

## ABSTRACT

Title of Document: THE EFFECTS OF NEISSERIAL SURFACE MOLECULES AND INITIAL BACTERIAL DOSE ON INTERACTIONS WITH HUMAN MONONUCLEAR PHAGOCYTES

Julia Beth Patrone, Ph.D., 2006

Directed By: Professor, Daniel C. Stein, CBMG

*Neisseria gonorrhoeae* is an important human pathogen which causes significant worldwide morbidity. The effects of two important neisserial surface molecules, LOS and H.8, as well as initial bacterial challenge dose, upon the interactions of *N. gonorrhoeae* with human monocytes and macrophages, were examined in this study. The data presented here demonstrate that alterations in the carbohydrate moiety of LOS do not directly impact production of proinflammatory cytokines. However, when specific LOS mutants were sialylated, upregulation of the chemokine MCP-2 was observed. The H.8 antigen was demonstrated to have little effect on monocytic cytokine production, but does appear to play a significant role during physical interactions with macrophages. In comparison with the parent strain, a greater percentage of the H.8<sup>-</sup> bacterial challenge population was shown to form large aggregates upon contact with human macrophages, and appeared to resist phagocytosis. I propose that host recognition of gonococcal surface structures, such

as IL-8 and sialylated LOS, may promote elicitation of monocytes and phagocytosis of the bacteria. This may contribute to gonococcal intracellular growth, and result in chronic and disseminated infection.

Finally, the experiments described here demonstrate the significance of bacterial dose during gonococcal challenge. While a high MOI was shown to cause elicitation of many proinflammatory cytokines and chemokines, a low MOI elicited a strong chemokine response in the absence of significant TNF $\alpha$  production. As the cytokine TNF $\alpha$  is thought to lead to tissue damage caused during gonococcal infection, these data may help to explain asymptomatic gonorrhea. Induction of IL-8 expression in low MOI challenges was produced independent of TNF $\alpha$  or IL-1 $\beta$ , and required NF- $\kappa$ B activation, though this activation was limited by the inoculating dose. Analysis of various MAP kinases indicated that low MOI challenges were able to efficiently activate both the ERK and p38 pathways, but failed to activate the JNK pathway. A lack of phosphorylated JNK leads to decreased production of AP-1 dimers, transcription factors that are critical for efficient transcription of TNF $\alpha$ . I therefore propose a mechanism where a low MOI gonococcal challenge results in diminished AP-1 activity and TNF $\alpha$  production while IL-8 levels remain constant.

THE EFFECTS OF NEISSERIAL SURFACE MOLECULES AND INITIAL  
BACTERIAL DOSE ON INTERACTIONS WITH HUMAN MONOCYTES.

By

Julia Beth Patrone

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Advisory Committee:  
Professor Daniel C. Stein, Chair  
Professor David Mosser  
Professor Wenxia Song  
Professor Louisa Wu  
Professor Philip DeShong

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## Dedication

To my husband, David and my son, Alexander,  
for helping me to always remember what is really important.  
To Mom, Dad, Becky, and Joe for all your support over the years.

I could not have done this without you.

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Finally, I would like to thank my family. Mom and Dad I could not have gotten this far without your love and encouragement every step of the way. It is a

wonderful thing to know that your parents will be proud of you no matter what you do. I also want to thank my sister, Becky for always listening to me complain and making me feel better, and my brother Joe, for being such a proud big brother. I would like to thank my 'second parents,' Nick and Lesley, for all their support over the years. Last but not least, my two guys: David, I would definitely not have made it this far without you. Thank you for being a wonderful husband and friend during all these years and for always understanding me. Alex, thank you for giving me perspective. One day I will tell you all about graduate school and the importance of a good night's sleep.

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## List of Abbreviations

activator protein-1	AP-1
asialoglycoprotein	ASGP
carcinoembryonic antigen-related cellular adhesion molecule	CEACAM
complement receptor type 3	CR3
cytidine 5'-monophospho- <i>N</i> -acetylneuraminic acid	CMP-NANA
diacylglycerol	DAG
disseminated gonococcal infection	DGI
granulocyte-macrophage colony stimulating factor	GM-CSF
growth regulated oncogene	GRO
heparin sulfate proteoglycan	HSPG
human immunodeficiency virus	HIV
immunoreceptor tyrosine inhibition motif	ITIM
interleukin	IL
jun amino-terminal kinase	JNK
lipooligosaccharide	LOS
lipopolysaccharide	LPS
lysosome/late endosome-associated membrane protein-1	lamp-1
macrophage-derived chemoattractant	MDC
macrophage infectivity potentiator	MIP
mitogen activated protein kinase	MAP kinase
monoclonal antibody	mAb
monocyte chemoattractant protein-1	MCP-1

monocyte chemoattractant protein-2	MCP-2
multiplicity of infection	MOI
<i>N</i> -tosyl-phenylalanine-chloromethyl ketone	TPCK
nuclear factor- $\kappa$ B	NF- $\kappa$ B
opacity-associated protein	Opa
p21-activated protein kinase	PAK
pathogen-associated molecular pattern	PAMP
pattern-recognition receptor	PRR
pelvic inflammatory disease	PID
phosphatidylinositol 4,5-bisphosphate	PIP <sub>2</sub>
phospholipase C	PLC
platelet-derived growth factor-B	PDGF-B
polymorphonuclear leukocyte	PMN
regulated upon activation, normal T-cell expressed, and presumably secreted	RANTES
room temperature	RT
tumor necrosis factor alpha	TNF $\alpha$
toll-like receptor	TLR

## Introduction

### Background and Significance

The genus *Neisseria* includes primarily commensal organisms which colonize the mucous membranes of mammals (1). These organisms are aerobic, Gram negative cocci, usually arranged in pairs (diplococci). Though several *Neisseria* species occasionally cause opportunistic infections in immunocompromised hosts, two species, *Neisseria gonorrhoeae* and *N. meningitidis*, can cause significant disease.

While *N. meningitidis* typically colonizes the nasopharynx of infected individuals, it often does not cause disease. It has been estimated that this organism is asymptotically carried by up to 25% of a population, where only a few individuals develop invasive infections (2). However, *N. meningitidis* can spread to the bloodstream, causing septicemia, and cross the blood-brain barrier where it induces meningitis. The principal means of transmission of *N. meningitidis* is through respiratory droplets of an infected individual (3). *N. gonorrhoeae* however, is the only obligate pathogen of the *Neisseriaceae* and is the causative agent of the sexually transmitted disease gonorrhea. This disease classically manifests as a local infection of the columnar epithelial cells of the mucosa (i.e. urethra, cervix, rectum) and is primarily transmitted through sexual contact. Occasionally, this organism causes ocular infections in neonates who acquire it upon passage through an infected birth canal (4, 5).

Gonorrhea is described by the U.S. Centers for Disease Control (CDC) as the “second most commonly reported notifiable disease in the United States” (CDC,

2004). Over 300,000 cases of gonorrhea were reported to the CDC in 2004, though this number likely underrepresents actual disease occurrence due to the prevalence of asymptomatic infections. Gonococcal infections lead to a significant worldwide public health burden which is complicated by increasing antibiotic resistance. The CDC reports that greater than 15% of isolates collected in 2004 from STD clinics by the Gonococcal Isolate Surveillance Project (GISP) were resistant to penicillin, tetracycline, or both (CDC, 2004). This situation results has significant financial implications regarding treatment of the disease.

Beyond the economic burden caused by *N. gonorrhoeae*, there exists an array of potential disease symptoms and sequelae that can be life-altering for the host. The severity of symptoms due to gonorrhea is strikingly different depending upon host gender. Disease in men is normally characterized by a massive infiltrate of polymorphonuclear leukocytes (PMNs, neutrophils) and monocytes to the site of infection, which results in pain and purulent discharge (6, 7). However, almost 80% of infections in women occur without noticeable symptoms (8). The reason for this gender-based discrepancy is unknown and individuals with asymptomatic gonorrhea often harbor infections which persist at subclinical levels. This is presumably advantageous for the bacterium, allowing continued transmission to other hosts. Prolonged gonococcal infections in women can lead to ascending infection and serious sequelae, such as pelvic inflammatory disease (PID) and disseminated gonococcal disease (DGI).

PID is an upper genital tract infection involving inflammation, and is often damaging to the structures involved in fertility. This condition can affect the uterus,



fallopian tubes, ovaries, and/or adjacent structures (9). The incidence of PID is difficult to estimate since it is not a reportable disease, it is treated in many different settings, and methods for its diagnosis are inconsistent. However, when considering reported hospitalizations, office visits, Emergency Department visits, and women who do not seek care, the number of individuals affected by PID is estimated to be at least one million per year (10). One study suggests that approximately one out of every ten women suffers from PID (11). *N. gonorrhoeae* is often isolated from the cervix and fallopian tubes of women experiencing this complication, though smaller numbers of organisms reach the fallopian tubes, making culture of bacteria from these sites difficult (9). The tissue damage resulting from PID is thought to be caused by the proinflammatory cytokine tumor necrosis factor alpha (TNF $\alpha$ ) (12), and this damage can predispose women to ectopic pregnancy or fallopian tube scarring and subsequent sterility [for review see (13)]. The pathology that mediates this infertility is caused by acute and chronic inflammation. Evidence of intraluminal exudates, microabscesses, and neutrophil/ monocyte infiltration can be seen upon microscopic examination of the fallopian tube tissue (9).

DGI is estimated to occur in 0.5-3% of Americans infected with gonorrhea each year (14). This condition is often characterized by dermatitis, tenosynovitis, and migratory polyarthritis. Diagnosis of DGI, as for other disease sequelae, is complicated by the frequency of asymptomatic gonococcal infections. The lack of symptoms from these silent infections provides no clues as to the disease etiology. Without prompt diagnosis and treatment, endocarditis, meningitis, perihepatitis and permanent joint damage may occur (14).

Aside from the direct complications associated with gonorrhea, recent work has indicated a correlation between gonococcal infection and increased transmission of the human immunodeficiency virus (HIV) (15-18). Gonococci are known to increase local expression of viral RNA and cause an intense inflammatory response, leading to a loss of mucosal integrity. Both of these disease characteristics increase susceptibility to HIV type 1 (15).

### **Human Innate & Adaptive Immunity**

The mucosal tissues of the urogenital tract serve as the host's initial line of defense against sexually transmitted pathogens such as *N. gonorrhoeae*. Epithelial cells connected by tight junctions provide an integral barrier function against invading microbes. These cells can produce a variety of antimicrobials including defensins, complement, cytokines, and immunoglobulin. [for review see (19)]. The complement system, involving interaction of serum and cell surface proteins with either microbial cell surfaces (alternative pathway) or antibodies bound to antigens (classical pathway) is often activated during these initial stages of invasion (20). Colonization or invasion of the epithelia signals response by a network of immune effector cells including macrophages, dendritic cells, monocytes, neutrophils, and T and B lymphocytes. Cutaneous immune surveillance by these cells is of central importance to the host's innate response to microbial infection. These effectors have the ability to release antimicrobial peptides, chemokines, and cytokines in order to contribute to an early immune response (21). Successful recognition of pathogens

during each of these interactions initiates a variety of innate and adaptive immune responses.

The innate immune response occurs upon initial recognition, by host cells, of several highly conserved structures found on microorganisms (22). These structures are termed pathogen-associated molecular patterns (PAMPs), and the cells of the immune system have evolved to recognize them through pattern-recognition receptors (PRRs). As PAMPs are made only by microbial pathogens, detection of these via highly specific receptors allows the host to differentiate between its own surface molecules and those of an invading microorganism. In addition, PAMPs are usually invariant and necessary for the survival of the pathogen, increasing the likelihood that host PRRs will remain effective over time (22). The PRRs of the immune system are encoded into the germ line and are expressed on many effector cells such as macrophages, dendritic cells, and B cells (22). There are three main functional classes of mammalian PRRs: secreted PRRs (e.g. mannan binding lectin), endocytic PRRs (e.g. the macrophage mannose receptor), and signaling PRRs [e.g. toll-like receptors (TLRs)]. These receptors play a major role in, respectively, opsonization of invading microbes, phagocytosis and killing of these microbes, and induction of the inflammatory response. PRRs allow for a very rapid host response to infection, which precedes the action of the adaptive immune response (22).

After initial recognition of a pathogen by the innate immune system, the adaptive immune system can respond in a variety of ways. The cell-mediated response occurs as a result of T lymphocytes which can help to destroy microbes residing in phagocytic cells or bring about lysis of infected host cells. The humoral

response, via specific antibodies, is generated by B lymphocytes (23). An important distinction between the innate and adaptive immune systems is that adaptive immune receptors (found on T cells and B cells) are generated somatically. This system results in a structurally unique receptor on the surface of each lymphocyte (22). These receptors are generated randomly and are not specific for any particular antigen. Those that are effective during infection (i.e. bind specifically to a pathogen) are selected for clonal expansion (22). Therefore the adaptive immune response requires cell proliferation after recognition of an antigen. This system results in an array of receptors which serve to protect the host from virtually any pathogen and allows the host a means to develop “memory” of past microbe encounters.

### **Inflammation and Cytokines**

Cytokines, often called interleukins (IL), are proteins which are produced by cells involved in host innate and adaptive immune responses. Production of these proteins occurs quickly in response to pathogens, serving to elicit an effective host response. Therefore, cytokine production is critical for efficient microbial clearing. These proteins can act in concert or alternately, one cytokine may have many functions. They can also promote the synthesis of other cytokines, thus sparking various signaling cascades, and can act both locally and systemically (23).

Some of the most studied cytokines are those involved in inflammation. They can act to either induce (proinflammatory) or suppress (anti-inflammatory) inflammation. These cytokines are commonly produced by epithelial cells and immune effector cells in response to bacterial infection. Some of these include  $\text{TNF}\alpha$ ,

interleukin-1 beta (IL-1 $\beta$ ), interleukin-12 (IL-12), interleukin-10 (IL-10), and a variety of chemotactic cytokines (chemokines) such as interleukin-8 (IL-8). TNF $\alpha$  and IL-1 $\beta$  are two of the first cytokines produced in response to invading bacteria, and can act synergistically. Their production is induced by a variety of bacterial PAMPs including endotoxin [for review see (24, 25)]. IL-12 is an important inducer of cell-mediated immunity and serves to stimulate natural killer cells and T lymphocytes [for review see (26)]. Anti-inflammatory cytokines, such as IL-10, act to suppress the cascade of cellular events initiated by cytokines such as TNF $\alpha$  and IL-1 $\beta$ . In addition, this cytokine inhibits further production of several proinflammatory cytokines and chemokines. Anti-inflammatory cytokines are therefore extremely important during the resolution of disease as the system returns to homeostasis. [for review see (23, 27)].

Chemokines are small cytokines characterized by four conserved cysteines, linked by disulfide bonds. The positioning of the first two cysteines, either adjacent or separated by one amino acid, is used to classify chemokines into the CC or CXC subfamilies (28). Chemokines are known to be produced by leukocytes as well as epithelial cells, endothelial cells, and fibroblasts (23). Chemokines bind to seven-transmembrane domain receptors and signal through heterotrimeric GTP-binding proteins (28). The best-characterized signal transduction pathway initiated by binding to this type of receptor involves activation of phospholipase C (PLC) which results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). This process generates the second messengers 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> stimulates the release of intracellular calcium stores while DAG activates protein kinase C

(PKC). Ultimately, this process leads to activation of the nuclear factor kappa B (NF- $\kappa$ B) family of transcription factors (23, 29).

A variety of chemokines are noted for their roles during bacterial infection, most notably IL-8. This chemokine is secreted by epithelial cells challenged with bacteria, such as *E. coli*, and has been shown to induce massive neutrophil migration *in vitro* (30, 31). IL-8 is also known to promote neutrophil exocytosis and induce the respiratory burst (32). Chemokines are therefore critical in promoting clearance of infectious bacteria by immune effector cells.

Several groups have investigated the production of cytokines during gonococcal challenge. One male challenge study using gonococcal strain MS11 found an increase in IL-8, IL-6 and TNF $\alpha$  levels in the urine samples of volunteers prior to the onset of symptoms. These cytokines were shown to increase concomitantly with the number of leukocytes found in urine sediment. At the onset of symptoms, IL-1 $\beta$  was detected (33). Fichorova, *et al.* measured IL-6, IL-8, and IL-1 $\beta$  production from challenged immortalized human cervical and vaginal epithelial cells using an MOI of 100:1, and also found that IL-1 $\beta$  was produced later than IL-6 and IL-8. However, TNF $\alpha$  was not measurable in these samples (34). Using a cervical epithelial cell line (ME180) as a model of infection, and a challenge dose of 5:1 (MOI), Naumann and coworkers demonstrated production of TNF $\alpha$  and IL-8 by 6 hours post infection (35). Though these experiments allow some prediction regarding cytokine production during gonorrhea, there is wide variety in the models, gonococcal strains, and challenge doses used.

Host inflammatory cascades are typically the first to be activated by bacterial or viral infections, and account for many aspects of disease symptoms. The current understanding of inflammatory processes is that mucosal inflammation can be triggered by epithelial cells after initial contact with a pathogen, or by more specialized cells once a pathogen has crossed the epithelial surface (36). After bacteria cross the epithelial layer, they interact with specialized cells, such as macrophages, and the inflammatory response is upregulated. A secondary response from the epithelial cells is then initiated (36). Within the subepithelial compartment a cytokine network is formed between responding epithelial cells, cells residing in the subepithelium, and those cells recruited to the site of infection (36).

### **Manipulation of Innate Immunity by Bacterial Pathogens**

Innate immunity is not sufficient for the prevention of all infections, since bacterial pathogens have evolved many mechanisms for evasion of host defenses. Bacteria are known to use a range of complex surface molecules to enhance their survival and replication within the human host. These molecules can cause a variety of results from altering host cell cytokine production and phagocytic ability to interfering with immune surveillance and innate signaling pathways.

Disruption or manipulation of normal host inflammatory cytokine production is a common strategy of bacterial pathogens, though the mechanisms are not always clear (37). Protein A from *Staphylococcus aureus* has been shown to bind directly to the TNF $\alpha$  receptor on respiratory epithelial cells, which elevates the cytokine response and thereby promotes disease (38). *Yersinia* species have been shown to

cause immunosuppression via a virulence antigen (LcrV) which causes induction of IL-10 (39). *Mycobacterium tuberculosis* invades host macrophages and downregulates production of IL-12, thereby suppressing the immune response (40). Another study has demonstrated that TNF $\alpha$  induction directly supports the growth of *M. tuberculosis* within host alveolar macrophages (41). Several cytokines including IL-1 and TNF $\alpha$  have been shown to stimulate replication of HIV-1 and contribute to its pathogenesis (42, 43). In addition, IL-10 can increase HIV-1 replication in the presence of TNF $\alpha$  (44). It has also been demonstrated that the parasite *Leishmania* can decrease host production of TNF $\alpha$  and IL-12, while inducing dramatic production of IL-10, thereby promoting survival of the parasite in host macrophages (45). Whatever the mechanism, alterations in cytokine production can lead to an inappropriate immune response to invading pathogens, and can be devastating to the host.

Many species of bacteria have developed ways in which to avoid detection by immune cells or subvert the actions of phagocytes (46). Production of a carbohydrate capsule is one way that organisms such as *Streptococcus pneumoniae* and *N. meningitidis* evade phagocytes. Capsules can prevent antibody and complement deposition, thus allowing the pathogen to escape opsonization and detection (47). Other bacterial pathogens, such as *Yersinia* species, secrete effector molecules that dampen host phagocytic ability (48). This mechanism allows avoidance of internalization by host phagocytes. However, bacteria that are readily phagocytosed have evolved ways in which to avoid intracellular killing once they are inside host cells. Some escape from the phagosome, a few are known to survive in



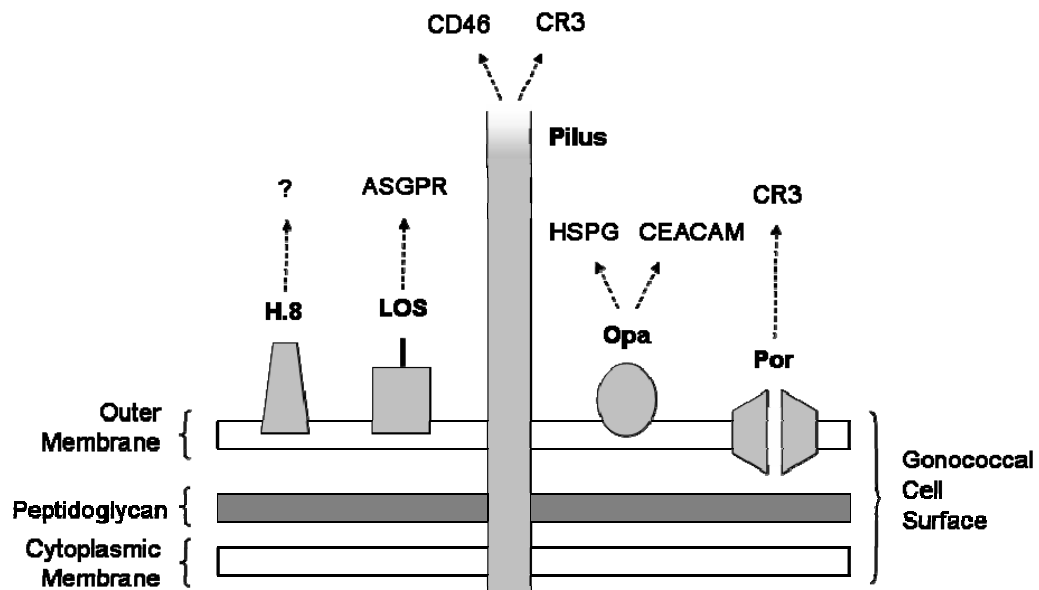
phagolysosomes, and most employ methods to inhibit phagosome-lysosome fusion (49).

Several pathogens secrete molecules that interfere with inflammatory signaling pathways. Two common targets of these pathogens are the NF- $\kappa$ B and mitogen activated protein (MAP) kinase signaling molecules (47). *Bacillus anthracis* produces a toxin which cleaves a MAP kinase and leads to blocked activation of genes downstream of NF- $\kappa$ B (50). *Shigella flexneri* produces an effector molecule, OspG, which has been shown to decrease NF- $\kappa$ B activation, thereby causing a decrease in inflammation (51). Modulation of TLR signaling is also an effective strategy for disruption of inflammation (39, 52). *Salmonella* species can modify their lipid A structures to decrease recognition by TLR2 and TLR4 (47).

### **Gonococcal Pathogenesis**

Gonococcal pathogenesis is a complicated process which is only partially understood. Tissue culture models of infection and male clinical challenge studies have helped to elucidate the mechanisms of gonococcal infection, as no animal model has been widely accepted. Several gonococcal PAMPs on the bacterial cell surface have been characterized and may play important roles in gonococcal invasion and disease progression.

Gonococci routinely exhibit phase variation of several important surface molecules including pili, colony opacity-associated proteins (Opas), and lipooligosaccharide (LOS) (Figure 1). This variation in surface expression is highly regulated through several genetic mechanisms and appears to assist the organisms in adapting to host environments. For example, when gonococci are initially transmitted

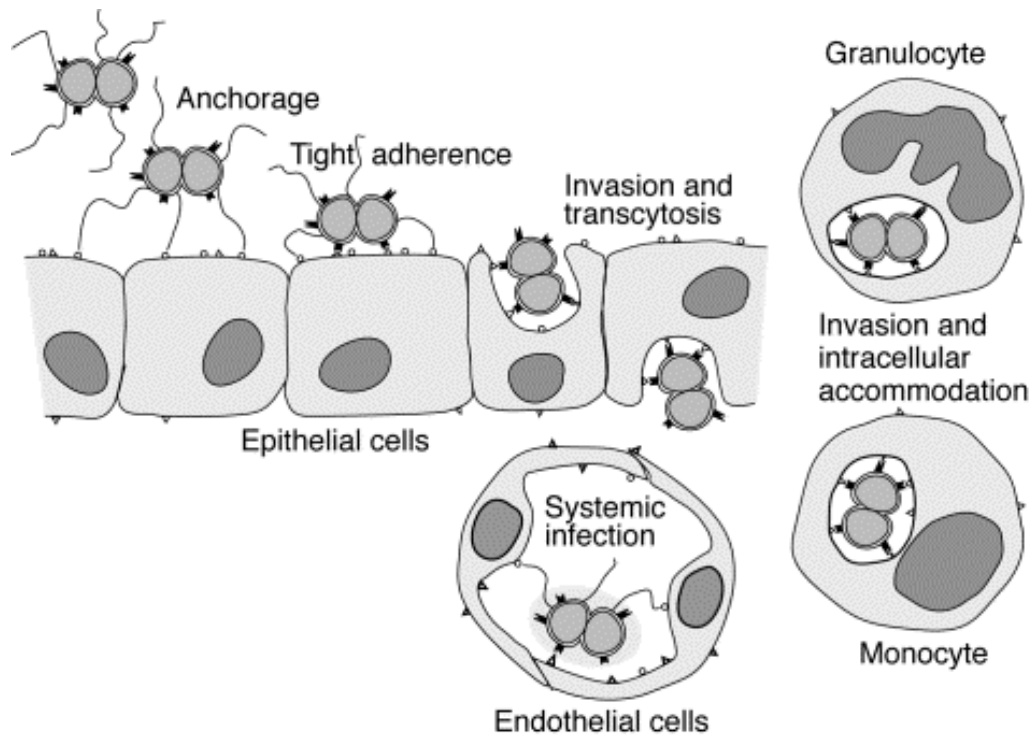


**Figure 1: Illustration of the gonococcal cell surface.**

Illustration of the gonococcal cell surface. Shown are several important surface-expressed molecules. Dashed lines indicate interactions with host cell receptors.

to the mucosal epithelial lining of the genital tract, they utilize type IV pili, which allow for bacterial attachment to host secretory epithelial cells (53). The cell-surface protein CD46 has been shown to act as a pilus receptor on host cells during gonococcal challenge (54). Antigenic variation in pilin sequences has been shown to cause cell-type-specific adherence and possibly cell tropism in vitro (55). It has been observed that between 8 and 16 hours post infection, gonococci appear to lose their pili as they bind tightly to host cell plasma membranes (53)). At this point in infection, classical “zippering” phagocytosis has been shown to occur (56). Subsequently, gonococci have been observed to survive within epithelial cells, and eventually traverse to the stromal matrix (57) (Figure 2).

Another important gonococcal surface structure is the Opa protein. A single gonococcal strain encodes up to eleven different *opa* alleles. Expression of each of these genes is independently phase-variable and Opa heterogeneity is common. In relation to gonococcal disease, it has been observed that clinical samples of purulent discharge (due to symptomatic infection) are mainly comprised of Opa<sup>+</sup> bacteria. Also, in clinical trials where individuals were inoculated with Opa<sup>-</sup> bacteria and allowed to develop symptoms, discharge was shown to contain almost entirely Opa<sup>+</sup> bacteria (58). These observations first led to the assumption that Opa proteins (and Opa phase variation) have a definite role in virulence. In the gonococcal strain MS11, Opa proteins have been shown to bind to two types of host cells receptors: the heparin sulfate proteoglycan (HSPG) receptors and the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) receptor family (59). Recently Boulton, *et al.* have demonstrated an interaction between a specific Opa (Opa<sub>52</sub>) and CEACAM1 on



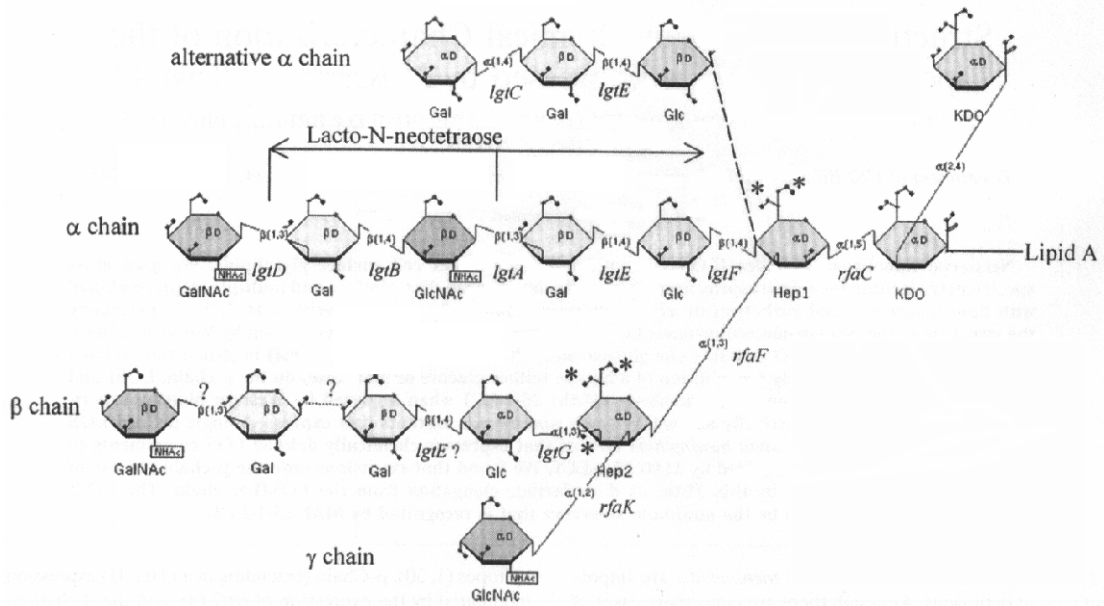
**Figure 2: Model of gonococcal colonization of host tissues.**

Schematic diagram illustrating the sequential interactions of *N. gonorrhoeae* and host cells: adherence, intimate contact, transcytosis, and interactions with professional phagocytes [image from (53)].

CD4<sup>+</sup> T lymphocytes. This receptor contains an immunoreceptor tyrosine inhibition motif (ITIM) and provides a possible mechanism by which gonococci may suppress T cell activation, thus evading the host immune response via Opa variation (8).

However, CEACAM binding does not appear necessary for the selection of Opa<sup>+</sup> variants during the early stages of infection (60). In addition, Song, *et al.* have demonstrated the Opa-independent invasion of polarized epithelial cells, indicating only a partial role for this molecule in gonococcal pathogenesis (61).

