 20 minutes. The sample was spun at 3500 rpm (Beckman Coulter, Fullerton, CA) for 15 minutes and the top layer (LPS and water) was removed to a fresh tube. Twenty mls of water was added to the LPS/phenol preparation and re-extracted as above. The final sample was dialyzed in a 10-14,000 MW cut-off dialysis tube against 3, 1L changes of water over two days and one change into 20 mM Tris, pH 8.0. The LPS was removed from the tubing, treated with 1 mg/ml DNaseI, 10 mg/ml RNaseI for several hours at 37ºC, and then 5 μg/ml Proteinase K for 3 hours at 37ºC. The sample was split in half and spun at 35,000 rpm in the ultracentrifuge (Sorval Ultracentrifuge, OTT65B) with rotor T 865 overnight at 4ºC. The supernatant was poured off and the LPS resuspended in 0.5 ml water. The LPS preparation was run on a 15% SDS-polyacrylamide gel and silver-stained to test its purity. This process was repeated to obtain more LPS.

Immunization of mice with heat-killed f11 CNF 1+ and serum collection. Strain f11 CNF 1+ (RM 945) was streaked onto a Trypticase Soy Agar (TSA) plate from a glycerol

stock and grown overnight at 37ºC. Several colonies were transferred to 500 mls of LB and grown for 48 hours at 37ºC with shaking. Bacteria were washed and resuspended to a concentration of 1 x 10^9 CFU/ml (OD = 1.1) in PBS. An aliquot was boiled for 30 minutes at 85 -100°C and then placed at 4°C until use. Blood was drawn from the tail vein of four wild type and five AOAH null mice that had previously been given UTIs with strain f11 CNF 1+. Immediately after the blood draw, mice were given a sub-cutaneous injection of 100 µls heat-killed bacteria into their back. Post-immunization serum was drawn 2 weeks post-immunization. Blood was stored at 4°C overnight, spun at 50% max speed at 4°C, and serum collected to a fresh tube.

f11-LPS ELISA. Ninety-six well, square-bottom, NUNC brand Maxisorb ELISA plates (Fisher Scientific) were coated in a 1:100 dilution of f11-LPS (diluted in 0.1M $NaHCO_3$) overnight at 4°C. Sample wells were washed 3 times in PBST (0.005% Tween 20 in PBS, pH 7.2) and blocked with 10% HI-FCS/PBS (block buffer) for 1 hr at 37°C. Samples (1:5 dilutions of urine) and standards (1:25, 1:100, or 1:400 dilutions of post-immune serum) were diluted in block buffer and incubated at 4°C overnight or at 37°C for 2 hrs. After washing, secondary antibody (goat anti-mouse IgM-HRP, goat anti-mouse IgG-HRP, or goat anti-mouse IgA-biotin) was added at a dilution of 1:1000 in block buffer for one hour at 37°C. After washing 5 times, the IgM and IgG plates were developed with TMB substrate reagent (PharMingen)(equal volumes of A and B mixed immediately before use). The IgA plates were incubated with 2 µg/ml alkaline phosphatase-conjugated streptavidin (Jackson Laboratories) in block buffer for 30 min at 37°C. The IgA plate was washed as above and developed with alkaline phosphatase

substrate (5 mM Sigma 104 and 0.1M Sigma Alkaline buffer 221 in water). Optical densities were read at 405 nm (IgA) and 450 nm test, 570 nm reference (IgG and IgM) and analyzed on an ELISA Plate Reader (MRX Revelation, Dynex Technologies, Chantilly, Virginia).

Discussion

Urinary tract infections are a significant cause of morbidity and account for thousands of medical visits each year in the developed world. In addition to the need for a better understanding of UTIs and their sequelae (ie. pyelonephritis, renal scarring, sepsis) for treatment purposes, the urinary system is also a useful model for studies of the innate epithelial cell responses to Gram-negative infection. While many scientists focus such studies on myeloid lineage cells (monocytes, macrophages, and dendritic cells), fewer have investigated the role that epithelial cells play in host defense. As discussed earlier, AOAH is highly expressed in the kidney and *in vitro* is able to associate with bladder cells, providing them with the ability to deacylate LPS. The enzyme is also present in the urine, where it is able to deacylate LPS. I aimed to assess the role of AOAH *in vivo*, and to do so, induced ascending UTIs in wild type and AOAH null mice. My results have shown that C57Bl/6 mice are susceptible to Gram-negative UTI. At the 0, 6, 12, and 24-hour time points, one can detect similar numbers of bacteria in the urine of both wild type and AOAH null mice. However, AOAH null mice have significantly fewer bacteria in their urine at 48 and 72 hours post-infection than do wild type animals.

One hypothesis to explain these data might be that due to their inability to deacylate LPS, AOAH null mice mount a more vigorous inflammatory response to LPS. Such a heightened response might in turn help the AOAH null mice to clear the infection faster than wild type mice. In apparent opposition to this hypothesis is the finding that C57BL/6 wild type and AOAH null mice have similar numbers of neutrophils in their

urine at all early time points. In fact, it is the wild type animals that eventually (48 and 72 hours post-infection) have significantly more neutrophils present in their urine. Analysis of bladder tissue at 24- and 72-hours post-infection also suggests a similar level of inflammation, since there is no difference in the mean histological score at either of these time points. To investigate further the immune response to invading uropathogens, urine was assayed for IL-6 and KC (the mouse IL-8 homolog) over the time course of infection. Unfortunately, any signal was below the level of detection. We are currently testing C3H/HeN urine for both IL-6 and KC, hoping this strain will have higher levels of cytokines. It is possible that such a response exists and I have been unable to detect it.

A second hypothesis is that AOAH null mice might produce more natural, LPS antibodies (that cross-react with the LPS present on uropathogens). Unlike an acquired immune response, natural antibody acts rather quickly and if present might explain the faster clearance of uropathogens by AOAH null mice. This hypothesis is based on preliminary results by Mingfang Lu, who has shown that AOAH null mice produce more polyclonal antibody in response to LPS or LOS immunization. Unfortunately, I was unable to detect any anti-f11 LPS in the urine of wild type or AOAH null C57Bl/6 or C3H/HeN mice by ELISA.

Recently, Martinez *et. al* have shown that type I piliated bacteria are able to invade and persist within epithelial cells of the murine bladder for months, avoiding recognition by the immune system [50]. It is speculated that these "bacterial factories" may be present in humans and account for recurrent UTIs, since many times the second infection is caused by the same bacterial strain that caused the first. How UPEC avoid clearance and remain quiescent is currently unknown. Importantly, the IL-6 response of

cultured bladder cells infected with UPEC is transient, peaking at two hours and returning to baseline thereafter. It is possible that AOAH plays a role in dampening the immune response to the LPS present on these intracellular bacteria, thereby allowing them to persist within the bladder epithelium for some time. In this way, the bacteria could be thought of as using the host machinery to their advantage, allowing them to escape immune recognition or clearance. However, my experiments do support this hypothesis. I see equal numbers of bacteria in the bladder tissue of both wild type and AOAH null mice at 72 hours post-infection (Figure 3.2). If the absence of AOAH allows bacteria to persist intracellularly, one might expect to fine more bacteria in the bladders of AOAH null mice at later time points. It might be interesting to follow infected mice for an extended period of time to see if wild type mice eventually clear intracellular bacteria faster than AOAH null.

If AOAH functions to dampen the innate immune response to LPS on intracellular uropathogens, the absence of AOAH might lead to a sustained LPS-induced inflammatory response within the bladder. It is possible that such a sustained response would benefit the host, helping to rid it of bacteria. On the other hand, one could imagine a sustained immune response as potentially harmful to the host, possibly causing inflammation induce damage to bladder or kidney tissue. Histological analysis of the bladder tissue from C3H/HeN mice infected with uropathogens might help us address this question. Because the mice are infected for such a long period of time (8 or more days), a potential immune mediated tissue injury might be detected, for C57Bl/6 mice clear pathogens in 2-3 days. Alternatively, one could induce a series of UTIs in the same mice

over a period of months, and assess the bladder and kidney histology after such repeated stress.

If the phenotype we are seeing is the result of AOAH deficiency, infecting both wild type and AOAH null mice with a strain of bacteria that has a lipid A structure similar to AOAH-treated LPS (dLPS), should result in a similar level of infection and clearance. Although a strain exists, the *msbB* mutant, it lacks the appropriate pili and virulence factors that enable it to cause significant infection in mice. Much work would be needed to render the bacterium infectious in an *in vivo* model.

Non-pathogenic strain FN414 was originally isolated from the stool of a healthy individual and does not express a type I pilus. For this reason, it is difficult to readily distinguish the infecting (inoculum) strain from normal flora or possible contaminants. I attempted to select for naladixic acid resistant (Nal^R) mutants by growing the bacteria on progressively more concentrated LB agar plates supplemented with naladixic acid (15 μg/ml → 100 μg/ml). I was unable to obtain viable, healthy Nal^R *E. coli* at any of the concentrations. The colonies were much smaller and of a different texture (more solid or stiff) than wild type FN414. When Gram-stained (after growing overnight on 15 μg/ml naladixic acid), they had an unusual elongated morphology and the bacteria seemed to form chains. In contrast, I obtained Nal^R colonies of strain f11 CNF 1+ with relative ease, and although less dramatic, they too had an elongated morphology when Gram-stained. Because strain f11 possess type I pili, they are easily distinguished from other, contaminating bacteria. Future studies where one would like to use a non-pathogenic strain of bacteria should address this issue and maybe try making a mutant resistant to a different antibiotic.

Chapter Four

AOAH-Fluorescent Fusion Protein

Introduction

While the role of AOAH in deacylating LPS is clear, where this reaction occurs is less clear. One of the most common questions regarding AOAH is, "where is it doing its job?" Little work has investigated the intracellular location of AOAH and the reason for this is several-fold. First, the abundance of AOAH in myeloid lineage cells is extremely low and the protein is nearly impossible to detect by immunoflourescence. Secondly, antibodies to murine AOAH proved very difficult to obtain, and have only recently been produced. Janet Staab conducted the only published study of AOAH's intracellular location. She showed diffuse, punctate, and cytoplasmic staining of AOAH-transfected BHK 570 cells (fibroblasts) by indirect immunofluorescence using a polyclonal rabbit anti-human antibody [33]. It was also demonstrated that, *in vitro*, the addition of 10mM ammonium chloride, which raises intracellular pH, blocks the processing of AOAH to its mature form. Ammonium chloride did not alter the localization of recombinant AOAH in the transfected fibroblasts. Deleting the entire small subunit or a critical portion of it (the first two cysteines) led to a more diffuse cytoplasmic staining pattern [33]. This, combined with *in vitro* data showing the ability of ammonium chloride to block

processing of AOAH to its mature form, led to the hypothesis that AOAH is localized in lysosome-like structures. Bacteria are also targeted for destruction within lysosomes. It is reasonable to think that AOAH may be found in lysosomes and act on bacteria there. It should be noted that all of these data were obtained from recombinant AOAH in fibroblasts, and may not apply to AOAH in its natural environment.

Macrophages are important innate immune cells that respond quickly and efficiently to invading bacteria. They express most of the pattern recognition molecules necessary for efficient recognition, internalization (phagocytosis), destruction of bacteria (within phagolysosomes), and recruitment of inflammatory mediators (ie. neutrophils) to the site of infection. AOAH protein is detected in human and murine macrophages [85], but in low abundance. Localization of AOAH within these important immune cells would help us to better understand AOAH's role in deacylating LPS.

Although uropathogenic *E. coli* were traditionally considered extracellular pathogens, it is now believed that both bladder and proximal tubule epithelial cells internalize them. Gram-negative bacteria have been detected *in vivo* and *in vitro* in both types of cells both by gentamicin protection assays and by electron microscopy [48;50;55;101-104]. Although the intracellular location of UPEC is currently unknown, it is believed that they evade phagosomes and lysosomes, and therefore escape death by the numerous anti-microbial factors located within these cellular compartments. Mulvey and colleagues have demonstrated the ability of UPEC to survive for extended periods of time within the bladder epithelium [50;75].

Today there are many tools for studying the localization of proteins within cells. We have purified a mouse anti-mouse AOAH monoclonal antibody that has been very

successful in Western blot analysis and in immunoprecipitating human, murine, and porcine AOAH. Unfortunately, this antibody has not been reliable in immunohistochemistry experiments. I decided to investigate the intracellular location of AOAH using a fluorescent-labeled AOAH fusion protein. My long-term goal was to co-localize AOAH with *E. coli* or purified LPS within cells. In addition, several attempts were made to localize AOAH within murine kidney sections using various anti-AOAH antibodies.

Results

The creation of AOAH-pTimer 1 fusion protein.

There are many fluorescent vectors to choose from when designing a fusion protein for localization studies, and even more have become available since I finished these experiments. BD Biosciences Clontech, which has a large variety of plasmids for these purposes, recently made available a plasmid called pTimer-1. This plasmid encodes DsRed1-E5, a mutant of the red fluorescent protein DsRed1, which has two amino acid substitutions that both increase its fluorescence and allow the protein to change color as it ages (from green to red approximately three hours after protein expression). I created a C-terminal (the 3' end of human AOAH fused to DsRed-1-E5) protein by placing the coding sequence of human AOAH upstream and in frame with the DsRed-1-E5 gene. The final product, pAF988, has the human CMV promoter driving the expression of human AOAH fused to DsRed1-E5 (Figure 4.3).

Transfection of pAF988 into CHO-CD14 cells.

I chose to express the AOAH fusion protein in CHO-CD14 cells because of their known ease of transfection and the presence of CD14, the receptor for LPS. In chapter two of this dissertation I demonstrated that exogenous AOAH (from LLC-PK1 or AOAH transfected BHK 570 cells) is taken up by CHO-CD14 cells in a mannose-6-phosphate dependent manner. For these reasons, CHO-CD14 cells seemed to be a good model for studying the effect of LPS on AOAH's intracellular location, whether AOAH and LPS co-localize, and the localization of AOAH after secretion and re-uptake.

