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MACROPHAGE MIGRATION INHIBITORY FACTOR POLYMORPHISMS AND INVASIVE *STREPTOCCUS PNEUMONIAE* INFECTIONS

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MACROPHAGE MIGRATION INHIBITORY FACTOR POLYMORPHISMS AND INVASIVE *STREPTOCCUS PNEUMONIAE* INFECTIONS

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Streptococcus pneumoniae (*S. pneumoniae*) causes a spectrum of disease severity, and human host factors likely play a role in this variation. One candidate factor is macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine and upstream regulator of innate immunity. The *MIF* promoter contains two functional polymorphisms, a tetranucleotide (CATT) repeat such that *MIF* expression increases with repeat number from 5-8 and a single nucleotide polymorphism (SNP) leading to a G-to-C transition, which results in increased *MIF* expression in cell line reporter assays. Emerging data suggest an association between high-expression *MIF* alleles and inflammatory disease. This study comprised two parts. For the *in vitro* portion, we hypothesized that peripheral blood monocytic cells (pBMCs) cultured from healthy individuals with low-expressing *MIF* genotypes (5-CATT alleles or SNP-GG) would have lower MIF content and release than those from individuals with high-expressing *MIF* genotypes (7-CATT or SNP-C alleles). For the *in vivo* study, we hypothesized that individuals with low-expressing *MIF* genotypes would have less severe systemic inflammatory responses than individuals with high-expressing *MIF* genotypes in response to *S. pneumoniae* infection. Blood samples and chart findings were collected prospectively at three Connecticut hospitals from 30 inpatients with documented invasive *S. pneumoniae* infections. Genomic DNA was isolated from host blood, amplified, and genotyped using fragment analysis (CATT repeat) and allelic discrimination (SNP) methods. Fisher's exact tests were used to compare genotypes and disease severity. For the *in vitro* experiments, there were no differences observed in serum MIF levels or MIF content or release from pBMCs based on *MIF* genotype. In the cohort of patients infected with *S. pneumoniae*, serum MIF levels among enrolled subjects were significantly higher than the reported normal values, but levels did not vary with genotype or disease severity. The SNP genotype was not correlated with disease severity or occurrence of meningitis. The CATT genotype did not correlate significantly with disease severity or occurrence of meningitis, although there was a trend suggesting an association between the 7-CATT allele and meningitis ($p = 0.1188$, 8% without meningitis had a 7-CATT allele vs. 40% with meningitis). More patient samples will need to be analyzed in order to definitively elucidate the role of *MIF* genetics in infection with *S. pneumoniae*.

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"It is our response to their presence that makes the disease"
Lewis Thomas, 1972 (1)

INTRODUCTION

The human immune system has evolved to protect against tissue invasion by infectious pathogens or host cellular antigens (e.g., tumor antigens). Although the host response is usually effective, disease can ensue if the response is unsuccessful or too robust. For example, an overactive immune response directed against self-antigens results in autoimmune disease; a strong response against invasive bacteria can result in complications such as septic shock (2). Over the past century, scientists have discovered how the immune system recognizes foreign antigens, recruits and influences immune cells, and destroys the invading agent (3). Recent efforts have focused on secreted proteins of the innate immune system, the host's first defense against foreign molecules, which result in cascades leading to an inflammatory response. Therapies directed against specific mediators in these cascades may be effective in preventing much of the damage of inflammatory and infectious diseases without the many systemic side effects that occur with broad-spectrum immunosuppressants such as steroids. Presently, these specific therapies only exist for chronic inflammatory diseases, such as rheumatoid arthritis, and have not yet been proven effective for infectious diseases (4, 5).

Immune response to severe infection: Sepsis

Systemic infection, or sepsis, is a major and growing problem that illustrates the central role for innate immunity in disease pathogenesis (6). It occurs in response to many types of infections and is characterized by systemic response to infection. This systemic response results in widespread inflammation with vasodilation, microvascular leakiness, and leukocyte invasion into organs distant from the initial insult. Clinical sepsis occurs as a spectrum of severity, which has now been defined for investigative and therapeutic purposes (7, 8). Sepsis is described first as infection along with clinical signs indicating the systemic inflammatory response syndrome

(SIRS), which requires the presence of abnormalities in at least two of the following categories: temperature, heart rate, respiratory rate, and white blood cell count. Severe sepsis involves the additional presence of end-organ damage as manifested by characteristics such as hypoxia, lactic acidosis, oliguria, disseminated intravascular coagulation, or altered mental status. Septic shock occurs when sepsis is associated with a hypotension that persists despite adequate fluid resuscitation.

Despite advances in antimicrobial therapy, sepsis continues to cause significant morbidity and mortality. One study based on a review of non-federal, acute hospitalizations across the United States over a 22-year period found the rate of sepsis increased almost 9% per year to 660,000 cases in 2000, which resulted in an incidence of 240.4 cases per 100,000 population (9). The authors of this study attributed this rise to higher rates of microbial resistance, more HIV-positive patients, growth in organ transplantation and chemotherapy programs, and greater use of invasive devices and procedures. Throughout this period, both males and non-whites showed higher rates of sepsis than females and white populations. Mortality rates declined among those with gram-negative sepsis but remained unchanged among those with gram-positive infections, which experienced increasing incidence throughout the study period. In addition, the study found that the mortality rate increased from 15% in those without any organ dysfunction to 70% in those with three or more failing organs. Several other epidemiological studies have also shown increasing incidences and high mortality rates over recent years (10, 11). Because its categories have been so well defined, sepsis is a useful way to study the progression of infectious diseases.

When a host is faced with an infectious insult, the immune response can be characterized as inadequate, sufficient, or excessive (12). A deficient response will result in overwhelming infection while an adequate one can result in eradication of the insult. This immune response to infection involves a coordinated series of events that may also end in an overactive, uncontrolled “cytokine storm,” often leading to vascular collapse, organ system failure, and possibly death.

From the time the microbe invades into the tissue, there are a number of points in both the innate and adaptive immune responses during which slight variation could contribute to a deleterious outcome (13, 14). The initial threat signal to the host involves membrane-bound extracellular receptors on mononuclear phagocytes, such as monocytes, macrophages, and dendritic cells, which recognize foreign molecules. These so-called pattern recognition receptors (PRRs) include the Toll-like receptor family, which recognize microbial products, or pathogen-associated microbial patterns (PAMPs), such as lipopolysaccharide, peptidoglycan, superantigens, viral nucleic acids, and endogenous heat-shock proteins (15). As outlined by Medzhitov and Janeway, binding of a PAMP to a PRR results in signal amplification and eventual mounting of an anti-microbial response. The specifics of this response are complex. For instance, this cascade often involves release of nuclear factor (NF)-κB from its inhibitor I-κB and translocation to the nucleus. Once in the nucleus, NF-κB influences transcription of numerous pro- and anti-inflammatory cytokines. In turn, these cytokines act through receptors and co-factors on specific cells to initiate their own signal transduction cascades resulting in specific products and actions of immune and endothelial cells. Many of these cytokines have multiple actions, resulting in production of vasoactive substances, oxygen free radicals, and other inflammatory molecules, leading to an intricate but controlled web of mediators and recruitment of other cell types to the area of injury or infection. The complement system is also activated and contributes to the outpouring of inflammatory mediators. In addition, there is systemic release of hormones such as catecholamines and corticosteroids, which modulates the overall systemic response to tissue invasion.