Lipopolysaccharides (LPS) constitute a family of toxic glycolipids which are integral in the outer membranes of Gram-negative organisms. These glycolipids are critical for the integrity and functioning of the outer membrane (62). They are also important surface antigens and are known to be highly immunostimulatory. Systemic distribution of LPS/LOS can lead to endotoxic shock (63). In addition, variation in LPS structure has been shown to affect bacterial virulence (64). The general structure of LPS is as follows: the highly hydrophobic Lipid A, a covalently attached core region (which can be divided into an inner and outer core), and finally a polymer of repeating saccharide subunits (Figure 3). This O-polysaccharide portion of the molecule, however, is not ubiquitous. Several Gram-negative strains, such as the gonococcus, express LPS with a truncated, non-repeating oligosaccharide chain, called LOS (65). The gonococcus routinely exhibits phase variation of its LOS which results in rapid and reversible alterations in oligosaccharide structure (66). A single strain of *N. gonorrhoeae* (and even a single gonococcal cell) can express several LOS structures at any given time (67). LOS from different strains shows antigenic diversity, yet LOS biosynthesis in gonococci and meningococci occurs via the same



**Figure 3: Schematic representation of the structure of LOS from *N. gonorrhoeae* strain F62.**

LOS biosynthesis genes are indicated by italics. Dotted lines indicate alternative LOS structures. Asterisks (\*) indicate possible site of PEA or phosphate attachment. The broken line indicates the addition of an alternate  $\alpha$  chain, joined to HepI as a  $\beta$ 1-4 linkage [Figure modified from (77)].

pathway and results in structurally identical molecules (68). Antigenic differences between LOS molecules from different strains can result from substitutions in various glucose units, alterations in sugar-sugar linkages, and additional decorations of the sugar backbone (69). The oligosaccharide extensions of some LOS species appear structurally and antigenically similar to human glycolipids, implying a role in molecular mimicry (70, 71). When lacto-*N*-neotetraose is expressed as the terminal tetrasaccharide, the resulting structure is identical to the carbohydrate portion of human paragloboside. Paragloboside is the precursor of the glycolipid antigens of erythrocytes in the human body (72). Other forms of LOS terminate in carbohydrates which match structures such as the precursor of lacto-*N*-neotetraose, the human p<sup>k</sup> glycosphingolipid, and the X<sub>2</sub> glycosphingolipid (73). In the same way as paragloboside, gonococcal LOS containing the terminal lacto-*N*-neotetraose has been shown to be readily sialylated *in vivo*. Gonococci use host-derived cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA), and their own sialyltransferase to add sialic acid to the terminal galactose of this LOS structure (74, 75). The resultant sialylated LOS has been shown to protect the bacterium from complement-mediated killing (76, 77).

Porins are the most abundant *Neisserial* outer membrane proteins. They function as channels to allow diffusion of small solutes between the bacteria and their environment (78). Most *Neisseria* only express one type of porin. The gonococcus has two different Por-encoding genes: *porA* and *porB*, though it expresses only alleles of PorB (79). These proteins have been shown to play a role in *Neisserial* pathogenesis. During *in vitro* interactions with human neutrophils, porins inhibited

actin polymerization, degranulation, opsonin receptor expression, and phagocytosis, yet primed neutrophils to increase their oxidative burst (80). In a urethral epithelial cell model, gonococcal porin has been shown to increase transcription of several antiapoptotic factors in an NF- $\kappa$ B-dependent manner (81). Another study suggests that gonococcal porin may increase invasion of the bacteria into epithelial cells and inhibit the response of phagocytes (82).

Another abundant neisserial surface molecule is the H.8 antigen (or Lip). Little is known about this lipoprotein other than it is conserved among the pathogenic *Neisseria* (83-85). H.8 has been shown to have unusual electrophoretic mobility, due to its complex nature (84, 85). It comprises 11-20 tandemly repeated pentameric sequences (AAEAP), a lipid-modified N-terminal cysteine residue, and lacks any aromatic residues (86, 87). These properties make H.8 difficult to detect in traditional protein assay and staining methods. This technical complication can lead to contamination of bacterial outer membrane preparations with H.8 antigen (88).

Gonococcal disease severity and symptoms differ based upon host gender. As published data are limited to male clinical trials and tissue culture models of infection, factors affecting colonization of women (uterine pH, hormonal cycles, vaginal secretions, normal flora, etc.) and their impact on infectious dose and subsequent disease sequelae, have not been fully explored. Specific physiological differences between genders have been shown to contribute to the complicated nature of gonococcal disease. For example, cervical epithelia in women express complement receptor type 3 (CR3) (89) and the female genital tract is a source of alternative pathway (but not classical) complement components (90, 91). Interestingly, the



neisserial outer membrane proteins porin and pilin have both been shown to bind CR3 (92), and the lipid A portion of LOS likely serves as a site for C3b deposition on the bacterial cell surface (93). In addition, epithelial cells derived from vaginal, ectocervical and endocervical tissues have been shown to lack TLR4 and MD2, which are critical during host detection of LPS (94).

Though some vaccine studies indicate the potential for the production of antibodies against gonococcal surface molecules, individuals who have had gonococcal infections can be re-infected by the very same strain (95-97). This occurs in spite of the fact that low gonococcal-specific antibody titers can sometimes be measured in previously infected individuals (98-100). This phenomenon indicates the lack of a protective memory response to gonococci (101).

Several aspects of host cell signaling in response to gonococcal contact have been examined. Gonococcal pili have been shown to bind CD46, inducing  $Ca^{2+}$  release from the intracellular stores of epithelial cells (102). Specific Opa proteins can bind HSPG and CEACAM (CD66) receptors, initiating internalization into epithelial cells. HSPG binding and uptake of gonococci has been shown to involve either phospholipase C and acid sphingomyelinase or the serum factors vitronectin or fibronectin (which can bind Opa<sub>50</sub> and mediate bacterial internalization via integrin receptors and protein kinase C) (103-106). Gonococcal Opa binding of CEACAM receptors in phagocytes leads to activation of Src family kinases and downstream signaling through the stress response kinases: p21-activated protein kinase (PAK) and jun amino-terminal kinase (JNK) (107). Neisserial PorB has been shown to interfere with phagocytosis by neutrophils and induce apoptotic signaling cascades in epithelial

cells (80, 108). Several signaling pathways activated by *N. gonorrhoeae* lead to activation of the transcription factors NF- $\kappa$ B (p50/p65 heterodimer) and activator protein 1 (AP-1) (35, 109, 110). Though TLR4/MD2 is important in recognition of neisserial LOS, activation of NF- $\kappa$ B during gonococcal challenge also occurs via TLR2 (94, 111-113).

## Specific Aims and Approaches

This study was designed to investigate modulation of the host immune response by *N. gonorrhoeae*. The central hypothesis for this work is that the outer membrane structures of *N. gonorrhoeae*, as well as the multiplicity of infection (MOI) can impact gonococcal interactions with human immune cells, contributing to different disease outcomes. Through the following dissertation I have explored the impact of gonococcal LOS, the H.8 antigen, and the initial bacterial dose during gonococcal challenge.

In this study, I developed a monocyte/macrophage model and used it to analyze the initial host response to gonococcal infection. While neutrophil influx is certainly important in gonococcal disease, the initial elicitation of host immune response (including neutrophil influx) is the focus of this work. Gonococci are known to first colonize host urogenital epithelial cells (where they are likely to encounter resident macrophages and dendritic cells). However, they have also been shown to invade this tissue. As such, these bacteria must then come into greater contact with monocytes and other immune cells. Though *in vitro* epithelial cell models have been shown to produce low levels of cytokines after gonococcal challenge, this study asks whether the early interaction with monocytes/macrophages at or beyond the initial epithelial barrier may be a significant source of cytokines. I further hypothesize that production of proinflammatory cytokines and chemokines by these monocytes/macrophages could impact the host response. Gonococcal characteristics having an impact on the production of cytokines/chemokines that contribute to tissue

damage (e.g. TNF $\alpha$ ) or the elicitation of neutrophils (i.e. IL-8) could dramatically affect disease presentation.

In the following work I utilized several gonococcal LOS variants. Strain F62 was originally isolated from the female urethra during uncomplicated gonococcal infection (114). F62 expresses the lacto-*N*-neotetraose LOS structure, which is associated with virulence (and is readily sialylated), and can also add a terminal *N*-acetylgalactosamine to its LOS structure (61). A variant of F62, F62 $\Delta$ lgtD, can only express the lacto-*N*-neotetraose LOS, with no phase variation. Strain F62 $\Delta$ lgtA is another naturally occurring disease isolate, which expresses a truncated form of LOS that is associated with asymptomatic gonorrhoea (61). A mutant strain of F62 $\Delta$ lgtD which does not express the intact H.8 antigen (F62 $\Delta$ lgtDH.8<sup>-</sup>) was also used in these studies.

Aim 1:

- Previous work in our lab has demonstrated a role for the carbohydrate portion of the LOS molecule during invasion of epithelial cells (61). The role of LOS in cytokine production was analyzed by challenging monocytes with several isogenic variants that were genetically manipulated to express invariant structures with defined sugars. The effects of LOS sialylation on cytokine production were also examined.

Aim 2:

- Creation of an H.8<sup>r</sup> mutant in our laboratory (described in Chapter 2) allowed examination of the effects of this molecule on interactions with human monocytes. There exists little published work describing any role for H.8, though its conservation among neisserial pathogens implicates its importance during the disease process. The effect of H.8 on cytokine production as well as gonococcal interactions with macrophages was examined.

Aim 3:

- The last part of this study was designed to investigate the effects of bacterial dose upon the host response. In several studies, only a small percentage of gonococci have been shown to invade challenged epithelial cell monolayers (56, 93). It was therefore hypothesized that this small number of bacteria might elicit a different immune response than the high MOI challenges typically used *in vitro*, leading to differential disease presentation.

## Materials and Methods:

**Bacterial strains and infection.** Gonococcal strain F62 was obtained from Dr. P. Frederick Sparling (University of North Carolina, Chapel Hill, NC). F62 $\Delta$ lgtD, a derivative of F62 that expresses a non-varying LOS, lacto-*N*-neotetraose, has been previously described (61). F62 $\Delta$ lgtA, also a derivative of F62, expresses a fixed lactosyl LOS structure and has been previously described (61). Prior to each experimental challenge, gonococci were grown on GCK agar and piliated, Opa<sup>-</sup> organisms were selected. Bacteria were suspended in GCP broth supplemented with Kellogg's solution (115), the concentration of cells was determined spectrophotometrically and verified via viable plate count. Cells were killed by incubation for three hours with 2 mg/ml gentamicin sulfate. Bacteria were stored at 4°C until challenge of human cells. Prior to bacterial challenge, gentamicin-killed bacteria were collected by centrifugation (12,000 rpm for 5 min.), resuspended in RPMI 1640 (Mediatech, Herndon, VA), and diluted as needed. Use of killed bacteria prevented growth and phase variation during the incubation period. For the preparation of bacterial lysates, live gonococci were diluted into PBS and sonicated. (Sonications were performed with a Branson Sonifier 250; 20 % duty cycle, output control = 4.) Sonication was carried out on ice, using twelve 15 second bursts, each followed by 30 seconds of cooling, for a total of 180 sec. Bacterial killing was confirmed by the absence of detectable bacteria on viable plate counts. For experiments involving *E. coli*, either strain DH5- $\alpha$  was used (New England Biolabs,

Ipswich, MA) or an Rc strain (*E. coli* Genetic Stock Center) as indicated. For experiments using LPS as a negative control, LPS was obtained from Sigma Chemical Co. (St. Louis, MO). Strain F62ΔlgtDH.8<sup>-</sup> was created as described in the results section of Chapter 2.

**LOS Extraction Procedure.** Quick preparations of gonococcal LOS were prepared from plated bacterial cultures as described by Hitchcock and Brown (116). Cells were collected from plates using sterile swabs and resuspended in PBS to a Klett reading of 100 (approximately  $1 \times 10^9$  bacteria/ml). An aliquot of 1 ml was centrifuged at 10,000 rpm and the pellet was re-suspended in 50  $\mu$ l lysing solution [1M Tris-HCl (pH = 6.8), 2% SDS, 4% 2-mercaptoethanol, 10% glycerol, bromophenol blue to saturation]. Proteinase K (10  $\mu$ l of 1mg/ml stock) was added and the sample was incubated at 60°C for one hour. Samples were then diluted 1:25 in lysing solution. Prior to SDS-PAGE analysis, samples were boiled for 10 min and 5  $\mu$ l was loaded onto the gel.

**H.8 purification and Western analysis.** [This procedure was carried out by Dr. Daniel Stein.] Protein extracts from the parent and H.8<sup>-</sup> mutant were prepared by resuspending cells, isolated from an agar plate, in PBS to a Klett reading of 100. Cells were collected from a 1 ml aliquot of this preparation, resuspended in 50 $\mu$ l of lysing buffer, boiled for 10 minutes and a small aliquot analyzed on a 15% SDS-PAGE gel (Usually 2.5  $\mu$ l of sample was analyzed). (Gel was run at 100V until the dye front reached the bottom of the gel.) A duplicate gel was run, the proteins transferred to a

nylon membrane, and reactivity of the proteins determined by Western blotting using monoclonal antibody (mAb) 2-1-CA3, obtained from Dr. Sanjay Ram, to detect H.8.

**PCR.** [This procedure was carried out by Dr. Daniel Stein.] The gene encoding H.8 was amplified from *N. gonorrhoeae* strain FA19 by PCR amplification using primers H8F (CCCGATCCTGCGGGATATAAACCTGCCCTTTTGCCAATCC) and H8R (CCCGGATCCAAATCTTTGGCGCACTGGCGGACAAAGTCGGC) to amplify a 3 kb fragment, with the H8 coding sequence located between bps 1623 and 1830. This PCR amplicon was digested with BamHI and cloned into the BamHI site of pUC19. After isolating an *E. coli* transformant containing the desired PCR amplicon, the resulting plasmid (puC19-H8) was used in a PCR reaction using primers H8IF (GGGCGTACGCATTTTCCGCTTGCAAAAAGC) and H8IR (GGGCGTACGCTAAGCAGCGGCAAACAGGGA). This PCR amplification deleted 239 bp (almost all of the coding sequence for H.8) from pUC19-H8. This PCR amplicon was digested with BsiWI, self ligated, and introduced into *E. coli*. The resulting plasmid was named pUC19-H8 $\Delta$ . A 2000 bp DNA sequence encoding the  $\Omega$  interposon was amplified from pHP45 using primer OmegaABC, digested with BsrGI and inserted into the BsiWI site of pUC19-H8 $\Delta$ . A spectinomycin resistant transformant of *E. coli* was isolated, and the plasmid was named pUC19-H8 $\Delta$ Spec. *N. gonorrhoeae* strain F62(lgtDfixoff) was transformed with pUC19-H8 $\Delta$ Spec, and spectinomycin resistant transformants were identified. DNA isolated from F62(lgtDfixoff)H8-spec7 and F62(lgtDfixoff) was used as a template for PCR, using primers H8F and H8R. The amplicon generated from F62(lgtDfixoff) generated a



fragment of 3000 bp, and yielded three fragments after digestion with HinCII (1595, 1168 and 237 bp).

**Cell Lines.** THP-1 cells used were originally from the American Type Culture Collection (Manassas, VA) and were donated to our lab courtesy of Dr. David Mosser. Cells were grown in RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine, supplemented with 0.05 mM 2-mercaptoethanol. Fresh medium was added every 2-3 days or when cell concentrations exceeded  $8 \times 10^5$  cells/ml. Prior to experimentation, cells were counted using a hemocytometer and plated at a concentration of  $1 \times 10^6$  per ml.

**Isolation and culture of human monocytes/macrophages.** Whole blood was drawn from healthy volunteers and peripheral blood mononuclear cells were prepared by density gradient centrifugation using Ficoll-Paque Plus. Briefly, blood was collected in heparinized tubes (BD Biosciences, San Jose, CA) and transferred to 50 ml conical tubes. The volume in each tube was brought to approximately 36 ml using warm PBS and cells were gently pipeted to ensure mixing. 14 ml of Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) was added beneath the blood suspension. (When necessary an untreated tube was used to collect blood for serum.) All blood tubes were centrifuged for 20 min at room temperature (RT), at 2500 rpm, and with the brake set to zero. This step was followed by transfer of the opaque layer (containing peripheral blood mononuclear cells) to a new 50 ml conical tube and dilution of the cells 1:1 with RPMI 1640 or removal of the serum layer into a separate

tube. (Serum was heat inactivated for 25 min at 56°C prior to use in sample media.) Cells were centrifuged for 10 min at 4°C at 1500 rpm. The pellet was resuspended in a small volume of RPMI 1640, transferred to a 15 ml conical tube, and centrifuged for 10 minutes at 4°C, at 2500 rpm. Supernatants were aspirated and cells counted on a hemocytometer. Prior to bacterial challenges, cells were seeded at  $1 \times 10^6$  cells/ml in tissue culture-treated plates in RPMI 1640 and incubated at 37°C, 5% CO<sub>2</sub> with humidity. After 30 min, the cells were washed to remove the nonadherent population and RPMI 1640 (supplemented with 10% autologous heat-inactivated serum) was added preceding bacterial challenge. Differentiated macrophages were utilized in order to represent a resident macrophage phenotype, and provided a clear model for visualization of both NF-κB translocation and gonococcal orientation. For differentiation into macrophages, cells were seeded at approximately  $5 \times 10^5$  cells/ml in untreated petri plates and maintained in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum, 20 mM HEPES for seven days. On day seven, cells were washed and macrophages were removed from petri plates with Cell Stripper (Cellgro/Mediatech, Herndon, VA) and re-seeded onto glass coverslips for immunofluorescence staining and confocal microscopy.

All studies employing human blood were approved by the University of Maryland's Institutional Review Board (IRB #01321, "Immune Response to Gonococcal Infection") prior to the initiation of any of these experiments.

**ELISA.** Prior to bacterial challenge, primary human monocytes were isolated and seeded as described above. Following challenge with the appropriate dilution (100  $\mu$ l) of gentamicin-killed gonococci, cells were incubated in RPMI 1640 supplemented with 10 % autologous human serum at 37°C 5 % CO<sub>2</sub> with humidity for the time specified. Cell supernatants were collected and assayed for the presence of cytokines by sandwich ELISA. Upon collection of supernatants, a protease inhibitor cocktail was added and each sample was stored at -80°C until analysis. Antibody pairs and recombinant standards for human TNF $\alpha$ , IL-8, IL-10, and IL-12p40 were purchased from BD Pharmingen (San Diego, CA). (Any reference to IL-12 within this text refers specifically to IL-12 p40.) Antibody pairs, recombinant standards, and neutralizing antibody for human IL-1 $\beta$  were purchased from R&D Systems (Minneapolis, MN). ELISAs were carried out according to protocols provided by BD Pharmingen. Streptavidin alkaline phosphatase and p-nitrophenyl phosphate substrate were purchased from Southern Biotech (Birmingham, AL) and used according to the manufacturer's specifications. Samples were read at 405 nm in 96-well, untreated, flat-bottom plates. In neutralizing experiments, 5  $\mu$ g/ml TNF $\alpha$  neutralizing antibody was shown to completely bind all available TNF $\alpha$  in cell supernatants. Similarly, 2  $\mu$ g/ml IL-1 $\beta$  neutralizing antibody was shown to completely bind all available IL-1 $\beta$  in cell supernatants.

**Immunofluorescence staining and confocal microscopy.** Human macrophages, differentiated from primary blood monocytes, were seeded overnight on sterile, acid-washed coverslips at a concentration of  $2 \times 10^5$  macrophages/coverslip. For NF- $\kappa$ B

translocation experiments: Macrophages were washed once in warm RPMI prior to the addition of 100  $\mu$ l of the appropriate dilution of gentamicin-killed bacteria in RPMI and 300  $\mu$ l RPMI supplemented with 10 % FCS. Cells were incubated at 37°C, 5 % CO<sub>2</sub> with humidity. At the specified time point, macrophages were washed once in warm PBS and fixed with cold methanol for 10 min. After incubation, macrophages were washed three times with PBS and blocked with PBS 5 % FCS for 15 min at 37°C with humidity. A mouse monoclonal anti-NF- $\kappa$ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to coverslips at a concentration of 1  $\mu$ g/ml in blocking buffer. Coverslips were incubated for 1 hr at RT with gentle agitation, washed twice in PBS and incubated with secondary antibody Alexa Fluor 633 goat anti-mouse (Molecular Probes, Carlsbad, CA) in blocking buffer at 1  $\mu$ g/ml, for 1 hr at RT with gentle agitation. Cells were washed twice in warm PBS and incubated with Alexa Fluor 546 phalloidin (1:50) for 30 min, washed once, and incubated with a 1:5000 dilution of SYTO 13 green fluorescent nucleic acid stain for 20 min. Coverslips were washed three times in PBS prior to mounting and analysis. During analysis, fields were randomly chosen and at least 50 macrophages per sample were categorized as positive or negative for the presence of activated NF- $\kappa$ B p65 in the nucleus. For experiments using H.8<sup>-</sup> mutant gonococci: Macrophages were washed once in warm RPMI prior to the addition of 100  $\mu$ l live bacteria (in RPMI 1640) and 300  $\mu$ l RPMI 1640 (supplemented with 10 % FCS) to give an MOI equal to 50. Cells were incubated at 37°C, 5 % CO<sub>2</sub> with humidity for the indicated time. At the specified time point, media was aspirated and cells were fixed in cold methanol for 10 min. Macrophages were washed three times with PBS and blocked with PBS 5

% FCS for 25 min at RT. Cells were stained with 0.008% Evans Blue (Sigma Aldrich, St. Louis, MO) for 30 min at RT, and incubated in a 1:5,000 dilution of SYTO 13 green fluorescent nucleic acid stain for 20 min at RT. Coverslips were washed four times with PBS prior to mounting.

All coverslips were mounted using Gel/Mount anti-fading mounting medium (Biomedex, Foster City, CA). Samples were analyzed via laser-scanning confocal microscopy (LSM 510; Zeiss, Oberkochen, Germany).

**Activation/Inhibition of MAPK in monocytes treated with gonococci.** Prior to infection, primary human monocytes were seeded overnight in 6-well tissue culture plates and incubated at 37°C, 5 % CO<sub>2</sub>. This overnight incubation was necessary in order to allow monocytes to return to expressing only basal levels of phosphorylated MAP kinases prior to detection by Western Blot. Post infection, the 6-well plates were placed on an ice bath and washed three times with ice-cold PBS. Lysates were collected by adding 100 µl of NP-40 lysis solution (1% NP40, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 µg/ml leupeptin and aprotinin protease inhibitors, 50 mM NaF, 1 mM sodium vanadate) to each well, scraping the cells from the plates, and pipetting the lysate into 1.5 ml microcentrifuge tubes. The lysates were placed on ice for 20-30 min and vortexed briefly every 7 min. All lysates were then centrifuged at 4°C for 30 min and the supernatants were transferred to a new 1.5 ml microcentrifuge tube and stored at -80°C. Protein concentration of the cellular extracts was determined using the Bio-Rad protein assay. MAP kinases were

inhibited by pretreatment with PD98059, SB203580, or SP600125, 30 min prior to challenge with gonococci (Biosource, Camarillo, CA).

**SDS-PAGE and immunoblotting.** Lysates were prepared for SDS-PAGE by combining them with 2X loading buffer (50 % glycerol, 5 % SDS, 0.25 M Tris-Cl pH 6.8, 0.04 % 2-mercaptoethanol, 25 µg/ml bromophenol blue) in a 1:1 ratio, boiling the samples for 5 min, and centrifuging them for 2 min at 12000 rpm. Equal volumes of each lysate were loaded into a gradient SDS-PAGE minigel and run at 0.01 Amp/gel until the dye front just ran off the bottom of the gel (~ 110 min). Gels were equilibrated in 1X transfer buffer (25 mM Tris, 192 mM glycine, 15 % methanol, pH 8.2) for 20 min while the PVDF transfer membranes (GE Osmonics, Trevose, PA) were prepared (10 sec in methanol, 5 min. in HPLC grade water, 15 min. in 1X transfer buffer). Proteins were transferred onto the PVDF membrane via wet, horizontal transfer at 100 V for 60 min at 4°C. The PVDF membranes were blocked overnight at RT in 1X PBS/0.05 % Tween 20%/ 1% fish skin gelatin (Sigma, St. Louis, MO). Membranes were washed twice in wash buffer (PBS/0.05 % Tween 20) and incubated in primary antibody solution (wash buffer, 1 % gelatin, 1:1000 rabbit anti-phospho p38 (Cell Signaling Technology, Beverly, MA), ERK, or JNK) for 90 min at RT. After 5-15 min. washes in wash buffer, membranes were incubated in secondary antibody solution (1X PBS/0.5 % gelatin, 1:2000 goat anti-rabbit IgG-HRP conjugate) (Cell Signaling Technology, Beverly, MA) for 60 min. at RT followed by 4, 15 min washes in wash buffer. Membranes were developed with luminal/peroxide chemiluminescence reagents (Perkin Elmer Western Lightning) and exposed to blue

autoradiography film (Marsh Bioproducts, Rochester, NY). To verify that equal amounts of lysate were loaded on the gel, the amount of  $\beta$ -actin in each lane was determined. The membrane was incubated in stripping buffer (62.5 mM Trizma base, 2 % SDS, 100 mM 2-mercaptoethanol, pH 6.7) for 45 min at 50°C, washed 5 times with wash buffer and reprobed for actin protein following the same procedure as listed above with the following exceptions. The primary antibody was rabbit anti- $\beta$ -actin (Sigma, St. Louis, MO) at 1:2000 and the secondary antibody was goat anti-rabbit (Sigma, St. Louis, MO) diluted 1:10000.

**Cytokine array.** Primary human monocytes were isolated as described above and seeded into 24-well tissue culture plates at  $1 \times 10^6$  cells/ml. The appropriate dilution (100  $\mu$ l) of gentamicin-killed gonococci was added to each sample monolayer, and cells were incubated in RPMI 1640 supplemented with 10 % autologous human serum at 37°C, 5 % CO<sub>2</sub> with humidity for 18 hrs. Supernatants from quadruplicate samples were pooled before addition to the array membrane. The RayBio Human Cytokine Array III was purchased from Raybiotech, Inc. (Norcross, GA) and used according to manufacturer's instructions. Array membranes were analyzed using a Gel Doc camera and Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA). The volume array tool (for 96 wells) was used to specify area intensity for each spot within an image. A "background" volume was placed over the internal negative control for each array. The average intensity of pixels in this volume was calculated and subtracted from each pixel in all other volumes. This process resulted in calculation of the "adjusted volume" (i.e. spot intensity minus the average intensity of

the internal negative control). Positive controls from several exposures were compared and the means were found to be statistically similar ( $P > 0.05$ ) using a paired, two-tailed, t test with a 95% confidence interval, thus allowing cross comparison of sample cytokine levels. Adjusted volumes (two for each cytokine) were then averaged and the standard deviation was determined.



# Chapter 1: The Effects of Lipooligosaccharide Structure on Human Monocytes

## Introduction

The role of lipooligosaccharide (LOS) has been shown by many groups to be important in gonococcal disease pathogenesis (61, 117, 118). Harvey, *et al.* have demonstrated an important interaction between those gonococci expressing lacto-*N*-neotetraose-terminal LOS and the asialoglycoprotein (ASGP) receptor of primary human urethral epithelial cells (119). Strains expressing lacto-*N*-neotetraose-terminal LOS were significantly more invasive than strains containing a deletion of the terminal Gal $\beta$ 1-4 and subsequent sugars (71). Song, *et al.* demonstrated that in the absence of Opa proteins, strains expressing specific LOSs (containing a terminal lacto-*N*-neotetraose) promoted increased gonococcal invasion of epithelial monolayers (61). In addition, gonococci predominantly expressing LOS with a specific carbohydrate structure have been associated with either symptomatology or a lack of symptoms in male clinical trials, further supporting the observation of pathogenic LOS phenotypes. Schneider, *et al.* demonstrated that after inoculating volunteers with low-molecular weight LOS variants, urethritis developed and primarily high molecular weight LOS (terminal lacto-*N*-neotetraose) was purified from the exudates (117). Thus, while the lipid A portion of the LOS molecule has historically been designated the immunogenic portion of the molecule, the carbohydrate structures of LOS also play an important function in pathogenesis.

Sialylation of LOS also impacts gonococcal interactions with host cells. Sialylated strains have been shown to be resistant to complement-mediated killing, to have a decreased ability to induce oxidative burst in neutrophils, and to possess a decreased ability to adhere to neutrophils in the absence of complement (62, 77). It has also been observed that the addition of CMP-NANA to gonococci (sialylation) reduces their ability to invade tissue culture epithelial cells (120). At present, further implications of LOS sialylation in the context of molecular mimicry remain unclear, though this process appears to be of significant biological importance. Since certain gonococcal LOS structures (potentially able to be sialylated) are associated with specific degrees of symptomatology, it is possible that LOS structure is integral in determining the severity of a gonococcal infection, and may serve to aid in evasion of the host immune system.

LPS is known to be highly immunostimulatory, and can elicit a variety of pro-inflammatory cytokines and chemokines from host cells during infection. However, the immunostimulatory effects of gonococcal LOS during infection remain somewhat controversial. Several studies differ in their observations regarding the types and amounts of cytokines elicited. Some of these contradictions may stem from use of different bacterial strains, phase variation of LOS molecules in culture, and different cellular models of infection. In addition, the biological significance of LOS antigenic variation during infection has not been determined. In general, it is accepted that the tissue damage resulting from gonococcal infections can be attributed to the cytokine  $\text{TNF}\alpha$  (12, 121). In addition, IL-8, IL-6, and IL-1 $\beta$  have been measured following gonococcal challenge of several models (33-35, 122). *In vivo* studies are rare, but

occasionally naturally occurring infections in women have been monitored. Gonococcal cervicitis in female sex workers has been shown to cause increases in interleukin-4 (IL-4), IL-6, IL-10, and TNF $\alpha$ , as well as a decline in CD4<sup>+</sup> T cell counts (123). Though IL-12 has not been detected in response to gonococcal challenge, this cytokine appears to be of significance during meningococcal infections (124). Cytokines such as TNF, IL-1 $\beta$ , IL-12, IL-10, MCP-1 (monocyte chemoattractant protein-1), IL-6, and RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) have also been measured during challenge with meningococcal LOS (124-127).