To determine the localization of AOAH within CHO-CD14 cells, I transfected pAF988 (hAOAH-DsRed1-E5 driven by the CMV promoter) and pAF987 (DsRed1-E5 driven by CMV promoter) with Superfect transfection reagent (Qiagen). After 24 hours, ~20% of cells transfected with the AOAH fusion protein were stained red (cytoplasmic), and few cells were green. Cells transfected with the control plasmid (DsRed1-E5 alone) were stained both green and red in both the cytoplasm and nucleus. I repeated this experiment, this time plating CHO-CD14 cells onto cover slips and studying under a better microscope at 24 hours post-transfection. Twenty to thirty percent of the cells transfected with the control plasmid (pAF987) were colored (green and red staining of nuclei and cytoplasm). Cells transfected with the AOAH-fusion protein (pAF988) were about 20% transfected, the cytoplasm was mostly red, with very little green staining. The experiment was repeated two times and each time, although the transfection efficiency was low, ~20%, the results were similar. In an attempt to increase transfection efficiency, I tested Lipofectamine 2000 (Invitrogen) transfection reagent and compared it to Superfect (Qiagen). Lipofectamine 2000 seemed to work better (~50% transfection

efficiency), did not require a medium change post-transfection, and was used in all subsequent experiments.

Mannose-6-Phosphate might block the re-uptake of AOAH by transfected CHO-CD14 cells.

In order to assess the effect of M6P on the localization of AOAH, CHO-CD14 cells were transfected with pAF987 (control, DsRed1-E5) and pAF988 (hAOAH-DsRed1-E5). Two hours post-tranfection, 10 mM M6P was added to both control and test cells in duplicate and the cells were incubated at 37°C, 5% CO_2 for an additional 24 hours. CHO-CD14 cells transfected with pAF987 alone had ~20% green (strong) and 20% red (strong) cytoplasmic and nuclear staining. Cells transfected with pAF988 were ~10% green and 5% red with both colors localized to the cytoplasm. Interestingly, the addition of 10 mM M6P abolished the red fluorescence in the cells transfected with the AOAH fusion protein, but had no effect on control cells (pAF987). The percentage of green cells did not change (~10%). Work presented earlier in this dissertation and by previous Munford lab members has characterized AOAH's ability to be secreted from cells as a precursor and to be processed by either the same cell or other cells (with M6P receptors) to form the mature, more enzymatically active form of the protein. It is possible that secretion and re-uptake is necessary for the maturation of AOAH. We know that M6P blocks the uptake of AOAH in CHO-CD14 cells and finding that M6P blocks the expression of red protein might imply that blocking the re-uptake of secreted AOAH blocks nearly all processing of AOAH. Because DsRed1-E5 changes color from green to red over time, seeing only green protein suggests that the cell no longer contains mature

(old) protein. M6P does not appear to affect the expression of proteins, for the control transfection (DsRed1-E5) was not affected.

In an attempt to localize AOAH in cells that have taken up AOAH, media from cells transfected with pAF988 (AOAH fusion protein) and pAF987 (control DsRed1-E5 alone) was overlaid onto confluent CHO-CD14 cells. This medium was collected 24 hours after the original transfection and after a change in medium, so that no DNA would be present). After a five-hour incubation with the media, both control and AOAH recipient cells were visualized under a fluorescent microscope and both sets had red cells. These results suggest that when overlaid onto CHO-CD14 cells, the fluorescent protein alone is able to enter cells. This experiment should be repeated, this time adding M6P to the medium to see if the uptake is inhibited.

The removal of DsRed1-E5 and replacement with DsRed2.

In order to reduce the complexity of the system and so that AOAH and LPS might be co-localized, the DsRed1-E5 gene was replaced with DsRed2 as described in Methods and used to transfect CHO-CD14 cells. Using the Lipofectamine 2000 transfection reagent, pAF1127 (AOAH-DsRed2) and control pRSM 978 (DsRed2 alone) were transfected into CHO-CD14 cells and visualized by fluorescence microscopy at 24 and 48 hours post-transfection. After numerous attempts and varying both the amount of DNA transfected and Lipofectamine 2000 used, I was never able to obtain a good transfection. Both the control and fusion protein killed the majority of cells. Approximately 5% of the cells were red, but even those cells looked sick. It was determined that in my system, DsRed2 is toxic and should not be used for future studies. One explanation for this

toxicity is the strong CMV promoter. Dr. Alan Varley has created several truncations of the CMV promoter, creating promoters that express reporter genes to various, increasing levels. It is possible that some of these constructs might be used to reduce the levels of protein in the cells and to reduce cellular toxicity. At the time of these experiments the truncated promoters were not well characterized and it was decided not to use these, but rather, to remove the DsRed2 gene and replace it with eGFP.

The removal of DsRed2 and replacement with eGFP.

The enhanced green fluorescent protein, eGFP (BD Biosciences Clontech), is well characterized, stable, and non-toxic. Plasmid pAF1224 (hCMV-hAOAH-eGFP), was generated and transfected into CHO-CD14 cells along with a control plasmid, pRK785, which expresses the eGFP protein under control of the human CMV promoter. The results of this experiment were promising. Twenty-four hours after transfection, cells transfected with both the AOAH fusion protein and control plasmid were green. The control cells were stained in both the cytoplasm and nucleus while the AOAH fusion protein was localized to the cytoplasm only. Although only a small percentage of the cells were transfected, it appeared as though the staining was in a specific location (not diffuse) near the nucleus (similar to proteins that localize to the golgi).

Construct	Strain ID	Fluorescence	AOAH Activity?	Location of fluorescence in CHO-CD14 cells
pTimer 1	RM 986	DsRed1-E5	No	N/A
hCMV-pTimer 1	RM 987	pTimer 1 (green→ red)	No	Green and red staining in the cytoplasm and nucleus.
pDrive + hAOAH	RM 1012	None	N/A	N/A
hCMV-hAOAH-pTimer1	RM 988	pTimer1 (green → red)	Yes (Lysates only)	The cells were red in the cytoplasm only. Cells were green when M6P was added.
hCMV-hAOAH-DsRed2	RM 1127	DsRed2 (Red)	No	Toxic to cells
hCMV-hAOAH-eGFP	RM 1224	eGFP (green)	No*	The cells were green in the cytoplasm only.
hCMV-.eGFP	RK785	eGFP (green)	No	The cells were green in the cytoplasm and nucleus.

Table 4.0 – *A list of the various AOAH fusion proteins and constructs I used to design them. N/A = not assayed; * The transfection efficiency was very low in this experiment and might explain the lack of AOAH activity.*

CHO K1 and CHO-CD14 cells phagocytose Bodipy-labeled *E.coli*.

Once we have localized AOAH within cells, we hope to co-localize it with whole bacteria or purified LPS. For these experiments to work, CHO-CD14 cells must be able to phagocytose bacteria. To test their ability to internalize Gram-negative bacteria, cells were plated onto 6-well dishes and infected with Bodipy-labeled *E. coli*. Bodipy is a green fluorophore, like FITC or GFP, that has a similar emission and excitation spectra, although it is brighter and more stable than either of these alternatives. Bacteria were spun onto the cells and incubated for one hour in the absence and presence of cytochalasin D, an inhibitor of phagocytosis. To our surprise, both CHO K1 and CHO-CD14 cells were able to internalize the K12 strain of bacteria (Bodipy-labeled). In both cases the internalization was inhibited by cytochalasin D (Figure 4.0). These results suggest that once transfected with AOAH-eGFP, CHO-CD14 cells can be infected with red labeled bacteria, allowing the localization studies to be performed.

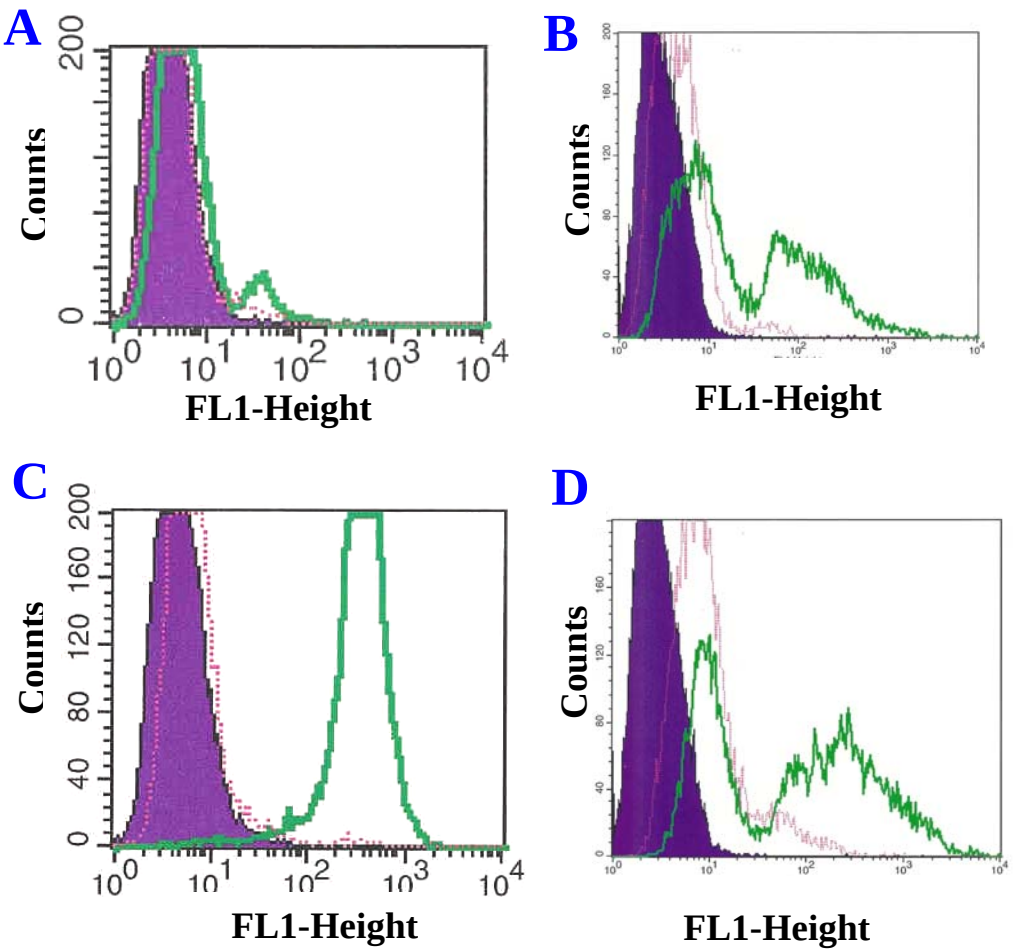


Figure 4.0 *– CHO K1 and CHO-CD14 cells are able to internalize Bodipy labeled E. coli. Panel A and C. CHO K1 (A) or CHO-CD14 (C) cells stained with murine anti-human CD14 antibody 63D3 (green) or control IgG2a (red). Cells alone are in purple. Only the CHO-CD14 cells express CD14. Panel B and D. CHO K1 (B) or CHO-CD14 (D) cells were incubated with Bodipy-labeled E. coli in the presence (red) or absence (green) of cytochalasin D, an inhibitor of phagocytosis. After a one hour incubation with bacteria, the cells were rinsed with PBS, lifted in 2 mM EDTA, and analyzed +/- 0.1% trypan blue by FACscan. Only the (+) trypan blue results are shown. Both CHO K1 and CHO-CD14 cells were able to internalize E.coli.*

Immunohistochemistry of AOAH in human and murine kidney.

In an attempt to localize AOAH within human or mouse kidney by immunohistochemistry, a collaboration with Dr. James Richardson (UT-Southwestern) was arranged. With his lab's assistance, sections of normal human kidney (medulla and cortex), excised from patients with renal tumors, were fixed, paraffin embedded, and cut (4μm slices). Slices were processed, using two murine anti-human AOAH monoclonal antibodies [3C5 (8F8) and 3G4 (3F3)], as described in Methods. To our delight, positive signal was detected in the proximal tubule cells of tissues that were incubated with a 1:200 dilution of primary antibody 3G4 (3F3). Slides that were incubated with a PBS, IgG control or the 3C5 (8F8) primary antibody were all negative. This experiment was repeated two times with similar results. After consulting with Dr. Richardson, it was determined that the staining was not strong enough to be convincing. Future experiments were never able to increase the level of staining in human kidney samples. When monoclonal antibody to murine AOAH became available, murine kidneys were tested as above. Antibody 2F3-2A4, a purified mouse anti-mouse monoclonal antibody, was moderately successful in these assays, and in one experiment stained the lumenal side of proximal tubule cells (Figure 4.1) . Unfortunately, these experiments were not very reproducible. I think that these experiments might be repeated, this time altering the way in which the tissue is processed. Because AOAH is a secreted protein, it is possible that the perfusion of the mouse flushed some of the protein out of the tubular lumens, effectively reducing the amount of AOAH available for detection. Immunohistochemistry on frozen sample or tissue fixed without prior perfusion might be more successful.

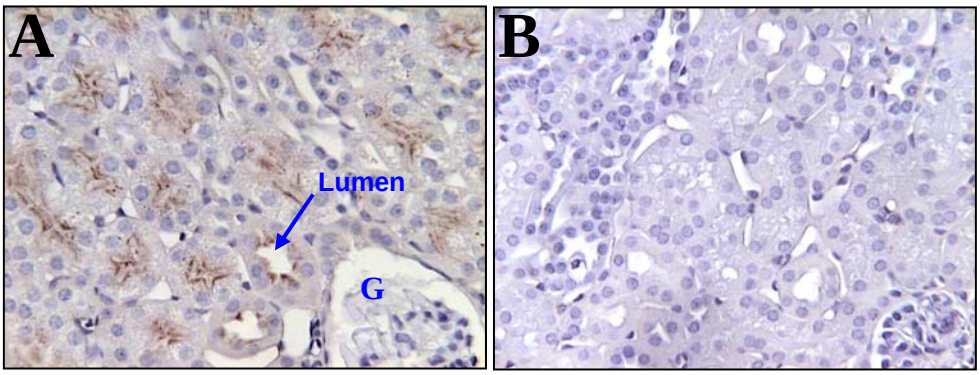


Figure 4.1 – *Immuno-localization of AOAH in the murine kidney. Sections were incubated with primary antibody 2F3-2A4 (A) and an IgG2a control (B) as described in Methods. AOAH is localized to the lumens of cortical tubule cells (the arrow) and absent from glomeruli (G).*

Materials and Methods

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Chemical Co, St. Louis, MO.