The coagulation and fibrinolytic cascades are closely tied into this inflammatory response, and sepsis results in a pro-coagulant state (16). As reviewed by Matthay, release of tissue factor as a result of direct cellular injury and the action of pro-inflammatory cytokines on monocytes and endothelial cells leads to a series of events resulting in thrombin production and

fibrin deposition, which in turn up-regulates much of the pro-inflammatory response. Increased levels of plasminogen-activator inhibitor type-1 (PAI-1) promote coagulation by preventing plasmin generation and degradation of fibrin clots. In turn, there is a relative deficiency of the anticoagulant molecules activated protein C (APC), antithrombin, and tissue factor pathway inhibitor. As a result, small vessels often clot off, resulting in decreased organ perfusion. Consequently, the pro-inflammatory response of sepsis often disrupts the homeostasis of the coagulation cascade, which further contributes to the organ damage.

There is an anti-inflammatory component that occurs simultaneously with the activation of the pro-inflammatory response (17). This counter-response includes anti-inflammatory molecules, such as the soluble TNF-α receptor and the IL-1 receptor antagonist, which act to downregulate cytokine activity, as well as anti-complement molecules and pro-inflammatory molecules such as IL-10. Lymphocyte apoptosis is upregulated in septic patients as compared with non-infected critically ill individuals (18). Whether this anti-inflammatory response sufficiently counterbalances the pro-inflammatory response or results in overcompensation and a worsening of morbidity and mortality may vary in different patients and be a feature of the dysregulated response in severe sepsis.

Ultimately if the response goes unchecked, the inflammatory and pro-coagulant cascades together contribute to vascular instability, tissue hypoxia, microvascular occlusion, and myocardial depression; organ compromise and irreversible tissue damage ensue (19). Despite the advances in understanding of the sepsis syndrome, there has been relatively little data to support specific treatments aimed at the underlying pathophysiology, though there is active research looking for clinical sub-populations that may benefit from targeted therapy (20). Recombinant activated protein C (APC; Drotrecogin Alpha), an anti-inflammatory and thrombin inhibitor molecule, is one such therapy that may benefit those with severe sepsis at very high risk of death (21). The lack of success with APC in patients with lower risk of death emphasizes the need for

accurate identification of candidate groups prior to these targeted treatments (22). It is still unclear how to identify accurately the appropriate patient group for such therapy, and genetic variation may play a role in defining candidate subgroups.

Immune response to severe infection: Meningitis

Bacterial meningitis is another serious infection in which the immune response plays a seminal role in disease pathophysiology. There are several ways in which meningeal infection can occur. Commonly, bacteria adhere to and colonize the nasopharyngeal mucosa, invade the intravascular space, enter through the blood-cerebrospinal fluid (CSF) barrier, and then survive within the subarachnoid space (23). Alternatively, infection can occur by direct spread from the sinuses or otitis media. Seeding of the CNS from bacteremia due to distant infections can also happen. In the age of antibiotics, community-acquired meningitis has a case-fatality rate of approximately 25% (24, 25). As with the sepsis syndrome, many of the complications of meningitis involve dysregulation of the host inflammatory response, which can lead to neurological as well as systemic sequelae (26). Bacterial products in the blood and the CSF engage endothelial PRRs, which leads to cytokine release, resulting in recruitment of inflammatory cells, increase in endothelial permeability, and upregulation of leukocyte adhesion molecules. This response interrupts the blood-brain barrier and results in cerebral edema, impaired cerebral blood flow, and vasculitis. Toxic free radicals released by both bacteria and inflammatory cells contribute to neuronal cell death. As a result, focal and global neurological deficits ensue. Patients with meningitis can die either from neurological complications, such as ischemia, hemorrhage, or edema, or from complications from the systemic response to a severe infection (27). As with sepsis, mortality remains high despite appropriate antibiotic use.

Evidence for genetic basis of infection

Variation between individuals with infectious insults provides a way to examine the role of the host response in disease severity and outcome. In disorders like sepsis and meningitis where the immune cascade plays a major role, it is no surprise that genetics are an important determination of disease course. While environmental exposure and microbial virulence factors undoubtedly play an important part, there is a genetic component that dictates whether an organism is able to cause clinically significant disease or not. For example, a longitudinal study of premature death in adoptees born between 1924 and 1926 and their biological and adoptive parents by the Danish Adoption Register demonstrated a strong genetic component to infection (28). The authors found a 5.81 (95% CI, 2.47 to 13.7) relative risk of death in an adopted child whose biological parent died before the age of 50 of an infection compared with an adoptee whose biological parents lived past 50 years of age. Death of an adoptive parent from infection before age 50 did not increase the risk of death in the adoptee compared with those whose adoptive parents survived past 50 years of age. Surprisingly, the converse was true with cancer where premature death of an adoptive, but not a biological, parent from cancer led to a 5.16 relative risk of death (95% CI, 1.20 to 22.2) in the child. Twin studies comparing dizygotic to monozygotic twins, groups that presumably have similar environmental exposures but different levels of genetic overlap, generally have shown a greater concordance to susceptibility or response to infection among monozygotes (29, 30, 31, 32). Although the evidence for a role of genetics in infectious diseases is strong, finding which specific genes play a determinant role will prove a great challenge. Variation in the host response to invading organisms likely accounts for the hereditary patterns since infection itself is not a genetic disease (33).

The classic way to identify hereditary single gene defects is through linkage analysis, which utilizes known chromosomal loci that are inherited in the same pattern as the gene of interest. However, in the case of infectious diseases, traditional Mendelian inheritance of a single

gene leading to susceptibility is rare. An example of this is immunodeficiency caused by the x-linked absence of Bruton's tyrosine kinase leading to agammaglobulinemia and susceptibility to infection by different extracellular organisms (34). Hereditary predispositions to specific infections, such as an autosomal recessive mutation of the interferon-γ receptor resulting in mycobacterial infections, have also been reported (35). Yet, despite the compelling evidence for genetic susceptibility to infectious diseases, such classic inheritance patterns are the exception. One theory to explain this disparity is the polygenic hypothesis, which posits that several common mutations, each of which has minor consequence alone, combine to result in increased susceptibility within a certain individual (36). It is likely that a mixture of subtle variations in the immune cascade can lead to certain susceptibilities in many individuals. In the cases where genetic mutations exert a large effect on the susceptibility to infection, classic linkage analysis is helpful, but when these alterations exert a smaller contributing effect, other strategies need to be implemented in order to identify involved genes.