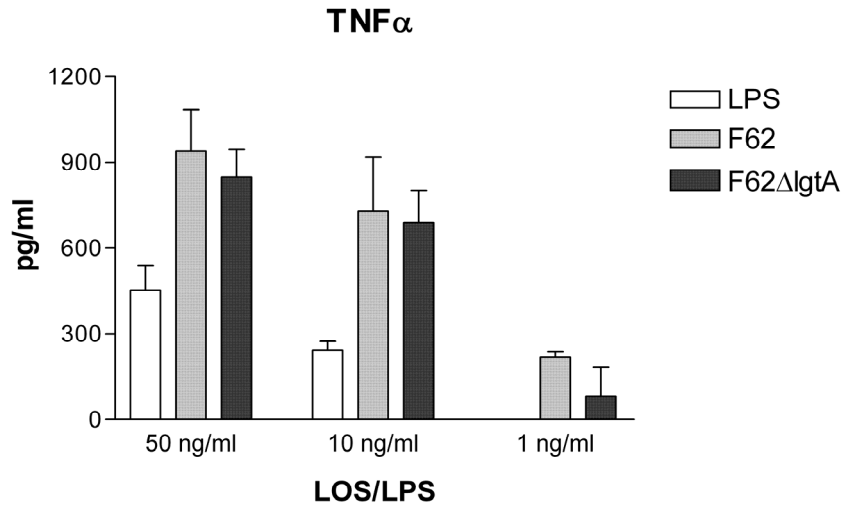
Different LOS structures have been shown to correlate with disease symptomatology: F62 (lacto-*N*-neotetraose LOS) is associated with symptomatic gonococcal infection, while F62 $\Delta$ lgtA (lactosyl LOS) is associated with asymptomatic gonococcal infection. In this study, I tested whether these naturally occurring LOS structures would differentially impact cytokine production by human monocytes. I hypothesized that compared with F62 and F62 $\Delta$ lgtD (lacto-*N*-neotetraose LOS) F62 $\Delta$ lgtA (and its lactosyl LOS) would elicit lower levels of the cytokines associated with symptoms (such as TNF $\alpha$ ). I further hypothesized that this strain might elicit greater production of IL-10, thereby suppressing pro-inflammatory responses. I investigated the ability of purified LOS and two distinct LOS variants to elicit cytokines, and also studied the effects of bacterial sialylation upon cytokine production. I chose to focus on measurement of the cytokines TNF $\alpha$ , IL-10, IL-12, and IL-8 due to both significance in published work and potential significance

regarding the overall disease model. During these studies both a human tissue culture cell model and a primary human cell model were utilized.

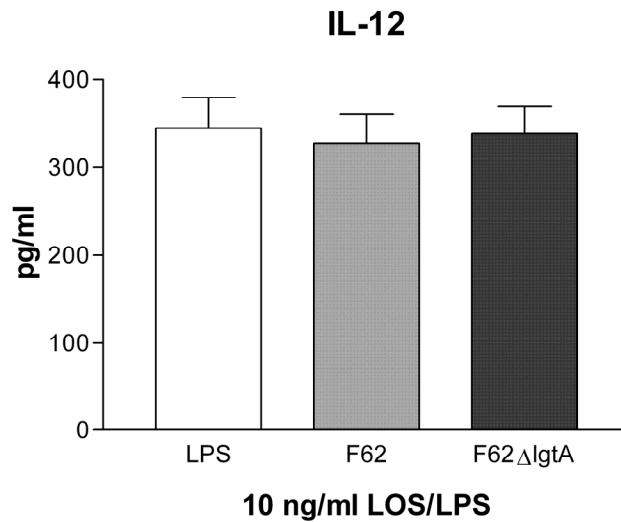
## Results

Challenge of THP-1 cells with equivalent amounts of LOS/LPS from different variants results in similar production of TNF $\alpha$  and IL-12. In order to directly study the effects of LOS variation on human monocytic cytokine production, I challenged THP-1 cells with purified preparations of LOS. Using *E. coli* LPS as a positive control and unchallenged samples as a negative control, I was able to compare the LOS purified from wild type F62 with truncated LOS, purified from the F62 $\Delta$ lgtA mutant. Analysis of cell supernatants by ELISA revealed no significant differences in the production of TNF $\alpha$  over a dose range, as seen in (Figure 4A). For subsequent experiments I chose to use 10 ng/ml LOS/LPS as an initial challenge dose, as it did not result in significant cell death, yet did result in measurable cytokine production. Measurement of IL-12 at two challenge doses similarly revealed no significant differences between F62 and the truncated mutant, as seen in (Figure 4B). Unchallenged samples did not result in measurable cytokine production. IL-10 expression was also analyzed, but no values above the limit of detection by ELISA were obtained (data not shown). While it does appear that gonococcal LOS elicits significantly more TNF $\alpha$  from THP-1 cells than does *E. coli* LPS (Figure 4A), I attribute this to differences in the molar ratios of the two molecules, when used at the same concentration.

A.



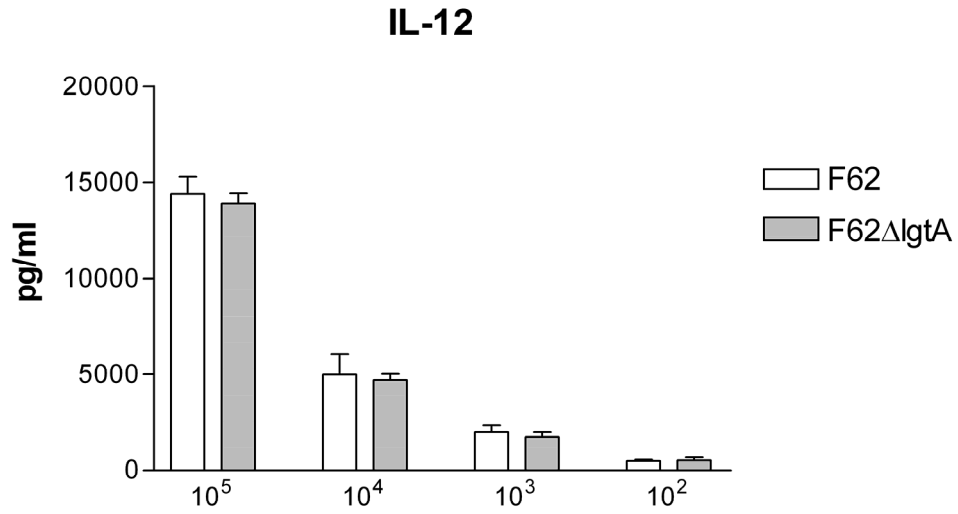
B.



**Figure 4: Production of TNF $\alpha$  and IL-12 by THP-1 cells challenged with purified LOS/LPS.**

(A) Sample supernatants were collected 7 hrs after challenge of THP-1 cells ( $1 \times 10^6$ /sample). These data are the result of two experiments, each performed in triplicate. Error bars indicate the standard deviation of the mean. (B) Sample supernatants were collected 16 hrs after challenge of THP-1 cells  $1 \times 10^6$ /sample. These data represent one experiment, performed in triplicate. Error bars indicate the standard deviation of the mean. (LPS = *E.coli* LPS)

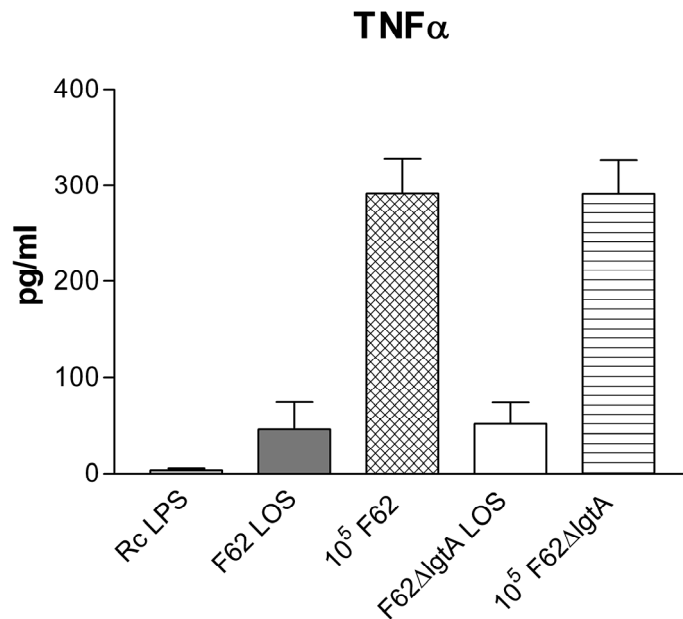
*Challenge with distinct gonococcal LOS variants elicits similar levels of TNF $\alpha$  and IL-12.* I hypothesized that the LOS on whole bacteria may be presented differently to monocytes than purified LOS. In order to test this, I challenged THP-1 cells with two variants of live gonococci. IL-12 was measured at 18 hours after challenge with a range of bacterial doses and levels were shown to be equal between variants (Figure 5). Similarly, no difference in TNF $\alpha$  was observed between challenge phenotypes. Challenge with whole-cell bacteria however, did result in greater production of TNF $\alpha$  when compared with the molar equivalent challenge dose of LOS (Figure 6). The same trend was evident for IL-12 (data not shown). (Molar equivalencies were previously determined by Dr. Daniel Stein: purified LOS preparations were weighed, and dilutions of these samples along with dilutions of LOS from whole cell preparations, were analyzed via SDS-PAGE.) Since this observation was likely to have resulted from growth of the bacteria *in vitro*, I eliminated this variable by using gentamicin sulfate to kill the gonococci prior to challenge. Gentamicin is an aminoglycoside antibiotic and binds directly to ribosomal RNA, thereby inhibiting protein synthesis (128). Use of this antibiotic does not cause lysis of the bacteria. Upon staining and visualization of killed bacteria, the cells appeared as normal diplococci (data not shown). As shown in Figure 7, killed gonococci elicited less IL-12 than live gonococci and no differences were observed between LOS variants. To avoid the complication of *in vitro* gonococcal growth as well as phase variation, experiments from this point forward mainly involved gentamicin-killed bacteria.



**Figure 5: Production of IL-12 by THP-1 cells challenged with live gonococcal variants over a dose range.**

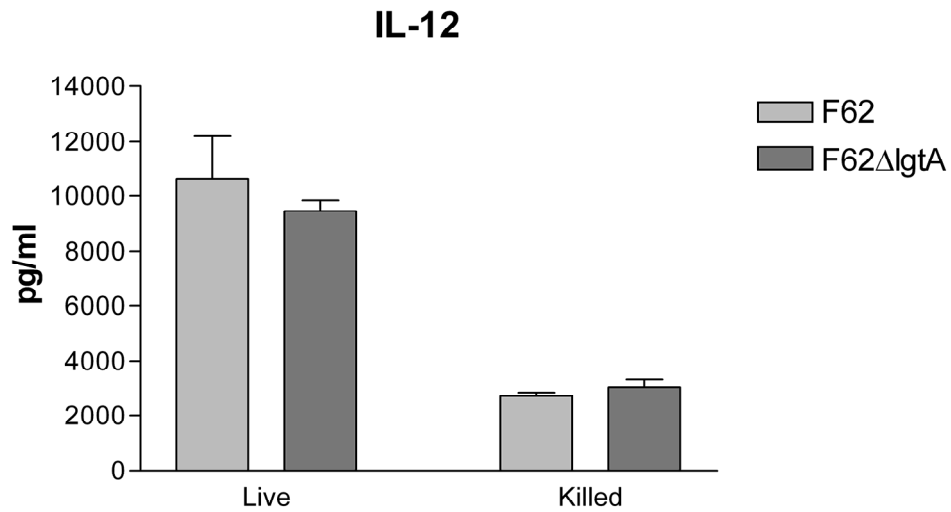
Various doses of live gonococci were added to THP-1 cells ( $1 \times 10^6$ /sample). At 18 hours post infection, supernatants were collected and analyzed by ELISA. Data shown are from one experiment, performed in triplicate. Error bars indicate the standard deviation.





**Figure 6: Comparison of TNF $\alpha$  production upon challenge with live gonococci vs. challenge with purified LOS.**

THP-1 cells ( $1 \times 10^6$ /sample) were challenged with either  $1 \times 10^5$  live gonococci or a molar equivalent amount of LOS/LPS (10 ng/sample). At three hours post challenge, sample supernatants were analyzed for TNF $\alpha$  by ELISA. These data represent two experiments, each performed in triplicate. Error bars indicate the standard deviation. (Rc LPS was purified from a rough LPS *E. coli* variant.)



**Figure 7: Production of IL-12 upon challenge with live vs. gentamicin-killed gonococci.**

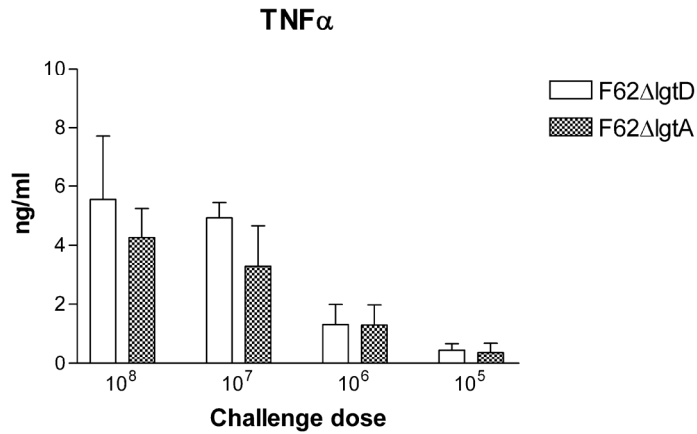
THP-1 cells ( $1 \times 10^6$ /sample) were challenged with either  $1 \times 10^5$  live or gentamicin-killed gonococci. At 18 hrs post challenge, IL-12 was measured by ELISA. These data represent one experiment, performed in triplicate. Error bars indicate the standard deviation.

*Challenge of primary human monocytes with two LOS variants reveals similar elicitation of TNF $\alpha$ , IL-10, IL-12, and IL-8.* In order to examine the effects of LOS variation in a primary cell model, we utilized monocytes derived from human whole blood. These primary cells were challenged with two variants of gentamicin-killed gonococci, F62 $\Delta$ lgtA (as described above) and F62 $\Delta$ lgtD which expresses a fixed terminal lacto-*N*-neotetraose structure (61). F62 $\Delta$ lgtD was used in order to ensure that no variation of LOS was occurring. After incubation, sample supernatants were tested for the presence of TNF $\alpha$ , IL-10, IL-12, and IL-8. Over the dose range tested, no differences in cytokine production were observed when comparing the two variants (Figure 8). However, these data demonstrate that gonococci can elicit all four of these cytokines from human monocytes. In addition, a large amount of the chemokine IL-8 was elicited by gonococci, implying a significant role for this chemokine during infection by *N. gonorrhoeae* (see chapter 3).

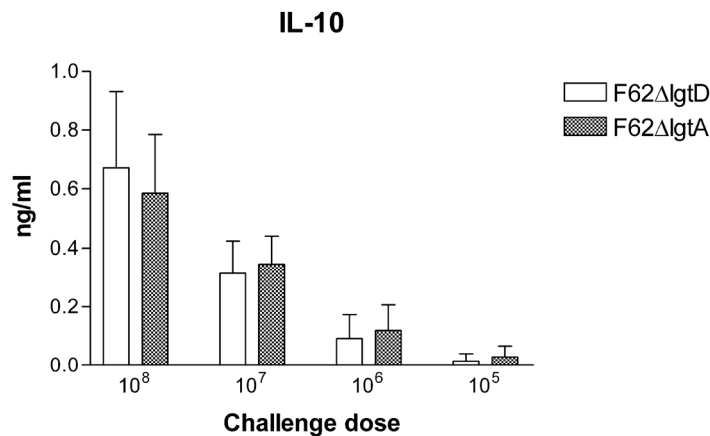
**Figure 8: Production of TNF $\alpha$ , IL-10, IL-12, and IL-8 upon challenge of primary human monocytes.**

Monocytes were isolated from human whole blood and cells ( $1 \times 10^6$ /sample) were challenged with varying doses of gentamicin-killed gonococci. Sample supernatants were collected at 18 hrs post challenge and cytokines were analyzed by ELISA. Clear bars indicate challenge with F62 $\Delta$ igtD and shaded bars indicate challenge with F62 $\Delta$ igtA. Challenge with media alone resulted in no measurable TNF $\alpha$  or IL-10 and less than 100 pg/ml IL-12; Approximately 1 ng/ml IL-8 was measurable from negative controls, with a standard error of 0.8 ng/ml. (A) TNF $\alpha$  (B) IL-10 (C) IL-8 (D) IL-12. These data represent three experiments, each performed in triplicate. Error bars indicate the standard error of the mean.

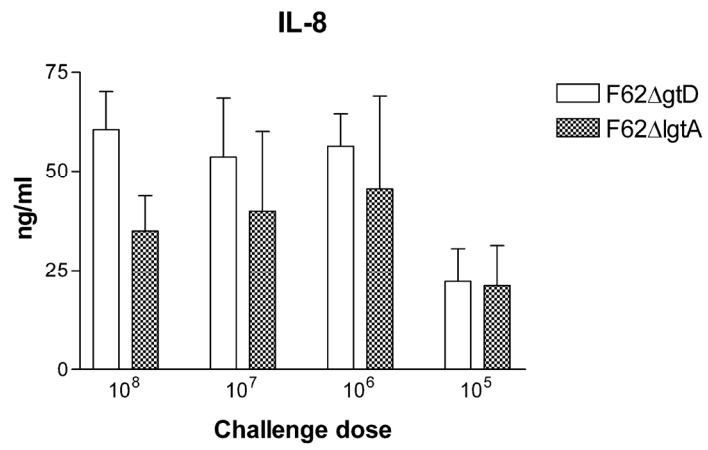
A.



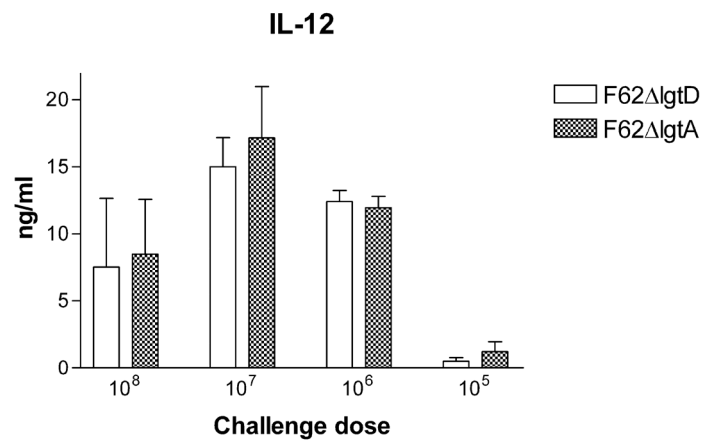
B.



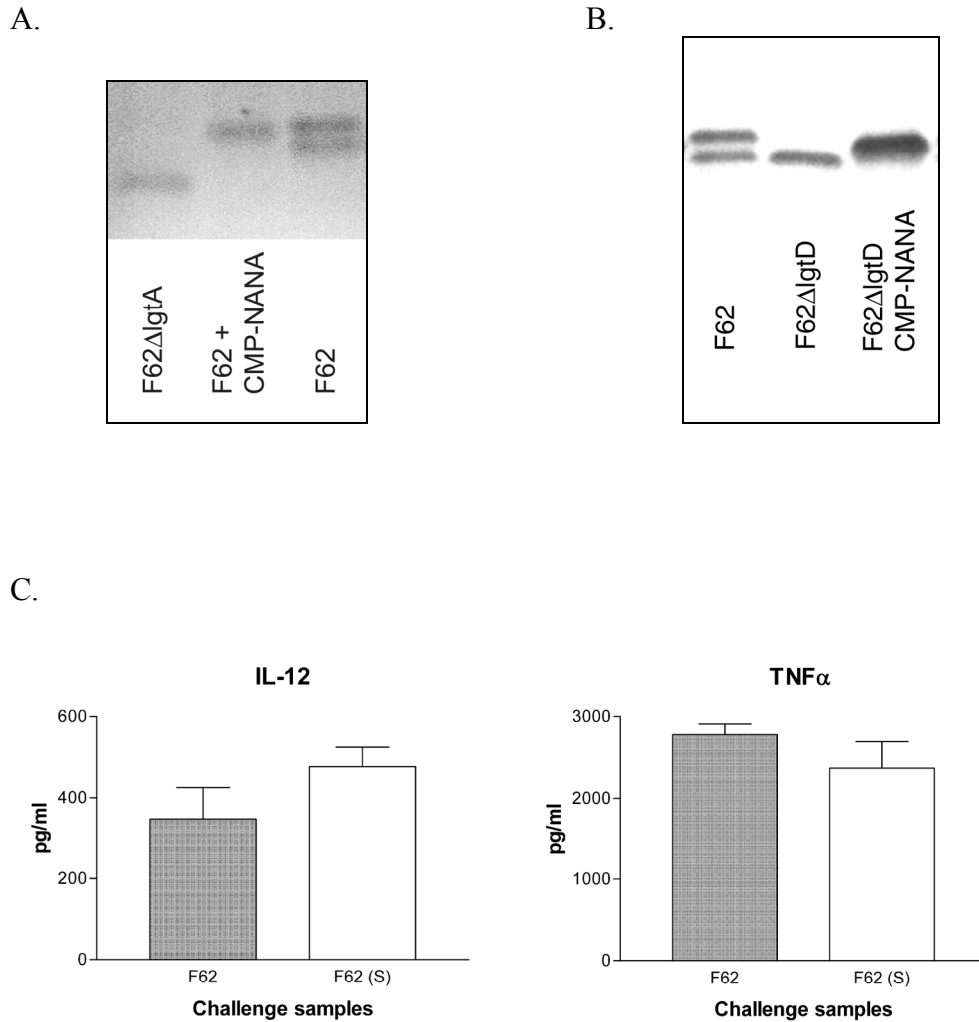
C.



D.



*Sialylation of gonococcal LOS does not alter production of TNF $\alpha$  or IL-12.* Since sialylation of gonococcal LOS is likely to play a role in natural infection, I determined its effects on monocytic cytokine production. After growing gonococci in the presence of CMP-NANA, the bacterial LOS was extracted and analyzed via polyacrylamide gel electrophoresis. This process confirmed the efficacy of the sialylation procedure. Figure 9A shows a low molecular weight band for F62 $\Delta$ lgtA (representing the lactosyl LOS), two bands for wild type F62 (one representing the terminal lacto-*N*-neotetraose, and one representing an increase in molecular weight as *lgtD* phase varies to add a terminal *N*-acetylgalactosamine). Sialylation of F62, represented by the center band, shows a shift in molecular weight of the lower band as sialic acid is added to the terminal sugar. Figure 9B shows wild type F62 and the F62 $\Delta$ lgtD gonococcal variant expressing only the fixed lacto-*N*-neotetraose LOS structure, and sialylated F62 $\Delta$ lgtD (a shift in molecular weight shows addition of the terminal sialic acid). Figure 9C shows the result of two challenge experiments utilizing the sialylated gonococci. There was no significant difference observed in either IL-12 or TNF $\alpha$  production as a result of challenge with sialylated bacteria. Therefore sialylation of gonococcal LOS does not appear to play a role in altering host proinflammatory cytokine production.



**Figure 9: Production of TNF $\alpha$  and IL-12 upon challenge with gonococci expressing sialylated LOS.**

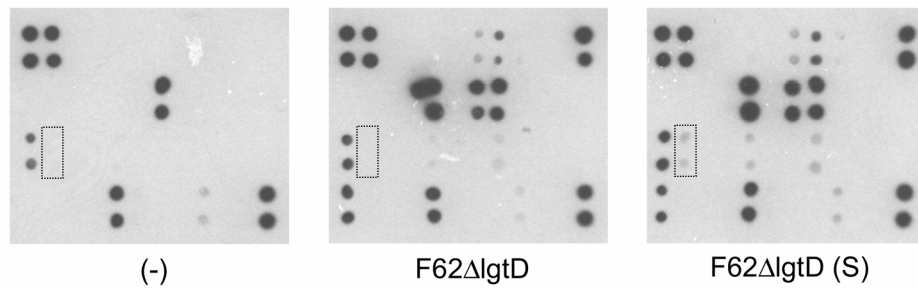
Bacterial LOS was analyzed via polyacrylamide gel electrophoresis. (A) LOS samples shown (from left): F62 $\Delta$ lgtA, F62 grown in the presence of CMP-NANA (sialylated), and wild type F62. (B) LOS samples shown (from left): wild type F62, F62 $\Delta$ lgtD, and F62 $\Delta$ lgtD grown in the presence of CMP-NANA (sialylated). (C) THP-1 cells ( $1 \times 10^6$ /sample) were challenged with sialylated and unsialylated *N. gonorrhoeae*. Cytokine production in sample supernatants was measured by ELISA. Left panel: IL-12 levels after an 18 hour challenge of THP-1 cells ( $1 \times 10^6$ /sample) with  $1 \times 10^2$  live *N. gonorrhoeae*. Right panel: TNF $\alpha$  levels after 7 hour challenge of THP-1 cells ( $1 \times 10^6$ /ml) with  $1 \times 10^8$  gentamicin-killed gonococci. (S) represents sialylated samples (grown in the presence of CMP-NANA). Challenge with media alone did not result in measurable production of IL-12 or TNF $\alpha$ .

*Gonococcal sialylation elicits production of MCP-2.* In order to further study whether sialylation of gonococcal LOS alters monocytic cytokine production, I utilized a cytokine array. This assay allowed simultaneous measurement of 42 different cytokines in samples. Challenge with gonococci resulted in upregulation of several cytokines including: GRO (growth regulated oncogene), IL-1 $\beta$ , IL-6, IL-10, GM-CSF (granulocyte-macrophage colony stimulating factor), TNF $\alpha$ , MDC (macrophage-derived chemoattractant), and RANTES when compared to samples challenged with media alone. Several cytokines [IL-8, MCP-1, and PDGF-B (platelet-derived growth factor-B)] were also measurable in unchallenged samples. There is a slight disconnect between cytokines measured in this assay compared with those measured previously by ELISA. No IL-12 was measurable during the array experiment and I attribute this to the different antibody pairs used in printing the array. In addition, IL-8 did not appear to be significantly upregulated between unchallenged and challenged samples since the spots were saturated on both arrays chosen for analysis. Even upon examination of a one second exposure, values for the IL-8 spots far exceeded positive control values. In addition, positive control values from these early exposures did not fall in the linear range, and therefore these arrays could not be included in the statistical analysis (data not shown). However, previous analysis by ELISA allowed me to quantify the IL-8 produced during challenge.

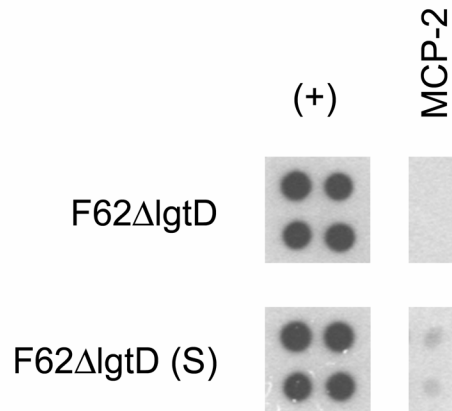
Interestingly, challenge with sialylated gonococci caused production of MCP-2 (monocyte chemoattractant protein-2) in comparison with unsialylated gonococci (Figure 10). MCP-2 was not detected in unchallenged samples or those challenged with unsialylated gonococci. Due to the limits of this type of assay, further analyses



A.



B.



**Figure 10: Comparison of MCP-2 production upon challenge with either F62ΔIgtD or sialylated F62ΔIgtD.**

Primary human monocytes ( $1 \times 10^6$ /ml) were challenged with gentamicin-killed gonococci ( $1 \times 10^7$ ) for 18 hours prior to analysis of sample supernatants using a protein array to detect the presence of cytokines. Densitometric analysis was carried out using a Gel Doc 2000 camera and Quantity One Software (BioRad Laboratories, Inc.). Analysis of internal positive controls (+) among array exposures using a paired, two-tailed, t test with a 95% confidence interval indicated that the means were statistically similar ( $P > 0.05$ ), allowing cross comparison of sample cytokine levels. (A) Whole array for each sample: (-) negative control, F62ΔIgtD, and F62ΔIgtD (S) Dashed boxes indicate the location of MCP-2 antibodies spotted on the array membrane. (B) Internal positive controls compared with MCP-2. Data shown are from one experiment. (S) indicates sialylated samples.

are necessary in order to quantitate the observed differences in MCP-2 production. A variety of immune cells are targets for this chemokine and chemotactic responses have been shown to differ depending upon the concentration of MCP-2 *in vivo*. Quantitation of MCP-2 regarding these gonococcal challenge experiments would allow greater speculation as to its biological significance (129).

## Discussion

Gonococcal LOS is an important surface molecule in disease. In this study, I measured the production of a variety of cytokines from monocytes during gonococcal challenge. The data demonstrate that alterations in the carbohydrate moiety of LOS do not impact the production of most cytokines by human monocytes. However, whole-cell bacterial challenge was more stimulatory than challenge with purified LOS, implying an important role for other surface structures with regard to cytokine elicitation. Interestingly, sialylation of LOS resulted in elicitation of the chemokine MCP-2 from challenged cells. As the LOS structure associated with symptomatic infection (lacto-*N*-neotetraose) is readily sialylated *in vivo*, differential elicitation of cytokines due to sialylation could significantly impact disease progression by increasing the response of specific immune cell types.

Though several groups have measured cytokine production during *in vitro* challenge with *N. gonorrhoeae*, controversy exists with regard to which cytokines are produced and in what quantities. In this study, I observed production of TNF $\alpha$ , IL-12, IL-10, and IL-8 by monocytes in response to whole-cell gonococcal challenge as well as in response to challenge with purified LOS. However, whole cell gonococci elicited a much greater response than purified LOS/LPS. Since these experiments were performed, this observation has been confirmed by several groups studying *N. meningitidis*. It has been demonstrated that *N. meningitidis* deficient in detectable LOS (LpxA<sup>-</sup>) causes substantial cytokine production from human peripheral blood mononuclear cells when compared to wild type bacteria (130, 131). Similarly, intact

meningococci have been shown to be more potent inducers of several cytokines than equal amounts of purified LPS (130). In contrast, Unkmeir, *et al.* reported that challenge of dendritic cells with an *N. meningitidis* strain lacking LOS resulted in almost undetectable levels of TNF $\alpha$ , IL-6, and IL-8 (132). However, this difference may be related to the use of dendritic cells as a model. These data, along with my observations, indicate that a substantial portion of the cytokines elicited from gonococcal interactions with monocytes, result from non-LPS components.