.

Cell Culture. CHO-CD14 (CHO K1 cells transfected with human CD14) and CHO K1 (ATCC, CCL-61) cells were cultured in 50:50 RPMI 1640 (Fisher Scientific): Ham's F

12 with L-glutamine (Fisher Scientific), 2% penicillin, streptomycin, glutamine (PSG, Invitrogen), and 10% heat-inactivated FCS (Hyclone, Logan, UT).

Mini-prep protocol. Colonies were picked into three mls LB-antibiotic medium in 10ml snap-top tubes (Falcon #2054) and grown at 37°C with shaking overnight. The bacteria was pelleted in 1.5ml Eppendorf tubes with a 30 second spin at maximum speed, room temperature, and resuspended in 100 µls TEG (25 mM Tris pH 8.0, 50 mM glucose, 10 mM EDTA, stored at 4°C). 200 µls of freshly prepared 1% SDS, 0.2N NaOH was added and samples were mixed by hand and incubated at room temperature for two minutes. 150 µls 3M potassium acetate (pH 5.0, with glacial acetic acid) was added, samples were mixed by hand, and incubated at room temperature for 2 minutes. 200 µls chloroform: isoamyl alcohol 24:1 were added and samples were vortexed and spun at maximum speed for 2 minutes. The upper layer was removed to a fresh 1.5 ml Eppendorf tube and two volumes of 100% EtOH were added and mixed by hand. Samples were spun at maximum speed at room temperature. The supernatant was carefully aspirated, the pellet washed in 70% EtOH, air-dried, and resuspended in 20 µls TE pH 7.6, with RNase (100 µg/ml) (Qiagen, Valencia, CA).

Generation of Plasmids.

P-timer.CMVpromoter (pAF987). The first cloning step in the creation of an AOAH-DsRed1-E5 fusion protein was the insertion of a CMV promoter in the pTimer-1 plasmid (Figure 4.2). To accomplish this, plasmid RSM 332, which contains the full length human IE CMV promoter in an eukaryotic expression vector from Invitrogen, was

digested with *Bgl*II and *Asp*718 (both from New England Biolabs, Beverly, MA) in Buffer B (Roche, Basel Switzerland) overnight at 37°C. Plasmid RSM 978 (pTimer-1, BD Biosciences Clontech, Palo Alto, CA) was similarly digested and both products were run on a 1% agarose gel with large combs. The products, a 900 bp CMV promoter and a 4 kb pTimer-1 vector, were excised from the gel and purified using Qiaquick gel purification system (Qiagen). The purified vector and insert were ligated using Rapid DNA Ligation kit (Roche) and transformed into DH5-alpha competent cells (Invitrogen). Seven potential clones were screened by mini-prep and digestion with *Bgl*II and *Asp*718. Clone one was streaked onto LB-Kan and stocked in glycerol as RM 987.

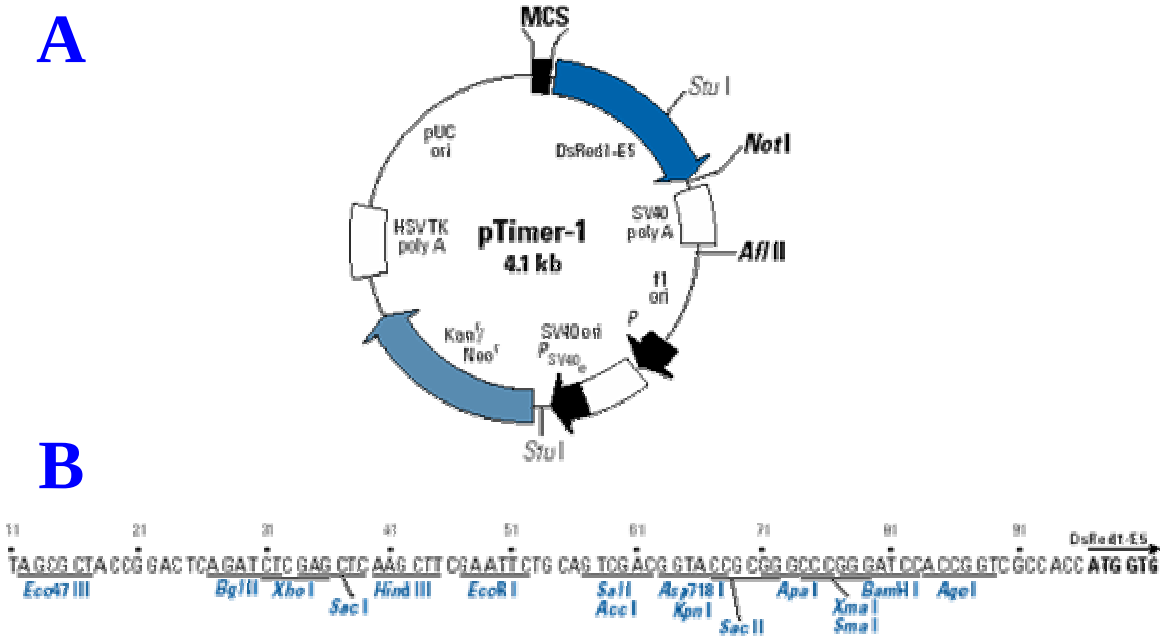


Figure 4.2 – *A.* *Vector map of pTimer-1 (BD Biosciences Clontech).* ***B.*** *The multiple cloning site of vector pTimer-1. The human IE CMV promoter was cloned into the BglII and Asp718 sites.*

PCR of human AOAH and creation of clone RM 1012. The coding region of human AOAH was amplified from plasmid pAF956 (pAF956 is the new name for pJSK2301, Janet Staab's wt hAOAH with 3 amino acid corrections in pSelect-1 downstream of T7 promoter. I re-stocked and gave it an accession number) using primers: AF3'hAOAH.*Age*I.23 (5'- ACC GGT TTG TGC CCG CCT TGG TC- 3') and AF5'hAOAH.*AgeI*.23 (5'- ACC GGT ATG CAG TCC CCC TGG AA-3') ordered from (Integrated DNA Technologies, Inc., Coralville, IA). The PCR was performed with High-Fidelity Taq polymerase (Roche), an annealing temperature of 65°C, and a 1.5-minute extension at 72°C, and 30 cycles. The final product was gel purified from a 1% agarose gel using Qiaquick Gel Purification System (Qiagen) and TA cloned into pDrive (Qiagen). Several clones were positive and three (1, 3, and 15) were streaked onto LB-Kan plates, glycerol stocked, and stored at -80°C in freezer position 2, 3, 2, 10 for future use. Clone number one was sequenced using the following primers: hAOAH.seq#1.af (5'- GGGTAGAGATGACACAATGG-3'); hAOAH.seq#2.af (5'-GCGCAGATATGAATGCTG-3'); hAOAH.seq#3.af (5'-CTGTAATGGCATTTGGGG-3'); hAOAH.seq#4.af (5'- GGTGTTGGACTATCCCG-3'); and hAOAHseq#5.af (5'-CTTCAGAGAGAGCAGAGC-3') (Integrated DNA Technologies, Inc., Coralville, IA). Clone one had 2 mutations, the first, an "A" to "G" at position 1206 which corresponded to a Thr → Ala amino acid change, and a second at position 1504, that did not change the amino acid. Both of these mutations were determined not to affect the activity of AOAH. This clone was given the accession number RM 1012 (plasmid = pAF1012).

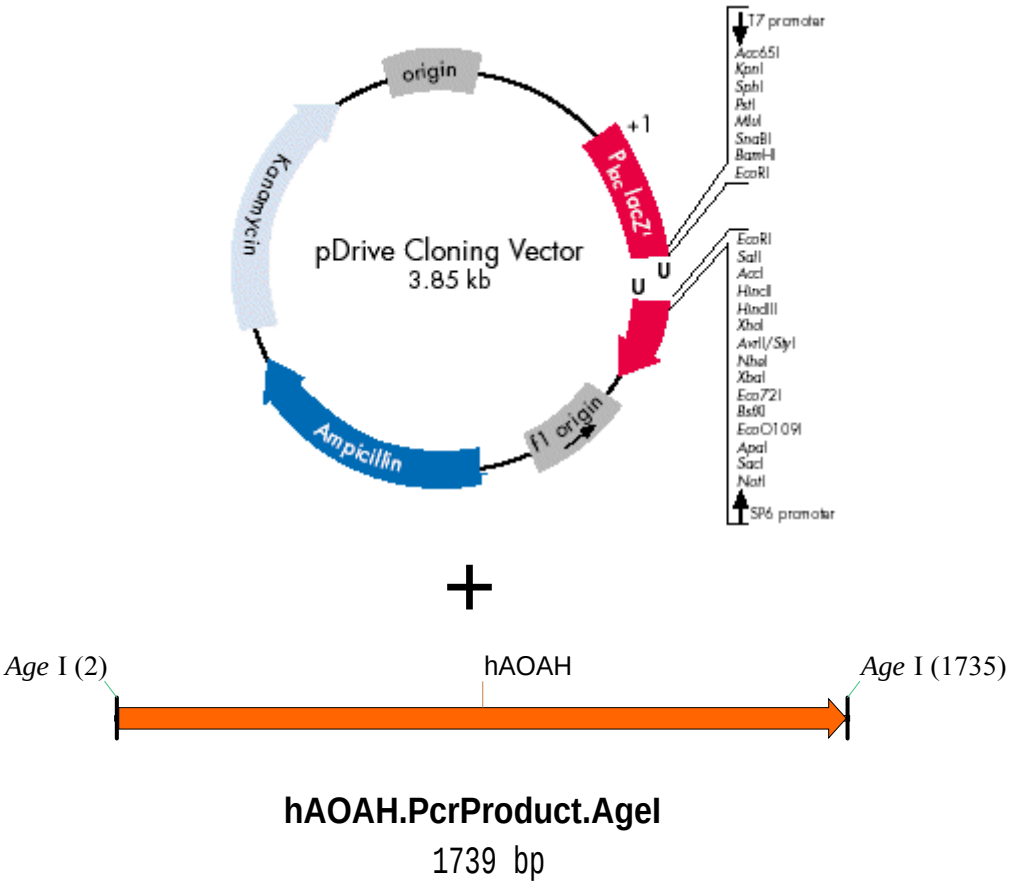


Figure 4.3 – *The cloning strategy of placing human AOAH into pDrive cloning vector. This insertion created plasmid pAF1012. It was sequenced and found to have only two mutations, one of which altered protein-coding sequences*

AOAH-DsRed1-E5 fusion protein (pAF988). The newly constructed pAF1012 and pAF 987 (pTimer.CMVpro) were digested with *Age*I overnight at 37°C and run on a 1% agarose gel. The vector (5 kb) and insert (1.7 kb) bands were excised and gel purified with Qiaquick gel extraction kit (Qiagen). The vector was SAP (shrimp alkaline phosphatase, Roche) treated for 30 minutes at 37°C and heat inactivated at 65°C for 15

minutes. The treated vector and insert were ligated with Rapid DNA Ligation kit (Roche) and transformed into DH5-alpha competent cells (Invitrogen). Positive clones number two and five (screened with *Age*I and *Age*I + *Bgl*II restriction enzymes) were streaked onto LB-Kan plates and glycerol stocked. They were given the accession number AF 988 clone 2 and AF 988 clone 2 (pCMV.p-Timer.hAOAH fusion).

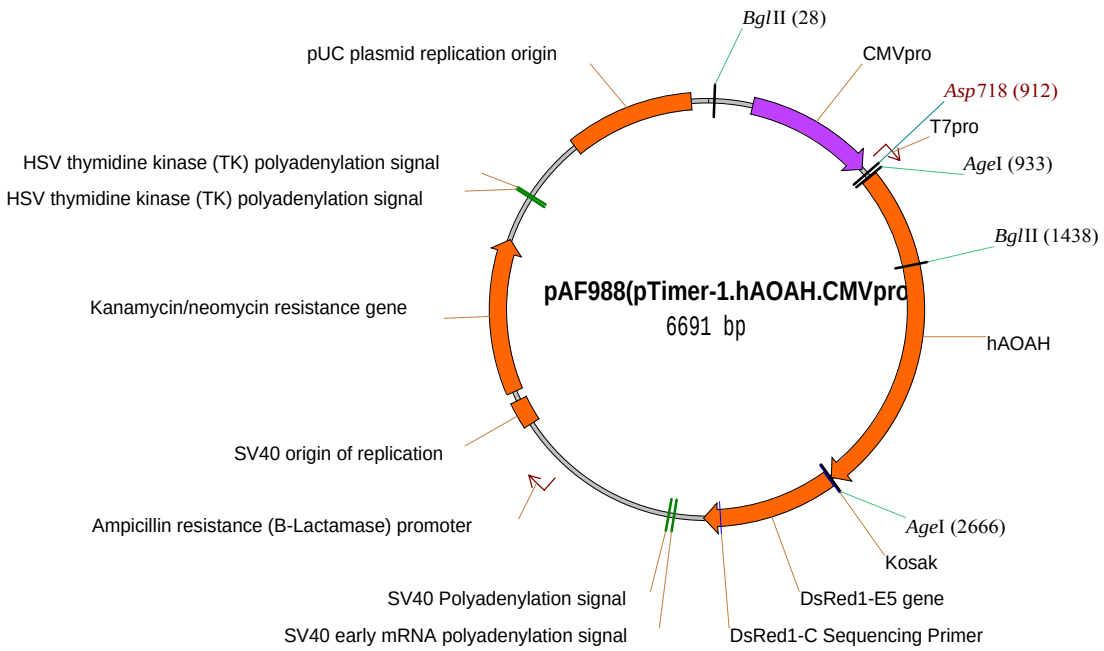


Figure 4.4 – *pAF988, the final hAOAH-DsRed1-E5 fusion protein. AOAH was digested with AgeI from pAF1012 and inserted into the AgeI site in the multiple cloning region of pAF987. Clones number two and five were glycerol stocked and stored at -80℃. Maxi-preps were also performed and used in future transfection experiments.*

AOAH-DsRed2 fusion protein (pAF1127 and pAF1128). To create this plasmid, the previously described pAF988 was partially digested with *Age*I (New England Biolabs)

for 0.10, 0.45, and 1.5 hours at room temperature, in hopes of cutting only one of the two *Age*I sites (the site at the 5' end of AOAH) and run on a 0.8% agarose gel for verification of a linear fragment. The linear fragment (6.69 kb) was excised from the gel and purified using Qiaquick Gel Purification System (Qiagen). Positive clones were treated with Klenow DNA polymerase I, large fragment at 20 u/µl (New England Biolabs) at 37°C for 20 minutes to fill in the 3' overhang created by the *Age*I digest and heat inactivated at 65°C for 5 minutes. Samples were ligated with Rapid DNA Ligation Kit (Roche) following the manufacturer's instructions and transformed into DH5-alpha competent cells (Invitrogen, Carlsbad CA). Approximately 20 clones were screened (mini-preps and digestion with *Age*I alone and then *Age*I and *Not*I combined). The correct sizes of the products after the double digest are 6 kb and 700 bp respectively. Clones number one and four were streaked onto LB-Kan plates and glycerol stocked. They were given the name RM 1125 (clone one) and RM 1126 (clone 4). These plasmids are the same as pAF988 with the 5' *Age*I site removed.