There are several related approaches to the elucidation of the complex genetics underlying the immune response to infection (37). Now that the human genome has been cloned, scientists have found many polymorphisms, alleles that co-exist in a population in a stable proportion at a frequency higher than would be accounted for by random mutation. The substitution of one base pair for another, known as a single nucleotide polymorphism (SNP), is the most common form of these polymorphisms, occurring every 300-600 base pairs (38). These SNPs arise both within a codon, causing an amino acid substitution that can alter protein structure, and in the 3' or 5' non-coding regions, where the modification can affect transcription factor binding affinity or efficiency. One common tactic in the study of the genetic basis for infection is use of associative studies that compare frequencies of polymorphisms in likely candidate genes between ethnically matched groups or in affected and non-affected family members. However, these studies must be carefully designed and analyzed because they are

difficult to reproduce, confounded by ethnic variation, subject to publication bias, and at risk for both type I and type II error (39). In addition, polymorphisms are often inherited preferentially alongside other neighboring polymorphisms, in a pattern known as linkage disequilibrium (40). Thus, once a polymorphism is shown to be associated with a particular disease, the specific combination polymorphism with neighboring polymorphisms, called a haplotype, needs to be examined to determine which polymorphism in that region specifically contributes to the phenotype under study.

A similar method utilizes the concept of linkage disequilibrium without relying on guessing a candidate gene that may be involved in disease. This strategy involves genome-wide scans that attempt to find SNPs that segregate with disease in a population or family (41). A related approach uses haplotypes, or collections of SNPs, in association studies without any assumptions of which specific SNP is the crucial contributor to disease variability. Because each polymorphism likely acts in concert with many others as well as with environmental and microbial factors, the mapping of complex diseases is not straightforward. Microarray technology is now allowing for analysis of multiple candidate genes based on differential levels of expression (42). Even in an ideal setting, these observational studies of candidate polymorphisms only demonstrate association and should be used as a starting point for further studies. Genetic studies knocking out genes in animals such as mice and fruit flies also have contributed to the identification of candidate genes for human studies (43).

The TNF-α molecule, which is encoded on the highly variable human leukocyte antigen (HLA) region on chromosome 6, is likely the best-studied part of the immune cascade with regards to the genetics of infections. The promoter of this cytokine contains an SNP at position –308, in which 30% of the Caucasian population has an A instead of a G (44). The less common version of the gene (A), referred to as TNF2, shows a significantly higher level of both spontaneous and induced transcriptional activity compared with the TNF1 version. This allele is

linked to the HLA-DR3 haplotype, which has in turn been associated with higher TNF-α levels *in vivo* and *in vitro* (45, 46). This cytokine plays an integral role in immune defense but in certain cases can be harmful when expressed in excess. It is increased in individuals with septic shock, and it can reproduce the shock syndrome in animal studies. The TNF-α polymorphism has been studied with respect to a number of infectious diseases. The high-expressing TNF2 allele was associated with a 7-fold increase in death or neurological disability in cerebral malaria, a 2.5-fold increase of death in meningococcal disease, and an amplified susceptibility to and 3.7-fold increase in death from septic shock (47, 48, 49, 50). Illustrating the challenges of association studies, these findings have not been corroborated by all investigators (51, 52). This discrepancy may be due to linkages with another HLA locus or could be due to a combination of other factors such as the spectrum of ethnicities studied.

Several other candidate genes also contain functional polymorphisms that have been hypothesized to play a role in sepsis, including TNF-β, interleukin-1 receptor antagonist, interleukin 6, the LPS receptor CD-14, and TLR4 (53, 54, 55, 56, 57, 58, 59, 60). Other candidate genes, such as tissue-plasminogen activator (t-PA), heat shock protein, and LPS-binding protein, have been studied and shown to have no association with infections (61, 62). Though the findings in these studies have not yet translated into improved outcomes, one can imagine a future where physicians have the capacity to identify individual polymorphisms and provide a gene-targeted therapy. For instance, preliminary studies of anti-TNF-α antibodies in individuals with sepsis have not proven effective (63, 64). In light of the studies that have shown the association of TNF-α promoter polymorphisms with septic shock, it may be that the antibodies could be helpful in a subset of infected individuals with the high-expressing polymorphism and severe disease.

Macrophage migration inhibitory factor as a candidate gene involved in severe infections

Macrophage migration inhibitory factor (MIF) is an upstream inflammatory mediator implicated in disease pathogenesis that remains a candidate for genetic studies similar to those with TNF-α. One of the first cytokine activities described in the 1960s, MIF initially was described as a soluble lymphocyte supernatant protein from tuberculin-hypersensitive guinea pigs that inhibited the migration of normal peritoneal macrophages *in vitro* (65, 66). Over the next decades of research, this lymphocyte substance was found to augment many macrophage functions, including adherence, spreading, motility, phagocytosis, and metabolism (67, 68). The initial studies did not identify MIF expressly as a source of these pro-inflammatory actions and, in fact, relied on T-cell supernatants, which contained a mixture of MIF and other mediators, including interleukin-4 and interferon-γ, substances that also exhibit migration inhibitory activity on monocytes. The specific role of MIF in triggering this macrophage activation was confirmed once the protein was molecularly cloned (69, 70, 71). Though these studies established MIF as a cytokine, this protein had more functions and more cellular sources than originally considered.

In addition to its role in the immune response, MIF was being studied simultaneously for its position in the endocrine system. The murine homologue of MIF was discovered as a major protein secreted by the anterior pituitary cell line AtT-20 and by whole pituitary glands in response to systemic lipopolysaccharide (LPS, gram negative endotoxin) injection (72). Pools of pre-formed MIF protein and increased pituitary MIF mRNA levels were measured in these cultured pituitary glands in response to LPS. To examine the role of systemic MIF in an *in vivo* murine model of septic shock, this same group also showed that co-injection of recombinant MIF with intraperitoneal LPS yielded a higher death rate. Anti-MIF antibodies protected from this lethal endotoxemia. Between its macrophage-activating and endocrine functions, it became clear that MIF had far-reaching and important effects on the early immune response to bacterial stimuli.

Though these early studies supported MIF as a product of pituitary and T cells, there was an unexpected initial MIF peak in the serum of T cell-deficient, hypophysectomized mice injected with LPS (73). Further experiments to elucidate the source of this initial release demonstrated that both human and mouse macrophage/monocyte cell lines contained significant pools of pre-formed MIF. The RAW 264.7 macrophage cell line demonstrated an increase in mRNA levels in response to LPS. Interestingly, MIF secretion and mRNA induction decreased at high concentrations of LPS. The authors of this study suggested that macrophage-derived MIF may work together with TNF in an initial response to infection and that pituitary- and T-cell-derived MIF may be responsible for a systemic response to a continued, invasive threat. The MIF protein is pre-made, stored intracellularly, and relies on an atypical protein-secretion pathway that does not use an amino-terminal leader sequence (74). Subsequent studies have supported the concept that MIF can override glucocorticoid-mediated anti-inflammatory actions. MIF expression occurs in most cells of the immune system as well as many tissues throughout the body, including the brain, lung, liver, adrenal gland, and skin (75). The constitutive expression across many cell types allows for a rapid pro-inflammatory response.