During these experiments I observed no difference in the elicitation of TNF $\alpha$ , IL-10, IL-12, or IL-8 between gonococcal LOS variants. Based on these observations, I believe that while the carbohydrate portion of the LOS molecule is important for pathogenesis of the organism, it does not play a significant role in determining host cytokine profile during gonococcal encounters with monocytes. Similar conclusions have been reached by other groups since these experiments were performed. Pridmore, *et al.* challenged THP-1 cells with purified LOS (200 ng) from different neisserial strains and measured TNF $\alpha$  production and signaling through TLR4/MD2 (113). Some variability was observed, although this did not correspond with oligosaccharide structure of the strains. Braun, *et al.* demonstrated that *N. meningitidis* variants expressing LOS immunotypes isolated from diseased patients induced significantly higher levels of TNF $\alpha$  and IL-6 when compared with immunotypes primarily associated with carriers of the bacteria (133). However, the immunotypes that differed in their ability to induce cytokine production each expressed identical oligosaccharide moieties. Interestingly, disruption of the *lpxLII* gene in *N. gonorrhoeae* was shown to alter the lipid A backbone of the LOS

molecule, resulting in mutants with a reduced ability to induce TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 from a human macrophage model (134).

Taken together, these data imply that differences in the immunostimulatory potential of gonococcal LOS variants during interactions with human monocytes originate from alterations in the lipid A structure. This hypothesis is supported by the fact that acylation patterns of the lipid A portion of meningococcal LOS have been demonstrated to be important in determining levels of TNF $\alpha$  elicited from a human macrophage cell line (135).

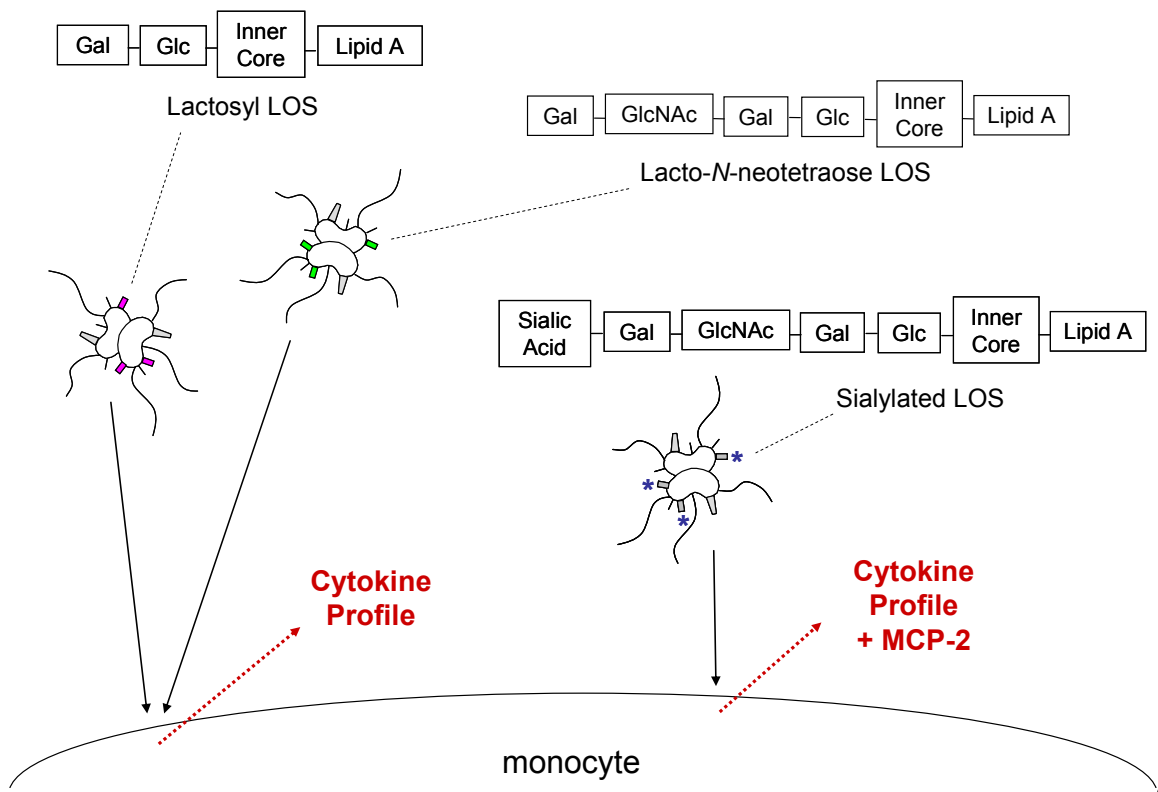
It has been previously shown that when dendritic cells are challenged with *N. meningitidis*, optimal production of TNF $\alpha$  and IL-12 only occurs upon internalization of the bacteria (124). In addition, this internalization appeared to be dependent on expression of LOS, as an LOS-deficient mutant was internalized poorly compared to wild type bacteria (124). In future studies, it would be interesting to study the effects of gonococcal oligosaccharide alterations during interactions with other cellular models, such as dendritic cells. In addition, measurement of internalization, as well as cytokine production during challenge of human macrophages with gonococcal lipid A mutants, may provide significant insight into gonococcal pathogenesis.

Data collected from these experiments allowed a broad look at the cytokine profile of monocytes after gonococcal challenge. A variety of cytokines and chemokines were measurable during infection including: GRO, IL-1 $\beta$ , IL-6, IL-10, IL-12, GM-CSF, TNF $\alpha$ , MDC, and IL-8. The cytokine profile I observed differed slightly from that observed by Makepeace, *et al.*, who utilized a differentiated human macrophage model during gonococcal challenge studies (136). Though production of

several cytokines (including TNF $\alpha$ , IL-6, MIP-1 $\alpha$ , and RANTES) was measured, no secretion of IL-1 $\beta$ , ENA-78, GM-CSF, IL-10, or IL-12 was reported. Since I was able to measure IL-1 $\beta$ , GM-CSF, IL-10 and IL-12 during challenge, it is possible that use of differentiated cells, a different gonococcal strain (P9), and a higher infectious dose (MOI = 400) by this group explains these differences with regard to my own observations. Further analysis of the roles of the cytokines and chemokines measured in this study could significantly contribute to our understanding of gonococcal disease.

Sialylation of gonococcal LOS has been previously shown to convert serum sensitive strains to a serum resistant phenotype (137-139). However, sialylation has also been shown to play a role in other aspects of gonococcal pathogenesis including interactions with antibodies and phagocytes [for review see (62, 73, 140)]. In these studies, sialylation of gonococcal LOS prior to challenge of THP-1 cells resulted in similar production of TNF $\alpha$  or IL-12. These experiments were followed by use of an array to examine cytokine production from challenged primary monocytes. This work demonstrated that sialylated gonococcal challenge samples exhibited upregulation of MCP-2 (in comparison to unchallenged samples and those challenged with unsialylated bacteria). MCP-2 is a low molecular weight monocyte chemotactic cytokine which is closely related to MCP-1 and MCP-3 (129). Target cells for MCP include monocytes, T lymphocytes, natural killer cells, eosinophils, and basophils, though monocytes are the most sensitive. The concentration of MCP-2 present *in vitro* has been shown to differentially activate specific target cell populations (129). Only one other group has reported MCP-2 production in response to gonococcal

infection. A human mononuclear cell model was utilized, though gonococci were not sialylated prior to challenge (141). As LOS molecules associated with symptomatic infection and increased invasiveness are readily sialylated *in vivo*, production of MCP-2 during infection may substantially increase the response of a specific cellular population during gonococcal infection. Future determination as to the quantities of MCP-2 produced during monocyte interactions with sialylated gonococci may provide insight into the pathogenesis of these more virulent strains. In addition, further analysis of the mechanism by which sialylated LOS causes production of MCP-2 may significantly add to our knowledge of the pathogenesis of *N. gonorrhoeae*. In summary, though alterations in the carbohydrate portion of LOS do not appear to effect cytokine production by human monocytes, sialylation of LOS may play an important role in gonococcal pathogenesis (Figure 11).



**Figure 11: Schematic diagram summarizing the observed interactions of human monocytes with gonococci expressing various LOS structures.**

Gonococci expressing the lactosyl LOS and the lacto-*N*-neotetraose LOS were observed to cause production of the same cytokines by human monocytes. Upon sialylation of gonococci expressing lacto-*N*-neotetraose LOS, an increase in production of the chemokine MCP-2 was observed.



## Chapter 2: Host Response to the Neisserial H.8 Antigen

### Introduction

*N. gonorrhoeae* expresses many surface molecules that undergo significant antigenic variation. However, the H.8 antigen (or Lip lipoprotein) is a surface-expressed lipoprotein that is conserved among Neisserial pathogens (84, 85). Immunogold-labeling of this antigen has demonstrated its relative abundance and uniform distribution on the bacterial cell surface (84). The H.8 antigen is named for a monoclonal antibody that recognizes an epitope common to *N. gonorrhoeae* and *N. meningitidis*, but not to most commensal Neisseria species (83). Not only is this lipoprotein abundantly expressed on the gonococcal cell surface, but it has also been found in membrane blebs (84, 142).

Though the cell membranes of Gram negative bacteria contain many different lipoproteins, few of these have been characterized biochemically. Most bacterial lipoproteins appear to be triacylated at the amino terminus, while the lipoproteins found on mycoplasmas are diacylated and have a free amino group (111). Lipid modification of the neisserial H.8 antigen has not been completely elucidated, however analysis by Fiset, *et al.* suggests that its lipid component can be composed of C<sub>16</sub> fatty acid and a shorter C<sub>8</sub>-C<sub>10</sub> lipid (111). The H.8 antigen has been difficult to characterize due to several unusual properties. It does not stain with Coomassie blue and migrates on SDS-PAGE gels as a cone-shaped band. In addition it migrates

aberrantly in two-dimensional gels (84). The purified protein component of Lip has been demonstrated to be rich in glutamate, alanine and proline, and lacks aromatic amino acids and methionine (143). The amino-terminus of Lip is a cysteine residue. Analysis of this protein from strain FA1090 reveals that it is 71 amino acids in length and is composed of a number of pentameric repeats (AAEAP). Perfect five-residue periodicity is maintained throughout (143). The H.8 lipoprotein shows some similarity to other microbial proteins with repetitive motifs such as the Streptococcal M protein and Lpp of *E. coli*. While these lipoproteins serve structural roles in the outer membrane, the Streptococcal M protein has also been shown to have an antiphagocytic function (143).

The role of H.8 in gonococcal pathogenesis is unclear. Several aspects of the host response to this molecule have been previously investigated. H.8-specific antibodies were measured in serum from patients with disseminated gonococcal and meningococcal infections (95). In addition, alterations in the molecular weight of H.8 have been shown to correspond with alterations in gonococcal serum sensitivity (84). Purified preparations of H.8 have been shown to elicit cytokine production from epithelial cells, and promote NF- $\kappa$ B activation (111).

The gonococcus is constantly altering many of its surface structures. However, since *N. gonorrhoeae* does not elicit a dramatic antibody response in infected hosts and re-infection with the same strain is common, the host response to H.8 does not appear to impede colonization/ disease progression. Therefore, it is unusual that the H.8 antigen is conserved among the pathogens and that it is invariable. This implies that H.8 may greatly impact Neisserial pathogenesis.

In this study, I investigated the role of the H.8 antigen in the elicitation of pro-inflammatory cytokines from monocytes and in the cell-cell interactions between gonococci and macrophages.

## Results

*Construction of an H.8 negative strain.* [The following section (including Figure 12) incorporates experiments performed by Dr. Daniel Stein.] The gene encoding H.8 was PCR-amplified from *N. gonorrhoeae* strain FA19. After isolating an *E. coli* transformant containing the desired PCR amplicon, the resulting plasmid (pUC19-H8) was used in a PCR reaction. This PCR amplification deleted 239 bp (almost all of the coding sequence for H.8) from pUC19-H8. This PCR amplicon was digested with BsiWI, self ligated, and introduced into *E. coli*. The resulting plasmid was named pUC19-H8 $\Delta$ . After successful insertion of a spectinomycin resistance-encoding gene (from the  $\Omega$  interposon) into this plasmid, a spectinomycin resistant transformant of *E. coli* was isolated (pUC19-H8 $\Delta$ Spec). After transformation of *N. gonorrhoeae* strain F62(lgtDfixoff) (with pUC19-H8 $\Delta$ Spec), one transformant (F62(lgtDfixoff)H8-spec7) was chosen for further analysis. The correct insertion of the  $\Omega$  interposon encoding sequence and deletion of the H.8 encoding sequence in F62(lgtDfixoff)H8-spec7 was verified by PCR analysis. DNA isolated from F62(lgtDfixoff)H8-spec7 and F62(lgtDfixoff) was used as a template for PCR and the amplicon generated from F62(lgtDfixoff) generated a fragment of 3000 bp, and yielded three fragments after digestion with HinCII (1595, 1168 and 237 bp). Since the  $\Omega$ -interposon encoding sequence correctly inserted into the chromosome of F62(lgtDfixoff)H8-spec7, a 4824 bp fragment was successfully amplified, and yielded three fragments after digestion with HinCII (3419, 1168 and 237 bp). The data presented in Figure 12A indicate that the expected profile for each amplicon was generated, indicating that the  $\Omega$

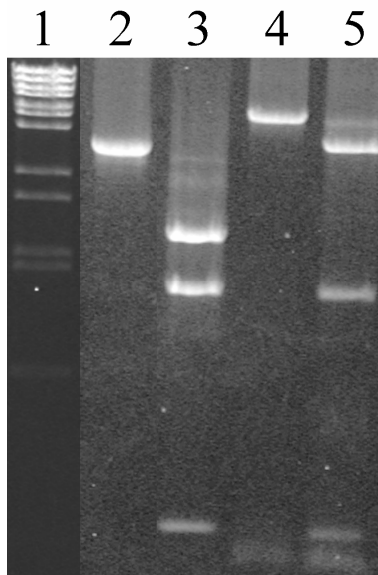
interposon correctly replaced the H.8 coding sequence. Protein extracts from the parent and H8<sup>-</sup> mutants were isolated and reactivity of the proteins was determined by Western blotting, using mAb 2-1-CA3, obtained from Dr. Sanjay Ram (Boston University), to detect H.8 (Figure 12B).

*H.8 does not affect human monocytic cytokine production.* The impact of the H.8 antigen during the initial stages of gonococcal disease is unknown. Since I have hypothesized that gonococcal interactions with monocytes may be an important step in determining disease progression, I chose to analyze the effect of H.8 on the production of several cytokines by these cells. Initially, THP-1 cells were challenged with either the parent strain (F62ΔlgtD) or the H.8<sup>-</sup> mutant (F62ΔlgtDH8<sup>-</sup>). Gentamicin-killing of bacteria prior to the challenge ensured that no phase variation or bacterial growth would occur during the 18 hr incubation. Sample supernatants were analyzed for the presence of TNFα and IL-12 (Figure 13). No differences in the levels of these cytokines were observed. After noting the apparent importance of IL-8 production during gonococcal challenges of primary human monocytes (see Ch.1, Figure 8), I chose to use this same model in order to study potential H.8-dependent differences in IL-8 production. However, I observed no effect upon the production of IL-8 due to challenge with the H.8<sup>-</sup> mutant in comparison with the parent strain (Figure 14). In order to more broadly analyze the cytokine production of primary human monocytes in response to our H.8<sup>-</sup> mutant, I utilized a cytokine array. Through use of this assay I was able to analyze sample supernatants for the presence of 42

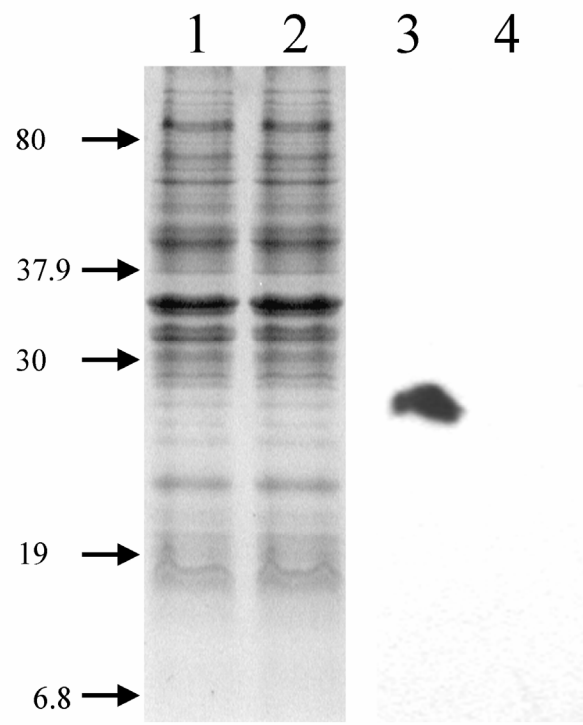
**Figure 12: Constuction of an H.8<sup>-</sup> strain.**

(A) Data indicate the replacement of the sequence for H.8 with the  $\Omega$  interposon. The lanes represent: 1)  $\lambda$  DNA digested with *Hinc*II, 2 ) amplicon generated from F62(*lgtD*fixoff) and 3) its digestion with *Hinc*II (1595, 1168 and 237 bp), 4)  $\Omega$ -interposon insertion and 5) its digestion with (*Hinc*II, 3419, 1168 and 237 bp). (B) SDS-PAGE and Western blot analysis of H.8 expressed by *N. gonorrhoeae* strain F62 $\Delta$ *lgtD*H.8<sup>-</sup> and its derivative. Lanes 1 and 2 represent SDS-PAGE analysis: 1) parent strain and 2) F62 $\Delta$ *lgtD*H.8<sup>-</sup>. Lanes 3 and 4 represent Western analysis using an anti-H.8 mAb: 3) F62 $\Delta$ *lgtD*, 4) F62 $\Delta$ *lgtD*H.8<sup>-</sup>.

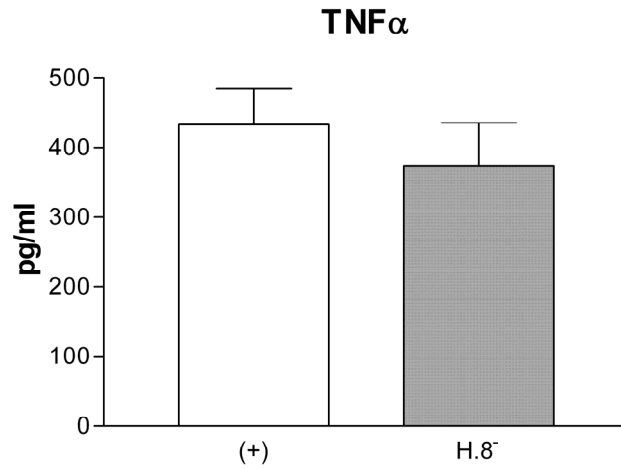
A.



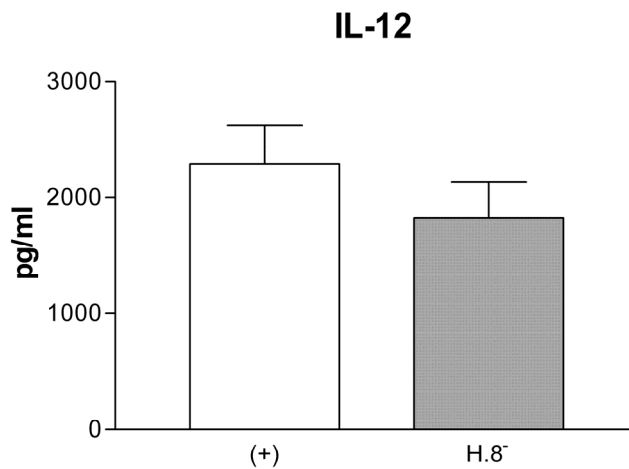
B.



A.



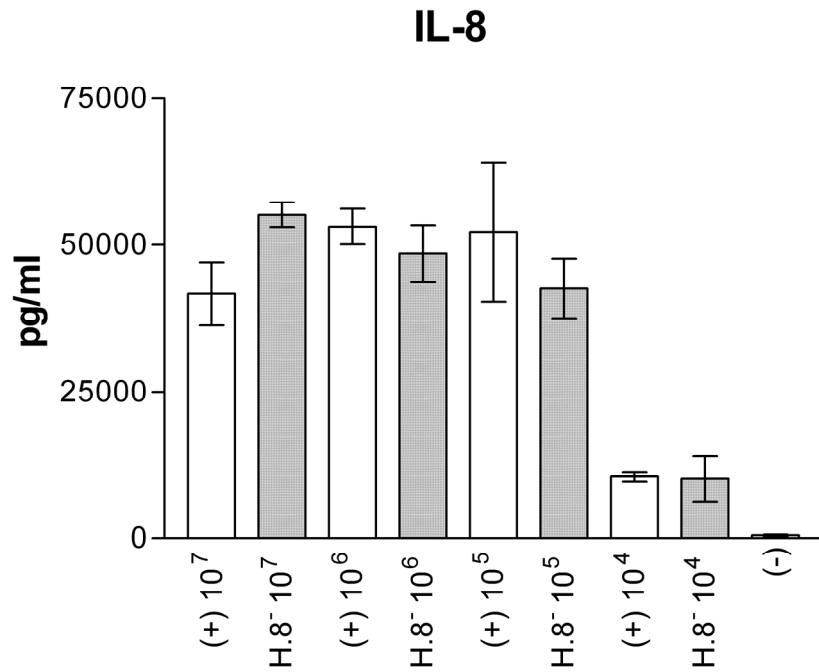
B.



**Figure 13: Challenge of THP-1 cells with an H.8<sup>-</sup> mutant.**

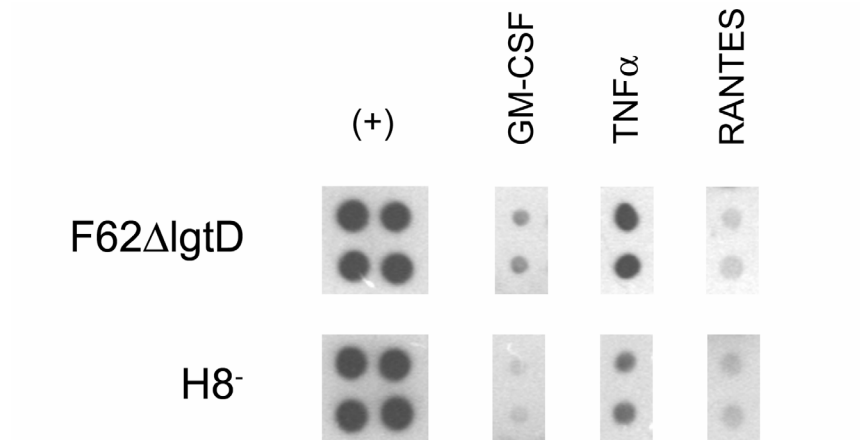
Parent strain (+) F62 $\Delta$ lgtD (clear bars), and its corresponding H.8<sup>-</sup> mutant (shaded bars) were gentamicin-killed and added to THP-1 ( $10^6$  per sample) at an MOI of 10. After an 18 hr incubation, sample supernatants were analyzed by ELISA for the presence of TNF $\alpha$  (A) and IL-12 (B). Data shown are from one experiment performed in triplicate and are representative of two independent experiments. Error bars represent the standard deviation of the mean.





**Figure 14: Challenge of primary human monocytes with H.8<sup>-</sup> gonococci.** Monocytes ( $10^6$  per sample) were challenged for 18 hrs with either gentamicin-killed (+) F62 $\Delta$ lgtD (clear bars) or the H.8<sup>-</sup> mutant (shaded bars). Sample supernatants were analyzed by ELISA for the presence of IL-8. Data shown are from one experiment, performed in triplicate, and are representative of two independent experiments. (-) indicates monocyte samples challenged with media alone. Error bars indicate the standard deviation of the mean.

different cytokines. As mentioned in Chapter 1, when comparing samples challenged with gonococci to those challenged with media alone I observed upregulation of several cytokines including: GRO, IL-1 $\beta$ , IL-6, IL-10, GM-CSF, TNF $\alpha$ , MDC, and RANTES. Some cytokines including IL-8, MCP-1 and PDGF-B were also measurable in unchallenged samples. When comparing between samples challenged with F62 $\Delta$ lgtD and the H.8<sup>-</sup> mutant I observed a difference between GM-CSF, TNF $\alpha$ , and RANTES (Figure 15). The level of RANTES was measured to be slightly higher in the H.8<sup>-</sup> samples, while GM-CSF and TNF $\alpha$  were measured to be lower in H.8<sup>-</sup> samples. However, none of these cytokines was determined to be significantly different between samples (2-fold change or greater). Therefore, I can conclude that production of most proinflammatory cytokines is not impacted by the H.8 antigen. As previously observed using this assay (Chapter 1), the level of IL-8 between challenged and unchallenged samples was not measured to be significantly different. I have however, previously shown a significant difference in production of this cytokine by ELISA (Figure 14). Since the cytokine array only allows determination of qualitative differences, it may be that the fold change between samples was not great enough to be reflected in my analysis. For example, the difference in IL-10 between challenged and unchallenged samples appeared, by array analysis, to be great. However quantitation by ELISA demonstrates very low levels of IL-10 present in samples. Therefore, intensity measured during array analysis does not reflect actual amounts of protein and may not always reflect biological significance.

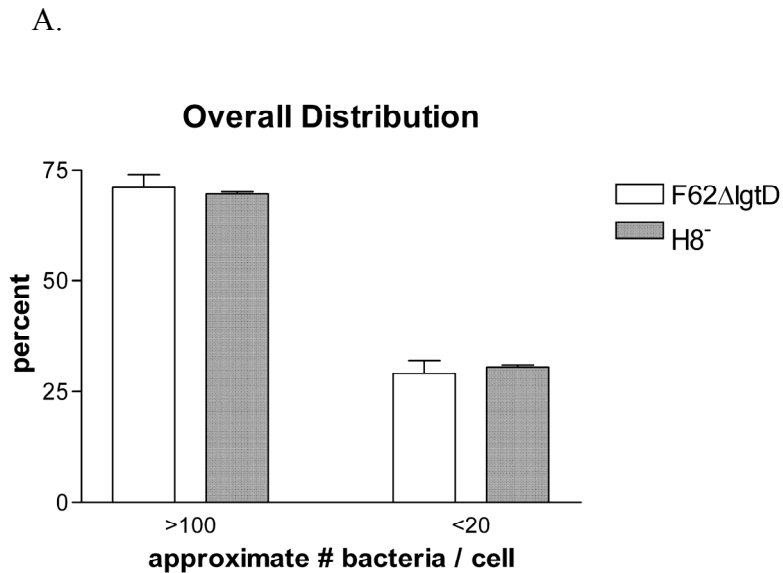


**Figure 15: Comparison of cytokine levels from challenge with either F62 $\Delta$ lgtD or the H.8 $^-$  mutant.**

Primary human monocytes ( $1 \times 10^6$ /sample) were challenged with gentamicin-killed gonococci for 18 hours prior to analysis of sample supernatants using a cytokine array. Densitometric analysis was carried out using a Gel Doc 2000 camera and Quantity One Software (BioRad Laboratories, Inc.). Analysis of internal positive controls (+) among array exposures using a paired, two-tailed, t test with a 95% confidence interval indicated that the means were statistically similar ( $P > 0.05$ ), allowing cross comparison of sample cytokine levels. Shown are internal positive controls and three cytokines measured to be slightly different between challenge samples during one experiment.

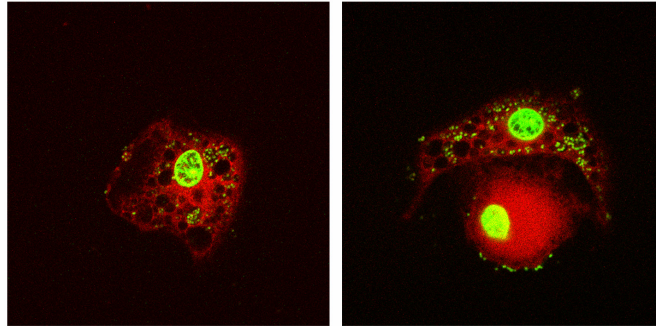
*H.8 alters bacterial distribution during cell-cell contact.* In order to examine the physical interactions between H.8<sup>-</sup> gonococci and human cells, I utilized a human monocyte-derived macrophage model. Use of these cells allowed clear visualization of cellular structures and relative orientation of the bacteria. Live bacteria (F62ΔlgtD and F62ΔlgtD H.8<sup>-</sup>) were diluted and added to macrophage monolayers at an MOI of 50. Cells were incubated at 37°C for 120 min. before fixation in methanol. Macrophages were stained and analyzed by confocal microscopy. During analysis, macrophages were examined and classified into two categories: those interacting with a large number of bacteria (>100 group) and those interacting with very few bacteria (<20 group). Initially, I compared the parent strain with the H.8<sup>-</sup> mutant and determined that approximately 70% of macrophages were interacting with large groups of bacteria and approximately 20% were interacting with only a few bacteria (Figure 16A). [A very small number of macrophages (<1%) did not appear to be interacting with any bacteria. These cells were not included in my analysis.] In the case of macrophages interacting with large numbers of bacteria, the bacterial cells appeared to be either evenly distributed or were arranged in distinct aggregates. Figure 16B shows several representative images of macrophages classified as interacting with “distributed” bacteria or “bacterial aggregates.” I observed a striking difference in bacterial orientation in samples challenged with the H.8<sup>-</sup> mutant. Examination of at least 60 macrophages per sample revealed that approximately 65% of these macrophages (in the >100 group) were in contact with large bacterial aggregates, compared with approximately 15% of those challenged with the parent strain (Figure 16C).

**Figure 16: Bacterial cell distribution upon challenge of human macrophages.** Live bacteria were added to macrophages (MOI = 50) as described in the methods, and incubated for 120 min. Cells were fixed with methanol and coverslips were stained with SYTO 13 green fluorescent nucleic acid stain (green) and Evan's Blue (red) prior to analysis by confocal microscopy. Macrophages were categorized after blind image capture of randomly selected fields. (A) Percentage of macrophages classified as interacting with more than 100 or less than 20 bacteria. (n > 60 for each sample.) Data represent the average of two independent experiments and error bars indicate the standard deviation of the mean. Clear bars indicate challenge with F62ΔlgtD and shaded bars indicate challenge with the H.8<sup>-</sup> strain. (B) Confocal images which represent the categories used to classify bacterial orientation in the >100 group. (C) Percent of macrophages interacting with evenly distributed bacteria vs. bacterial cell aggregates in the >100 group. (n > 60 macrophages categorized in each sample.) Data represent the average of two independent experiments and error bars indicate the standard deviation.