DsRed1-E5 was replaced with DsRed2 by digesting plamsids pAF1125 and RSM 978 (DsRed2, BD Biosciences Clontech) with *Age*I and *Not*I. The products were run on a 1% agarose gel with large combs and the vector (6 kb) and insert (700 bp-eGFP) were excised, gel purified as above, ligated with Rapid DNA Ligation Kit (Roche), and transformed into DH5-alpha competent cells (Invitrogen). After mini-prep and *Age*I→ *Not*I digest, the products were run on a 1% agarose gel. Clones one and two were streaked onto LB-Kan plates and glycerol stocked as RM 1127 and RM 1128 respectively (Figure 4.5).

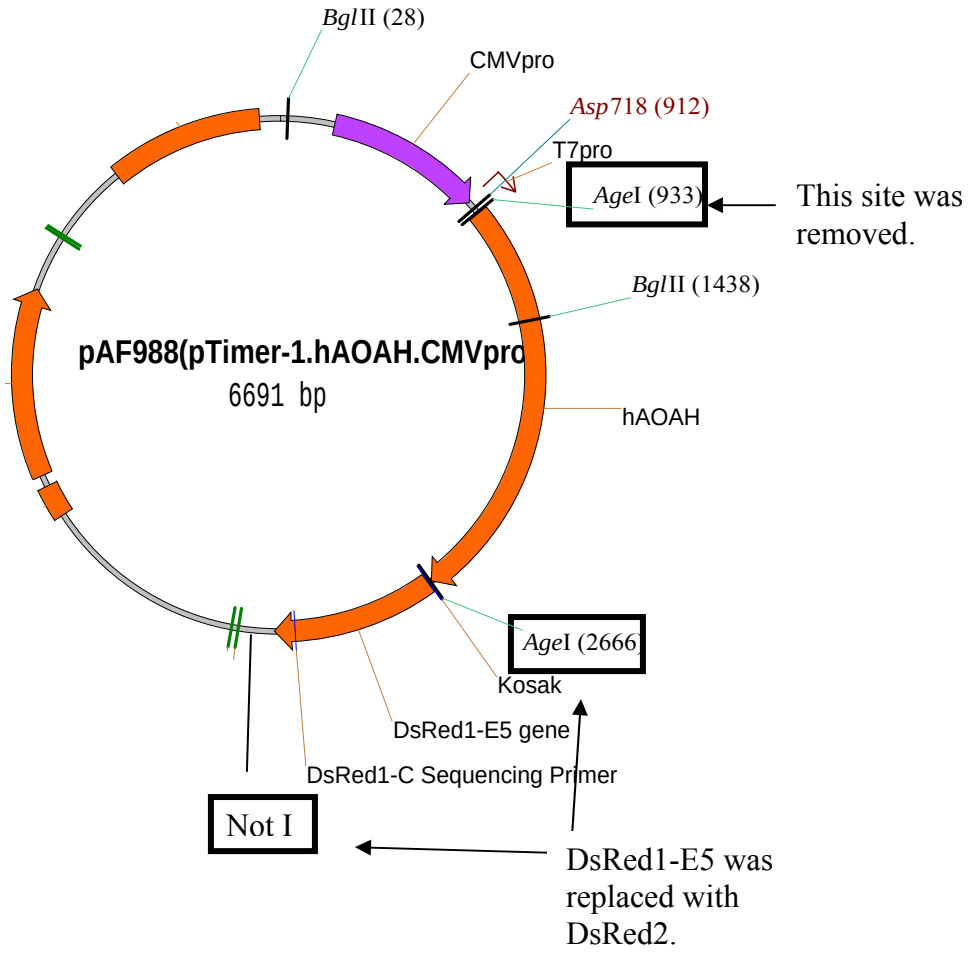


Figure 4.5 – *Cloning on pAF 1127. The 5' Age I site from plasmid pAF988 was removed by partial digest and re-ligation. The DsRed1-E5 gene was replaced with the DsRed2 gene by double digestion with AgeI and Not I.*

AOAH-eGFP fusion protein (pAF1224). Faith Sharp, a rotation student, under my supervision performed the following cloning steps. Plasmid AF1127 (DsRed2-AOAH fusion protein) and pRK785 (plasmid DsRed1-N1 from BD Biosciences Clontech with the DsRed1 gene replaced with the enhanced green fluorescence gene, eGFP) were digested with *Age*I and *Not*I overnight at 37°C. Products were run on a 1% agarose gel and the 6 kb vector (pAF1127 minus the DsRed2 gene), and 717 bp insert (eGFP), were excised and purified with Qiaquick gel extraction kit (Qiagen). Vector and insert were ligated using Rapid DNA Ligation kit (Roche) and transformed into DH10B electro competent cells (Invitrogen). Colonies were screened with several restriction enzyme digests: *Age*I, *Not*I, and *Kpn*I to linearize the plasmid at 6.7 kb, *Not*I + *Age*I to give 6.0 kb and 735 bp fragments, and finally with *Kpn*I + *Age*I to give 5.0 kb and 1.7 kb fragments. Positive clone #2 was streaked onto LB-Kan plates, grown overnight at 37°C, and glycerol stocked. It was given the accession number RM 1224 and stored in the lab's bacterial strain stocks at -80°C. Two plasmid preps were made and one stored in the labs plasmid stocks at -80°C and the other in Y9.330, -20°C, AF Fusion Protein box.

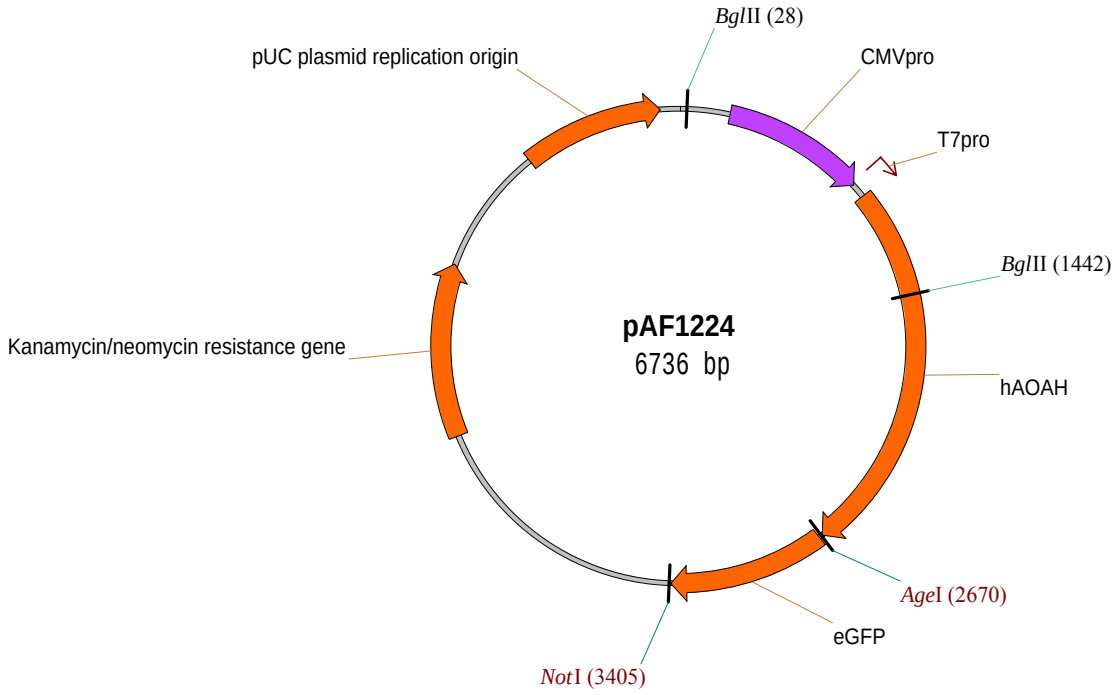


Figure 4.6 – Plasmid pAF1224 (hAOAH-eGFP). *Plasmid pAF1224 was created by replacing the DsRed2 gene from pAF1127 with eGFP from pRK785.*

Transfections. CHO-CD14 cells were plated at ~ 2 x 10^5 cells per well in 12-well plates on 18 mm circle, glass cover slips at #1 thickness (Fisher Scientific cat # 12-545-100), that had been flamed prior to putting into wells. Transfection was conducted when the cells reached ~80% confluency (usually in 2 days) using either Superfect (Qiagen) or Lipofectamine 2000 (Invitrogen) transfection reagents. For Superfect transfections, one µg of DNA was diluted in 100 µl serum and antibiotic-free CHO medium; four µl of Superfect reagent was then added slowly and incubated at room temperature for ten

minutes. During the incubation, the CHO-CD14 cells were washed twice with PBS pH 7.2, and 0.6 mls of fresh serum and antibiotic-free CHO medium was added to the cell monolayer. After the ten-minute incubation, 100 µl of diluted DNA/Superfect was added to each well. Cells were incubated for two hours at which time the medium was changed and the cells were re-incubated for 24 or 48-hours.

Transfections using the Lipofectamine 2000 Reagent (Invitrogen) were performed as follows. One µg of DNA was diluted into 50 µl serum and antibiotic-free CHO medium. Two µl of Lipofectamine 2000 reagent was diluted in 50 µl of the serum and antibiotic-free medium and incubated at room temperature for five minutes. During the incubation, CHO-CD14 cells were washed two times with PBS and 0.5 ml of fresh serum and antibiotic-free medium was added to each well. After the five-minute incubation, the diluted DNA and Lipofectamine were combined and incubated at room temperature for 20 minutes. One hundred µl of this mixture was added to each well of CHO-CD14 cells. It was determined that changing the media at 5 hours post-transfection did not increase the efficiency of transfection. The cells can be cultured in serum free media for about two days before significant cell death is seen. This may actually increase the transfection efficiency.

Phagocytosis Experiments. Two days prior to the experiment, CHO-CD14 cells were passed into 6-well plates at 2 x 10^5 cells per well. XS52 cells were used as a positive control and were plated at 1 x 10^6 cells per well two days prior to the experiment. On the day of the experiment, duplicate negative control cells were treated with 10 µM cytochalasin D (1:200 from 2M stock) and all cells were given fresh medium and

incubated at 37°C, 5% CO_2 for 30 minutes to one hour. During this incubation, Bodipy-labeled *E. coli* (Molecular Probes, Eugene, OR, cat # E-2864) were thawed on ice, sonicated for 30 seconds at 6-watts in a water-bath sonicator (Braunsonic 1510, B. Braun, Melsungen, AG), and analyzed under a fluorescent microscope to ensure there were no large clumps of bacteria. 100 µl of Bodipy were added to each well and spun at 800 rpm (~55 x g) at room temperature for 5 minutes on the Sorvall tabletop centrifuge (RT6000B, Kendro LabProducts, Newton, CT). Cells were re-incubated at 37°C, 5% CO_2 for 1.5 hours. The media was removed, cells washed once in 1x PBS, and lifted into 3 ml FACS tubes (Falcon) with 2 mM EDTA diluted in PBS. Cells were run through a Fluorescence activated cell sorter (FACS) +/- trypan blue and data were analyzed by Cell Quest software (Becton Dickinson).