In a somewhat surprising discovery, low levels of glucocorticoids, molecules that generally result in immunosuppression and decreased inflammation, promoted MIF release (76). This release from murine macrophages occurred in a bell-shaped dose-response curve, and rats injected with dexamethasone also showed increased serum MIF levels. MIF treatment of human peripheral blood monocytic cells (pBMCs) overcame glucocorticoid-mediated immunosuppression of the macrophage cytokines TNF-α, IL-1, IL-6, and IL8, supporting the pro-inflammatory function of MIF. At higher glucocorticoid levels, MIF lost the ability to counterbalance the steroid-induced immune suppression, which may indicate a natural defense against an overwhelming pro-inflammatory response. Further experiments with animal models demonstrated that recombinant MIF injection blocked dexamethasone protection from lethal LPS endotoxemia. As with many other homeostatic hormone systems such as insulin and glucagon or

PTH and calcitonin, MIF acts to counter-regulate the anti-inflammatory effects of glucocorticoids.

Subsequent studies have supported the idea of MIF overcoming glucocorticoid anti-inflammatory actions. In fact, many of this cytokine's pro-inflammatory effects directly antagonize glucocorticoid actions. In human volunteers, MIF and cortisol showed a similar circadian pattern, and ingestion of cortisone led to increased levels of MIF (77). Hydrocortisone normally blunts LPS-induced increases in NF-κB, TNF-α-stimulated augmentation of arachidonic acid, and T-cell proliferation and cytokine production; MIF prevents these effects (78, 79, 80). In critically ill patients, MIF levels correlate closely with glucocorticoid levels, and the levels of MIF increase with increasing severity of sepsis or ARDS (81). MIF released from monocytes/macrophages and T-cells likely acts in a local manner to balance the anti-inflammatory effects of glucocorticoid hormones.

In addition to its ability to check the action of corticosteroids through stimulation of macrophage cytokine expression, MIF has many other pro-inflammatory roles in the innate and adaptive immune responses, including generation of matrix metalloproteinases, arachidonic acid products, and T-cell cytokines (82, 83, 84). MIF increases insulin release and glucose utilization and activates transcription factors for several pro-inflammatory mediators (85). It inhibits LPS-induced p53-dependent apoptosis and directly increases responsiveness to endotoxemia by augmenting the expression of TLR4, the signal transducer for LPS (86, 87). MIF appears to be involved in an early part of the inflammatory cascade. It is likely that MIF acts as part of a network of cytokines that normally functions to control invasive stimuli; in certain situations this network can be dysregulated and contribute to, rather than protect against, disease. As a part of this network, MIF and TNF-α appear to function in a feedback loop with each cytokine in turn provoking increased release of the other.

Bacterial invasion is likely one stimulus that this cytokine network acts to control. The role of MIF in animal models of infection has been looked at most closely with gram-negative

bacteria. Normally, MIF plays an essential role in control of infection, but as with TNF-α, excess expression can be maladaptive. As discussed above, LPS stimulates MIF release both *in vivo* and *in vitro*. In turn, recombinant MIF exacerbates while anti-MIF antibodies prevent LPS-induced lethality in mouse models (88). *Mif* knockout mice showed resistance to LPS-lethality, cleared intra-tracheal *Pseudomonas aeruginosa*, and had lower serum TNF-α levels compared with wild-type mice (89). Wild-type mice showed high local and systemic levels of MIF release in response to intraperitoneal *Escherichia coli* injection or cecal ligation and puncture (CLP), a model of polymicrobial sepsis (90). In addition, TNF-α-deficient mice, which model immunodeficiency, showed a 62% survival rate when injected with anti-MIF antibodies following CLP compared with a 0% survival rate when injected with control antibodies. This result helped establish the intrinsic effects of MIF in the absence of TNF-α-mediated effects. The anti-MIF antibodies remained protective up until 8 hours after infection in wild-type mice undergoing CLP or *E. coli* injection as well. When co-injected into mice with *E. coli*, MIF led to a 40% increase in mortality. Not all studies support the above data correlating MIF to lethality from infection. For example, one group reported that neutralization of MIF with antibodies enhanced susceptibility to bacterial superinfection after CLP while treatment with recombinant MIF prevented infection (91). In addition, all MIF-deficient mice (*Mif* -/-) inoculated with *Salmonella typhimurium*, an intracellular bacterium, died within 25 days compared with only 50% of wild-type mice, and this increased mortality was associated with a decreased Th1 cytokine response (92). Clearly, the role of MIF in gram-negative infections is not easy to simplify, and likely there is variation in the response to specific bacteria.

The position of MIF in the response to gram-positive bacterial infections has been studied as well, especially the host response to gram-positive exotoxins. Treatment of mouse macrophage cell lines (RAW 264.7), primary murine peritoneal macrophages, and mouse corticotrophic cell lines (AtT-20) with either *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) or *Streptococcus pyogenes* exotoxin A (SPEA), both T-cell superantigens, led to a bell-shaped MIF

response (93). As with LPS and glucocorticoid stimulation of macrophages, MIF was released at lower concentrations of the exotoxins but not in response to higher concentrations. Similar to the bell-shaped dose-response curve to LPS, this prevention of release at high concentrations of toxin may be a natural protection against an overwhelming pro-inflammatory response to infection or trauma. On the other hand, the inhibition could also be an effect of the bacteria to paralyze the immune system. Injection of anti-MIF antibodies prior to a lethal dose of TSST-1 in BALB/c mice increased survival from 8 to 54%. Another series of experiments with *Mif* -/- mice demonstrated low levels of TNF-α release and resistance to a normally lethal injection of *Staphylococcus aureus* enterotoxin B (94). While some gram-positive organisms produce shock through exotoxins (e.g. staphylococcal and streptococcal toxic shock syndromes), others isolated during sepsis do not produce such superantigens and act via different means. Though more research needs to be completed in this area, one unpublished study found that mice with *Streptococcus pneumoniae* pneumonia, a non-toxin driven disease, treated with anti-MIF antibodies demonstrated increased survival associated with lower cytokine production (unpublished data, referenced in 95).