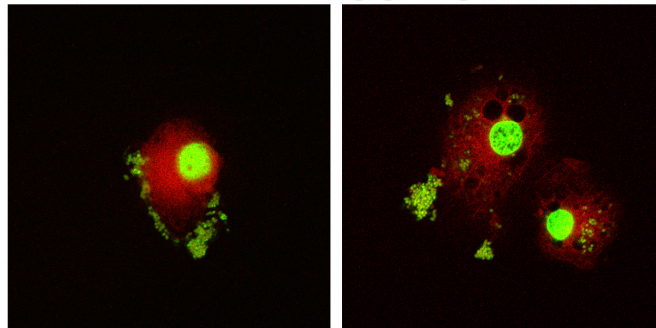


B.

distributed bacteria

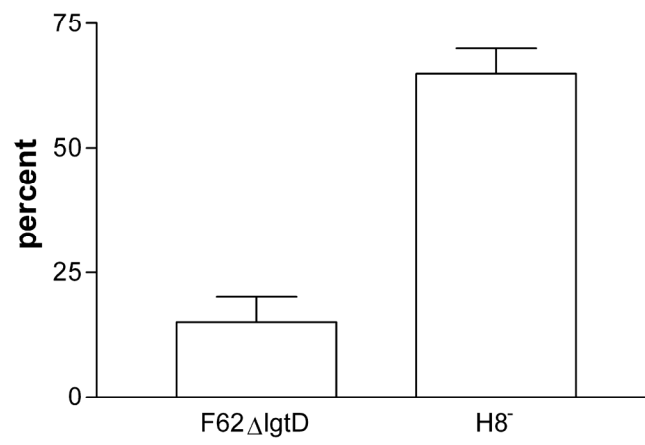


bacterial aggregates



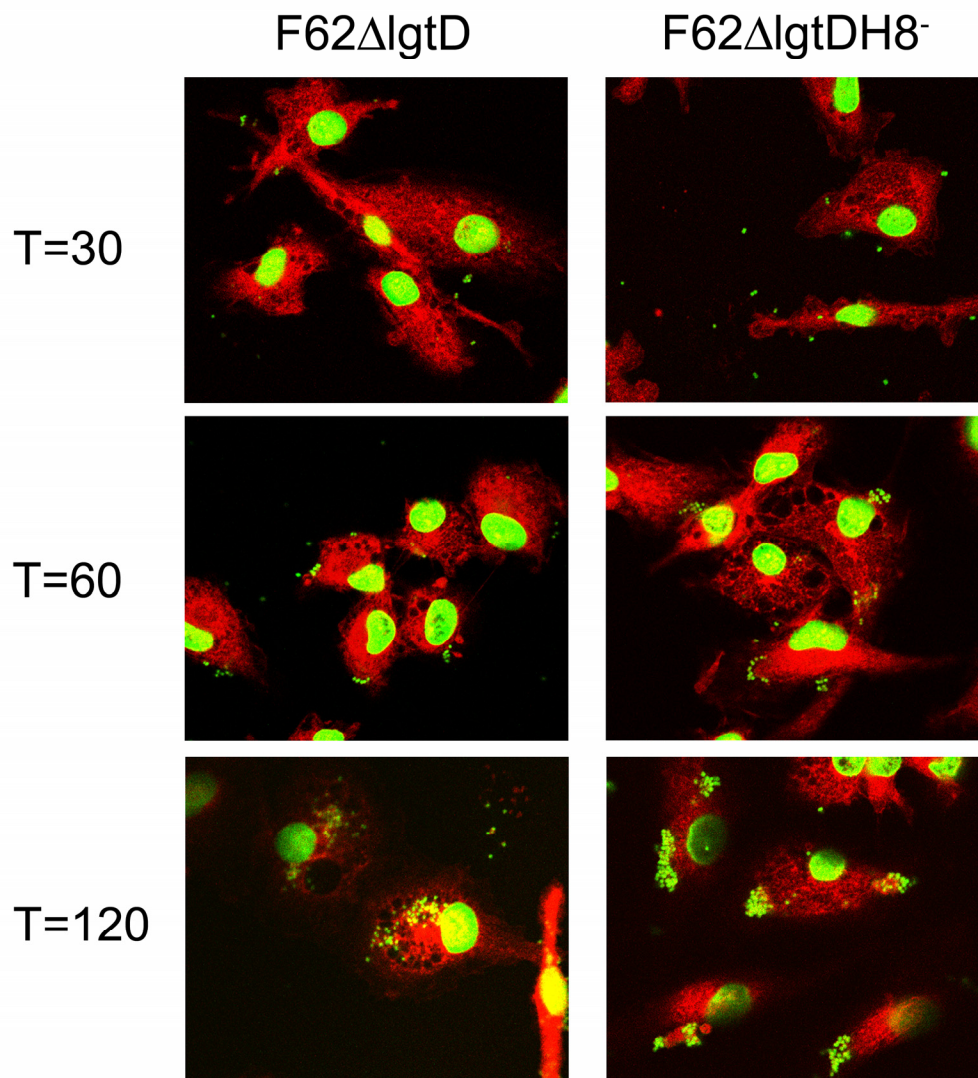
C.

Observed Aggregation



*Bacterial cell orientation is not altered prior to contact with macrophages.* After observing the increased aggregation of H.8<sup>-</sup> mutants, I hypothesized that since H.8 has an overall negative charge, this observation may be due to the altered surface charges of the H.8<sup>-</sup> mutants. As a result, the mutant gonococci would be more likely to aggregate in general, independent of contact with macrophages. In order to test this hypothesis, I challenged human macrophage monolayers over a time course. Samples were incubated at 37°C for 5, 30, 60, and 120 min post challenge, prior to fixation with methanol. Samples were then stained and analyzed via confocal microscopy. Figure 17 shows two representative images for each time point, with the exception of 5 min. In the 5 min samples, no bacteria were visible. I attribute this observation to the fact that the minimum time for bacterial attachment in this system must be greater than 5 min. Therefore, most of the bacteria were likely removed upon aspiration of the samples, and during post-fixation washing. At the 30, 60, and 120 min time points, H.8<sup>-</sup> gonococci can be seen arranged as diplococci when they are not interacting with macrophages. However, the majority of those bacteria in direct contact with macrophages were observed to form aggregates or microcolonies. In comparison, the parent strain appeared to be taken up by the macrophages over time, and few large aggregates were observed. These data indicate a possible role for the H.8 antigen during recognition and phagocytosis by immune cells.

In order to further study the distribution of the H.8<sup>-</sup> mutants in contact with human macrophages, I examined the three-dimensional arrangement of the bacteria. Cross-sectional images of a cell interacting with a large number of evenly distributed bacteria (F62ΔlgtD) are shown in Figure 18A. In these images, bacteria are evident



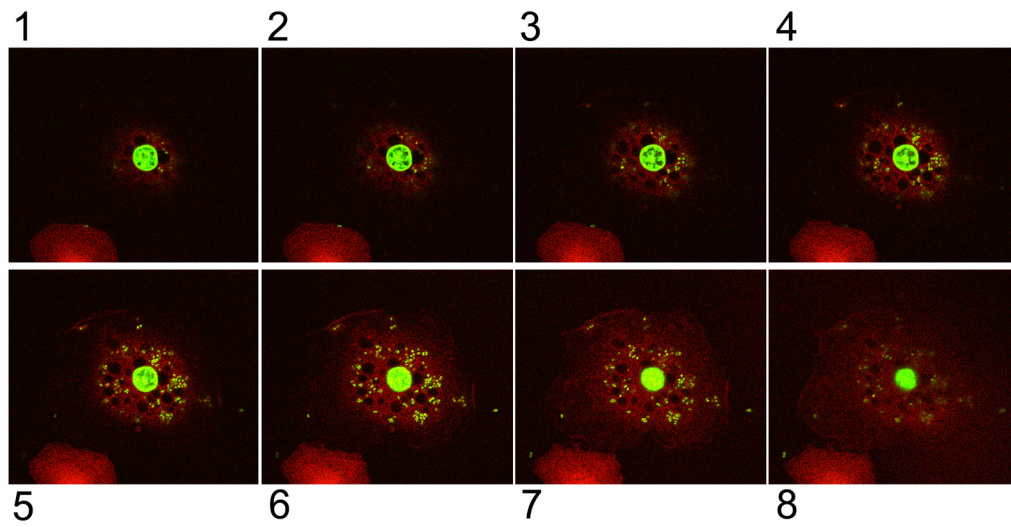
**Figure 17: Bacterial cell distribution over time.**

Live bacteria were added to macrophages (MOI = 50) and incubated over a time course. Cells were fixed with methanol and coverslips were stained with SYTO 13 green fluorescent nucleic acid stain (green) and Evan's Blue (red) prior to analysis by confocal microscopy. One representative image per time point is shown for both F62ΔlgtD and F62ΔlgtD H.8<sup>-</sup>.

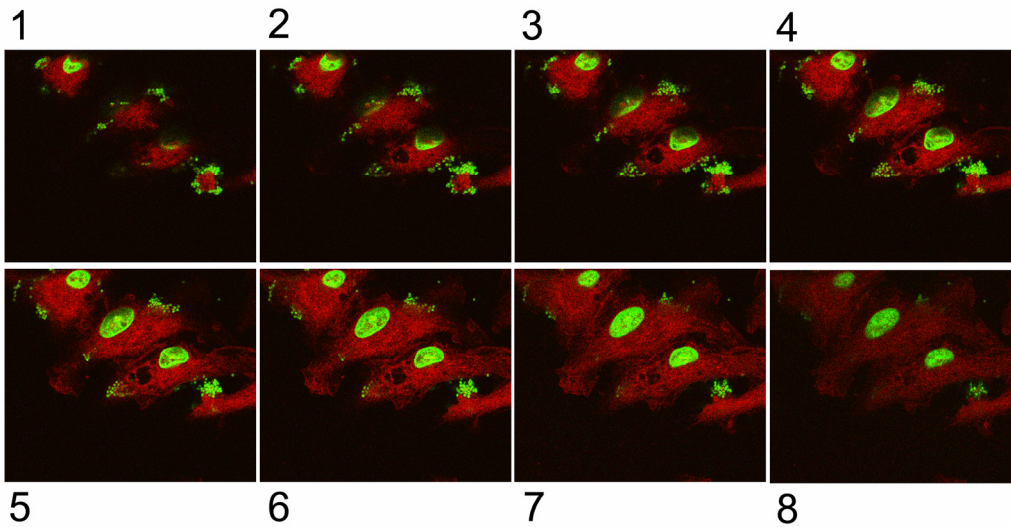


throughout the macrophage, and many appear to be localized within vacuoles. In contrast, several macrophages interacting with large bacterial aggregates (F62ΔlgtDH.8<sup>-</sup>) are shown in Figure 18B. These data indicate that the H.8<sup>-</sup> gonococci are not readily taken up by macrophages in comparison with wild type bacteria. This implies that the H.8 antigen may be recognized by macrophages, serving as an important PAMP.

A.



B.



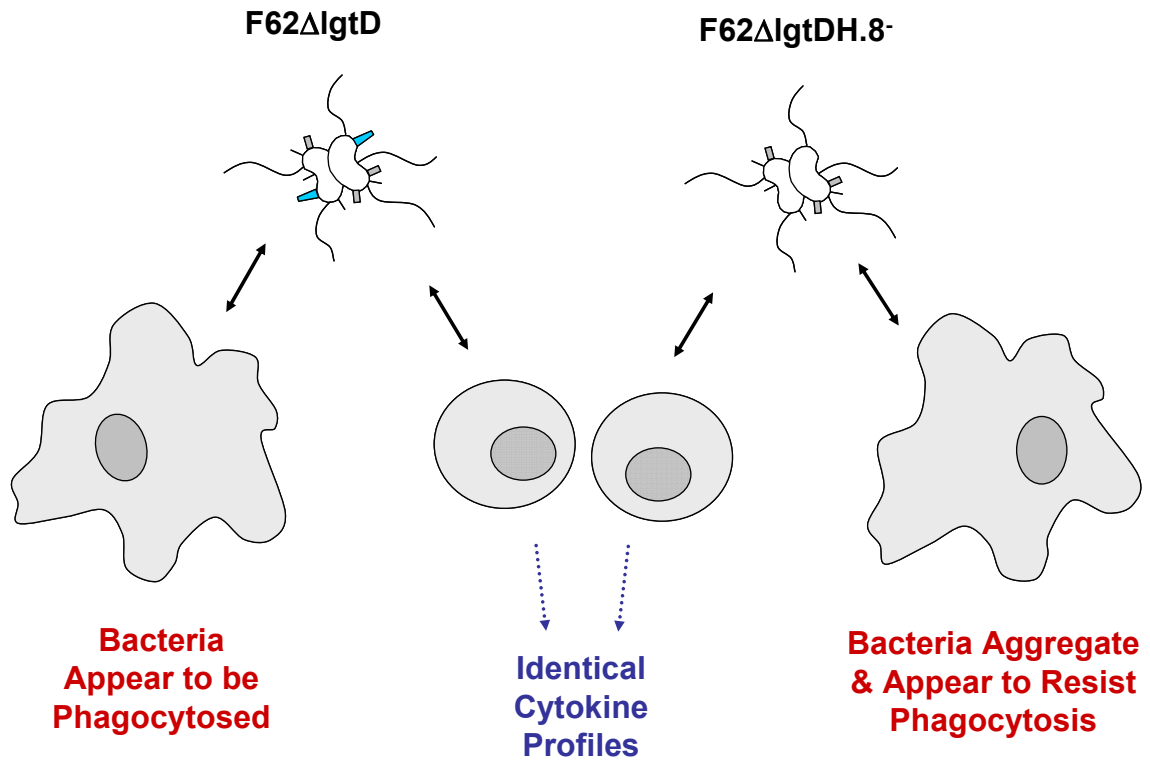
**Figure 18: Cross-sectional analysis of challenged macrophages.**

Live bacteria were added to macrophages (MOI = 50) and incubated for 120 min. Cells were fixed with methanol. Cells were stained with SYTO 13 green fluorescent nucleic acid stain (green) and Evan's Blue (red) prior to analysis by confocal microscopy. Images from 1 to 8 show cross-sections from the surface of the cells (1) down to their point of attachment on a glass coverslip (8). (A) F62ΔlgtD (B) F62ΔlgtDH.8<sup>-</sup>.

## Discussion

The function of the neisserial H.8 antigen remains undefined, though its role in disease is likely to be significant. The structural properties of this molecule have been studied, but few groups have performed experiments to characterize its impact upon the human immune system. Only one other group has created a gonococcal mutant lacking H.8, however no studies regarding interactions of this mutant with host cells have been reported (87). Generation of an H.8<sup>-</sup> strain in our laboratory allowed direct comparison of the effects of this antigen on gonococcal interactions with human monocytes and macrophages. Though I observed no significant role for H.8 in cytokine elicitation, this molecule appears important during recognition by host macrophages (Figure 19).

Initially, I measured the elicitation of the cytokines TNF $\alpha$ , IL-12, IL-10, and IL-8 from monocytes challenged with both wild type *N. gonorrhoeae* and H.8<sup>-</sup> mutants. I observed no differences in the production of these cytokines when using either a human monocytic cell line or human primary monocytes. After more broadly analyzing this response using a cytokine array, RANTES was measured to be slightly higher in the H.8<sup>-</sup> samples, while GM-CSF and TNF $\alpha$  were measured to be lower in H.8<sup>-</sup> samples. However, these differences were not determined to be significant. Therefore, I concluded that the H.8 antigen does not appear to impact the elicitation of cytokines from this cellular model. Fisette, *et al.*, have demonstrated that challenge of epithelial cells with purified H.8 antigen elicited IL-8 and IL-6 in a TLR2-dependent manner (111). This apparent difference from my findings may be



**Figure 19: Schematic diagram summarizing the observed interactions of F62ΔlgtD and F62ΔlgtDH.8<sup>-</sup> with human monocytes and macrophages.** Strain F62ΔlgtD and F62ΔlgtDH.8<sup>-</sup> were observed to elicit similar levels of cytokines from human monocytes. However, during challenge of human macrophages, strain F62ΔlgtDH.8<sup>-</sup> was more likely to form large aggregates over time, upon contact with human macrophages. This aggregation appeared to inhibit phagocytosis.

explained by the use of a different model, but also by the fact that the epithelial cells were challenged with dilutions of purified antigen. It is possible that the challenge concentration of H.8 over-represents the amount found on the bacterial cell surface during natural infection. Additionally, use of purified H.8 is prone to contamination by outer membrane components (88). Finally, the presentation of cell surface antigens to immune cells may be altered when purified molecules are used rather than whole-cell bacterial deletion mutants.

Upon examination of the physical interactions of the H.8<sup>-</sup> mutant and human macrophages, I found a marked difference in bacterial cell aggregation. Upon examination of macrophages in contact with large numbers of gonococci (approximately 70% of the total population), a greater percentage of the H.8<sup>-</sup> bacteria were observed to form large aggregates in comparison with the parent strain. Analysis of cell-cell interactions over time demonstrated that both parent strain and H.8<sup>-</sup> mutant were initially arranged in diplococci. Over time, the mutant bacteria appeared to form aggregates or microcolonies as they interacted with the human cells. This indicates that elimination of H.8 from the gonococcal cell surface does not simply cause a difference in surface charge, leading to increased aggregation of the bacteria in general. Cross-sectional analysis revealed that macrophages interacting with large bacterial aggregates did not contain large numbers of evenly distributed bacteria (as seen with the parent strain). In fact, analysis of several cells demonstrated that the macrophages in contact with large aggregates did not appear able to take up the bacteria efficiently. Historically, aggregation of *N. gonorrhoeae* has been shown to loosely correlate with colony opacity, independent of piliation (144, 145). However,

the bacteria used in this study were all opa<sup>-</sup> and pil<sup>+</sup>. Additionally, there exist no studies of gonococcal aggregation upon initial contact with macrophages.

Though the mechanism behind the observed gonococcal aggregation is unknown, these data imply that the H.8 antigen may serve as an important PAMP during recognition by host macrophages. However, since H.8 is well-conserved among the neisserial pathogens, this molecule must in some way contribute to pathogenesis of the bacterium.

The process of gonococcal phagocytosis has been studied, but the occurrence of intracellular survival by gonococci remains controversial. Reports differ based upon bacterial characteristics, experimental conditions, and cellular models (146-153). Some studies have demonstrated that human monocytes/ macrophages are capable of efficiently killing gonococci *in vitro* (154, 155). However, other reports indicate both gonococcal resistance to phagocytosis and intracellular survival through use of a human blood model (156-158). More recent studies intended to characterize gonococcal virulence factors have also implicated gonococcal intracellular survival. Recently, a macrophage infectivity potentiator (MIP) has been described and characterized. This lipoprotein (Ng-MIP) was shown to promote the intracellular survival of *N. gonorrhoeae* in human macrophages (159). It has also been demonstrated that gonococcal IgA protease cleaves the lysosome/late endosome-associated membrane protein-1 (lamp-1). Since gonococci have been detected in lamp-1-positive vacuoles after uptake by phagocytes and epithelial cells, secretion of IgA protease may significantly contribute to gonococcal escape of the phagosome and intracellular survival (160). In addition, the gonococcal surface protein porin has been

shown to arrest phagosome maturation within macrophages and inhibits the release of reactive oxygen species within human peripheral blood neutrophils and monocytes (161, 162). In the context of these findings, I hypothesize that the H.8 antigen has a role in increasing the efficiency of phagocytosis by human macrophages and may also contribute to intracellular survival.

In future studies, it will be necessary to use additional staining procedures to more definitively examine intracellular vs. extracellular gonococci. In addition, it would be interesting to use our human macrophage model to study intracellular survival of H.8<sup>r</sup> gonococci in comparison with the parent strain. Analysis of the interactions of the H.8<sup>r</sup> strain with other cell types, such as human neutrophils, would also be important, as these cells are commonly observed in association with gonococci during the symptomatic stages of gonorrhea.

# Chapter 3: TNF $\alpha$ -independent IL-8 Expression: Alterations in Bacterial Challenge Dose Cause Differential Human Monocytic Cytokine Response

## Introduction

While it is known that some Gram-negative pathogens such as *Salmonella spp.* are able to establish infections via an extremely low inoculum (163), the infectious dose for *N. gonorrhoeae* is unknown and likely differs depending upon host gender and the site of colonization. Schneider, *et al.* demonstrated that an initial inoculum of only 250 gonococci was sufficient to establish infection in 3 of 7 healthy male volunteers in a clinical challenge (164). The best estimates of the number of bacteria transferred to women suggest an inoculating dose for women ranging from approximately  $2 \times 10^4$  to  $6 \times 10^6$  gonococci (165). Though data are limited to male clinical trials and tissue culture models of infection, it is probable that even given a high inoculating dose, only a very small number of gonococci will successfully invade the reproductive tract.

During infection, the degree of inflammation usually reflects disease severity and symptoms, and serves to clear the host of infectious microbes. Mucosal inflammation can occur as a result of the direct interaction of a pathogen with surface epithelial cells or with specific immune cells beneath the epithelial layer. The production and regulation of inflammatory cytokines is a critical component of the



human innate response to bacterial infections. The cytokines produced as a result of infection can have both local and systemic effects including alteration of vascular permeability, recruitment of immune cells, accumulation of toxins, and tissue damage (36). Inflammation is widely accepted as the classic host response to gonococcal colonization; however the intricacies of the human immune response to this bacterium are unknown. In the following study, I chose to measure the production of several proinflammatory cytokines such as  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$ , as well as chemokines such as IL-8. These cytokines are associated respectively, with gonococci-induced tissue damage/inflammation and neutrophil/monocyte influx to the site of infection.

I have hypothesized that in naturally occurring gonococcal infections, colonization and subsequent invasion by a small number of bacteria can result in a dramatic innate immune response. I show here that *N. gonorrhoeae* is remarkably efficient at eliciting significant levels of IL-8 in our low dose challenge model, in the absence of  $\text{TNF}\alpha$ . The following data indicate that this IL-8 production is likely caused by direct contact of gonococcal surface molecules with monocytes and is  $\text{NF-}\kappa\text{B}$ -dependent. In addition, the data demonstrate that the MAP kinase JNK is not activated in the low dose model. These results demonstrate the importance of chemokines in gonococcal infection and imply that lowering the initial bacterial dose causes a striking per cell increase in the production of specific chemokines/cytokines. I propose a mechanism where a low dose challenge results in diminished AP-1 activity and  $\text{TNF}\alpha$  production while overall IL-8 levels remain constant.

The following work has been published in the Journal of Immunology (166).

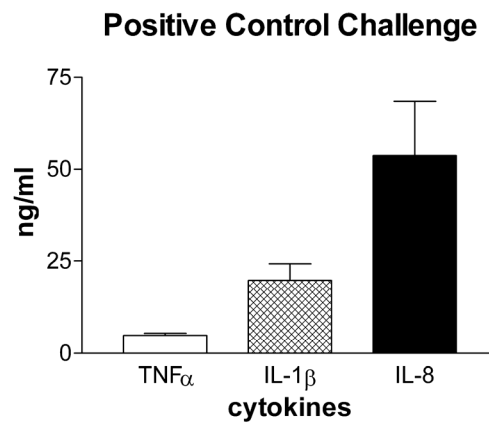
## Results

*Challenge dose of N. gonorrhoeae affects cytokine production by monocytes.* Primary human monocytes were challenged with a range of gonococcal doses and the production of several cytokines predicted to be important in gonococcal infections was measured. Gentamicin-killed bacteria were determined to be most appropriate for use in these experiments, removing the problems associated with gonococcal multiplication in the invasion media, and preventing phase variation of the challenge inoculum. In positive control samples (MOI = 10), TNF $\alpha$ , IL-1 $\beta$ , and IL-8 were all measurable in large quantities at 18 hours post infection (Figure 20A). In the low dose challenge (one bacterium per ten monocytes), approximately 20 ng/ml IL-8 was measurable, while the other cytokines tested were produced at very low levels or were immeasurable (Figure 20B). The difference in IL-8 production between the positive control and the low dose challenge was statistically insignificant, while TNF $\alpha$  and IL-1 $\beta$  respectively resulted in a 20-fold and 10-fold reduction subsequent to low dose challenge (Figure 20C). These data indicate that during a low inoculum gonococcal challenge, large amounts of IL-8 are produced by human monocytes. This occurs in the absence of significant TNF $\alpha$ , demonstrating that the initial infectious dose can greatly impact the overall host cytokine profile. I next examined whether a similar dose-dependent differential for TNF $\alpha$  would result from monocytes challenged with either killed *E. coli* or killed *S. typhimurium*. The average fold reduction in TNF $\alpha$  between doses was shown to be significant for *E. coli* samples, yet insignificant for *S. typhimurium* (Figure 20D). As in the case of the gonococcal challenge, no significant

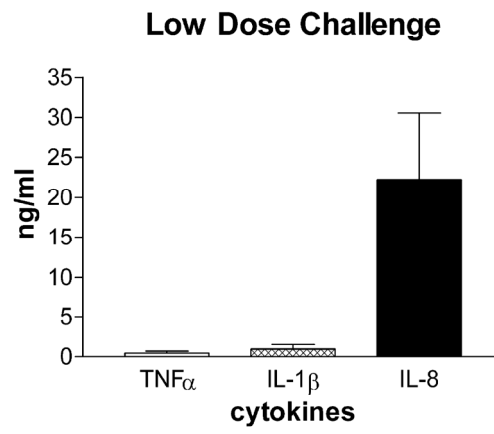
**Figure 20: Production of cytokines by primary human monocytes after *in vitro* challenge.**

Cytokine profile of monocytes ( $10^6$  per sample) after an 18-hr challenge with killed *N. gonorrhoeae*, strain F62ΔigtD. Shown are TNF $\alpha$ , IL-1 $\beta$  and IL-8 levels as measured by ELISA. Data is representative of at least three independent experiments, each performed in triplicate. Error bars represent the standard error of the mean. Challenge with media alone resulted in no detectable TNF $\alpha$  or IL-1 $\beta$ , and approximately 1 ng/ml IL-8 (SEM= 0.8 ng/ml). (A) MOI = 10; positive control samples (B) MOI = 0.1; low dose challenge samples. (C) Statistical analysis of positive control and low dose samples using a paired, two-tailed, t test with a 95% confidence interval indicates that low dose inoculations resulted in significantly lower levels of TNF $\alpha$  (10-fold) and IL-1 $\beta$  (20-fold), but not IL-8 (2-fold). Asterisks (\*) denote a statistically significant difference between doses: P < 0.05. (D) Statistical analysis of positive control and low dose challenges using *E. coli* or *S. typhimurium* in comparison to *N. gonorrhoeae*. Results of a paired, two-tailed, t test with a 95% confidence interval demonstrate that low dose inoculations resulted in significantly lower levels of TNF $\alpha$  for *E. coli* (as for *N. gonorrhoeae*) but not *S. typhimurium*. Asterisks (\*) denote a statistically significant difference between doses: P < 0.05.

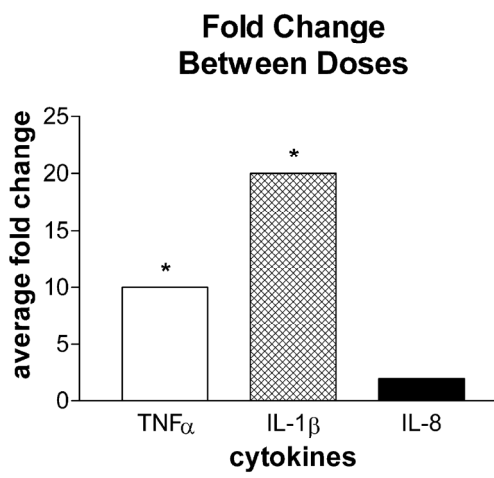
A.



B.

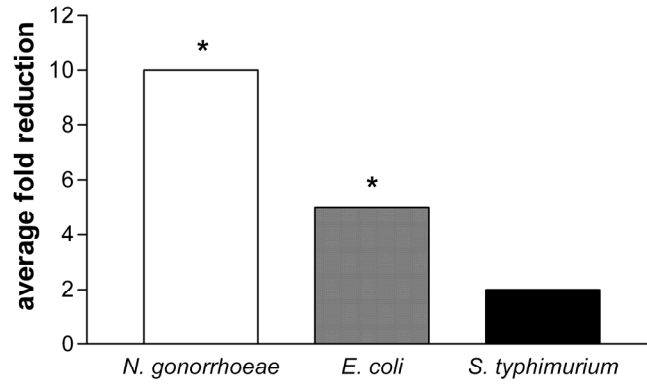


C.



D.

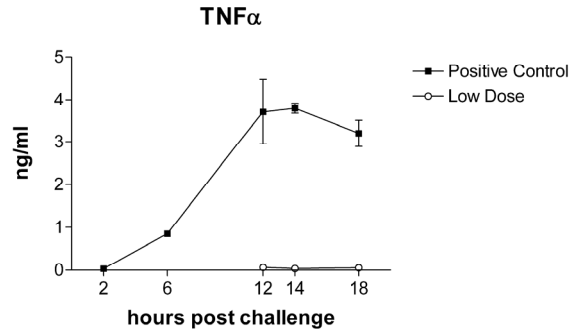
### Fold Reduction in $\text{TNF}\alpha$ Between Doses



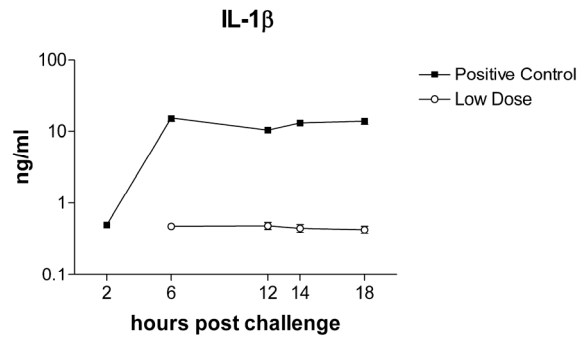
differences in IL-8 production were observed between doses for either species (data not shown). These data may indicate that the cytokine differential I observed for *N. gonorrhoeae* is species-specific. Experiments using live gonococci, where gentamicin (100 µg/ml) was added at 30 min post challenge, resulted in a slight increase in production of TNF $\alpha$  when compared with challenge by killed gonococci (data not shown). I attribute this difference to growth of the bacteria, as complete killing of the gonococci under these conditions did not occur in less than two hrs. These experiments revealed no significant difference in the production of IL-8 (data not shown). Since the concentration of IL-8 in these samples was quite high at both doses, it is logical that bacterial growth during gentamicin treatment did not result in significantly different IL-8 levels during live bacterial challenge. In addition, when monocytes were challenged with sonicated bacteria (MOI = 10 and MOI = 0.1), no significant differences in TNF $\alpha$  or IL-8 production were observed when compared with killed bacterial challenge samples (data not shown). I attribute this observation to the fact that gonococci routinely shed membrane blebs (containing DNA) and may not elicit alternate signaling patterns when lysed (167).