Immunohistochemistry. Sections of normal human kidney (medulla and cortex) were excised from renal tissue after their removal from patients undergoing radical nephrectomy. Sections about the size and thickness of a quarter were immediately placed in 4% formaldehyde/DEPC-PBS (freshly prepared from paraformaldehyde) and incubated at 4°C for 16 hours. The formaldehyde was replaced with sterile PBS, pH 7.2 and incubated at 4°C for an additional 16 hours. Kidneys were dehydrated and paraffin-embedded, and 4 µm sections were placed onto microscope slides treated with Vectabond (Vector Laboratories, Burlingame, CA). Slides were stored desiccated at 4ºC until use. Murine kidneys were obtained from 129 mice (Harlan) that were anesthetized (ketamine-acepromazine) and transcardially perfused with cold heparinized DEPC-saline. Tissues were deparaffinized twice in xylene for 5 minutes and then 3 times in 100% EtOH 2

minutes. Slides were dipped in water and then incubated in PBS for 5 minutes. Tissues were permeabilized in 0.3% Triton X-100/ PBS for 2 incubations, each 5 minutes, followed by 2 PBS washes. PBS was used as the buffer in all subsequent steps. Non-specific secondary interactions were blocked with 3% normal horse serum (Vector Laboratories) for 30 minutes at room temperature. Excess blocking serum was decanted off the slide by tapping on a paper towel and the areas around the tissue were wiped with a Kim-wipe. Dilutions of primary antibody (see below) were placed over the tissue and slides were incubated overnight at 4ºC in a covered dish. After washing in PBS, a biotinylated horse anti-mouse antibody (Vector Laboratories) was diluted 1:200 in PBS and applied to the tissue for 30 minutes at room temperature. After a PBS wash, horseradish peroxidase streptavidin (Vector Laboratories) was diluted 1:500 and incubated for 30 minutes at room temperature. During this incubation, fresh DAB-chromagen (Dako, Caprinteria, CA) was prepared by placing 1 tablet into 10 mls of sterile 0.5 M Tris-Cl, pH 7.6 and allowing it to dissolve. Just prior to use, hydrogen peroxide (3%, Sigma-Aldrich) was added to a final concentration of 0.3%. After a PBS wash, DAB-chromagen was added in 2, five-minute incubations. The slides were placed under running water for approximately 5 minutes and then counter-stained with filtered hematoxylin for one second. Sections were washed under running water for 5 minutes, dehydrated 3 times in 100% EtOH for 3 minutes each, and equilibrated 3 times in xylene for 3 minutes each. Slides were mounted with Permount (Fisher Scientific) and cover-slips were applied. Human kidney sections were stained with murine monoclonal antibodies 3C5 (8F8) and 3G4 (3F3). Various concentrations of primary antibody were tested (1:10 → 1:10,000). A 1:200 dilution of 3G4 (3F3) was successful in detecting

human AOAH in kidney samples. Murine kidneys were incubated with primary antibody 4E5-B3 and B6 (ascites fluid antibody) and purified monoclonal antibody 2F3-2A4. Only a 1:100 dilution of 2F3-2A4 monoclonal antibody was successful in the detection of AOAH in the murine kidney.

Discussion

In this chapter, I have discussed the generation of three separate AOAH-fusion proteins. The first, a C-terminal fusion of human AOAH to DsRed1-E5 (pTimer-1), was an apparent success. Upon transfection, both green and red cytoplasmic staining could be visualized in transfected CHO-CD14 cells. The addition of 10mM M6P abolished all red fluorescence in cells transfected with the AOAH-fusion protein, but had no effect on cells transfected with pTimer-1 alone. This potentially interesting result, if repeated, might give further evidence to our hypothesis that AOAH must be secreted and subsequently re-enter the cell in order to mature to the more enzymatically active form of AOAH. Because DsRed1-E5 changes color from green to red as it matures, finding only green protein within the cell implies that "old" protein must either be secreted from the cell or destroyed. Differentiating between these two events should be relatively easy. One could collect lysates and supernatants from transfected cells and assay them for AOAH activity as well as for fluorescence on a flourometer. If AOAH is indeed secreted and taken back up again, the AOAH obtained from the supernatants might initially be green, and if removed from the cells it would change fluorescence to red over time. I have

never detected mature AOAH in the supernatants of LLC-PK1 or AOAH-transfected BHK 570 cells. If AOAH is destroyed within the cell, one should not be able to detect AOAH activity in the supernatant. One would assume that red fluorescence would not be detectable at any early time points, but only after the protein ages.

The second fusion protein, a C-terminal fusion of human AOAH to DsRed2, did not work. This fusion was created to reduce the complexity of the previous pTimer-1 system. If successful, all red fluorescence would be derived from the AOAH-fusion protein. Co-staining transfected cells with various organelle-specific markers would allow us to determine better AOAH's intracellular location. The single color fusion protein should also allow us to use our available sources of green fluorescent *E. coli* and purified LPS. Once successfully transfected, CHO-CD14 cells could be infected with Bodipy-labeled *E. coli* or GFP-labeled purified LPS and one could search for the co-localization of green and red fluorescence. Unfortunately, this fusion-protein (pAF1127) was toxic and killed the vast majority of transfected cells. It was nearly impossible to find a red cell at any time after transfection and the experiments using this plasmid were terminated.

The third fusion protein, a C-terminal fusion of human AOAH to enhanced green fluorescent protein (eGFP), is probably a success. Preliminary experiments suggest that transfection efficiency in CHO-CD14 cells is approximately 20-30% and that it is non-toxic. These experiments need to be repeated. If consistent results are obtained, experiments to determine the exact intracellular location of AOAH should be conducted. I suggest starting with a marker for lysosomes. Molecular Probes Inc., have many of these transfection reagents and the experiments should be relatively simple. CHO-CD14

cells phagocytose Bodipy-labeled *E. coli* and should therefore phagocytose red-fluorescent labeled bacteria, although a preliminary experiment should be done to prove that this is the case. Once we know that the cells phagocytose red-labeled bacteria, we can begin co-localization studies. One could also do this experiment with fluorescent-labeled purified LPS. At this time, we do not know if AOAH acts on intact bacteria or LPS that has been shed from Gram-negative bacteria. I believe it will act on both, but may do so in different sub-cellular locations. The above experiments will help us to answer such questions.

Renal cortical tubule cells express abundant AOAH activity. While initial attempts at immuno-localizing AOAH within these cells were discouraging, I believe it warrants further investigation. While we only have one antibody capable of recognizing murine AOAH, several antibodies, both human and mouse, recognize porcine (pig) AOAH. Immunohistochemical experiments on pig kidneys may allow us to localize AOAH within proximal tubule cells. If successful, immuno-electron microscopy may be utilized to localize better AOAH within specific cellular organelles. Bladder cells do not express AOAH, but *in vitro* they are able to acquire exogenous AOAH from the supernatants of AOAH-producing cells. It may not be possible to detect such small quantities of AOAH in the bladder tissue, although it would be an interesting experiment.

While no definitive results have come from the work presented in this chapter, I believe we are well on our way to discovering the intracellular location of AOAH. The plasmids described here should provide a starting point for others to create more useful fusion proteins. Whoever takes over these projects should be prepared to spend numerous hours on a good fluorescence microscope. The good microscopes are, at this

time, very costly and one needs to spend a lot of time to understand truly how to use them to their fullest advantage.

Chapter Five

Discussion

Acyloxyacyl hydrolase (AOAH) is a lipopolysaccharide-deacylating enzyme that until recently has been found only in myeloid lineage cells. By cleaving the secondary fatty acyl chains from the bioactive center of LPS, lipid A, AOAH reduces the toxicity of LPS and greatly reduces its ability to stimulate human endothelial cells *in vitro* as well as in several models of inflammation [12;24;29]. In addition to this function, AOAH may also modulate innate immune responses to invading Gram-negative bacteria. In the work presented here, I have described several important discoveries that contribute to a better understanding of AOAH. There are many future directions to pursue regarding this work and I hope to outline some of those here.

AOAH Expression in Renal Proximal Tubule Cells

One of my first discoveries in the lab was the finding that AOAH is expressed in renal cortical tubule cells. This came as a surprise, for AOAH had never been localized to a non-myeloid lineage cell before. Not only was AOAH present in murine tubule cells, but its activity was also detected in lysates of human kidney and in first-passage

primary murine and human renal proximal tubule cells. AOAH expression was lost as the primary cells differentiated in culture. One of the most commonly used human proximal tubule cell-lines, A498, used extensively by other labs to study the LPS-induced renal inflammatory response, does not express AOAH. Because this cell line lacks the only enzyme capable of deacylating and detoxifying LPS, one must conclude that it is not an appropriate model for such studies. In an attempt to find a renal proximal tubule cell line that expresses AOAH , I tested numerous cell lines for AOAH activity. Only LLC-PK1, porcine proximal tubule cells, had AOAH activity. LLC-PK1 cells were able to secrete the precursor form of AOAH (Figure 2.9B and 2.9C) into their medium as was previously demonstrated for recombinant AOAH stably expressed in BHK 570 fibroblast cells [33]. LLC-PK1 cells retain many properties of native proximal tubule cells [86;105] and therefore are a representative cell line to use for *in vitro* studies. The available rabbit and murine monoclonal anti-AOAH antibodies were able to recognize porcine AOAH, which allowed me to perform immunoprecipitation and Western blot analyses. As described in chapter two of this dissertation, the lack of appropriate antibodies to murine AOAH has made immunohistochemical studies in mouse tissues difficult. An alternative might be to use pig tissue for such studies.

Several lines of evidence, including gentamicin protection and electron microscopic assays, suggest that Gram-negative bacteria are able to bind to, and invade, proximal tubule cells [55;56;106-108]. Studies in cynomogus monkeys have suggested that bacteria must possess p-pili in order to bind to proximal tubule cells [53]. This work, combined with the finding that bacteria without such pili are rarely detected in the kidneys of humans or mice with Gram-negative UTI, has led to the hypothesis that the

presence of uropathogens in the kidney (in particular proximal tubule cells) is dependent upon p pili. On the other hand, both pyelonephritic (pap operon positive) and fecal (pap operon negative) isolates were able to bind to and invade human first-passage renal cortical tubule cells [55;56]. Regardless of the necessity for p pili-mediated invasion of the kidney, once bacteria have gained access to the kidney it is evident that proximal tubule cells are their targets. It seems reasonable that proximal tubule cells should contain enzymes, such as AOAH, that degrade the bacteria that they ingest.

In order to test the hypothesis that AOAH functions in renal proximal tubule cells to deacylate LPS contained in internalized bacteria, one could utilize the porcine LLC-PK1 cells. The first step in these studies would be to perform internalization assays (either by the traditional gentamicin-protection assay or by allowing cells to internalize fluorescently-labeled bacteria and analyzing uptake by FACS) using various strains of *E. coli*. The experiments conducted by Donnenberg and Warren [55;56] suggest that the presence of a p pilus is not necessary for the bacteria to gain entry into the tubule cells and therefore it is reasonable to assume that *E.coli* lacking p pili might be utilized. I tested the ability of LLC-PK1 cells to phagocytose Bodipy-labeled (K12) *E. coli* and found that while XS52 dendritic cells were able to internalize these bacteria, LLC-PK1 cells were not. I next wondered if a pyelonephritic strains of *E. coli* (expressing p pili) might be internalized by LLC-PK1 cells. After labeling pyelonephritic strains RSM 947 (DS17) and RSM 953 (AAEC185/pPil110-35) and their non-piliated control, RSM 948 (DS17-8) [53], and RSM 954 (AAEC185/pPil110-35, G-) with Bodipy, I assayed their ability to be internalized by both XS52 dendritic and LLC-PK1 cells. Neither the pyelonephritic or control strains were internalized by LLC-PK1 cells. All strains were

internalized by XS52 dendritic cells, but not as effiecintly as commercially prepared Bodipy-*E.coli* (K12).

Murine renal proximal tubule cells possess several toll-like receptors (TLRs 1, 2, 3, 4, and 6), CD14 and MD-2 [79] and therefore have the pattern recognition receptors necessary for LPS recognition. Using our double-labeled [^{3}H/^{14}C]LPS substrate, I performed an experiment to investigate the ability of LLC-PK1 cells to deacylate cell-associated LPS. Unfortunately, I was unable to show significant deacylation of purified LPS by LLC-PK1 cells. These experiments should be repeated, using fresh LPS substrate and if successful, experiments that test the ability of LLC-PK1 cells to deacylate LPS on internalized Gram-negative bacteria might then be performed. While the experiments using purified LPS will be relatively straightforward, preparing the substrate for the internalization of whole *E. coli* might prove more difficult, especially if p pili are necessary for invasion. LCD25 *E. coli*, a rough Ra mutant, is unable to produce acetate or utilize it as a carbon or energy source. When one incubates it with [^{14}C]acetate, the label is incorporated exclusively into the fatty acids [94]. Although not tested, we have no reason to believe that LCD25 bacteria express p pili. If need be, one can transform LCD25 with a plasmid that contains the pap operon, test for agglutination of sheep erythrocytes, and then label the fatty acyl chains with [^{14}C]acetate. By using positive (XS52 dendritic) and negative (A498 proximal tubule cells which do not express AOAH) controls, one might show the dependence on AOAH for the deacylation of internalized Gram-negative bacteria in proximal tubules.

The Secretion of AOAH by Proximal Tubule Cells *In Vitro* and *In Vivo*

As mentioned above, AOAH is secreted from renal proximal tubule cells *in vitro*. As has been previously demonstrated for recombinant AOAH in transfected BHK 570 fibroblasts, the secreted form of the protein runs as a single band on SDS-PAGE at an apparent molecular weight of 70,000. It is termed pro-AOAH. Cell lysate fractions from LLC-PK1 and AOAH-transfected BHK 570 cells contain both pro-AOAH and the mature, more enzymatically active form of AOAH. Mature AOAH has an apparent molecular weight of 60,000 on a non-reducing SDS-polyacrylamide gel and 50,000 on a reducing gel.

AOAH is also present in cortical tubule cells *in vivo*. This was demonstrated by AOAH activity assays on human and murine kidney lysates (Figures 2.1, 2.6, and 2.7) and by *in situ* hybridization experiments in murine kidney tissue (Figures 2.3 and 2.4). The presence of AOAH in renal proximal tubules and their known ability, *in vitro*, to secrete AOAH, led me to speculate that AOAH would also be secreted from proximal tubule cells *in vivo*. A small volume (5 µl) of urine from wild type, but not AOAH -/- mice, was able to remove ^{3}H fatty acyl chains from the double labeled [^{3}H/^{14}C]LPS substrate (Figure 2.10 and 2.11) and only secondary (acyloxyacyl-linked) chains were removed (Figure 2.2). While these data suggest that AOAH is being released from tubule cells *in vivo*, they do not indicate which form (pro- or mature AOAH) is present. To determine this, AOAH was immunoprecipitated from both wild type and AOAH-/- mice, run on a reducing and non-reducing polyacrylamide gel, and immunoblotted with

antibodies specific for murine AOAH. To our surprise, only mature AOAH was detected in murine urine (Figure 2.12).