In humans, median serum MIF levels are elevated in septic shock (17.8 ng/ml in 9 patients) and severe sepsis (12.2 ng/ml in 7 patients) when compared with healthy individuals (2.2 ng/ml: 96, 97). Bronchoalveolar lavage samples from patients with acute respiratory distress syndrome (ARDS) show higher levels of MIF than normal volunteers. High MIF levels also have been associated with fatal outcomes in sepsis (98, 99). PBMCs from patients with sepsis showed increased spontaneous and induced (in response to LPS and heat-killed bacteria) release of MIF when compared with those from healthy controls (100). Interestingly, pBMCs from septic patients treated with glucocorticoids/mineralocorticoids released less MIF in response to LPS, peptidoglycan, and heat-killed *E. coli* compared with septic patients not given corticosteroids. This evidence varies from the *in vitro* experiments discussed above where steroid exposure led to a bell-shaped MIF response and may be increasingly relevant as the use of steroids in sepsis has

come back *en vogue* and has been shown to increase survival in certain subpopulations (101). Adrenal insufficiency most likely results in higher MIF levels because the corticosteroid-MIF dyad is thrown off, and this increased MIF level could contribute to morbidity from the inflammatory response to infection. The improved outcomes in patients with sepsis and adrenal insufficiency treated with corticosteroids could be due in part to the suppression of MIF release that is seen with the pBMC data.

MIF levels have also been examined in non-infectious human diseases, and in general, as with infection, higher levels correlate with worse disease. High serum MIF levels have been found in diverse diseases such as rheumatoid arthritis, systemic lupus erythematosus, cancer, and pre-eclampsia when compared with control populations (102, 103, 104). Furthermore, these elevated serum MIF levels have been correlated with disease activity in several inflammatory diseases, including Crohn's disease, multiple sclerosis, and Guillain-Barré syndrome (105, 106, 107). These data, along with the basic science results placing MIF centrally in the pro-inflammatory cascade, make MIF a good candidate for studies of disease pathogenesis and for therapy. MIF has the capacity to tautomerise several non-physiological substrates, and this catalytic site serves as a target for therapy with inhibitors (108). In addition, anti-MIF antibodies remain a potential source of therapy as well.

Genetics of *MIF*

With its central role in inflammation, individual variability in MIF expression may help contribute to susceptibility to or outcome of inflammatory diseases, akin to TNF-α. The *MIF* gene is found on chromosome 22q11.2 and, as discussed above, is expressed constitutively by many cell types. Recent work has identified CD74, a transmembrane protein, as a receptor for MIF, which is required for many, if not all, downstream activities such as activation of the extracellular signal-regulated kinase-1/2 (ERK-1/2)-mitogen-activated protein kinase (MAPK) cascade, cellular proliferation, and prostaglandin production (109). MIF has been reported to work via

intracellular pathways as well, and its diverse actions most likely occur through diverse pathways (110).

The promoter region of the *MIF* gene contains two functional polymorphisms (see Figure I-1 below). The first polymorphism described is a series of five to eight CATT repeats (referred to henceforth by numbers from 5-8) occurring in the –794 position of the *MIF* promoter (111). As summarized by Baugh and colleagues, gene reporter assays showed decreased transcription of the alleles with 5 CATT repeats compared to the 6, 7, and 8 CATT repeat constructs. The initial case-control association study with this polymorphism found that Caucasian rheumatoid arthritis patients with two 5-CATT alleles had lower MIF expression and less severe disease than those with at least one 6-, 7-, or 8-CATT allele. In addition, the proportion of individuals carrying at least one of the 5-CATT alleles decreased incrementally from the normal population to the mild arthritis group and the severe arthritis group. The promoter region contains a series of transcription factor binding sites, including the pituitary specific factor known as pit-1, although the role that this factor plays in differential expression of MIF is currently unknown.

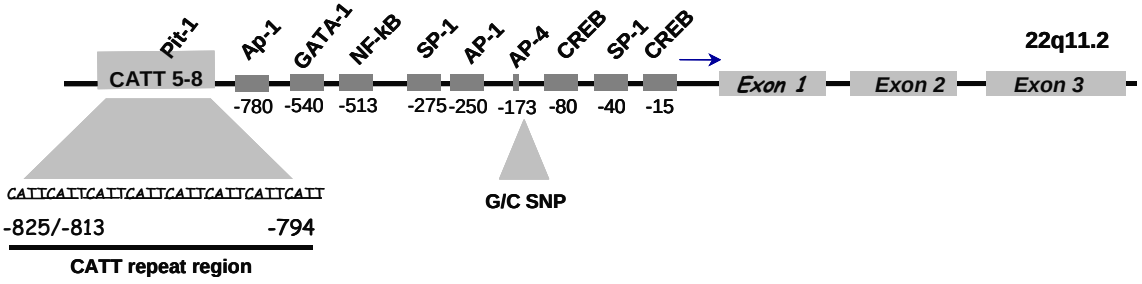

Figure I- 1. MIF promoter and gene. The CATT-repeat polymorphism at position –794 and the G/C SNP at position –173 are illustrated in gray. Modified with permission from Richard Bucala.

The second *MIF* promoter polymorphism described is a G-to-C transition occurring at position –173 in the *MIF* promoter that was associated with an increased risk of systemic-onset juvenile idiopathic arthritis (JIA) in an initial case-control study of Caucasian children from the UK (112). In a follow-up study using the same population, this group confirmed the association between the SNP-C allele and JIA and showed that elevated levels of MIF were present in the

sera of healthy individuals with the C allele (113). Another study of a juvenile population with JIA again demonstrated that those individuals with an SNP-C allele had higher serum and synovial fluid MIF levels (114). Additionally, those with at least one SNP-C allele were less responsive to steroid therapy. The C allele introduces an activator protein-4 transcription factor-binding site, which may be responsible for increased MIF levels (115). Study of the functionality of the SNP-C allele *in vitro* by this group yielded contradictory results with increased levels of expression found in a T lymphoblast line but decreased levels found in an epithelial cell line. This discrepancy could be due to differences in transcription factor binding in different cell lines. There is strong linkage disequilibrium between the rare 7-CATT and SNP-C polymorphisms, which adds another layer of complexity to the association studies looking at one polymorphism or the other. The prevalence of these polymorphisms varies among different populations (116, 117, 118). As reviewed by Zhong *et al*., the frequencies of the 5, 6, and 7-CATT alleles in the Caucasian population are 27.3%, 60.3%, and 11.0%, respectively. In a healthy African population, the frequencies of the 5, 6, and 7-CATT alleles have been reported as 41.3%, 45.0%, and 13.0%, respectively. For the SNP, in the Caucasian population, the frequencies are 88.1% for the G allele and 11.9% for the C allele. In the African population, the G allele frequency is 38.7% and the C allele frequency is 61.3%.