In order to determine if the low inoculum was simply resulting in slower kinetics of cytokine production, I measured cytokine levels produced over time (Figure 21). In positive control samples, all three cytokines tested were detectable at 2 hours post challenge. TNF $\alpha$  and IL-1 $\beta$  both increased over time until reaching a maximal level at about 6-12 hours post challenge. IL-8 expression continued to increase throughout the entire time course. During the low dose challenge (MOI = 0.1) TNF $\alpha$  and IL-1 $\beta$  were detected much later than IL-8 (12 and 6 hours

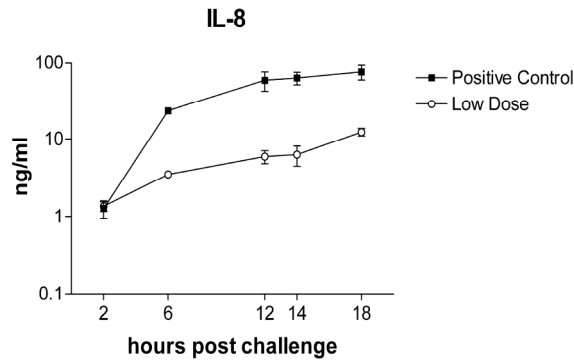
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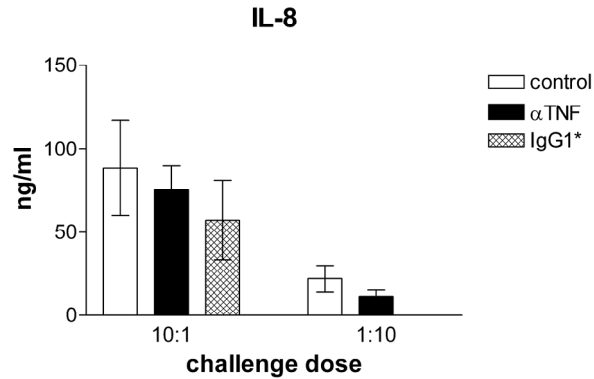
**Figure 21: Cytokine production by primary human monocytes over time.** Monocytes ( $10^6$  per sample) were challenged with killed *N. gonorrhoeae*, strain F62 $\Delta$ lgtD. In the positive control challenge time course an MOI of 10 was used. All three cytokines tested were measurable during the entire time course. In the low dose time course an MOI of 0.1 was used. These experiments were performed twice in triplicate. Error bars represent the standard deviation of the mean.

respectively) and when measurable, peaked at very low levels. TNF $\alpha$  and IL-1 $\beta$  measured after a longer time period (24 hrs) never approached levels seen in the positive control (data not shown), demonstrating that the low inoculum challenge did not affect cytokine kinetics. As in the positive control, IL-8 continued to increase during the time course at levels far above the other cytokines measured. In addition, since the low dose challenge results in the production of IL-8 in the absence of measurable TNF $\alpha$ , it suggests that IL-8 is not produced as a result of primary production of TNF $\alpha$ .

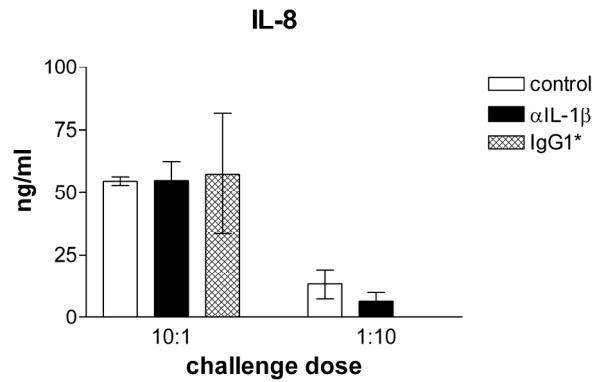
*IL-8 production is independent of TNF $\alpha$  and IL-1 $\beta$ .* Since IL-8 production is often attributed to prior production of TNF $\alpha$  and IL-1 $\beta$  during bacterial infections, I tested whether small amounts of these cytokines in culture supernatants were driving production of IL-8 during low dose gonococcal infections. Primary cultures of human monocytes were pretreated with neutralizing antibodies against TNF $\alpha$  and IL-1 $\beta$  (the antibody concentration was experimentally shown to neutralize all of the TNF $\alpha$  or IL-1 $\beta$  in culture supernatants), and challenged with either an MOI of 10 (positive control) or a low MOI of 0.1. Resulting IL-8 production was measured by ELISA (Figure 22). Pretreatment of monocytes with the two antibodies had no effect on the amount of IL-8 expressed at each dose. These data indicate that IL-8 production by monocytes challenged with *N. gonorrhoeae* is likely a result of direct stimulation by a bacterial product and not due to autocrine stimulation by TNF $\alpha$  or IL-1 $\beta$ .



A.



B.

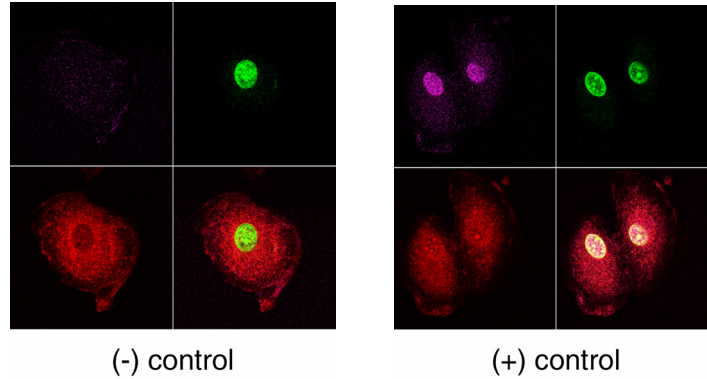


**Figure 22: Cytokine production by challenged primary human monocytes after pretreatment with neutralizing antibodies.**

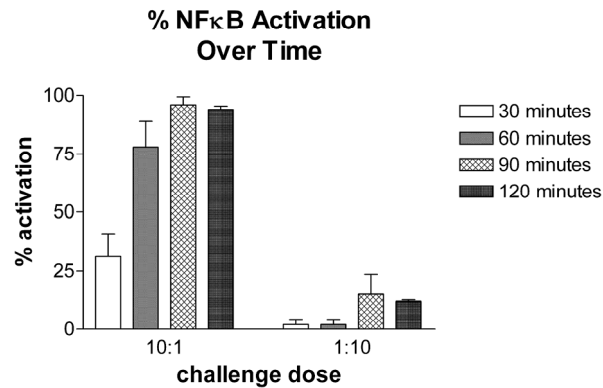
Monocytes ( $10^6$  per sample) were pretreated with neutralizing antibodies for thirty mins prior to challenge with killed *N. gonorrhoeae*, strain F62 $\Delta$ lgtD. Monocytes were challenged with an MOI equal to either 10 or 0.1. (A) IL-8 measurement at 18 hrs post challenge (in the presence of antibody able to neutralize all available TNF $\alpha$ ). (B) IL-8 measurement at 18 hrs post challenge (in the presence of antibody able to neutralize all available IL-1 $\beta$ ). Data represent the average of at least two independent experiments, each performed in triplicate. Error bars represent the standard deviation of the mean. Asterisk (\*) denotes 3  $\mu$ g/ml isotype control (mouse IgG1).

*NF-κB Activation is Proportional to the Bacterial Challenge Dose.* The TNFα, IL-1β, and IL-8 genes can all be transcriptionally regulated via NF-κB binding to specific DNA sequences within their promoters (168-170). However the importance of NF-κB in IL-8 expression is controversial (170-174). I hypothesized that NF-κB may not be activated during the low dose challenge. In order to test this hypothesis, primary human monocytes were differentiated into macrophages and challenged with different doses of gonococci (MOI = 10 and MOI = 0.1). Immunofluorescence staining and laser scanning confocal microscopy were employed to visualize the degree of NF-κBp65 translocation into macrophage nuclei. Random fields at each time point and dose were examined. At least fifty cells from each sample were analyzed and categorized as positive or negative for activated NF-κB staining in their nuclei (Figure 23A). The positive control challenge (MOI = 10) resulted in NF-κB activation in nearly 100% of the macrophages by 90 min. post challenge. In the low dose challenge (MOI = 0.1) at 90 min., NF-κB was translocated to the nucleus in approximately 12% of the macrophage population. At 120 min. post infection this percentage did not change significantly (Figure 23B). The percentage of activated cells in the low dose is reflective of the 10% of the macrophage population expected to come into direct contact with bacteria. NF-κB activation therefore, appears to result from direct contact between macrophages and gonococci. These data demonstrate that NF-κB is translocated into macrophage nuclei in low dose challenges and that the activated cell population is limited by the initial dose of infection. Based on these findings, it is likely that NF-κB is driving at least a portion of the IL-8 response. These data as well as the previous cytokine measurements imply

A.



B.



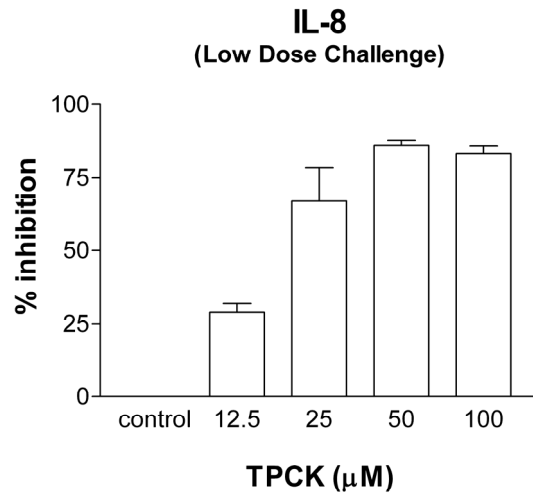
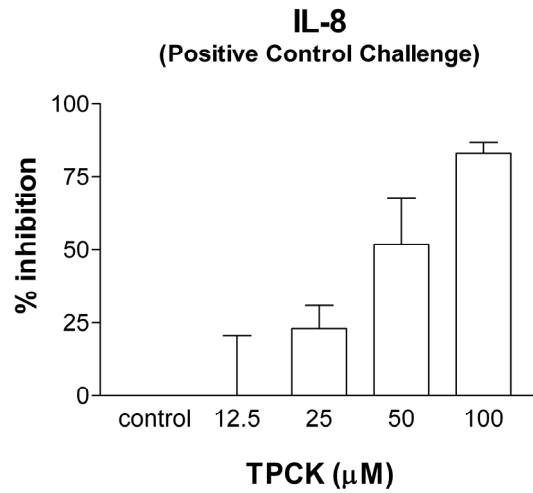
**Figure 23: Analysis of challenged primary human macrophages by confocal microscopy.**

Differentiated macrophages were challenged with killed *N. gonorrhoeae*, strain F62ΔlgtD, over time. Macrophages were challenged with an MOI of either 10 or 0.1, stained, and analyzed via confocal microscopy. Staining included phalloidin (red), SYTO-13 nucleic acid stain, and anti-NF-κBp65 (purple). Red shows cellular actin (lower left), green shows nuclear material (upper right), and purple shows activated NF-κBp65 (upper left). An overlay of the three colors is shown in the lower right panel. At least fifty cells per time point were analyzed and placed into one of two categories: those with NF-κBp65 in the nucleus and those without. (A) Confocal images showing unstimulated (- control) and activated (+ control) macrophage controls. (B) The percentage of cells with NF-κBp65 in the nucleus at 30, 60, 90, and 120 mins post challenge for each MOI. Data shown represent the average of at least two independent experiments. Error bars indicate the standard deviation of the mean.

that the low dose challenge results in a marked increase in IL-8 production on a per cell basis.

*Effects of the NF- $\kappa$ B Inhibitor TPCK on IL-8 Production.* In order to directly test the effects of NF- $\kappa$ B translocation on production of IL-8, I pretreated monocytes with TPCK, a chemical that blocks the activation of NF- $\kappa$ B by blocking phosphorylation and therefore subsequent degradation of I $\kappa$ B. Primary human monocytes were pretreated with TPCK over a dose range for 45 min. prior to challenge with gonococci. A positive control challenge (MOI = 10) was compared with the low dose challenge (MOI = 0.1) and supernatants were collected for cytokine ELISA analysis (Figure 24). This concentration of inhibitor was able to inhibit ~ 80 % of the IL-8 produced in both low dose and positive control samples. TNF $\alpha$  production was completely inhibited by TPCK pretreatment (data not shown), as has been demonstrated previously (175, 176). These experiments demonstrate that inhibition of NF- $\kappa$ B almost completely inhibits the IL-8 production resulting from gonococcal challenge. Therefore, NF- $\kappa$ B is critical for the production of IL-8 during a gonococcal infection.

*Phosphorylated JNK is Undetectable in Low Dose Infections.* AP-1 is a collective term which refers to a family of transcription factors. AP-1 exists as a dimer and is comprised of proteins from the Jun, Fos, Maf, and ATF sub-families. These molecules contribute to the regulation of many cytokine genes, including IL-8 and TNF $\alpha$  (177-179). Activation of this transcription factor family is regulated by the



**Figure 24: Inhibition of cytokine expression by TPCK.**

IL-8 measured after TPCK pretreatment of primary human monocytes challenged for 18 hrs with killed *N. gonorrhoeae*, strain F62 $\Delta$ lgtD. TPCK is a serine protease inhibitor that has been shown to block LPS- or cytokine-mediated activation of NF- $\kappa$ B. Positive control samples were challenged with an MOI of 10, while low dose samples were challenged with an MOI of 0.1. Data shown are from one experiment performed in triplicate and are representative of three independent experiments. Error bars represent the standard deviation of the mean.

MAP kinases [for review see (179, 180)]. Since *N. gonorrhoeae* has been shown to activate AP-1 in an epithelial cell tissue culture model through activation of JNK (110), I chose to examine the roles of this and other MAP kinases in our low dose monocyte model of infection. I hypothesized that differential MAP kinase regulation may be responsible for our observed dose-dependent cytokine profile. I challenged monocytes with both low dose and positive control doses of gonococci for various lengths of time, collected lysates and analyzed the level of phosphorylation of several kinases by SDS-PAGE/western blotting experiments, using antibodies specific for phosphorylated p38, JNK, and ERK (Figure 25A). [Western blotting for this experiment was performed by Samuel Bish.] These data provide evidence that, unlike p38 and ERK, JNK is not phosphorylated in low dose challenge samples.

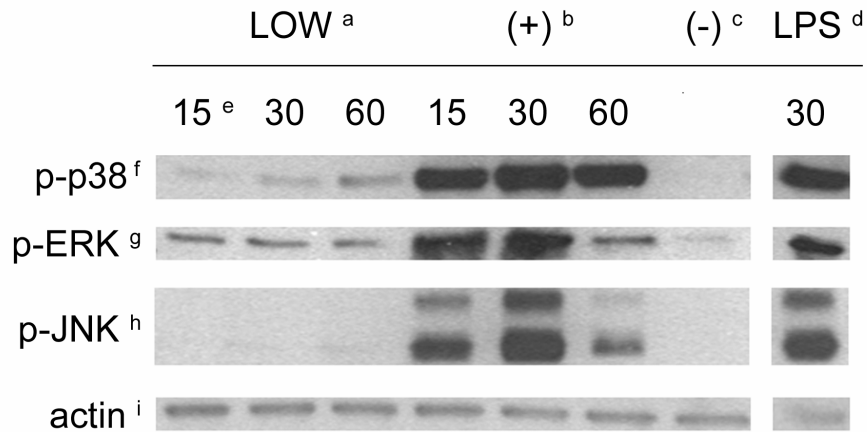
[Overexposure of the JNK blot did not result in significant signal when compared with negative controls (data not shown)]. This indicates a dose-dependent difference in the MAP kinase signaling pathway.

Three MAP kinase inhibitors were used to examine the roles of p38, ERK, and JNK in low dose challenge IL-8 production. The specificity of these inhibitors has been previously demonstrated (181-183). The data in Figure 25B show the percent inhibition of IL-8 after pretreatment with the inhibitors and subsequent bacterial challenge. The p38 inhibitor SB203580 inhibited most of the IL-8 production, while the ERK inhibitor PD98059 inhibited about 30%. The JNK inhibitor SP600125 had almost no effect on IL-8 production. These data further indicate that the activation of the JNK pathway (and thus AP-1 production/activation)

**Figure 25: Phosphorylation levels of MAP kinases from challenged primary human monocytes.**

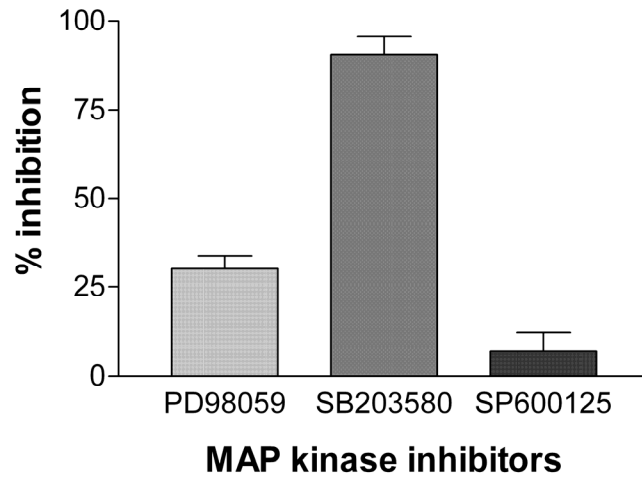
Monocytes were challenged for the indicated time period with killed *N. gonorrhoeae*, strain F62ΔlgtD. (A) Resting monocytes ( $2 \times 10^6$  per sample) were challenged with an MOI of 10 or 0.1 over time. Monocyte whole cell lysates were collected and analyzed by immunoblot to detect the phosphorylation levels of p38 (f) ERK 1/2 (g), and JNK 1/2/3 (h). (a) MOI = 0.1 (b) MOI = 10 (positive control) (c) untreated primary human monocytes (negative control) (d) LPS-treated monocytes (100 ng/ml) (e) mins, post-challenge (f) probing monocyte lysates with phospho-p38 polyclonal antibody (g and h) same as f, except phospho-ERK and phospho-JNK, respectively (i) F-actin levels (loading control). Data are representative of three independent experiments. (B) Percent inhibition of IL-8 in low dose challenge samples after pretreatment with three specific MAP kinase inhibitors (PD98059/ ERK, SB203580/ p38, and SP600125/ JNK) and subsequent 18 hr challenge. These inhibitors were tested over a dose range and data shown are the result of the pretreatment of monocytes with 5  $\mu$ M SB203580, 50  $\mu$ M SP600125, and 50  $\mu$ M PD98059, thirty mins prior to bacterial challenge. Levels of inhibitors used were experimentally determined to have reached maximal inhibition (data not shown).

A.



B.

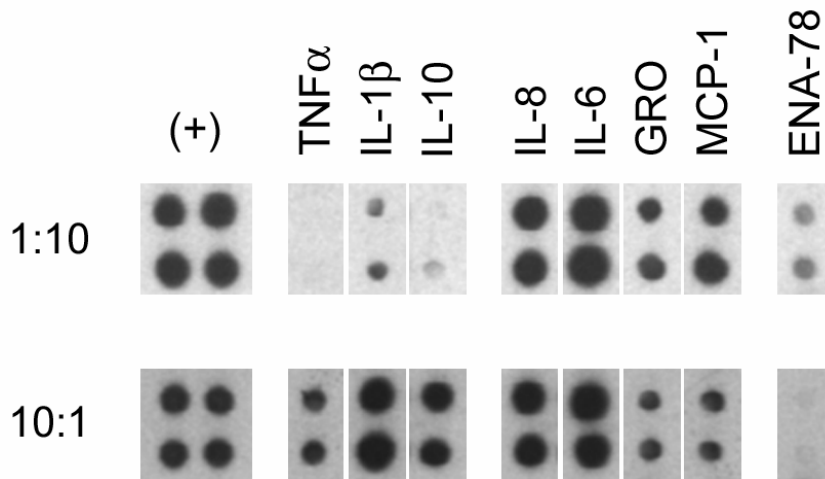
### IL-8 in Low Dose Challenge Samples





is not contributing significantly to IL-8 production resulting from the low dose challenge.

*Overall Cytokine Profile is Altered Depending upon the Initial Challenge Dose.* In order to gain a greater understanding of overall gonococcal-induced dose-dependent cytokine expression, I employed a cytokine expression array. Monocytes were challenged with a specific dose of gonococci, incubated for 18 hrs, and supernatants were collected for array analysis (Figure 26). Several cytokines demonstrated dose-dependent differences in expression (two-fold change or greater, see Table 1). Included in this group were TNF $\alpha$ , IL-1 $\beta$ , and IL-10. This correlates with previous ELISA data where TNF $\alpha$ , IL-1 $\beta$ , and IL-10 were all upregulated as a result of the higher challenge dose. Similar to IL-8, the cytokines IL-6, GRO, and MCP-1 were also substantially upregulated compared to negative control samples, yet did not differ significantly between doses. Interestingly, the chemokine epithelial neutrophil-activating protein-1 (ENA-78) was measured to have a negative fold change. In other words, the low dose challenge resulted in greater production of this chemokine than did the high dose challenge. (No ENA-78 was measurable in unchallenged samples.) This finding demonstrates that a chemokine can be elicited specifically upon challenge with a low bacterial inoculum and not upon challenge with a higher inoculum. This phenomenon has never been reported, presumably since most *in vitro* experiments typically utilize higher challenge doses than those employed here. ENA-78 is similar to IL-8 in structure and function, and may have significant disease implications (184). (I chose not to include data regarding the detection of ENA-78 for



**Figure 26: Comparison of cytokine levels from both low dose and positive control inoculations during one experiment.**

Primary human monocytes were challenged for 18hrs with killed *N. gonorrhoeae*, strain F62 $\Delta$ lgtD. Monocytes were challenged with an MOI of either 10 or 0.1. Densitometric analysis was carried out using a Gel Doc 2000 camera and Quantity One Software (BioRad Laboratories, Inc.). Internal positive controls were compared among array exposures and the means were found to be statistically similar ( $P=0.7548$  according to a paired, two-tailed t test) prior to cross comparison of sample cytokine levels. A fold change greater than or equal to two-fold was determined to be significant.

Table 1:

	<b>Average fold change between doses</b>
<b>TNF<math>\alpha</math></b>	<b>+ 5.3</b>
<b>IL-1<math>\beta</math></b>	<b>+ 8.6</b>
<b>IL-10</b>	<b>+ 17.5</b>
<b>IL-8</b>	+ 1.8
<b>IL-6</b>	+ 1.0
<b>GRO</b>	+ 1.0
<b>MCP-1</b>	+ 1.8
<b>ENA-78</b>	<b>- 2.9</b>

**Table 1: Average fold change between doses.**

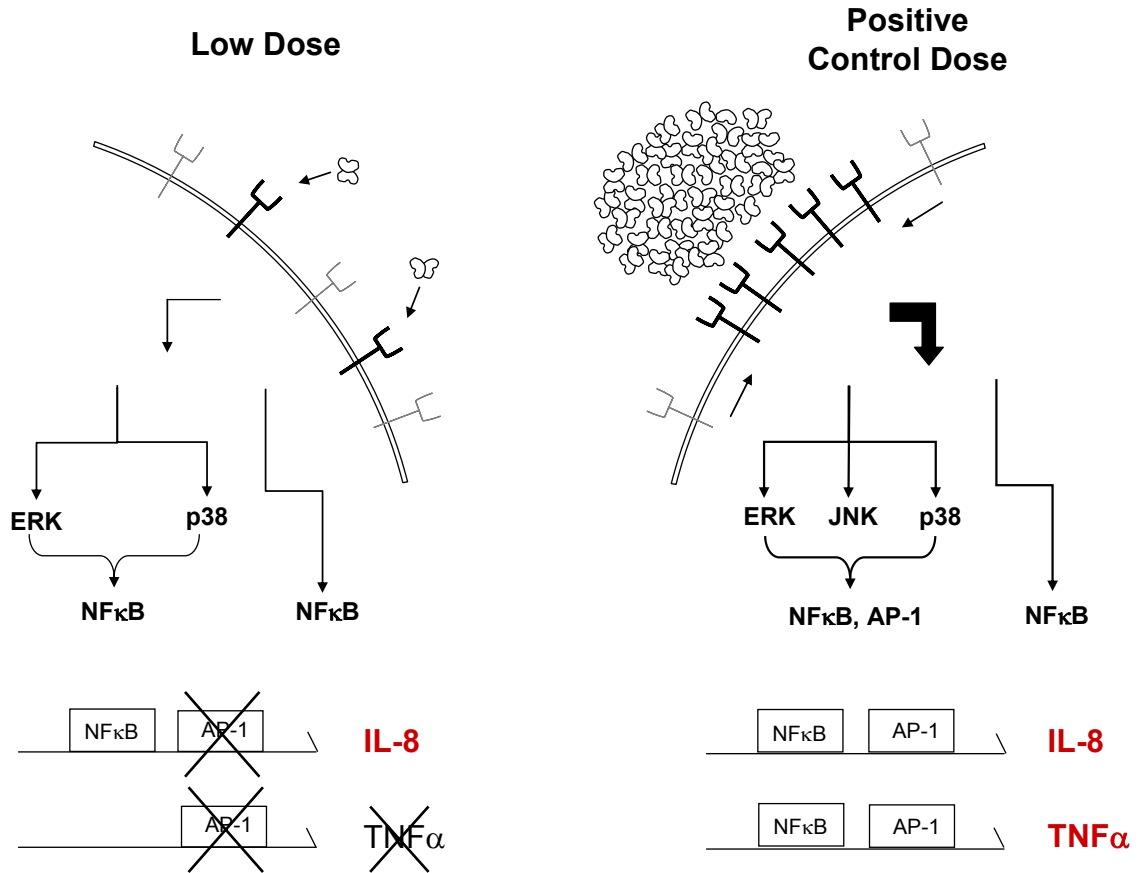
Table values represent an average of two independent array experiments. Bold numbers indicate significant values (greater than or equal to a two-fold change).

publication as further study is necessary to determine the significance of this chemokine during gonococcal challenge.) Taken together, these data indicate that the lower bacterial challenge elicits a dramatic immune response and that significant differences in the overall cytokine expression profile can result from two different inoculating doses of bacteria.

## Discussion

Bacterial infections typically result in the production of specific pro-inflammatory cytokines and chemokines, leading to clearance of the invading microbes. In this study, I have shown that when confronted with a low challenge dose of *N. gonorrhoeae*, human monocytes are extremely efficient producers of IL-8 as well as several other chemokines/cytokines. In this scenario, host cells do not produce significant levels of TNF $\alpha$ . This overall cytokine profile could result in an influx of neutrophils and monocytes to the site of colonization, without causing the pain associated with symptomatic infection. I therefore propose that a low percentage of invasive bacteria can elicit a dramatic innate immune response without causing substantial cytokine-mediated tissue damage .

Cytokine response to *N. gonorrhoeae* has been investigated by several groups, but never in the context of a low dose infection (MOI = 0.1). The monocytic inflammatory response to a low dose gonococcal challenge has not been documented, though this dose is almost certainly physiologically relevant. Subsequent to this low dose challenge, I measured levels of IL-8 that were statistically similar to those of high dose challenge samples, yet were produced in the absence of significant amounts of TNF $\alpha$ . In addition, this work supports the conclusion that IL-8 production does not result from autocrine action by IL-1 $\beta$ . Makepeace, *et al* inoculated primary monocyte-derived human macrophages with *N. gonorrhoeae* at an MOI of 400 and measured the resultant cytokine response. In contrast to my findings, no increase in



**Figure 27: Schematic diagram summarizing the proposed model for the signaling differential induced by a low gonococcal MOI.**

Upon challenge with a low MOI vs. the positive control MOI, phosphorylated JNK was not detectable in low dose challenge samples. In addition, TNF $\alpha$  production was only observed upon challenge with the positive control MOI, where IL-8 was measurable upon challenge with both doses. I propose a mechanism by which AP-1 dimers are not available to drive production of TNF $\alpha$  in low dose challenge samples, while NF- $\kappa$ B is sufficient to drive IL-8 production subsequent to both challenge doses.

IL-8 was observed when compared with controls, though negative control samples produced 10 ng/ml IL-8. Additionally, no IL-1 $\beta$  was detected from challenged samples within these experiments (136). Though these findings seem to be at odds with my results, the challenge dose that they employed greatly exceeded my high dose challenge. Therefore these results further support my observation of dose-dependent alterations in cytokine gene expression. Analysis of two other bacterial species (*E. coli* and *S. typhimurium*) indicates that the dose differential we observed for *N. gonorrhoeae* may be species-specific, indicating an interesting avenue for future study.

Through the use of neutralizing antibodies against TNF $\alpha$  and IL-1 $\beta$  (two of the strongest activators of IL-8) (172), I verified that the IL-8 measured was not being produced in response to either of these cytokines. I hypothesize that IL-8 production results from a direct interaction between a bacterial structure or product, with host monocytes. Lorenzen *et al.* have shown that Neisserial immunoglobulin (Ig)A1-specific serine protease plays a role in eliciting IL-8 from peripheral blood mononuclear cells (185). In addition, recombinant Neisserial PorA has been shown to elicit several pro-inflammatory cytokines including IL-8. The Neisserial surface lipoprotein Lip has also been shown to elicit IL-8 and IL-6 in an immortalized epithelial model (111). Though LPS/ LOS activation is known to cause IL-8 production from human monocytes(186), Pridmore, *et al.* demonstrated that both wild type and lpxA mutant (LOS deficient) meningococci were similarly able to induce IL-8 promoter activity (131). Similarly, pili, LOS and gonococcal outer membrane vesicles were shown to have no effect on IL-8 production in one study using a human

macrophage model (136). A conflicting study showed that piliated gonococci were associated with increased production of IL-8 in an *ex vivo* human endometrial cell model (187). Recently, peptidoglycan has been shown to interact with mannose binding lectin, ultimately resulting in an increase in chemokine production and a decrease in TNF $\alpha$  production by macrophages (188). Specific gonococcal LOS molecules have also been shown to avidly bind mannose binding lectin *in vitro* (189). It is possible that one or a combination of several gonococcal surface components may be responsible for the cytokine differential we have observed in low dose challenge samples.