There are several explanations for this finding, some of which have already been tested. It is possible, although not very likely, that proximal tubule cells secrete the mature form of AOAH *in vivo*. If culture conditions for primary murine proximal tubule cells were identified, one could argue that their supernatant might contain AOAH in its true, *in vivo*, state. However, these cells have been manipulated and probably do not represent the true *in vivo* state of tubule cells. In addition, it has been very difficult to culture primary tubule cells; even in our successful attempts, cells lost their AOAH activity relatively quickly. A second explanation for the presence of mature AOAH in the urine is that AOAH might be converted to the mature form by some component of the urine during its transit through the kidney and into the bladder. The low pH of the urine may provide an environment that promotes proteolytic cleavage of AOAH into its mature form. To address this possibility, urine from AOAH -/- mice was incubated with concentrated pro-AOAH *ex-vivo*. Our attempts to show conversion of pro-AOAH to mature AOAH by Western blot were unsuccessful. It's possible that the conditions of this experiment were not optimized or that Western blot is not a sensitive enough assay. Thirdly, it is possible that AOAH is being converted to the mature form by cells in the urinary tract as it transits into the bladder. These cells might, then, re-secrete the mature form into the urine. This is also difficult to test, since it must be done *in vitro*, where there are extensive numbers of cell types to test and conditions might not be true to the *in vivo* state. Nevertheless, I attempted to address this hypothesis in cultured T24 bladder cells. Upon incubation with pro-AOAH, T24 bladder cells were unable to secrete the

mature form of AOAH into their culture medium, although they were able to mature cell-associated AOAH (Figure 2.15). It's possible that another cell type (distal tubule, collecting duct, or ureter cell) is responsible for such maturation or that this is not the maturation mechanism.

Importantly, only the precursor form of AOAH (pro-AOAH) can be taken up by bladder cells *in vitro*, and therefore it seems unlikely that the mature form of AOAH is becoming cell-associated *in vivo*. Because we find AOAH activity in washed bladder tissue, I am inclined to believe that we do not yet understand the mechanism of uptake and maturation *in vivo*. It is possible that proximal tubule cells are indeed secreting pro-AOAH, but that all of it is becoming cell-associated during its transit in the urinary tract, allowing for little or none to be detected in our assays.

As discussed in Chapter Two, AOAH activity can also be detected in lysates of human cortex and medulla (Figure 2.7). Despite repeated attempts, we were unable to detect AOAH activity in normal or infected (cystitis) human urine (data not shown). Attempts to concentrate the urine did not allow for the detection of AOAH activity. It is possible that the kidneys of human and mouse differ in respect to AOAH expression or secretion or that we were unable to detect the AOAH present in human urine. Firstly, it's possible that AOAH is not secreted from human renal proximal tubule cells. This is unlikely due to the fact that we detect AOAH activity in both the human cortex and medulla and because all cell types tested to date have secreted the precursor form of AOAH into their culture medium *in vitro* or murine urine *in vivo*. Secondly, AOAH might be expressed in very low abundance in the human urinary tract making it difficult for us to detect. A third, related, explanation is that the bladder (or other downstream

cells) is taking up nearly all of the secreted AOAH, leaving little enzyme available for detection in our assays.

Understanding the mechanisms by which AOAH is secreted by tubule cells and matured within the urinary tract might prove difficult, but this information is very important for understanding AOAHs role in deacylating the LPS in Gram-negative uropathogens (or commensal bacteria).

Enzyme Sharing in the Urinary Tract

Another important finding presented in this dissertation is the observation that AOAH, secreted from proximal tubule cells *in vitro*, can be "shared" with other cells. As presented in Chapter two, AOAH protein (as measured by activity) and mRNA (as measured by real-time PCR) were present in isolated murine renal cortex. However, renal medulla and urinary bladder samples had AOAH activity but lacked AOAH mRNA (Figures 2.5 and 2.6). Although the activity in the medulla might be explained by the presence of urine within the tissue, the activity in the washed bladder tissue argues that the cells are able to take up AOAH from the urine. This finding, combined with data presented in Chapter Two (Figure 2.19 A-C), suggests that bladder cells are able to take up, and subsequently mature, pro-AOAH *in vitro*. In addition to finding that pro-AOAH is taken up by bladder cells, it was also demonstrated that 10 mM M6P and ammonium chloride, but not G6P or mannose, could block such uptake. It is not surprising that the uptake of AOAH, a heavily N-glycosylated protein, is mediated by the M6P receptor. Kornfeld, in a 1989 review, suggested that the lysosome is the final destination for

soluble proteins internalized by endocytosis. He also noted that this transport is dependent on recognition of M6P residues by M6P receptors [39]. It makes sense then that AOAH, known for its role in detoxifying LPS on Gram-negative bacteria, should traffic to lysosomes, where bacteria are known to be targeted for destruction.

There are several experiments that might strengthen the evidence for the role of M6P residues and receptors in the uptake of AOAH. Cells can be transiently depleted of M6P receptors by an infection with an adenovirus expressing a ribozyme (a RNA molecule that can cleave RNA in a sequence specific way) specific for M6P/IGF2R mRNA [109]. One could use such cells (or cells treated with siRNAs specific for the M6P receptor mRNA) to test their ability to take up pro-AOAH from their culture medium. One would expect that cells lacking M6P receptors would be unable to associate with pro-AOAH. Another relatively simple experiment would be to use phosphatases to remove phosphates from AOAH and subsequently test the modified protein's ability to become associated with cells. Again, if the association is mediated by M6P receptors, one would expect phosphatase treatment to inhibit such associations. A third experiment, which would require the acquisition of M6P receptor antibodies, would be to test the ability of such antibodies to block the association of AOAH with recipient cells.

BHK cells that have been transfected with CD-M6PR secrete nearly all newly synthesized M6P-containing proteins. When co-transfected with both the CD- and CI-M6P/IGFII receptor, cells restore the intracellular targeting of M6P-containing proteins to endosomes and reduce their secretions [110]. These findings suggest that the CD-M6P receptor is responsible for the secretion of nascent polypeptides with M6P moieties and that in the absence of the CI-M6P/IGFII receptor, very few of these proteins are found

intracellularly. We wondered if P388D1 murine macrophage-like cells, which lack the CI-M6P/IGFII receptor (but possess the 46 kDa CD-M6PR)(14), would express AOAH and if so, whether or not there would be an abundance of AOAH in the culture media but not in the cells. We hypothesized that, due to the lack of CI-M6P receptors at the plasma membrane, any AOAH activity would be detected in the culture medium. There was some confusion about our available P388D1 cell-line, since it had been obtained from another lab and we could not verify that the cells were indeed P388D1 cells (ATCC literature claims that P388D1 cells should be in suspension and our cells were not). We obtained new P388D1 cells from ATCC (CCL-46) and assayed both the cell lysate and medium for AOAH activity. The results were highly variable and therefore inconclusive. The first assay, done in duplicate, suggested that both cell lysate and supernatant samples from P388D1 cells contain a significant amount of AOAH activity. However, a second and third assay, done 3 and 12 days later, suggested that the supernatant did indeed have more AOAH than the cell lysate fraction. In two attempts to assay the P388D1 cells again, months later, neither the supernatant or cell lysate expressed AOAH. This might suggest that expression of AOAH is lost as the cells are passed in culture over time.

We believe this to be the first description of potential "enzyme sharing" in the urinary tract. Although other proteins (such as hormones) act at locations distant from their origin, we were unable to find another such example in the urinary system. In addition, hormones are signaling molecules whose function is to transmit information from one site to another. AOAH, however, is not known to be a signaling molecule; rather, it is part of the cellular machinery. There is no (known) reason why bladder cells shouldn't make their own AOAH. Although this is mere speculation, the original (and

main) role for AOAH might have been within proximal tubule cells. Here, the enzyme might function to deacylate internalized Gram-negative bacteria or to perform tasks completely separate from those involved in host defense against invading uropathogens. Bladder cells might have taken advantage of the AOAH present in urine, thus allowing them to associate with and use AOAH to degrade the LPS on internalized Gram-negative bacteria. By allowing bladder cells to deal with invading pathogens before they make their way to the kidneys, AOAH might help to prevent kidney infections (pyelonephritis), which are usually more serious diseases.

While "enzyme sharing" is certainly an interesting concept, it should be noted that *in vitro*, recombinant AOAH and murine urine have been shown to deacylate purified LPS. This suggests that *in vivo*, AOAH might function to deacylate LPS shed from Gram-negative bacteria even in the absence of cells (in the urine, for example). Indeed, an association with bladder cells might not be necessary for the deacylation and detoxification of bacteria in the urinary tract.

The Role of AOAH in Ascending Urinary Tract Infection

The above-described observations, combined with the fact that the majority of UTIs are the result of colonization of the urinary tract with uropathogenic *E. coli* [44], led me to speculate about possible roles for AOAH in ascending urinary tract infections. Initially, we wondered if AOAH, by degrading the LPS in invading bacteria, might contribute to the bacteriostatic nature of murine urine. If so, one would expect infected

AOAH null mice to have a more prolonged infection (as assessed by positive urine, bladder, or kidney cultures) than wild type mice. This did not turn out to be the case. In fact, as described in Chapter three (Figures 3.0 and 3.1), wild type mice had positive urine cultures for days longer than did AOAH null animals.

We next wondered if the inflammatory response to UTI might be more intense or of longer duration in AOAH null mice. Because AOAH is the only known enzyme capable of removing secondary acyl chains from lipid A, it seems reasonable that animals without this ability (AOAH null mice) would be more likely to possess fully acylated (highly stimulatory) LPS than would wild type animals. In fact, data from several studies where C3H/HeN and C3H/HeJ (hyporesponsive to LPS due to a TLR4 mutation) were infected with UPEC suggest that LPS is necessary for the influx of neutrophils into both the urine and bladder tissue. Although both strains of mice were infected to similar levels, C3H/HeJ mice were unable to recruit neutrophils to either their urine or bladder, had no IL-6 present in their urine [111] and failed to clear bacteria from their bladders as efficiently as did C3H/HeN mice [42;58-61]. It seems reasonable, then, that overstimulation of the immune system by fully acylated LPS might cause a more sustained inflammatory response and possible immune-mediated tissue injury.

In light of these data, we wondered whether, upon inducing experimental ascending UTIs in both wild type and AOAH null mice, AOAH null mice might exhibit a more intense (duration or intensity) inflammatory response to the infection. To assess this, I analyzed the number of neutrophils present in the urine following ascending UTI with a pathogenic strain of *E. coli*. I found that there was no significant difference in the number of neutrophils present in the urine of wild type or AOAH null mice at any of the

early time points (6, 12, 24 hours post-infection) examined. As the bacteria were cleared from wild type animals (48 and 72 hours post-infection), I also saw a drop in neutrophil numbers in the urine (Figure 3.4).

Although enumeration of urinary neutrophils is commonly used to assess inflammatory responses within the urinary tract, we wondered whether this was indeed an appropriate measure of local inflammatory responses. As discussed previously, UPEC invade both bladder epithelium and renal proximal tubule cells [55;56;106-108] and both tissues either express or have acquired AOAH activity. We wondered if differences between wild type and AOAH null mice might be better seen in the bladder or kidney. To test this hypothesis, these tissues were collected at 72 hours post-infection, when both wild type and AOAH null mice had cleared bacteria from their urine. Tissues were fixed, paraffin embedded, cut, and stained with hematoxylin and eosin and scored by Dr. Joseph Zhou, a renal pathologist at UT-Southwestern, for the extent of inflammation present. I chose the 72 hour time point because it allowed me to follow the entire course of infection without sacrificing any mice. Our results, described in Chapter three and Figure 3.5, indicate that there is no significant difference in the level of inflammation in either the bladder or kidney at this time point. In fact, the kidney showed no signs of inflammation in either wild type or AOAH null mice. Because we infected with a cystitis, rather than a pyelonephritic, strain of *E. coli*, we did not expect to see a phenotype in the kidney. We wondered whether this 72 hour time point might be too late -- if we had missed a critical early time point that would explain the clearance of bacteria from AOAH null mice, since one might expect a correlation between clearance of bacteria from the urinary tract and a preceding inflammatory response. To address this concern, the experiment was repeated

and four wild type and five AOAH null mice were sacrificed at 24 hours post-infection. The results indicated that, at 24 hours post-infection, there is no significant difference in the inflammatory response between wild type and AOAH null mice in the bladder (Figure 3.6). I believe there were too few mice to accurately address this question and that this experiment should be repeated with more mice in each group.

Because these experiments require the use of a large number of animals, it is difficult to obtain an appropriate sample number for statistical analysis. Ideally, one would induce infection in approximately 55 mice, collecting urine from all and sacrificing 10 test and 1 control at each 6, 12, 24, 48, and 72-hour time point. Such an experiment would allow us to obtain important information about the inflammatory responses in the bladder and kidney throughout the duration of the experiment without compromising our ability to assess the final outcome of the infection. Ten mice at each sacrifice point should be sufficient to see any statistically significant difference that might be present. Such an experiment would require a coordinated effort on the part of several individuals in the lab.

It should also be noted here that C57BL/6 mice respond very differently to ascending UTI than do C3H/HeN mice. While both strains are susceptible to and show similar immune responses to the invading bacteria, C3H/HeN mice have a more sustained infection and inflammatory response than do C57BL/6 mice. As described in Chapter 3, bacteria were present in the urine and bladders of C3H/HeN mice up to 8 days post-infection, whereas C57BL/6 mice had cleared their infections by day two. While I believe both strains are potentially useful for our studies, we might be better able to

detect an inflammatory phenotype in the C3H/HeN strain of mice due to the higher base-line level of infection.