These two *MIF* promoter polymorphisms have been studied in a variety of different settings and have been associated with a number of human diseases. These studies often analyze genotypes in different ways and in varying populations, so it is important to examine the way in which the study was conducted. In reviewing these association studies, it becomes clear that there is no single clear-cut "inflammatory" genotype associated with all diseases that have been studied, but certain patterns have emerged. For example, a Spanish case-control study of individuals with erythema nodosum (EN) demonstrated an association between the *MIF* SNP-C allele and EN secondary to sarcoidosis but not other forms of EN (119). In a study of Japanese individuals with alopecia areata, the –173-C allele was associated with early onset of severe

disease, which had previously been associated with elevated serum MIF (120). Japanese patients with either the –173-GC genotype or who had no –794 5-CATT alleles had a greater than 3-fold relative risk of atopy, though not asthma, when compared with controls (121). On the other hand, a recent study revealed that the 5-CATT allele was associated with lower asthma severity in a Caucasian group of patients (122). In a case-control study of Turkish children, higher frequencies of the –173-GC and –173-CC genotypes were found in children with idiopathic nephrotic syndrome (123). Interestingly, the –173-CC genotype was significantly higher among those with steroid-resistant disease. In another study of Japanese subjects, non-carriers of the –794 5-CATT allele were more likely to be obese while the –173 genotype did not significantly correlate with obesity (124). However, the CATT-5/-173-G haplotype was associated with decreased risk while the CATT-6/-173-G haplotype was associated with increased risk of obesity. The –173-CC genotype was associated with pancolitis-type ulcerative colitis versus more limited types in a population of Japanese patients (125). A case-control study of Caucasian individuals showed an association between the CATT-7 allele, -173-C allele, or the CATT-7/-173-C haplotype with chronic plaque psoriasis (126). As illustrated by these diverse studies, certain *MIF* alleles appear to be associated with many inflammatory diseases, though the exact role for MIF in pathogenesis of disease remains elusive.

While MIF has a clear role in the immune response to infectious stimuli in experimental studies, only recently have published studies begun to link the *MIF* polymorphisms to infectious diseases. A small preliminary study of Zambian children admitted to the hospital with malaria demonstrated that individuals with at least one 5-CATT allele had lower parasitemia loads than those children with no CATT-5 alleles (127). A case-control study of Irish adults with stable cystic fibrosis revealed that individuals with a 5-CATT allele had lower incidence of colonization with *Pseudomonas aeruginosa*, a common respiratory pathogen in this population, but no difference in rates of *Staphylococcus aureus* or *Candida albicans* carriage (128). It is unclear what mechanism underlies these differences. As with inflammatory diseases, the relationship

between MIF genetics and infectious disease is not consistent or clear-cut. This is most likely due to the complexity of the host-microbe interaction and the potential for multiple contributing factors, including host and microbe variation, to disease susceptibility.

Severe infection with *Streptococcus pneumoniae* as a model for genetic studies

Because both sepsis and meningitis can be caused by many pathogens, elucidating the precise genetic determinants for these types of infections is difficult. In order to simplify this question, it is helpful to pick one particular cause of these syndromes. Infection with *Streptococcus pneumoniae* is a specific example of the complex of interaction between host and microbe factors. *Streptococcus pneumoniae* is a gram positive, aerobic diplococcus that is a very common cause of a variety of disease states in the human host, including otitis media, sinusitis, bronchitis, pneumonia, bacteremia, and meningitis (129). Pneumococci reside in the nasopharynx of many individuals, especially during winter, without causing disease (130). What determines whether one person will develop fulminant disease as opposed to occult bacteremia or nasopharyngeal carriage is not clear, and it would seem unlikely for the strain of bacteria to fully explain the variability. Because of the clinical variation in pneumococcal disease, it is a good model to explore genetic polymorphisms that may influence the host response to infection. Two vaccines (one polysaccharide and one conjugated) are available for prevention of pneumococcal disease, and individuals with polymorphisms that contribute to high risk of invasive disease could be candidates for earlier vaccination (131, 132, 133).

The incidence of pneumococcal disease in 1998 was estimated from a population-based surveillance of 9 states to be 23.2 cases per 100,000 people, with higher rates among the very young and very old as well as among blacks of all ages (134). Case-fatality was 10% among all cases and was highest in the elderly (20.6%) and in those with meningitis (16.7%). In 2002-2003, there were an estimated 23,600 cases and 4500 deaths per year in American adults over 50 years old, a decrease of 6250 cases and 550 deaths from the 1998 pre-childhood vaccination data (135).

The higher rates of pneumococcal disease that have been reported among blacks and native Americans could be due in part to higher prevalence of underlying co-morbidities, such as HIV, sickle cell disease, and diabetes, or to disparities in vaccination rates (136, 137, 138, 139). Yet, these disparities have not been fully examined or accounted for, and the fact that many of the genetic polymorphisms show racial and ethnic variation suggests that these may also play a role in the different rates of pneumococcal infection among blacks and Native Americans. Currently, nothing is known about the prevalence of the *MIF* polymorphisms in groups of patients with these co-morbidities. The incidence of invasive pneumococcal disease rises sharply during the winter months, possibly due to a concomitant increase in respiratory viral illnesses (140). Despite effective vaccinations, pneumococcal disease remains a significant challenge.

To fully understand the complexity of pneumococcal disease, the microbe and the host need to be examined. Pneumococci have played an important historical role in the discovery and elucidation of principles of humoral immunity and of molecular genetics (141). As gram-positive bacteria, pneumococci do not act through LPS but instead act via an oligosaccharide capsule, cell wall, and surface proteins to gain access into the host and incite an inflammatory response through extracellular receptors (142). More than 90 capsular serotypes, each stimulating the appearance of specific antibodies, have been identified (143). The cell wall is composed largely of peptidoglycan (PGN) and teichoic acid and has a number of associated surface proteins, and there is an underlying three-layer cell membrane containing lipids and teichoic acid.

In order for the pneumococcus to cause disease, it must invade into areas where it does not normally inhabit, avoid immune clearance, replicate, and cause tissue damage. Once bound to cells by molecules known as adhesins, the bacteria are cleared by, colonize, or invade the host. In order to cause disease, the bacteria avoid phagocytosis through multiple mechanisms, including the capsule and production of certain toxins, such as pneumolysin. Once the pneumococci have invaded, they activate the complement cascade. In addition, peptidoglycan and techoic acid bind to toll-like receptor 2 and pneumolysin to toll-like receptor 4 to upregulate proinflammatory

cytokine production (144, 145). This inflammatory response is often responsible for the clinical disease caused by pneumococci. Individual genetic variability likely plays a role in severity and type of invasive streptococcal disease. For instance, certain variants in the *MBL* gene, which activates the complement cascade, correlate with increased susceptibility to invasive infection (146). Another study found that certain polymorphisms in IL-10, a key anti-inflammatory molecule, were associated with septic shock from pneumococci (147).

Many other conditions also have been shown to predispose individuals to pneumococcal infection (148). Because clearance of the bacteria relies on humoral immunity, any congenital or acquired deficiency of antibody production contributes to invasive disease. Other immune deficiencies, including complement defects, neutropenia, neutrophil dysfunction, and asplenia, also predispose to infection. The increased incidence of *Streptococcus pneumoniae* infections among certain populations, including those with liver disease, pulmonary disease, diabetes, old age, viral infections, and alcoholism, is often multifactorial. For instance, older individuals produce fewer and faulty immunoglobulins and have poor gag reflexes, many comorbidities, and, often, malnutrition. When considered in the context of these diverse bacterial and host factors, pneumoccal infection is pathophysiologically complex. Its ability to cause a wide variety of clinical syndromes, including sepsis and meningitis, makes it a good candidate infection to examine underlying genetic predispositions to particular disease phenotypes.