It is known that IL-8 gene expression is affected by the binding of NF- $\kappa$ B, AP-1, and C/EBP (NF-IL-6) to regions of its promoter [for review see (174)]. The role that each of these transcription factors plays in the overall expression of IL-8 remains unclear. TNF $\alpha$  gene expression is even more complex and varies among different cell types. In human monocytes, transcription factor binding sites for NF- $\kappa$ B, AP-1 (Jun-ATF-2), Sp1, Egr-1, NFAT, and Ets/Elk have been identified in the TNF $\alpha$  promoter (178, 190-192). Interestingly, distinct stimuli (viral antigen, a calcium ionophore, *Mycobacterium tuberculosis*, and LPS) have been shown to result in the binding of different sets of transcriptional activators to shared binding sites in the TNF $\alpha$  promoter, in a cell-type specific manner (191-193). Specific combinations of these regulatory elements are required for recruitment of the coactivator proteins CBP/p300 and subsequent enhanceosome formation (190-192). Though NF- $\kappa$ B binding motifs have also been identified in the upstream regions flanking the TNF $\alpha$  gene (168), the role of these sites in TNF $\alpha$  transcription is controversial. Deletion of



these sequences produces little effect on TNF $\alpha$  induction by several distinct stimuli (191, 192). However, simultaneous mutations in each of the NF- $\kappa$ B sites of the mouse TNF promoter resulted in a 90% reduction in promoter response to LPS (194). These differences may be stimulus-specific or reflective of differences among cellular models.

While NF- $\kappa$ B was historically thought to be essential for IL-8 expression in many cell types (170) NF- $\kappa$ B-independent IL-8 expression has been reported (171-174). The confocal microscopy data shown here (Figure 23) demonstrate that NF- $\kappa$ Bp65 is translocated into the nucleus in low inoculum samples, though activation is limited by the initial dose. Though differentiated cells certainly have some altered sensitivity in comparison with monocytes, the NF- $\kappa$ B data generated from use of this model was reflective of the percentage of macrophages expected to come into direct contact with bacteria. These percentages correlate well with the cytokine differential observed in Figure 20 where monocyte cytokine production was measured. These data support the hypothesis that NF- $\kappa$ B activation and IL-8 production in the low dose are the result of direct contact between bacteria and macrophages. Inhibition of NF- $\kappa$ B translocation through use of the serine protease inhibitor TPCK significantly inhibited IL-8 expression. This indicates the importance of NF- $\kappa$ B in our model of gonococcal disease.

AP-1 has clearly been shown to contribute to both TNF $\alpha$  and IL-8 expression. Since AP-1 abundance, binding, and activity are impacted by the activity of various MAP kinases, I examined the roles of ERK, JNK, and p38 in our low dose challenge model. The data shown here demonstrate that under these experimental conditions,

the JNK pathway is not activated. Therefore, available c-Jun cannot be phosphorylated, which leads to a failure in further *c-jun* induction [for review see (180)]. Since c-Jun is a central component of all AP-1 complexes (179), the data suggest that in my model, AP-1 dimers are not available to bind to the DNA. The protein ATF2 is also phosphorylated in part by JNK (195). Thus in my model, fewer phosphorylated ATF2 proteins are available. Since AP-1 dimers (Jun-ATF-2) are important for enhanceosome formation and efficient transcription of the TNF $\alpha$  gene, it is not surprising that in the absence of phosphorylated c-jun, and with little available phosphorylated ATF-2, TNF $\alpha$  production is limited. I hypothesize that the IL-8 produced as result of the low dose gonococcal infection is mediated by NF- $\kappa$ B binding, independent of AP-1. In support of this, the AP-1 binding site has been shown to be dispensable for IL-8 production in some studies (196, 197). Similarly, differential transcription of cytokine genes has been demonstrated in the IL-1 $\beta$  promoter, where fibronectin was shown to induce AP-1 binding, but not NF- $\kappa$ B (198). Additionally, different TLR agonists have been shown to elicit distinct cytokine responses (i.e. induction of TNF $\alpha$  but not IL-1 $\beta$ ) though both AP-1 and NF- $\kappa$ B were activated (199). I believe that in response to a low dose challenge, the TNF $\alpha$  promoter is not efficiently transcribed. Though the NF- $\kappa$ B inhibitor TPCK resulted in complete inhibition of TNF $\alpha$  production, I feel that this is due to TPCK-induced inhibition of TNF $\alpha$  mRNA expression. This effect by TPCK has been previously documented in a model of LPS-stimulated macrophages (175, 176).

The results of my cytokine array experiment provide further insight into the global cytokine response of human monocytes in response to gonococcal infection.

Several cytokines and chemokines were upregulated (in both low and high dose challenge samples) when compared to untreated controls, and the dose differential was apparent for TNF $\alpha$ , IL-1 $\beta$ , and IL-10 (Fig 7). Both the chemokines GRO and MCP-1 were shown to be produced in large quantities when compared to negative controls. Interestingly, their production, just as that of IL-8, was not significantly altered in the low dose challenge. Since both GRO and MCP-1 are also transcriptionally regulated in part by NF- $\kappa$ B it is possible that these genes are still efficiently transcribed, as is IL-8, without the presence of AP-1 (200-202). Since chemokine regulation is critical for appropriate regulation of leukocyte infiltration, it is also essential for regulation of the intensity of the inflammatory response. In the context of the low dose, one could imagine an asymptomatic gonococcal infection where a significant chemokine response is generated in the absence of substantial TNF $\alpha$  production. This would result in continued bacterial transmission and gradual microbial clearance without the host experiencing noticeable symptoms. These experiments also demonstrated greater elicitation of the chemokine ENA-78 subsequent to low dose challenges when compared to positive controls. As this chemokine causes neutrophil influx and activation, similar to IL-8, it may have a significant impact during gonococcal infection (184).

I further propose that gonococci in different doses can appear as distinct stimuli to host monocytes. When gonococci exist in sufficient numbers, multicellular aggregates form. These aggregates may physically alter the scope of the interaction between gonococcal PAMPs such as Por, Lip, and LOS, and their corresponding Pattern Recognition Receptors. In turn, distinct recognition and/or phagocytosis by

host cells may occur, in comparison with that of individual diplococci. In the future, it would be interesting to determine which gonococcal surface molecules contribute to the observed signaling differential.

Taken together the results of this study indicate that *N. gonorrhoeae* is remarkably efficient at inducing the production of several cytokines/chemokines from host monocytes. This response can occur in the absence of damaging cytokines (TNF $\alpha$ ) and appears to result from direct gonococcal/monocyte surface contact in an NF- $\kappa$ B-dependent manner. This observation may help to explain gonococcal disease in women, which is so often asymptomatic. While these studies are helpful in understanding gonococcal pathogenesis, I also feel that the implications are broadly applicable to our understanding of the development of immune responses to any infectious disease.

## Chapter 4: Conclusions

*Neisseria gonorrhoeae* is an obligate pathogen which has an important global impact on human health. Significant disease sequelae can result from infection by *N. gonorrhoeae*, including PID in women, which often results in sterility. A correlation between gonococcal infection and increased transmission of HIV also contributes to the need for greater understanding of gonococcal pathogenesis. This organism achieves colonization and invasion through a multi-step process, presumably involving many types of host cells. Inflammation and neutrophil influx are two important diagnostic symptoms of gonorrhea, and there is very little antibody response generated against this bacterium. Diagnosis of gonorrhea is complicated by many factors including the large percentage of asymptomatic infections in women. The experiments described herein relate to two important gonococcal surface molecules, LOS and H.8, and highlight the importance of bacterial dose upon disease outcome.

In order to analyze the impact of antigenic variation in gonococcal LOS, I utilized gonococcal mutants with specific LOS structures to challenge human monocytes. These mutants expressed LOS structures associated with either symptomatic (F62/ F62ΔlgtD) or asymptomatic (F62ΔlgtA) infection. These specific LOS structures have been previously shown to affect gonococcal interaction with epithelial cell models (61). Upon challenge of monocytes during this study, the levels of several proinflammatory cytokines were measured. The data generated demonstrate that alterations in the carbohydrate moiety of LOS do not directly impact

the production of these cytokines. From my data, as well as several other reports, it seems likely that differences in the immunostimulatory potential of LOS variants during interactions with human monocytes may stem from alterations in the structure of lipid A (113, 133-135). In addition, whole-cell bacterial challenge was shown to be much more stimulatory than challenge with purified LOS. This implies that other surface structures may play an important role with regard to cytokine elicitation. Sialylation of LOS was shown to result in upregulation of the chemokine MCP-2 from challenged cells. As MCP-2 is associated with recruitment of monocytes, this finding may indicate a role for sialylation during further interactions with this cell type. A greater understanding of the mechanism behind MCP-2 elicitation by gonococci, as well as quantitation of this cytokine during challenge, will be important questions to explore in the future.

Through use of an H.8<sup>-</sup> strain generated in our laboratory, I was able to directly compare the effects of this antigen on gonococcal interactions with human monocytes. Though I observed only insignificant differences in cytokine elicitation by H.8<sup>-</sup> mutants, this molecule appeared important during interactions with host macrophages. A greater percentage of mutant gonococci were shown to form large aggregates upon contact with host macrophages over time, in comparison with the parent strain. These bacterial aggregates also appeared to resist phagocytosis. As recent studies have implicated the ability of gonococci to survive within host immune cells, I hypothesize that H.8 antigen may serve as an important PAMP, thereby promoting phagocytosis (160-162).

After exploration of the effects of dose on gonococcal challenge, I have demonstrated a differential response by human monocytes. A low challenge dose of *N. gonorrhoeae* was demonstrated able to efficiently elicit a significant amount of IL-8, as well as several other chemokines/cytokine. During this scenario, monocytes did not produce significant levels of TNF $\alpha$ , the cytokine associated with gonococci-induced tissue damage. In addition, levels of the chemokine ENA-78 were measured to be approximately three-fold greater in the low dose challenge when compared with positive controls. I hypothesize that this overall cytokine profile could result in an influx of neutrophils and monocytes to the site of colonization, without causing the pain associated with symptomatic infection. I therefore propose that a low percentage of invasive bacteria can elicit a dramatic innate immune response without causing substantial cytokine-mediated tissue damage.

IL-8 induction was demonstrated to be NF- $\kappa$ B-dependent, though this activation was limited by the inoculating dose, implying that direct contact between bacteria and host cells is necessary for induction of this chemokine. Analysis of various MAP kinases indicated that low MOI challenges were able to efficiently activate both the ERK and p38 pathways but failed to activate the JNK pathway. A lack of phosphorylated JNK leads to decreased production of AP-1 dimers, transcription factors that are critical for efficient transcription of TNF $\alpha$ . I therefore hypothesize that a low MOI gonococcal challenge dose results in diminished AP-1 activity and TNF $\alpha$  production while IL-8 levels remain constant. I further propose that gonococci in different doses can appear as distinct stimuli to host monocytes. Multicellular aggregates, which can form when gonococci are concentrated, might

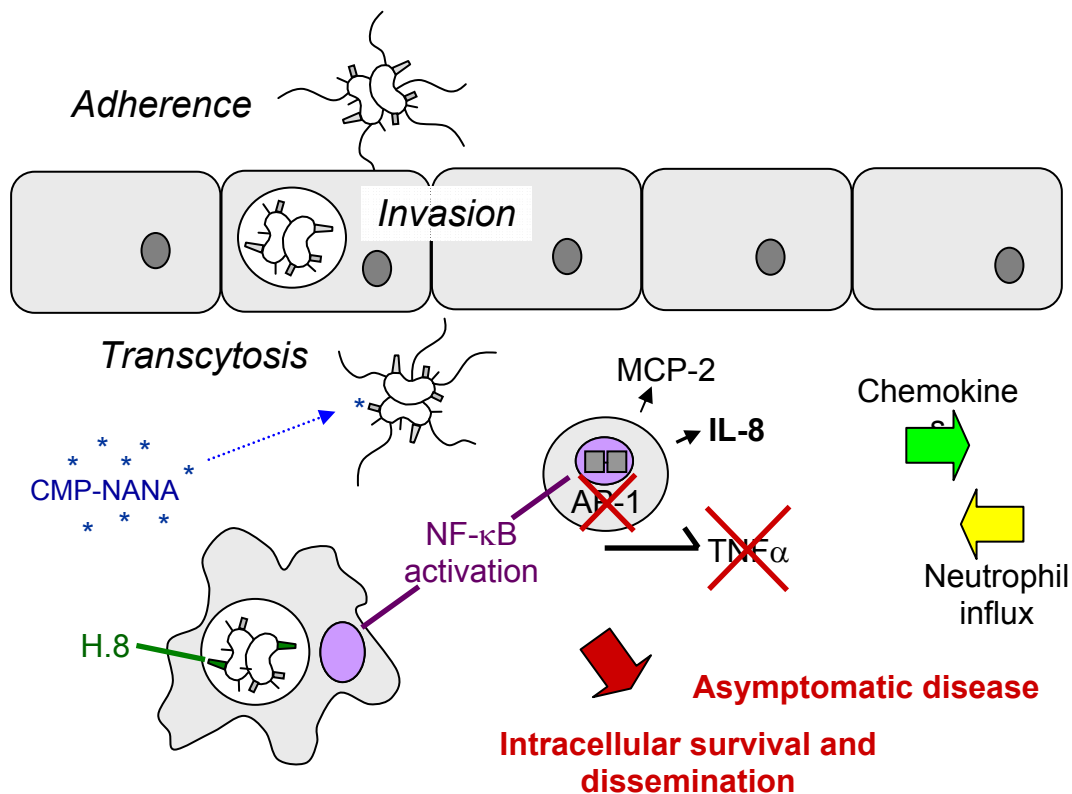
physically alter the interaction between gonococcal PAMPS and their corresponding PRRs. In this way, distinct recognition and/or phagocytosis by host cells may occur, in comparison with that of individual diplococci.

The data generated from these studies contribute to our current understanding of gonococcal pathogenesis and may have future importance in the development of successful gonococcal vaccines. My results highlight the importance of monocyte/macrophage interactions with *N. gonorrhoeae*. In addition, the implication that bacterial dose can significantly alter disease outcome is broadly applicable to our understanding of the development of immune responses not only to infection by *N. gonorrhoeae*, but to all infectious microbes.

From the data presented in this dissertation and using data from the published literature, I would like to propose the following overall model of gonococcal infection (Figure 28). Since gonococci expressing invasive LOS structures (lacto-*N*-neotetraose) are readily sialylated, I hypothesize that after initial colonization of host epithelial cells, these sialylated gonococci encounter monocytes and resident macrophages and elicit MCP-2 production. Elicitation of this chemokine contributes to the specific recruitment of additional host monocytes. In addition, only a portion of the initial gonococcal inoculum is able to transcytose the epithelial tissues. This small number of bacteria interacts with immune cells, leading to phosphorylation of ERK and p38 and NF- $\kappa$ B translocation, yet no phosphorylation of c-Jun. Therefore, a lack of AP-1 dimers exists and cytokine signaling (i.e. TNF $\alpha$  production) is limited. Though chemokine production is induced from this low MOI, TNF $\alpha$  levels are not sufficient to cause pain and tissue damage. Recognition of gonococcal surface



structures (such as H.8) by infiltrating monocytes and neutrophils may allow efficient phagocytosis of these bacteria. Once distributed within immune cell vacuoles, the gonococci employ several virulence factors such as Ng-MIP, IgA protease, and porin to allow intracellular survival. This scenario could result in a persistent asymptomatic disease or disseminated infection.



**Figure 28: Proposed Model of Gonococcal Disease in Women.**

Schematic diagram illustrating several aspects of gonococcal disease in women. Shown are the three main stages of gonococcal infection (adherence, invasion, and transcytosis), a summary of the observed interactions between gonococci and human primary monocytes and macrophages (LOS sialylation with host CMP-NANA, NF- $\kappa$ B activation, and cytokine/chemokine production), and proposed disease implications (asymptomatic infection and intracellular survival and dissemination).

## Reference List

1. Knapp, J. S., and E. W. Hook, III. 1988. Prevalence and persistence of *Neisseria cinerea* and other *Neisseria spp.* in adults. *J. Clin. Microbiol.* 26:896-900.
2. Hart, C. A., and T. R. Rogers. 1993. Meningococcal disease. *J. Med. Microbiol.* 39:3-25.
3. Apicella, M. 1995. *Neisseria meningitidis*. In *Principles and practice of infectious diseases*. G. L. Mandell, J. E. Bennett, and R. Dolin, eds. Churchill Livingstone, New York, pp. 1896.
4. Fransen, L., H. Nsanze, V. Klauss, S. P. Van der, L. D'Costa, R. C. Brunham, and P. Piot. 1986. Ophthalmia neonatorum in Nairobi, Kenya: the roles of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. *J. Infect. Dis.* 153:862-869.
5. Fransen, L., and V. Klauss. 1988. Neonatal ophthalmia in the developing world. Epidemiology, etiology, management and control. *Int. Ophthalmol.* 11:189-196.
6. Koomey, M. 2001. Implications of molecular contacts and signaling initiated by *Neisseria gonorrhoeae*. *Curr. Opin. Microbiol.* 4:53-57.
7. Naumann, M., T. Rudel, and T. F. Meyer. 1999. Host cell interactions and signalling with *Neisseria gonorrhoeae*. *Curr. Opin. Microbiol.* 2:62-70.
8. Boulton, I. C., and S. D. Gray-Owen. 2002. Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nat. Immunol.* 3:229-236.
9. Ness, R. B. 1999. Anatomic Influences on Sexually Transmitted Bacterial Diseases. In *Health and Disease Among Women: Biological and Environmental Influences* Oxford Univerisy Press, New York, pp. 314.
10. Sweet, R. L. 1987. Sexually transmitted diseases. Pelvic inflammatory disease and infertility in women. *Infect. Dis. Clin. North Am.* 1:199-215.
11. Aral, S. O., W. D. Mosher, and W. Cates, Jr. 1991. Self-reported pelvic inflammatory disease in the United States, 1988. *JAMA* 266:2570-2573.
12. McGee, Z. A., R. L. Jensen, C. M. Clemens, D. Taylor-Robinson, A. P. Johnson, and C. R. Gregg. 1999. Gonococcal infection of human fallopian tube mucosa in organ culture: relationship of mucosal tissue TNF-alpha concentration to sloughing of ciliated cells. *Sex Transm. Dis.* 26:160-165.

13. Burnakis, T. G., and N. B. Hildebrandt. 1986. Pelvic inflammatory disease: a review with emphasis on antimicrobial therapy. *Rev. Infect. Dis.* 8:86-116.
14. Kerle, K. K., J. R. Mascola, and T. A. Miller. 1992. Disseminated gonococcal infection. *Am. Fam. Physician* 45:209-214.
15. Chen, A., I. C. Boulton, J. Pongoski, A. Cochrane, and S. D. Gray-Owen. 2003. Induction of HIV-1 long terminal repeat-mediated transcription by *Neisseria gonorrhoeae*. *AIDS* 17:625-628.
16. Mostad, S. B., J. Overbaugh, D. M. DeVange, M. J. Welch, B. Chohan, K. Mandaliya, P. Nyange, H. L. Martin, Jr., J. Ndinya-Achola, J. J. Bwayo, and J. K. Kreiss. 1997. Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. *Lancet* 350:922-927.
17. Anzala, A. O., J. N. Simonsen, J. Kimani, T. B. Ball, N. J. Nagelkerke, J. Rutherford, E. N. Ngugi, J. J. Bwayo, and F. A. Plummer. 2000. Acute sexually transmitted infections increase human immunodeficiency virus type 1 plasma viremia, increase plasma type 2 cytokines, and decrease CD4 cell counts. *J. Infect. Dis.* 182:459-466.
18. Kaul, R., S. L. Rowland-Jones, G. Gillespie, J. Kimani, T. Dong, P. Kiama, J. N. Simonsen, J. J. Bwayo, A. J. McMichael, and F. A. Plummer. 2002. Gonococcal cervicitis is associated with reduced systemic CD8+ T cell responses in human immunodeficiency virus type 1-infected and exposed, uninfected sex workers. *J. Infect. Dis.* 185:1525-1529.
19. Wira, C. R., K. S. Grant-Tschudy, and M. A. Crane-Godreau. 2005. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *Am. J. Reprod. Immunol.* 53:65-76.
20. Carroll, M. C. 1998. The role of complement and complement receptors in induction and regulation of immunity. *Annu. Rev. Immunol.* 16:545-568.
21. Kupper, T. S., and R. C. Fuhlbrigge. 2004. Immune surveillance in the skin: mechanisms and clinical consequences. *Nat. Rev. Immunol.* 4:211-222.
22. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. *N. Engl. J. Med.* 343:338-344.
23. Abbas, A. K., A. H. Lichtman, and J. S. Pober. 2000. *Cellular and Molecular Immunology*, Vol. 1. W.B. Saunders Company, Philadelphia.
24. Beutler, B. 1995. TNF, immunity and inflammatory disease: lessons of the past decade. *J. Investig. Med.* 43:227-235.
25. Dinarello, C. A. 1997. Interleukin-1. *Cytokine Growth Factor Rev.* 8:253-265.

26. Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu. Rev. Immunol.* 16:495-521.
27. Dinarello, C. A. 2000. Proinflammatory cytokines. *Chest* 118:503-508.
28. Baggiolini, M., B. Dewald, and B. Moser. 1997. Human chemokines: an update. *Annu. Rev. Immunol.* 15:675-705.
29. Ben-Baruch, A., D. F. Michiel, and J. J. Oppenheim. 1995. Signals and receptors involved in recruitment of inflammatory cells. *J. Biol. Chem.* 270:11703-11706.
30. Godaly, G., A. E. Proudfoot, R. E. Offord, C. Svanborg, and W. W. Agace. 1997. Role of epithelial interleukin-8 (IL-8) and neutrophil IL-8 receptor A in *Escherichia coli*-induced transuroepithelial neutrophil migration. *Infect. Immun.* 65:3451-3456.
31. Agace, W. W., S. R. Hedges, M. Ceska, and C. Svanborg. 1993. Interleukin-8 and the neutrophil response to mucosal gram-negative infection. *J. Clin. Invest* 92:780-785.
32. Baggiolini, M., and I. Clark-Lewis. 1992. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* 307:97-101.
33. Ramsey, K. H., H. Schneider, A. S. Cross, J. W. Boslego, D. L. Hoover, T. L. Staley, R. A. Kuschner, and C. D. Deal. 1995. Inflammatory cytokines produced in response to experimental human gonorrhea. *J. Infect. Dis.* 172:186-191.
34. Fichorova, R. N., P. J. Desai, F. C. Gibson, III, and C. A. Genco. 2001. Distinct proinflammatory host responses to *Neisseria gonorrhoeae* infection in immortalized human cervical and vaginal epithelial cells. *Infect. Immun.* 69:5840-5848.
35. Naumann, M., S. Wessler, C. Bartsch, B. Wieland, and T. F. Meyer. 1997. *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors nuclear factor kappaB and activator protein 1 and the induction of inflammatory cytokines. *J. Exp. Med.* 186:247-258.
36. Svanborg, C., G. Godaly, and M. Hedlund. 1999. Cytokine responses during mucosal infections: role in disease pathogenesis and host defence. *Curr. Opin. Microbiol.* 2:99-105.
37. Tato, C. M., and C. A. Hunter. 2002. Host-pathogen interactions: subversion and utilization of the NF-kappa B pathway during infection. *Infect. Immun.* 70:3311-3317.

38. Gomez, M. I., A. Lee, B. Reddy, A. Muir, G. Soong, A. Pitt, A. Cheung, and A. Prince. 2004. Staphylococcus aureus protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat. Med.* 10:842-848.
39. Sing, A., D. Rost, N. Tvardovskaia, A. Roggenkamp, A. Wiedemann, C. J. Kirschning, M. Aepfelbacher, and J. Heesemann. 2002. Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196:1017-1024.
40. Nau, G. J., J. F. Richmond, A. Schlesinger, E. G. Jennings, E. S. Lander, and R. A. Young. 2002. Human macrophage activation programs induced by bacterial pathogens. *Proc. Natl. Acad. Sci. U. S. A* 99:1503-1508.
41. Engele, M., E. Stossel, K. Castiglione, N. Schwerdtner, M. Wagner, P. Bolcskei, M. Rollinghoff, and S. Stenger. 2002. Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent *Mycobacterium tuberculosis*. *J. Immunol.* 168:1328-1337.
42. Tesmer, V. M., A. Rajadhyaksha, J. Babin, and M. Bina. 1993. NF-IL6-mediated transcriptional activation of the long terminal repeat of the human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. U. S. A* 90:7298-7302.
43. Farrar, W. L., M. Korner, and K. A. Clouse. 1991. Cytokine regulation of human immunodeficiency virus expression. *Cytokine* 3:531-542.
44. Finnegan, A., K. A. Roebuck, B. E. Nakai, D. S. Gu, M. F. Rabbi, S. Song, and A. L. Landay. 1996. IL-10 cooperates with TNF-alpha to activate HIV-1 from latently and acutely infected cells of monocyte/macrophage lineage. *J. Immunol.* 156:841-851.
45. Kane, M. M., and D. M. Mosser. 2001. The role of IL-10 in promoting disease progression in leishmaniasis. *J. Immunol.* 166:1141-1147.
46. Celli, J., and B. B. Finlay. 2002. Bacterial avoidance of phagocytosis. *Trends Microbiol.* 10:232-237.
47. Finlay, B. B., and G. McFadden. 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* 124:767-782.
48. Mota, L. J., and G. R. Cornelis. 2005. The bacterial injection kit: type III secretion systems. *Ann. Med.* 37:234-249.
49. Rosenberger, C. M., and B. B. Finlay. 2003. Phagocyte sabotage: disruption of macrophage signaling by bacterial pathogens. *Nat. Rev. Mol. Cell Biol.* 4:385-396.

50. Park, J. M., F. R. Greten, Z. W. Li, and M. Karin. 2002. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science* 297:2048-2051.
51. Kim, D. W., G. Lenzen, A. L. Page, P. Legrain, P. J. Sansonetti, and C. Parsot. 2005. The *Shigella flexneri* effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proc. Natl. Acad. Sci. U. S. A* 102:14046-14051.
52. Underhill, D. M. 2004. Toll-like receptors and microbes take aim at each other. *Curr. Opin. Immunol.* 16:483-487.
53. Merz, A. J., and M. So. 2000. Interactions of pathogenic Neisseriae with epithelial cell membranes. *Annu. Rev. Cell Dev. Biol.* 16:423-457.
54. Kallstrom, H., G. D. Blackmer, B. Albiger, M. K. Liszewski, J. P. Atkinson, and A. B. Jonsson. 2001. Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. *Cell Microbiol.* 3:133-143.
55. Jonsson, A. B., D. Ilver, P. Falk, J. Pepose, and S. Normark. 1994. Sequence changes in the pilus subunit lead to tropism variation of *Neisseria gonorrhoeae* to human tissue. *Mol. Microbiol.* 13:403-416.
56. Merz, A. J., D. B. Rifken, C. G. Arvidson, and M. So. 1996. Traversal of a polarized epithelium by pathogenic Neisseriae: facilitation by type IV pili and maintenance of epithelial barrier function. *Mol. Med.* 2:745-754.
57. McGee, Z. A., D. S. Stephens, L. H. Hoffman, W. F. Schlech, III, and R. G. Horn. 1983. Mechanisms of mucosal invasion by pathogenic Neisseria. *Rev. Infect. Dis.* 5 Suppl 4:S708-S714.
58. Swanson, J., O. Barrera, J. Sola, and J. Boslego. 1988. Expression of outer membrane protein II by gonococci in experimental gonorrhea. *J. Exp. Med.* 168:2121-2129.
59. Dehio, C., S. D. Gray-Owen, and T. F. Meyer. 1998. The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol.* 6:489-495.
60. Simms, A. N., and A. E. Jerse. 2006. *In vivo* selection for *Neisseria gonorrhoeae* opacity protein expression in the absence of human carcinoembryonic antigen cell adhesion molecules. *Infect. Immun.* 74:2965-2974.
61. Song, W., L. Ma, R. Chen, and D. C. Stein. 2000. Role of lipooligosaccharide in Opa-independent invasion of *Neisseria gonorrhoeae* into human epithelial cells. *J. Exp. Med.* 191:949-960.

62. Moran, A. P., M. M. Prendergast, and B. J. Appelmelk. 1996. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol. Med. Microbiol.* 16:105-115.
63. Heine, H., E. T. Rietschel, and A. J. Ulmer. 2001. The biology of endotoxin. *Mol. Biotechnol.* 19:279-296.
64. Raetz, C. R., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan. 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* 5:2652-2660.
65. Erridge, C., E. nett-Guerrero, and I. R. Poxton. 2002. Structure and function of lipopolysaccharides. *Microbes. Infect.* 4:837-851.
66. Shafer, W. M., A. Datta, V. S. Kolli, M. M. Rahman, J. T. Balthazar, L. E. Martin, W. L. Veal, D. S. Stephens, and R. Carlson. 2002. Phase variable changes in genes *lgtA* and *lgtC* within the *lgtABCDE* operon of *Neisseria gonorrhoeae* can modulate gonococcal susceptibility to normal human serum. *J. Endotoxin. Res.* 8:47-58.
67. Burch, C. L., R. J. Danaher, and D. C. Stein. 1997. Antigenic variation in *Neisseria gonorrhoeae*: production of multiple lipooligosaccharides. *J. Bacteriol.* 179:982-986.
68. Arking, D., Y. Tong, and D. C. Stein. 2001. Analysis of lipooligosaccharide biosynthesis in the Neisseriaceae. *J. Bacteriol.* 183:934-941.
69. Griffiss, J. M., H. Schneider, R. E. Mandrell, R. Yamasaki, G. A. Jarvis, J. J. Kim, B. W. Gibson, R. Hamadeh, and M. A. Apicella. 1988. Lipooligosaccharides: the principal glycolipids of the neisserial outer membrane. *Rev. Infect. Dis.* 10 Suppl 2:S287-S295.
70. Mandrell, R. E., J. M. Griffiss, and B. A. Macher. 1988. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. *J. Exp. Med.* 168:107-126.
71. Harvey, H. A., W. E. Swords, and M. A. Apicella. 2001. The mimicry of human glycolipids and glycosphingolipids by the lipooligosaccharides of pathogenic neisseria and haemophilus. *J. Autoimmun.* 16:257-262.
72. Preston, A., R. E. Mandrell, B. W. Gibson, and M. A. Apicella. 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev. Microbiol.* 22:139-180.