Finally, although it can be assumed that any phenotypic difference in the responses of wild type and AOAH null mice are the result of the absence (or presence) of AOAH, it might be interesting to test this assumption directly. Using a strain of *E. coli* which lacks secondary fatty acyl chains (the *msbB* mutant), one could induce ascending infections in wild type and AOAH null mice. One would expect that the level of infection in the wild type and AOAH null mouse would be virtually identical, since neither animal would have the "advantage" of being able to deacylate the LPS present on invading bacteria. These experiments might be difficult because to my knowledge, such strains do not yet exist.

Malakoplakia and Interstitial Cystitis

Malakoplakia is an uncommon inflammatory disease of unknown etiology that is most frequently diagnosed in middle-aged women with histories of recurrent Gram-negative urinary tract infections [112]. It is believed that the disease begins, possibly after a transient period of immunosuppression, with the ingestion of bacteria or bacterial products by macrophages within the urinary tract (kidney > bladder). Once internalized, the bacteria seem to be contained within the phagolysosomes, eventually persisting as dense amorphous aggregates that become encrusted with calcium phosphate and/or calcium hydroxyapatite. Such calcified structures are termed Michaelis-Gutmann bodies

(MGBs). Prolonged antibiotic therapies resolve the lesions and suggest a bacterial etiology [112].

Xanthogranulomatous pyelonephritis (XGP) is another uncommon disease that has been speculated to be a more severe or chronic form of malakoplakia. Patients with XGP have characteristic "stuffed macrophages" that form nodular lesions in the kidneys and other organs [112]. Several investigators have been successful in inducing both malakoplakia and XGP in animal models. One of the most common methods of inducing these diseases in rats is ureteral obstruction followed by intravenous *E. coli* injections [113]. Interestingly, maintaining ureteral obstruction for months after the intravenous dose of LPS seems to produce kidney lesions that resemble those in XGP patients [114]. In each case, the manifestation of disease seems to be the result of Gram-negative bacterial infection within the urinary tract and inadequate destruction of bacterial products within macrophages.

Because of AOAH's presence throughout the urinary tract and its previously described expression in macrophages, it might be possible that a deficiency in AOAH would make one more susceptible to malakoplakia (or XGP). To test this hypothesis, one could obstruct one ureter with a ligature (using the opposite kidney as a control) while injecting an intravenous, sub-lethal, dose of *E. coli* [adapted from [113]]. The ligature is removed two days later and wild type and AOAH null mice are assessed at various time points (3 days to a month post-infection) for difference in the size, number, or extent of tissue involvement between the groups. Even if we do not detect the presence of MGBs, the experiments might uncover other roles for AOAH in a more chronic inflammatory-disease model. In fact, AOAH might have a greater role in chronic infections, where

regulating the responses to LPS might be extremely important so as not to induce immune-mediated tissue damage.

Conclusions

AOAH is highly conserved throughout evolution and plays an important role in the detoxification of LPS on Gram-negative bacteria. Although somewhat surprising, the discovery of AOAH in renal proximal tubule cells seems to make sense. Proximal tubule cells are equipped with several receptors (CD14, TLR4, and MD-2) that aid in the recognition of Gram-negative bacteria and they are known targets for such uropathogens during ascending UTI. One might expect such cells to contain enzymes, such as AOAH, that help to defend them from overwhelming infection and inflammation. The presence of AOAH in murine urine, combined with its ability to deacylate purified LPS in the absence of added detergents, suggests that AOAH might also be able to deacylate free LPS that is shed from Gram-negative bacteria in the urine. Although this suggests that bacteria do not have to become cell-associated in order for AOAH to detoxify their LPS, the ability of AOAH to function in bladder cells would be an added advantage to the host. The discovery of "enzyme sharing" *in vitro* suggests that AOAH will be able to deacylate the LPS on internalized uropathogens in the bladder *in vivo*. The findings described in this dissertation help us understand how our immune system responds to Gram-negative

bacterial LPS and should give insight into the ways in which our bodies deal with ascending urinary tract infections.

Reference List

1. **Rietschel,E.T. and O.Westphal. 1999. Endotoxin: Historical perspectives. *In* Endotoxin in Health and Disease. H.Brade, S.M.Opal, S.N.Vogel, and D.C.Morrison, editors. Marcel Dekker, Inc., New York. 1-30.**

2. **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.**

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

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

5. **Luderitz,O., A.M.Staub, and O.Westphal. 1966. Immunochemistry of O and R antigens of *Salmonella* and related Enterobacteriaceae. *Bacteriol.Rev.* 30:192-255.**

6. **Luderitz,O., C.Galanos, H.J.Risse, E.Ruschmann, S.Schlecht, G.Schmidt, H.Schulte-Holthausen, R.Wheat, O.Westphal, and J.Schlosshardt. 1966.**

Structural relationship of *Salmonella* O and R antigens. *Ann.N.Y.Acad.Sci.* 133:349-374.

7. **Holst,O. and H.Brade. 1992. Chemical structure of the core region of lipopolysaccharides. *In* Bacterial Endotoxic Lipopolysaccharides. D.C.Morrison and J.L.Ryan, editors. CRC Press, Boca Raton, FL. 135-170.**

8. **Brade,H. and E.T.Rietschel. 1984. Alpha-2----4-interlinked 3-deoxy-D-manno-octulosonic acid disaccharide. A common constituent of enterobacterial lipopolysaccharides. *Eur.J.Biochem.* 145:231-236.**

9. **Mukerjee,P., M.Kastowsky, S.Obst, and K.Takayama. 1999. Lipopolysaccharide preparations in aqueous media: implications for solution vs. suspension. *In* Endotoxin in Health and Disease. H.Brade, S.M.Opal, S.N.Vogel, and D.C.Morrison, editors. Marcel Dekker, Inc., New York. 221-228.**

10. **Qureshi,N., K.Takayama, D.Heller, and C.Fenselau. 1983. Position of ester groups in the lipid A backbone of lipopolysaccharides obtained from *Salmonella typhimurium*. *J.Biol.Chem.* 258:12947-12951.**

11. **Wollenweber,H.-W., K.W.Broady, O.Luderitz, and E.Th.Rietschel. 1982. The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. *Eur.J.Biochem.* 124:191-198.**

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

13. **Imoto,M., H.Yoshimura, N.Sakaguchi, S.Kusumoto, and T.Shiba. 1985. Total synthesis of *Escherichia coli* lipid A. *Tetrahedron Lett.* 26:1545-1548.**

14. **Kotani,S., H.Takada, M.Tsujimoto, T.Ogawa, I.Takahashi, T.Ikeda, K.Otsuka, H.Shimauchi, N.Kasai, J.Mashimo, S.Nagao, A.Tanaka, S.Tanaka, K.Harada, K.Nagaki, H.Kitamura, T.Shiba, S.Kusumoto, M.Imoto, and H.Yoshimura. 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.**

15. **Galanos,C., O.Luderitz, E.T.Rietschel, O.Westphal, H.Brade, B.Brade, M.Freudenberg, U.Schade, M.Imoto, H.Yoshimura, S.Kusumoto, and T.Shiba. 1985. Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. *Eur.J.Biochem.* 148:1-5.**

16. **Takada,H. and S.Kotani. 1992. Structure-function relationships of lipid A. *In* Bacterial Endotoxic Lipopolysaccharides, Vol. I. D.C.Morrison and J.L.Ryan, editors. CRC Press, Boca Raton. 107-135.**

17. **Imoto,M., H.Yoshimura, M.Yamamoto, T.Shimamoto, S.Kusumoto, and T.Shiba. 1984. Chemical synthesis of phosphorylated tetra-acyl disaccharide**

corresponding to a biosynthetic precursor of lipid A. *Tetrahedron Lett.* 25:2667-2670.

18. Galanos,C., V.Lehmann, O.Luderitz, E.T.Rietschel, O.Westphal, H.Brade, L.Brade, M.A.Freudenberg, T.Hansen-Hagge, T.Luderitz, G.McKenzie, U.Schade, W.Strittmatter, K.Tanamoto, U.Zahringer, M.Imoto, H.Yoshimura, M.Yamamoto, T.Shimamoto, S.Kusumoto, and T.Shiba. 1984. Endotoxic properties of chemically synthesized lipid A part structures. *Eur.J.Biochem.* 140:221-227.

19. Homma,J.Y., M.Matsuura, S.Kanegasaki, Y.Kawakubo, Y.Kojima, N.Shibukawa, Y.Kumazawa, A.Yamamoto, K.Tanamoto, T.Yasuda, M.Imoto, H.Yoshimura, S.Kusumoto, and T.Shiba. 1985. Structural requirements of lipid A responsible for the functions: A study with chemically synthesized lipid A and its analogues. *J Biochem* 98:395-406.

20. Kanegasaki,S., K.Tanamoto, T.Yasuda, J.Y.Homma, M.Matsuura, M.Nakatsuka, Y.Kumazawa, A.Yamamota, T.Shiba, S.Kusumoto, M.Imoto, H.Yoshimura, and T.Shimamoto. 1986. Structure-activity relationship of Lipid A: Comparison of biological activities of national and synthetic Lipid A's with different fatty acid compositions. *J.Biochem.* 99:1203-1210.

21. Takada,H., S.Kotani, M.Tsujimoto, T.Ogawa, I.Takahashi, K.Harada, C.Katsukawa, S.Tanaka, T.Shiba, S.Kusumoto, M.Imoto, H.Yoshimura, M.Yamamoto, and T.Shimamoto. 1985. Immunopharmacological activities of

a synthetic counterpart of a biosynthetic lipid A precursor molecule and of its analogs. *Infect.Immun.* 48:219-227.

22. Erwin,A.L. and R.S.Munford. 1992. Processing of LPS by phagocytes. *In* Bacterial Endotoxic Lipopolysaccharides. Molecular Biochemistry and Cellular Biology. D.C.Morrison and J.L.Ryan, editors. CRC Press, Boca Raton, FL. 405-434.

23. Wollenweber,H.-W., U.Seydel, B.Lindner, O.Luderitz, and E.Th.Rietschel. 1984. Nature and location of amide-bound (R)-3-acyloxyacyl groups in lipid A of lipopolysaccharides from various gram-negative bacteria. *Eur.J.Biochem.* 145:265-272.

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

25. Lynn,W.A. and D.T.Golenbock. 1992. Lipopolysaccharide antagonists. *Immunol.Today* 13:271-276.

26. 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.

27. 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.

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

29. Somerville,J.E., Jr., L.Cassiano, B.Bainbridge, M.D.Cunningham, and R.P.Darveau. 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J.Clin.Invest.* 97:359-365.

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

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

32. 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.

33. 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.

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

35. 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.

36. 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.

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

38. von Figura,K. and A.Hasilik. 1986. Lysosomal enzymes and their receptors. *Annu.Rev.Biochem.* 55:167-193.

39. Kornfeld,S. and I.Mellman. 1989. The biogenesis of lysosomes. *Annu.Rev.Cell Biol.* 5:483-525.

40. Kornfeld,S. 1992. Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu.Rev.Biochem.* 61:307-330.

41. Burkitt,H.G., B.Young, and J.W.Heath. 1993. Urinary System. *In* Wheater's Functional Histology. Churchill Livingstone, New York. 282-303.

42. Hopkins,W.J., A.Gendron-Fitzpatrick, E.Balish, and D.T.Uehling. 1998. Time course and host responses to *Escherichia coli* urinary tract infection in genetically distinct mouse strains. *Infect.Immun.* 66:2798-2802.

43. Mulvey,M.A. 2002. Adhesion and entry of uropathogenic *Escherichia coli*. *Cell Microbiol.* 4:257-271.

44. Mühldorfer,I., W.Ziebuhr, and J.Hacker. 2001. *Escherichia coli* in urinary tract infections. *In* Molecular Medical Microbiology. M.Sussman, editor. Academic Press, London. 1515-1540.

45. Hooton,T.M. and W.E.Stamm. 1997. Diagnosis and treatment of uncomplicated urinary tract infection. *Infect.Dis.Clin.North Am.* 11:551-581.

46. Wagenlehner,F.M. and K.G.Naber. 2000. Hospital-acquired urinary tract infections. *J.Hosp.Infect.* 46:171-181.

47. Ronald,A. 2002. The etiology of urinary tract infection: traditional and emerging pathogens. *Am.J.Med.* 113 Suppl 1A:14S-19S.

48. Martinez,J.J., M.A.Mulvey, J.D.Schilling, J.S.Pinkner, and S.J.Hultgren. 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *EMBO J.* 19:2803-2812.

49. Johnson,J.R. 2003. Virulence factors in *Escherichia coli* urinary tract infections. *Clin.Microbiol.Rev.* 4:80-128.

50. Mulvey,M.A., Y.S.Lopez-Boado, C.L.Wilson, R.Roth, W.C.Parks, J.Heuser, and S.J.Hultgren. 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science* 282:1494-1497.

51. Johanson,I., R.Lindsted, and C.Svanborg. 1992. Roles of the pap- and prs-encoded adhesins in *Escherichia coli* adherence to human uroepithelial cells. *Infect.Immun.* 60:3416-3422.

52. Sampaio,E.P., E.N.Sarno, R.Galilly, Z.A.Cohn, and G.Kaplan. 1991. Thalidomide selectively inhibits tumor necrosis factor α production by stimulated human monocytes. *J.Exp.Med.* 173:699-703.

53. Roberts,J.A., B.-I.Marklund, D.Ilver, D.Haslam, M.B.Kaack, G.Baskin, M.Louis, R.Möllby, J.Winberg, and S.Normark. 1994. The Gal(α1-4)Gal-specific tip adhesin of *Escherichia coli* P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract. *Proc.Natl.Acad.Sci.USA* 91:11889-11893.

54. Wullt,B., G.Bergsten, H.Connell, P.Rollano, N.Gebretsadik, R.Hull, and C.Svanborg. 2000. P fimbriae enhance the early establishment of *Escherichia coli* in the human urinary tract. *Mol.Microbiol.* 38:456-464.