SPECIFIC AIMS AND HYPOTHESIS

This study comprised *in vitro* and *in vivo* components. The goals of the *in vitro* portion were to confirm MIF release from monocytic cells stimulated with bacterial products and to examine stimulated MIF release from peripheral blood monocytic cells (pBMCs) harvested from healthy individuals with varying genotypes. We hypothesized that pBMCs cultured from individuals with low-expressing *MIF* genotypes (5-CATT alleles or SNP-GG) would have less

MIF release at baseline or in response to microbial product stimulation than those individuals with high-expressing *MIF* genotypes (7-CATT or SNP-C alleles).

The goal of the *in vivo* translational study was to examine whether *MIF* promoter genotypes affect severity and type of invasive pneumococcal disease. Our hypothesis was that individuals with the low-expressing *MIF* genotypes (5-CATT alleles or SNP-GG) would have less severe systemic inflammatory responses while individuals with the high-expressing *MIF* genotypes (7-CATT or SNP-C alleles) would have a more severe systemic inflammatory response to infection with *Streptoccus pneumoniae*.

MATERIALS AND METHODS

Contributions: The W.M. Keck facility at Yale University ran the DNA fragment analysis assays and generated primers for PCR reactions. Members of the Bucala Laboratory carried out determination of the healthy control MIF genotypes and generated reagents for the human MIF ELISA. The author of this thesis completed all other experiments. A presentation of the preliminary data from this report was given at the Infectious Disease Society of America 43rd Annual Meeting in October 2005.

Reagents: LPS from *E. coli* serotype 011:B4 (Fluka) was resuspended in pyrogen-free water at a concentration of 200 μg/ml, aliquoted, and stored at –20˚C. PGN from *Staphylococcus aureus* (Fluka) was resuspended in pyrogen-free water at a concentration of 2 mg/ml, aliquoted, and stored at –20 ˚C.

Cells: All cells were maintained at 37°C in a humidified environment with 5% CO_2. THP-1 human monocytes (American Type Culture Collection) were cultured in Dulbecco's Modified Eagles medium (DMEM, Gibco), which contained 10% fetal bovine serum (FBS, Gibco) unless otherwise noted. Peripheral blood monocytes (pBMCs) were purified in a sterile fashion using the Ficoll-Paque plus method as follows (Amersham Biosciences). Five ml of venous blood in 1 mg/ml K_2EDTA was collected in Vacutainer tubes (Fisher) from 10 healthy donors with *MIF*

genotypes previously ascertained by members of the Bucala Laboratory. Blood was diluted to 35 ml with cold phosphate buffered solution (PBS) in a 50 ml conical tube. Fifteen ml of sterile Ficoll-Paque Plus was layered at the bottom of each tube with careful maintenance of an intact blood:Ficoll interface. Tubes were centrifuged at 1800 revolutions per minute for 30 minutes at 18°C without brakes. The white blood cell layer was removed from the Ficoll-Paque Plus:plasma interface, transferred with a sterile Pasteur pipette to a 15 ml conical tube, and washed twice with PBS. For each wash step, tubes were centrifuged at 1800 RPM for 5 minutes at 4°C with brakes, and the supernatant was discarded carefully. Cells were resuspended to 10^5 cells/ml in DMEM with 10% FBS, plated in 96-well tissue culture plates (Becton-Dickenson), and cultured overnight. The adherent cells were then washed three times with PBS and used for experiments described below. For experiments comparing genotypes, these cells were prepared in parallel to minimize variation.

Stimulation of cells with LPS and PGN: THP-1 cells were cultured overnight prior to experiments at 5 x 10^5 cells/ml and plated at 2 x 10^4 cells/well in 96-well tissue culture plates (Becton-Dickenson). THP-1 cells or adherent pBMCs were treated with DMEM + 10% FBS ± 10 ng/ml-10 μg/ml LPS or 100 ng/ml-100 μg/ml PGN for 6-24 hours. Supernatants from selected wells were collected, and cellular debris was centrifuged into a pellet. The supernatants were stored in 1.5 ml centrifuge tubes at –20°C until further analysis. Other wells with stimulated THP1 or pBMCs were used for LDH assays or lysis studies, as described below.

Cell lysis: To determine intracellular MIF stores, both THP-1 cells and pBMCs were lysed after stimulation with LPS or PGN with a 1:10 ratio of Cell Lysis Solution (Promega) to volume. The lysate was centrifuged to eliminate debris, and the supernatants were stored at –20°C until further analysis.

Lactate dehydrogenase (LDH) assay: To examine cell death, CytoTox 96 Non-radioactive Cytotoxicity Assays (Promega) were carried out. Cell supernatants were collected at the specified time-points. One-hundred μl of supernatant from the above experiments was incubated with 50 μl

Substrate Solution for 30 minutes in the dark. The reaction was arrested with the Stop Solution and analyzed for colorimetric changes with a spectrophotometer.

Enzyme-linked immunosorbant assay (ELISA) for detection of MIF: Levels of MIF in cell supernatants and in the plasma of subjects were obtained by sandwich ELISA (149). Ninety-six well microtiter plates (Nunc) were coated overnight at 4°C with 1 μg/well of mouse monoclonal IgG antibody directed against human MIF IgG. Plates were washed 3 times with wash buffer containing 0.05% Tween20 (Sigma) in PBS, blotted dry with clean paper towels, and blocked overnight at 4°C with 250 μl of buffer containing 1% BSA and 1% sucrose in PBS. Before applying samples, the blocking buffer was removed, and plates were blotted dry with paper towels. Recombinant human MIF was prepared by serial dilution from 1.6-100 ng/ml for the standard curve. The assay was sensitive for concentrations of MIF from 0 to 50 ng/ml. Fifty μl sample or standard plus 50 μl of conjugate mouse anti-human MIF-HRP diluted 1:250 in reaction buffer containing 1% BSA, 1 mM EDTA, and 0.05% Tween20 (Sigma) in PBS was applied to each well. The plate was incubated for two hours in the dark at room temperature and then washed 5 times with 250 μl wash buffer. One-hundred μl TMB substrate-chromagen (Dako Corporation) was added to each well, and plates were incubated for 30 minutes at room temperature in the dark. One hundred μl stop solution containing 2 M H_2SO_4 and 0.5 M HCl was added to each well, and the optical density of each plate was determined with a microplate reader set to 450 nm and 590 nm.

Bradford protein assay: To assess total protein content in pBMCs, Bradford Assays (Bio-Rad) were carried out in serum-free conditions. After cell stimulation, adherent cells were washed three times with PBS and lysed in a solution of 10^5 cells/100 μl 0.1% Triton-X (Sigma). Protein standards were made with serial dilutions of 1 mg/ml bovine serum albumin (BSA) in water with concentrations ranging from 1-10 μg/ml BSA. One to 50 μl of each sample or standard was mixed with dH_2O to 800 μl and then with 200 μl Bradford Solution. Samples were incubated at room

temperature for 5 minutes. Samples were read in triplicate, and absorbance at 595 nm was calculated using a spectrophotometer.