73. Mandrell, R. E., and M. A. Apicella. 1993. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. *Immunobiology* 187:382-402.
74. Apicella, M. A., R. E. Mandrell, M. Shero, M. E. Wilson, J. M. Griffiss, G. F. Brooks, C. Lammel, J. F. Breen, and P. A. Rice. 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. *J. Infect. Dis.* 162:506-512.
75. McGee, D. J., and R. F. Rest. 1996. Regulation of gonococcal sialyltransferase, lipooligosaccharide, and serum resistance by glucose, pyruvate, and lactate. *Infect. Immun.* 64:4630-4637.
76. Parsons, N. J., A. Curry, A. J. Fox, D. M. Jones, J. A. Cole, and H. Smith. 1992. The serum resistance of gonococci in the majority of urethral exudates is due to sialylated lipopolysaccharide seen as a surface coat. *FEMS Microbiol. Lett.* 69:295-299.
77. Wetzler, L. M., K. Barry, M. S. Blake, and E. C. Gotschlich. 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. *Infect. Immun.* 60:39-43.
78. Nikaido, H. 1992. Porins and specific channels of bacterial outer membranes. *Mol. Microbiol.* 6:435-442.
79. Unemo, M., O. Norlen, and H. Fredlund. 2005. The *porA* pseudogene of *Neisseria gonorrhoeae*--low level of genetic polymorphism and a few, mainly identical, inactivating mutations. *APMIS* 113:410-419.
80. Bjercknes, R., H. K. Guttormsen, C. O. Solberg, and L. M. Wetzler. 1995. Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but prime the neutrophils to increase their oxidative burst. *Infect. Immun.* 63:160-167.
81. Binnicker, M. J., R. D. Williams, and M. A. Apicella. 2004. Gonococcal porin IB activates NF-kappaB in human urethral epithelium and increases the expression of host antiapoptotic factors. *Infect. Immun.* 72:6408-6417.
82. Bauer, F. J., T. Rudel, M. Stein, and T. F. Meyer. 1999. Mutagenesis of the *Neisseria gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness. *Mol. Microbiol.* 31:903-913.
83. Cannon, J. G., W. J. Black, I. Nachamkin, and P. W. Stewart. 1984. Monoclonal antibody that recognizes an outer membrane antigen common to the pathogenic *Neisseria* species but not to most nonpathogenic *Neisseria* species. *Infect. Immun.* 43:994-999.

84. Hitchcock, P. J., S. F. Hayes, L. W. Mayer, W. M. Shafer, and S. L. Tessier. 1985. Analyses of gonococcal H8 antigen. Surface location, inter- and intrastain electrophoretic heterogeneity, and unusual two-dimensional electrophoretic characteristics. *J. Exp. Med.* 162:2017-2034.
85. Strittmatter, W., and P. J. Hitchcock. 1986. Isolation and preliminary biochemical characterization of the gonococcal H.8 antigen. *J. Exp. Med.* 164:2038-2048.
86. Baehr, W., E. C. Gotschlich, and P. J. Hitchcock. 1989. The virulence-associated gonococcal H.8 gene encodes 14 tandemly repeated pentapeptides. *Mol. Microbiol.* 3:49-55.
87. Woods, J. P., S. M. Spinola, S. M. Strobel, and J. G. Cannon. 1989. Conserved lipoprotein H.8 of pathogenic *Neisseria* consists entirely of pentapeptide repeats. *Mol. Microbiol.* 3:43-48.
88. Woods, J. P., J. R. Black, D. S. Barritt, T. D. Connell, and J. G. Cannon. 1987. Resistance to meningococemia apparently conferred by anti-H.8 monoclonal antibody is due to contaminating endotoxin and not to specific immunoprotection. *Infect. Immun.* 55:1927-1928.
89. Edwards, J. L., E. J. Brown, K. A. Ault, and M. A. Apicella. 2001. The role of complement receptor 3 (CR3) in *Neisseria gonorrhoeae* infection of human cervical epithelia. *Cell Microbiol.* 3:611-622.
90. Price, R. J., and B. Boettcher. 1979. The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement-dependent sperm-immobilizing antibodies. *Fertil. Steril.* 32:61-66.
91. Vanderpuye, O. A., C. A. Labarrere, and J. A. McIntyre. 1992. The complement system in human reproduction. *Am. J. Reprod. Immunol.* 27:145-155.
92. Edwards, J. L., E. J. Brown, S. Uk-Nham, J. G. Cannon, M. S. Blake, and M. A. Apicella. 2002. A co-operative interaction between *Neisseria gonorrhoeae* and complement receptor 3 mediates infection of primary cervical epithelial cells. *Cell Microbiol.* 4:571-584.
93. Edwards, J. L., and M. A. Apicella. 2002. The role of lipooligosaccharide in *Neisseria gonorrhoeae* pathogenesis of cervical epithelia: lipid A serves as a C3 acceptor molecule. *Cell Microbiol.* 4:585-598.
94. Fichorova, R. N., A. O. Cronin, E. Lien, D. J. Anderson, and R. R. Ingalls. 2002. Response to *Neisseria gonorrhoeae* by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signaling. *J. Immunol.* 168:2424-2432.

95. Black, J. R., W. J. Black, and J. G. Cannon. 1985. Neisserial antigen H.8 is immunogenic in patients with disseminated gonococcal and meningococcal infections. *J. Infect. Dis.* 151:650-657.
96. Boslego, J. W., E. C. Tramont, R. C. Chung, D. G. McChesney, J. Ciak, J. C. Sadoff, M. V. Piziak, J. D. Brown, C. C. Brinton, Jr., S. W. Wood, and . 1991. Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine* 9:154-162.
97. Zhu, W., C. E. Thomas, C. J. Chen, C. N. Van Dam, R. E. Johnston, N. L. Davis, and P. F. Sparling. 2005. Comparison of immune responses to gonococcal PorB delivered as outer membrane vesicles, recombinant protein, or Venezuelan equine encephalitis virus replicon particles. *Infect. Immun.* 73:7558-7568.
98. Hedges, S. R., M. S. Mayo, J. Mestecky, E. W. Hook, III, and M. W. Russell. 1999. Limited local and systemic antibody responses to *Neisseria gonorrhoeae* during uncomplicated genital infections. *Infect. Immun.* 67:3937-3946.
99. Plummer, F. A., H. Chubb, J. N. Simonsen, M. Bosire, L. Slaney, I. Maclean, J. O. Ndinya-Achola, P. Waiyaki, and R. C. Brunham. 1993. Antibody to Rmp (outer membrane protein 3) increases susceptibility to gonococcal infection. *J. Clin. Invest* 91:339-343.
100. Plummer, F. A., H. Chubb, J. N. Simonsen, M. Bosire, L. Slaney, N. J. Nagelkerke, I. Maclean, J. O. Ndinya-Achola, P. Waiyaki, and R. C. Brunham. 1994. Antibodies to opacity proteins (Opa) correlate with a reduced risk of gonococcal salpingitis. *J. Clin. Invest* 93:1748-1755.
101. Shafer, W. M., and R. F. Rest. 1989. Interactions of gonococci with phagocytic cells. *Annu. Rev. Microbiol.* 43:121-145.
102. Kallstrom, H., M. S. Islam, P. O. Berggren, and A. B. Jonsson. 1998. Cell signaling by the type IV pili of pathogenic *Neisseria*. *J. Biol. Chem.* 273:21777-21782.
103. Duensing, T. D., and J. P. van Putten. 1997. Vitronectin mediates internalization of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. *Infect. Immun.* 65:964-970.
104. Gomez-Duarte, O. G., M. Dehio, C. A. Guzman, G. S. Chhatwal, C. Dehio, and T. F. Meyer. 1997. Binding of vitronectin to opa-expressing *Neisseria gonorrhoeae* mediates invasion of HeLa cells. *Infect. Immun.* 65:3857-3866.
105. Grassme, H., E. Gulbins, B. Brenner, K. Ferlinz, K. Sandhoff, K. Harzer, F. Lang, and T. F. Meyer. 1997. Acidic sphingomyelinase mediates entry of *N. gonorrhoeae* into nonphagocytic cells. *Cell* 91:605-615.

106. van Putten, J. P., T. D. Duensing, and R. L. Cole. 1998. Entry of OpaA+ gonococci into HEp-2 cells requires concerted action of glycosaminoglycans, fibronectin and integrin receptors. *Mol. Microbiol.* 29:369-379.
107. Hauck, C. R., T. F. Meyer, F. Lang, and E. Gulbins. 1998. CD66-mediated phagocytosis of Opa52 *Neisseria gonorrhoeae* requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. *EMBO J.* 17:443-454.
108. Muller, A., D. Gunther, F. Dux, M. Naumann, T. F. Meyer, and T. Rudel. 1999. Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. *EMBO J.* 18:339-352.
109. Muenzner, P., M. Naumann, T. F. Meyer, and S. D. Gray-Owen. 2001. Pathogenic *Neisseria* trigger expression of their carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1; previously CD66a) receptor on primary endothelial cells by activating the immediate early response transcription factor, nuclear factor-kappaB. *J. Biol. Chem.* 276:24331-24340.
110. Naumann, M., T. Rudel, B. Wieland, C. Bartsch, and T. F. Meyer. 1998. Coordinate activation of activator protein 1 and inflammatory cytokines in response to *Neisseria gonorrhoeae* epithelial cell contact involves stress response kinases. *J. Exp. Med.* 188:1277-1286.
111. Fiset, P. L., S. Ram, J. M. Andersen, W. Guo, and R. R. Ingalls. 2003. The Lip lipoprotein from *Neisseria gonorrhoeae* stimulates cytokine release and NF-kappaB activation in epithelial cells in a Toll-like receptor 2-dependent manner. *J. Biol. Chem.* 278:46252-46260.
112. Muenzner, P., O. Billker, T. F. Meyer, and M. Naumann. 2002. Nuclear factor-kappa B directs carcinoembryonic antigen-related cellular adhesion molecule 1 receptor expression in *Neisseria gonorrhoeae*-infected epithelial cells. *J. Biol. Chem.* 277:7438-7446.
113. Pridmore, A. C., G. A. Jarvis, C. M. John, D. L. Jack, S. K. Dower, and R. C. Read. 2003. Activation of toll-like receptor 2 (TLR2) and TLR4/MD2 by *Neisseria* is independent of capsule and lipooligosaccharide (LOS) sialylation but varies widely among LOS from different strains. *Infect. Immun.* 71:3901-3908.
114. West, S. E., and V. L. Clark. 1989. Genetic loci and linkage associations in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Clin. Microbiol. Rev.* 2 Suppl:S92-103.
115. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and D. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* 85:1274-1279.

116. Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154:269-277.
117. Schneider, H., J. M. Griffiss, J. W. Boslego, P. J. Hitchcock, K. M. Zahos, and M. A. Apicella. 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. *J. Exp. Med.* 174:1601-1605.
118. Harvey, H. A., N. Porat, C. A. Campbell, M. Jennings, B. W. Gibson, N. J. Phillips, M. A. Apicella, and M. S. Blake. 2000. Gonococcal lipooligosaccharide is a ligand for the asialoglycoprotein receptor on human sperm. *Mol. Microbiol.* 36:1059-1070.
119. Harvey, H. A., M. P. Jennings, C. A. Campbell, R. Williams, and M. A. Apicella. 2001. Receptor-mediated endocytosis of *Neisseria gonorrhoeae* into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. *Mol. Microbiol.* 42:659-672.
120. van Putten, J. P., H. U. Grassme, B. D. Robertson, and E. T. Schwan. 1995. Function of lipopolysaccharide in the invasion of *Neisseria gonorrhoeae* into human mucosal cells. *Prog. Clin. Biol. Res.* 392:49-58.
121. McGee, Z. A., C. M. Clemens, R. L. Jensen, J. J. Klein, L. R. Barley, and G. L. Gorby. 1992. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. *Microb. Pathog.* 12:333-341.
122. Harvey, H. A., D. M. Post, and M. A. Apicella. 2002. immortalization of human urethral epithelial cells: a model for the study of the pathogenesis of and the inflammatory cytokine response to *Neisseria gonorrhoeae* infection. *Infect. Immun.* 70:5808-5815.
123. Anzala, A. O., J. N. Simonsen, J. Kimani, T. B. Ball, N. J. Nagelkerke, J. Rutherford, E. N. Ngugi, J. J. Bwayo, and F. A. Plummer. 2000. Acute sexually transmitted infections increase human immunodeficiency virus type 1 plasma viremia, increase plasma type 2 cytokines, and decrease CD4 cell counts. *J. Infect. Dis.* 182:459-466.
124. Uronen-Hansson, H., L. Steeghs, J. Allen, G. L. Dixon, M. Osman, L. P. van der, S. Y. Wong, R. Callard, and N. Klein. 2004. Human dendritic cell activation by *Neisseria meningitidis*: phagocytosis depends on expression of lipooligosaccharide (LOS) by the bacteria and is required for optimal cytokine production. *Cell Microbiol.* 6:625-637.
125. Brandtzaeg, P., K. Bryn, P. Kierulf, R. Ovstebo, E. Namork, B. Aase, and E. Jantzen. 1992. Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. *J. Clin. Invest* 89:816-823.

126. Zughailer, S. M., Y. L. Tzeng, S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens. 2004. *Neisseria meningitidis* lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. *Infect. Immun.* 72:371-380.
127. Zughailer, S., S. Agrawal, D. S. Stephens, and B. Pulendran. 2006. Hexa-acylation and KDO(2)-glycosylation determine the specific immunostimulatory activity of *Neisseria meningitidis* lipid A for human monocyte derived dendritic cells. *Vaccine* 24:1291-1297.
128. Yoshizawa, S., D. Fourmy, and J. D. Puglisi. 1998. Structural origins of gentamicin antibiotic action. *EMBO J.* 17:6437-6448.
129. Proost, P., A. Wuyts, and D. J. Van. 1996. Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. *J. Leukoc. Biol.* 59:67-74.
130. Sprong, T., N. Stikkelbroeck, L. P. van der, L. Steeghs, A. L. van, N. Klein, M. G. Netea, J. W. van der Meer, and D. M. van. 2001. Contributions of *Neisseria meningitidis* LPS and non-LPS to proinflammatory cytokine response. *J. Leukoc. Biol.* 70:283-288.
131. Pridmore, A. C., D. H. Wyllie, F. Abdillahi, L. Steeghs, L. P. van der, S. K. Dower, and R. C. Read. 2001. A lipopolysaccharide-deficient mutant of *Neisseria meningitidis* elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. *J. Infect. Dis.* 183:89-96.
132. Unkmeir, A., U. Kammerer, A. Stade, C. Hubner, S. Haller, A. Kolb-Maurer, M. Frosch, and G. Dietrich. 2002. Lipooligosaccharide and polysaccharide capsule: virulence factors of *Neisseria meningitidis* that determine meningococcal interaction with human dendritic cells. *Infect. Immun.* 70:2454-2462.
133. Braun, J. M., C. C. Blackwell, I. R. Poxton, O. E. Ahmer, A. E. Gordon, O. M. Madani, D. M. Weir, S. Giersen, and J. Beuth. 2002. Proinflammatory responses to lipo-oligosaccharide of *Neisseria meningitidis* immunotype strains in relation to virulence and disease. *J. Infect. Dis.* 185:1431-1438.
134. Ellis, C. D., B. Lindner, C. M. njam Khan, U. Zahringer, and H. R. marco de. 2001. The *Neisseria gonorrhoeae* lpxLII gene encodes for a late-functioning lauroyl acyl transferase, and a null mutation within the gene has a significant effect on the induction of acute inflammatory responses. *Mol. Microbiol.* 42:167-181.
135. van der, L. P., L. Steeghs, H. J. Hamstra, H. J. ten, B. Zomer, and A. L. van. 2001. Modification of lipid A biosynthesis in *Neisseria meningitidis* lpxL

- mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infect. Immun.* 69:5981-5990.
136. Makepeace, B. L., P. J. Watt, J. E. Heckels, and M. Christodoulides. 2001. Interactions of *Neisseria gonorrhoeae* with mature human macrophage opacity proteins influence production of proinflammatory cytokines. *Infect. Immun.* 69:1909-1913.
  137. Mandrell, R. E., A. J. Lesse, J. V. Sugai, M. Shero, J. M. Griffiss, J. A. Cole, N. J. Parsons, H. Smith, S. A. Morse, and M. A. Apicella. 1990. *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. *J. Exp. Med.* 171:1649-1664.
  138. Nairn, C. A., J. A. Cole, P. V. Patel, N. J. Parsons, J. E. Fox, and H. Smith. 1988. Cytidine 5'-monophospho-N-acetylneuraminic acid or a related compound is the low Mr factor from human red blood cells which induces gonococcal resistance to killing by human serum. *J. Gen. Microbiol.* 134:3295-3306.
  139. Parsons, N. J., C. Constantinidou, J. A. Cole, and H. Smith. 1994. Sialylation of lipopolysaccharide by CMP-NANA in viable gonococci is enhanced by low Mr material released from blood cell extracts but not by some UDP sugars. *Microb. Pathog.* 16:413-421.
  140. Smith, H., N. J. Parsons, and J. A. Cole. 1995. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. *Microb. Pathog.* 19:365-377.
  141. Rarick, M., C. McPheeters, S. Bright, A. Navis, J. Skefos, P. Sebastiani, and M. Montano. 2006. Evidence for cross-regulated cytokine response in human peripheral blood mononuclear cells exposed to whole gonococcal bacteria *in vitro*. *Microb. Pathog.*
  142. Robinson, E. N., Jr., Z. A. McGee, T. M. Buchanan, M. S. Blake, and P. J. Hitchcock. 1987. Probing the surface of *Neisseria gonorrhoeae*: simultaneous localization of protein I and H.8 antigens. *Infect. Immun.* 55:1190-1197.
  143. Cannon, J. G. 1989. Conserved lipoproteins of pathogenic *Neisseria* species bearing the H.8 epitope: lipid-modified azurin and H.8 outer membrane protein. *Clin. Microbiol. Rev.* 2 Suppl:S1-S4.
  144. Swanson, J. 1977. Surface components affecting interactions between *Neisseria gonorrhoeae* and eucaryotic cells. *J. Infect. Dis.* 136 Suppl:S138-S143.
  145. Swanson, J. 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. *Infect. Immun.* 19:320-331.

146. Agerer, F., S. Waeckerle, and C. R. Hauck. 2004. Microscopic quantification of bacterial invasion by a novel antibody-independent staining method. *J. Microbiol. Methods* 59:23-32.
147. Booth, J. W., D. Telio, E. H. Liao, S. E. McCaw, T. Matsuo, S. Grinstein, and S. D. Gray-Owen. 2003. Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related cellular adhesion molecule-mediated internalization of *Neisseria gonorrhoeae*. *J. Biol. Chem.* 278:14037-14045.
148. Post, D. M., N. J. Phillips, J. Q. Shao, D. D. Entz, B. W. Gibson, and M. A. Apicella. 2002. Intracellular survival of *Neisseria gonorrhoeae* in male urethral epithelial cells: importance of a hexaacetyl lipid A. *Infect. Immun.* 70:909-920.
149. Williams, J. M., G. C. Chen, L. Zhu, and R. F. Rest. 1998. Using the yeast two-hybrid system to identify human epithelial cell proteins that bind gonococcal Opa proteins: intracellular gonococci bind pyruvate kinase via their Opa proteins and require host pyruvate for growth. *Mol. Microbiol.* 27:171-186.
150. Lin, L., P. Ayala, J. Larson, M. Mulks, M. Fukuda, S. R. Carlsson, C. Enns, and M. So. 1997. The *Neisseria* type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. *Mol. Microbiol.* 24:1083-1094.
151. Casey, S. G., D. R. Veale, and H. Smith. 1979. Demonstration of intracellular growth of gonococci in human phagocytes using spectinomycin to kill extracellular organisms. *J. Gen. Microbiol.* 113:395-398.
152. Veale, D. R., M. Goldner, C. W. Penn, J. Ward, and H. Smith. 1979. The intracellular survival and growth of gonococci in human phagocytes. *J. Gen. Microbiol.* 113:383-393.
153. Rest, R. F. 1979. Killing of *Neisseria gonorrhoeae* by human polymorphonuclear neutrophil granule extracts. *Infect. Immun.* 25:574-579.
154. Mezzatesta, J. R., and R. F. Rest. 1983. Phagocytic killing of *Neisseria gonorrhoeae* by human monocytes. *Infect. Immun.* 42:99-105.
155. Cooper, M. D., and S. A. Floyd. 1982. In vitro kinetics of phagocytosis and intracellular killing of gonococci by peritoneal macrophages from mice deficient in complement component 5. *Infect. Immun.* 36:363-370.
156. Veale, D. R., H. Finch, and H. Smith. 1976. Penetration of penicillin into human phagocytes containing *Neisseria gonorrhoeae*: intracellular survival and growth at optimum concentrations of antibiotic. *J. Gen. Microbiol.* 96:353-363.



157. Witt, K., D. R. Veale, H. Finch, C. W. Penn, D. Sen, and H. Smith. 1976. Resistance of *Neisseria gonorrhoeae* grown *in vivo* to ingestion and digestion by phagocytes of human blood. *J. Gen. Microbiol.* 96:341-350.
158. Witt, K., D. R. Veale, and H. Smith. 1976. Resistance of *Neisseria gonorrhoeae* to ingestion and digestion by phagocytes of human buffy coat. *J. Med. Microbiol.* 9:1-12.
159. Leuzzi, R., L. Serino, M. Scarselli, S. Savino, M. R. Fontana, E. Monaci, A. Taddei, G. Fischer, R. Rappuoli, and M. Pizza. 2005. Ng-MIP, a surface-exposed lipoprotein of *Neisseria gonorrhoeae*, has a peptidyl-prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. *Mol. Microbiol.* 58:669-681.
160. Hauck, C. R., and T. F. Meyer. 1997. The lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of *Neisseria gonorrhoeae*. *FEBS Lett.* 405:86-90.
161. Lorenzen, D. R., D. Gunther, J. Pandit, T. Rudel, E. Brandt, and T. F. Meyer. 2000. *Neisseria gonorrhoeae* porin modifies the oxidative burst of human professional phagocytes. *Infect. Immun.* 68:6215-6222.
162. Mosleh, I. M., L. A. Huber, P. Steinlein, C. Pasquali, D. Gunther, and T. F. Meyer. 1998. *Neisseria gonorrhoeae* porin modulates phagosome maturation. *J. Biol. Chem.* 273:35332-35338.
163. Srinivasan, A., J. Foley, R. Ravindran, and S. J. McSorley. 2004. Low-dose *Salmonella* infection evades activation of flagellin-specific CD4 T cells. *J. Immunol.* 173:4091-4099.
164. Schneider, H., A. S. Cross, R. A. Kuschner, D. N. Taylor, J. C. Sadoff, J. W. Boslego, and C. D. Deal. 1995. Experimental human gonococcal urethritis: 250 *Neisseria gonorrhoeae* MS11mkC are infective. *J. Infect. Dis.* 172:180-185.
165. Isbey, S. F., T. M. Alcorn, R. H. Davis, J. Haizlip, P. A. Leone, and M. S. Cohen. 1997. Characterisation of *Neisseria gonorrhoeae* in semen during urethral infection in men. *Genitourin. Med.* 73:378-382.
166. Patrone, J. B., S. E. Bish, and D. C. Stein. 2006. TNF- $\alpha$ -Independent IL-8 Expression: Alterations in Bacterial Challenge Dose Cause Differential Human Monocytic Cytokine Response. *J. Immunol.* 177:1314-1322.
167. Dorward, D. W., C. F. Garon, and R. C. Judd. 1989. Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *J. Bacteriol.* 171:2499-2505.

168. Goldfeld, A. E., C. Doyle, and T. Maniatis. 1990. Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide. *Proc. Natl. Acad. Sci. U. S. A* 87:9769-9773.
169. Cogswell, J. P., M. M. Godlevski, G. B. Wisely, W. C. Clay, L. M. Leesnitzer, J. P. Ways, and J. G. Gray. 1994. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J. Immunol.* 153:712-723.
170. Hoffmann, E., O. ttrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 72:847-855.
171. Kunsch, C., and C. A. Rosen. 1993. NF-kappa B subunit-specific regulation of the interleukin-8 promoter. *Mol. Cell Biol.* 13:6137-6146.
172. Hipp, M. S., C. Urbich, P. Mayer, J. Wischhusen, M. Weller, M. Kracht, and I. Spyridopoulos. 2002. Proteasome inhibition leads to NF-kappaB-independent IL-8 transactivation in human endothelial cells through induction of AP-1. *Eur. J. Immunol.* 32:2208-2217.
173. Akhtar, M., J. L. Watson, A. Nazli, and D. M. McKay. 2003. Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NFkappaB-independent pathway. *FASEB J.*
174. Roebuck, K. A. 1999. Regulation of interleukin-8 gene expression. *J. Interferon Cytokine Res.* 19:429-438.
175. Jeong, J. Y., K. U. Kim, and D. M. Jue. 1997. Tosylphenylalanine chloromethyl ketone inhibits TNF-alpha mRNA synthesis in the presence of activated NF-kappa B in RAW 264.7 macrophages. *Immunology* 92:267-273.
176. Lo, C. J., M. Fu, and B. Kim. 1997. Macrophage TNF mRNA expression is modulated by protease inhibitors. *J. Surg. Res.* 69:408-412.
177. Foletta, V. C., D. H. Segal, and D. R. Cohen. 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. *J. Leukoc. Biol.* 63:139-152.
178. Chinenov, Y., and T. K. Kerppola. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* 20:2438-2452.
179. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr. Opin. Cell Biol.* 9:240-246.
180. Karin, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* 270:16483-16486.

181. Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young, and J. C. Lee. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 364:229-233.
182. Bennett, B. L., D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S. S. Bhagwat, A. M. Manning, and D. W. Anderson. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U. S. A* 98:13681-13686.
183. Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* 270:27489-27494.
184. Walz, A., P. Schmutz, C. Mueller, and S. Schnyder-Candrian. 1997. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J. Leukoc. Biol.* 62:604-611.
185. Lorenzen, D. R., F. Dux, U. Wolk, A. Tsirpouchtsidis, G. Haas, and T. F. Meyer. 1999. Immunoglobulin A1 protease, an exoenzyme of pathogenic *Neisseriae*, is a potent inducer of proinflammatory cytokines. *J. Exp. Med.* 190:1049-1058.
186. Marie, C., S. Roman-Roman, and G. Rawadi. 1999. Involvement of mitogen-activated protein kinase pathways in interleukin-8 production by human monocytes and polymorphonuclear cells stimulated with lipopolysaccharide or *Mycoplasma fermentans* membrane lipoproteins. *Infect. Immun.* 67:688-693.
187. Christodoulides, M., J. S. Everson, B. L. Liu, P. R. Lambden, P. J. Watt, E. J. Thomas, and J. E. Heckels. 2000. Interaction of primary human endometrial cells with *Neisseria gonorrhoeae* expressing green fluorescent protein. *Mol. Microbiol.* 35:32-43.
188. Nadesalingam, J., A. W. Dodds, K. B. Reid, and N. Palaniyar. 2005. Mannose-binding lectin recognizes peptidoglycan via the N-acetyl glucosamine moiety, and inhibits ligand-induced proinflammatory effect and promotes chemokine production by macrophages. *J. Immunol.* 175:1785-1794.
189. vyatyarova-Johnson, M., I. H. Rees, B. D. Robertson, M. W. Turner, N. J. Klein, and D. L. Jack. 2000. The lipopolysaccharide structures of *Salmonella enterica* serovar Typhimurium and *Neisseria gonorrhoeae* determine the attachment of human mannose-binding lectin to intact organisms. *Infect. Immun.* 68:3894-3899.

190. Yao, J., N. Mackman, T. S. Edgington, and S. T. Fan. 1997. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J. Biol. Chem.* 272:17795-17801.
191. Tsai, E. Y., J. V. Falvo, A. V. Tsytsykova, A. K. Barczak, A. M. Reimold, L. H. Glimcher, M. J. Fenton, D. C. Gordon, I. F. Dunn, and A. E. Goldfeld. 2000. A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol. Cell Biol.* 20:6084-6094.
192. Barthel, R., A. V. Tsytsykova, A. K. Barczak, E. Y. Tsai, C. C. Dascher, M. B. Brenner, and A. E. Goldfeld. 2003. Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhanceosome dependent on the coactivator proteins CBP/p300. *Mol. Cell Biol.* 23:526-533.
193. Falvo, J. V., A. M. Uglialoro, B. M. Brinkman, M. Merika, B. S. Parekh, E. Y. Tsai, H. C. King, A. D. Morielli, E. G. Peralta, T. Maniatis, D. Thanos, and A. E. Goldfeld. 2000. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. *Mol. Cell Biol.* 20:2239-2247.
194. Drouet, C., A. N. Shakhov, and C. V. Jongeneel. 1991. Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-alpha promoter in primary macrophages. *J. Immunol.* 147:1694-1700.
195. Gupta, S., D. Campbell, B. Derijard, and R. J. Davis. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267:389-393.
196. Mukaida, N., S. Okamoto, Y. Ishikawa, and K. Matsushima. 1994. Molecular mechanism of interleukin-8 gene expression. *J. Leukoc. Biol.* 56:554-558.
197. Wu, G. D., E. J. Lai, N. Huang, and X. Wen. 1997. Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. The role of Oct-1 as a transcriptional repressor. *J. Biol. Chem.* 272:2396-2403.
198. Roman, J., J. D. Ritzenthaler, M. J. Fenton, S. Roser, and W. Schuyler. 2000. Transcriptional regulation of the human interleukin 1beta gene by fibronectin: role of protein kinase C and activator protein 1 (AP-1). *Cytokine* 12:1581-1596.
199. Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.* 69:1036-1044.

200. Wood, L. D., and A. Richmond. 1995. Constitutive and cytokine-induced expression of the melanoma growth stimulatory activity/GRO alpha gene requires both NF-kappa B and novel constitutive factors. *J. Biol. Chem.* 270:30619-30626.
201. Viedt, C., R. Dechend, J. Fei, G. M. Hansch, J. Kreuzer, and S. R. Orth. 2002. MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and activating protein-1. *J. Am. Soc. Nephrol.* 13:1534-1547.
202. Lim, S. P., and A. Garzino-Demo. 2000. The human immunodeficiency virus type 1 Tat protein up-regulates the promoter activity of the beta-chemokine monocyte chemoattractant protein 1 in the human astrocytoma cell line U-87 MG: role of SP-1, AP-1, and NF-kappaB consensus sites. *J. Virol.* 74:1632-1640.