55. Donnenberg,M.S., B.Newman, S.J.Utsalo, A.L.Trifillis, J.R.Hebel, and J.W.Warren. 1994. Internalization of *Escherichia coli* into human kidney

epithelial cells: comparison of fecal and pyelonephritis-associated strains. *J.Infect.Dis.* 169:831-838.

56. Warren,J.W., H.L.T.Mobley, and A.L.Trifillis. 1988. Internalization of *Escherichia coli* into human renal tubular epithelial cells. *J.Infect.Dis.* 158:221-223.

57. Hedlund,M., B.Frendeus, C.Wachtler, L.Hang, H.Fischer, and C.Svanborg. 2001. Type 1 fimbriae deliver an LPS- and TLR4-dependent activation signal to CD14-negative cells. *Mol.Microbiol.* 39:542-552.

58. Hagberg,L., R.Hull, S.Hull, J.R.McGhee, S.M.Michalek, and E.C.Svanborg. 1984. Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infect.Immun.* 46:839-844.

59. Haraoka,M., L.Hang, B.Frendeus, G.Godaly, M.Burdick, R.Strieter, and C.Svanborg. 1999. Neutrophil recruitment and resistance to urinary tract infection. *J.Infect.Dis.* 180:1220-1229.

60. Linder,H., I.Engberg, I.M.Baltzer, K.Jann, and C.Svanborg-Eden. 1988. Induction of inflammation by *Escherichia coli* on the mucosal level:requirement for adherence and endotoxin. *Infect.Immun.* 56:1309-1313.

61. Shahin,R.D., I.Engberg, L.Hagberg, and C.Svanborg-Eden. 1987. Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local gram-negative infection. *J.Immunol.* 138:3475-3480.

62. Frendeus,B., C.Wachtler, M.Hedlund, H.Fischer, P.Samuelsson, M.Svensson, and C.Svanborg. 2001. *Escherichia coli* P fimbriae utilize the Toll-like receptor 4 pathway for cell activation. *Mol.Microbiol.* 40:37-51.

63. Gentschev,I., G.Dietrich, and W.Goebel. 2002. The *E. coli* alpha-hemolysin secretion system and its use in vaccine development. *Trends Microbiol.* 10:39-45.

64. Gadeberg,O.V., I.Orskov, and J.M.Rhodes. 1983. Cytotoxic effect of an alpha-hemolytic *Escherichia coli* strain on human blood monocytes and granulocytes *in vitro*. *Infect.Immun.* 41:358-364.

65. Falzano,L., C.Fiorentini, G.Donelli, E.Michel, C.Kocks, P.Cossart, L.Cabanie, E.Oswald, and P.Boquet. 1993. Induction of phagocytic behavior in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Mol.Microbiol.* 9:1247-1254.

66. Fiorentini,C., G.Donelli, P.Matarrese, A.Fabbri, S.Paradisi, and P.Boquet. 1995. *Escherichia coli* cytotoxic necrotizing factor 1: evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect.Immun.* 63:3936-3944.

67. De Rycke,J., P.Mazars, J.P.Nougayrede, C.Tasca, M.Boury, F.Herault, A.Valette, and E.Oswald. 1996. Mitotic block and delayed lethality in HeLa epithelial cells exposed to *Escherichia coli* BM2-1 producing cytotoxic necrotizing factor type 1. *Infect.Immun.* 64:1694-1705.

68. Carbonetti,N.H. and P.H.Williams. 1984. A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. *Infect.Immun.* 46:7-12.

69. Jann,B., A.S.Shashkov, D.S.Gupta, and K.Jann. 1992. The O18 antigens (lipopolysaccharides) of *Escherichia coli*. Structural characterization of the O18A, O18A1, O18B and O18B1-specific polysaccharides. *Eur.J.Biochem.* 210:241-248.

70. Svanborg,C., M.Hedlund, H.Connell, W.Agace, R.D.Duan, A.Nilsson, and B.Wullt. 1996. Bacterial adherence and mucosal cytokine responses. Receptors and transmembrane signaling. *Ann.N.Y.Acad.Sci.* 797:177-190.

71. Schilling,J.D., M.A.Mulvey, C.D.Vincent, R.G.Lorenz, and S.J.Hultgren. 2001. Bacterial invasion augments epithelial cytokine responses to *Escherichia coli* through a lipopolysaccharide-dependent mechanism. *J.Immunol.* 166:1148-1155.

72. Hagberg,L., I.Engberg, R.Freter, J.Lam, S.Olling, and C.Svanborg-Eden. 1983. Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin. *Infect.Immun.* 40:273-283.

73. 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.

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

75. Mulvey,M.A., J.D.Schilling, and S.J.Hultgren. 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. *Infect.Immun.* 69:4572-4579.

76. Schilling,J.D., S.M.Martin, C.S.Hung, R.G.Lorenz, and S.J.Hultgren. 2003. Toll-like receptor 4 on stromal and hematopoietic cells mediates innate resistance to uropathogenic *Escherichia coli*. *Proc.Natl.Acad.Sci.U.S.A* 100:4203-4208.

77. Bäckhed,F., M.Söderhäll, P.Ekmann, S.Normark, and A.Richter-Dahlfors. 2001. Induction of innate immune responses by *Escherichia coli* and purified lipopolysaccharide correlate with organ- and cell-specific expression of Toll-like receptors within the human urinary tract. *Cellular Microbiology* 3:153-158.

78. Schilling,J.D., S.M.Martin, D.A.Hunstad, K.P.Patel, M.A.Mulvey, S.S.Justice, R.G.Lorenz, and S.J.Hultgren. 2003. CD14- and toll-like receptor-dependent activation of bladder epithelial cells by

lipopolysaccharide and type 1 piliated *Escherichia coli*. *Infect.Immun.* 71:1470-1480.

79. Tsuboi,N., Y.Yoshikai, S.Matsuo, T.Kikuchi, K.Iwami, Y.Nagai, O.Takeuchi, S.Akira, and T.Matsuguchi. 2002. Roles of toll-like receptors in C-C chemokine production by renal tubular epithelial cells. *J.Immunol.* 169:2026-2033.

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

81. 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.

82. 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.

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

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

85. 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.

86. Nielsen,R., H.Birn, S.K.Moestrup, M.Nielsen, P.Verroust, and E.I.Christensen. 1998. Characterization of a kidney proximal tubule cell line, LLC-PK1, expressing endocytotic active megalin. *J.Am.Soc.Nephrol.* 9:1767-1776.

87. Trifillis,A.L., A.L.Regec, and B.F.Trump. 1985. Isolation, culture and characterization of human renal tubular cells. *J.Urol.* 133:324-329.

88. Brown,W.J., E.Constantinescu, and M.G.Farquhar. 1984. Redistribution of mannose-6-phosphate receptors induced by tunicamycin and chloroquine. *J.Cell Biol.* 99:320-326.

89. Brown,W.J., J.Goodhouse, and M.G.Farquhar. 1986. Mannose-6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. *J.Cell Biol.* 103:1235-1247.

90. Shimomura,I., Y.Bashmakov, and J.D.Horton. 1999. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J.Biol.Chem.* 274:30028-30032.

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

92. Shelton,J.M., M.H.Lee, J.A.Richardson, and S.B.Patel. 2000. Microsomal triglyceride transfer protein expression during mouse development. *J.Lipid Res.* 41:532-537.

93. 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.

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

95. Baur,M., T.-O.Schmid, and B.Landauer. 1989. Role of phospholipase A in multiorgan failure with special reference to ARDS and acute renal failure (ARF). *Klin.Wochenschr.* 67:196-202.

96. Schilling,J.D., M.A.Mulvey, and S.J.Hultgren. 2001. Dynamic interactions between host and pathogen during acute urinary tract infections. *Urology* 57:56-61.

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

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

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

100. Johnson,D.E., C.V.Lockatell, R.G.Russell, J.R.Hebel, M.D.Island, A.Stapleton, W.E.Stamm, and J.W.Warren. 1998. Comparison of *Escherichia coli* strains recovered from human cystitis and pyelonephritis infections in transurethrally challenged mice. *Infect.Immun.* 66:3059-3065.

101. Fukushi,Y., S.Orikasa, and M.Kagayama. 1979. An electron microscopic study of the interaction between vesical epithelium and *E. coli*. *Invest.Urol.* 17:61-68.

102. McTaggart,L.A., R.C.Rigby, and T.S.Elliott. 1990. The pathogenesis of urinary tract infections associated with *Escherichia coli*, *Staphylococcus saprophyticus* and *S. epidermidis*. *J.Med.Microbiol.* 32:135-141.

103. Palmer,L.M., T.J.Reilly, S.J.Utsalo, and M.S.Donnenberg. 1997. Internalization of *Escherichia coli* by human renal epithelial cells is associated with tyrosine phosphorylation of specific host cell proteins. *Infect.Immun.* 65:2570-2575.

104. Springall,T., N.S.Sheerin, K.Abe, V.M.Holers, H.Wan, and S.H.Sacks. 2001. Epithelial secretion of C3 promotes colonization of the upper urinary tract by *Escherichia coli*. *Nat.Med.* 7:801-806.

105. Dowland,L.K., V.A.Luyckx, A.H.Enck, B.Leclercq, and A.S.L.Yu. 2000. Molecular cloning and characterization of an intracellular chloride channel in the proximal tubule cell line, LLC-PK1. *J.Biol.Chem.* 275:37765-37773.

106. Kabore,A.F., M.Simard, and M.G.Bergeron. 1999. Local production of inflammatory mediators in an experimental model of acute obstructive pyelonephritis. *J.Infect.Dis.* 179:1162-1172.

107. Chippendale,G.R., J.W.Warren, A.L.Trifillis, and H.L.T.Mobley. 1994. Internalization of *Proteus mirabilis* by human renal epithelial cells. *Infect.Immun.* 62:3115-3121.

108. Cotran,R.S. 1969. The renal lesion in chronic pyelonephritis: immunofluorescent and ultrastructural studies. *J.Infect.Dis.* 120:109-117.

109. Chen,Z., Y.Ge, N.Landman, and J.X.Kang. 2002. Decreased expression of the mannose 6-phosphate/insulin-like growth factor-II receptor promotes growth of human breast cancer cells. *BMC Cancer* 2:18.

110. Chao,H.H., A.Waheed, R.Pohlmann, A.Hille, and K.von Figura. 1990. Mannose 6-phosphate receptor dependent secretion of lysosomal enzymes. *EMBO J* 9:3507-3513.

111. de Man,P., C.van Kooten, L.Aarden, I.Engberg, H.Linder, and C.Svanborg-Eden. 1989. Interleukin-6 induced at mucosal surfaces by gram-negative bacterial infection. *Infect.Immun.* 57:3383-3388.

112. Dobyan,D.C., L.D.Truong, and G.Eknoyan. 1993. Renal malacoplakia reappraised. *Am.J.Kidney Dis.* 22:243-252.

113. Tan,H.K. and R.H.Heptinstall. 1969. Experimental pyelonephritis -- a light and electron microscopic study of the periodic acid-Schiff positive interstitial cells. *Lab Invest* 20:62-69.

114. Povysil,C. and L.Konickova. 1972. Experimental xanthogranulomatous pyelonephritis. *Invest.Urol.* 9:313-318.

ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Robert Munford for providing me with an excellent scientific education and for his constant support, and advice. His attentions to detail, persistence, ethics, and scientific know-how have taught me what a great scientist should aspire to. I would also like to thank several members of the Munford lab (past and present) who have been a joy to work with: Dr. Richard Kitchens, Dr. Alan Varley, John Hardwick, Hong Li, Susan Lin, Dr. Julye Nesbitt, Nikki Reading, Faith Sharp, and Patty Thompson. I would especially like to thank Mingfang Lu and Jami Cowan Robinson. I could not have made it through graduate school without the two of you and I will forever be proud to have spent such valuable time with you.

I would like to thank Dr. Nicolai van Oers and Dr. Justin Radolf for allowing me to rotate in their labs. Thanks to Dr. Timothy Selatti, who taught me to be thorough and honest. Thanks to the Microbiology Graduate Program and my committee members, Dr. Leon Eidels, Dr. Christopher Lu, Dr. Kevin McIver, and Dr. Nicolai van Oers for your advice and commitment during my training.

Thanks to Dr. Philip Perlman, Dr. Nancy Street and Nancy McKinney for giving me such wonderful opportunities to serve on committees and advisory panels and always treating me as a colleague and friend.

Finally, I'd like to thank my family and friends for their support and encouragement during my time in Dallas. I especially thank my Grandma Feulner, Alice and Grandpa Ron for all of the letters and long-distance love. Thank you to all of my friends (I hope you all know who you are). Without you this journey would have been so

long! I will miss all of you dearly. To Joe, Darren, Jeremiah, Carilee, Stephanie and Jeffrey Milush: good luck in your future endeavors, you are all such wonderful people and I am fortunate to have met you!

VITAE

J. Amelia Feulner was born in Saratoga Springs, New York on May 18, 1976 and is the only daughter of Gene Artell Feulner and Candace Rickett Feulner. She has an older brother, Kenneth Feulner, who lives in St. Louis, Missouri with his wife Colleen Moses Feulner and their son, Logan Paul Feulner. After graduating from Schuylerville Central High School she attended Russell Sage College in Troy, New York. In May 1998 she graduated, *Summa Cum Laude*, highest honors, with a Bachelor of Arts degree in Biology. In August of 1998 she entered the Graduate School of Biomedical Sciences at The University of Texas Southwestern Medical Center at Dallas where she joined the Molecular Microbiology program. During her time at UT-Southwestern, she has been awarded the S. Edward Sulkin Student Scholarship in Microbiology, the Dean's Discretionary Award for excellence in leadership (three years in a row), a Graduate Student Organization Travel Scholarship, and a Microbiology journal-club award.

Permanent Address: 127 Grant Avenue Extension
Queensbury, NY 12804