Patient samples: From September 2004 to September 2005, hospitalized patients over 18 years of age with positive cultures for *Streptococcus pneumoniae* isolated from sterile body fluids (e.g. blood, CSF, pleural fluid, joint aspirates, peritoneal fluid) were identified through the microbiology laboratories at the Hospital of Saint Raphael, Saint Francis Hospital, and Yale-New Haven Hospital. Permission to approach the potential subjects for enrollment and assessment of capacity to consent was obtained from the attending physician. Written informed consent was obtained from all subjects or surrogate decision-makers. The respective hospital investigation review boards and the Yale Human Investigation Committee approved the study protocol. Blood samples were collected into EDTA-coated Vacutainer tubes (Fisher) from subjects by venopuncture or from central lines. Alternatively, blood samples previously collected during the hospitalization and stored at 4°C by the hospital chemistry laboratories were obtained. Samples were transferred to 15-ml conical tubes and separated by centrifugation for 10 minutes at 2,000 rpm. Serum and buffy coats were collected and stored at –80°C until further analysis. Certain whole blood samples were spotted onto filter paper, dried, and stored at –20°C.

Data collected: Information from the patient's admission record, including presenting signs and symptoms, physical findings, laboratory and imaging results, and outcomes, was collected onto data-extraction forms and entered into Microsoft Excel spreadsheets for statistical analyses.

DNA extraction: Genomic DNA was isolated per manufacturer protocol from patient serum using DNAzol Reagent (Invitrogen) or from dried, whole blood-spotted filter paper using the QIAmp DNA Blood Mini kit (Qiagen). Briefly, for the DNAzol protocol, 900 µl of DNAzol Reagent was added to 100 µl of patient serum and incubated for 3 minutes at room temperature. The DNA was mixed by inversion with 500 µl of 100% ethanol, incubated for 3 minutes at room temperature, and centrifuged at 4,000 x *g* for 1 minute at room temperature to precipitate and pellet the DNA. The pellet was then resuspended twice with 1 ml of 70% ethanol. After

resuspension, the DNA was re-pelleted at 4,000 x *g*, and the ethanol was decanted. After the second wash, the ethanol was removed by pipette, and the DNA was air dried for 15 seconds in an open tube under a fume hood. The DNA was then resuspended with 200 µl of 8 mM NaOH and stored at –20°C. For the QIAmp method, Qiagen supplied all reagents. Briefly, the dried blood spot from the filter paper was placed into a 1.5-ml microcentrifuge tube with 180 µl of Buffer ATL and incubated at 85°C for 10 minutes. Twenty µl Proteinase K stock solution was added, vortexed, and incubated at 56°C for 1 hour. Two-hundred µl Buffer AL was added, vortexed, and incubated at 70°C for 10 minutes. Two-hundred µl of 100% ethanol was added and vortexed. This solution was applied to a QIAamp Spin Column in a 2 ml collection tube and centrifuged at 6,000 x *g* for 1 minute. The column was placed in a new collection tube, 500 µl Buffer AW1 was added, and the sample was centrifuged at 6,000 x *g* for 1 minute. The column was placed into another collection tube, 500 µl Buffer AW2 was added, and the sample was centrifuged at 20,000 x *g* for 3 minutes. The column was then placed in a 1.5-ml microcentrifuge tube, and 150 µl of dH2O was added. The sample was incubated at room temperature for 1 minute, and then centrifuged at 6,000 x *g* for 1 minute, and stored at –20°C.

Multiple displacement amplification: Genomic DNA was amplified by multiple displacement amplification overnight at 31°C in a 99 µl solution containing: 37.5 mM pH 7.5 Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM each dNTP, 0.05 mM thiophosphate-modified primer (5'-NNNN*N*N, *=phosphorothioate), 0.2 units yeast pyrophosphatase (Roche), 1 unit Phi29 polymerase, and 1-5 ng genomic DNA (150). The reaction was stopped at 75°C for five minutes, and the product was analyzed on a 1% ethidium bromide-stained agarose gel.

Fragment analysis protocol for CATT repeat polymorphism: The *MIF* gene was amplified specifically from the genomic MDA products by polymerase chain reaction (PCR) using the following reagents: 22.5 µl Invitrogen Supermix, 1 µl dH_2O, 0.5 µl CATT forward primer (TGCAGGAACCAATACCCATAGG), 0.5 µl CATT reverse primer (XAATGGTAAACTCGGGGAC, X=6-FAM label), and 0.5 µl of the DNA product from the

MDA reaction. The reactions were amplified with the following thermal profile: 1 cycle at 95°C for 12 min followed by 40 cycles of 95°C for 30 sec, 54°C for 30 sec, and 72°C for 60 sec, and one cycle of 72°C for 10 min. Samples were then cooled to 4°C. To confirm that the amplified gene products were the anticipated sizes of 340-352 base pairs, the PCR products were resolved on 1.5 % agarose gels stained with ethidium bromide. One µl of each product was then diluted in 9 µl of dH_2O and loaded into a 96-well Applied Biosystems MicroAmp Optical Plate. Samples were resolved using an ABI 3730XL DNA Sequencer (Applied Biosystems) with the LIZ500 dye size standards. The data was analyzed using Genemapper version 3.7 software (Applied Biosystems).

Single nucleotide polymorphism analysis: DNA from MDA reactions was purified using the Qiagen Protocol for Cleanup of Genomic DNA as follows. One hundred µl of genomic DNA, 10 µl of buffer AW1, and 250 µl of buffer AW2 were mixed by pulse-vortexing and transferred to a QIAmp MinElute Column (Qiagen). Each column was centrifuged at 6,000 x *g* for 1 minute and placed in a clean collection tube. Five hundred µl of buffer AW2 was added, and the column was centrifuged again at 6,000 x *g* for 1 minute and placed in another clean collection tube. Each column was centrifuged at 20,000 x *g* for 3 minutes and placed in a clean 1.5 ml microcentrifuge tube. Each column was eluted with 20 µl of buffer AE for one minute and then centrifuged at 20,000 x *g* for one minute. The concentration of DNA was calculated using the 260/280 spectrophotometer absorbance ratio. DNA was diluted in dH_2O to 5 ng/µl. For the allelic discrimination PCR reaction, the following components were combined: 2.5 µl 2X TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 0.25 µl 20X Assays-On-Demand SNP Genotyping Assay Mix for the MIF –173 G/C SNP (Applied Biosystems), and 2.25 µl genomic DNA diluted to 5 ng/ml. Samples were loaded into 384 well clear optical reaction plates (Applied Biosystems) and covered with optical adhesive covers (Applied Biosystems). The PCR reaction was completed at the Yale University Keck Affymetrix Facility using an ABI Prism 7900 machine. The reaction parameters were: 1 hold cycle for 10 minutes at 95°C followed by 45

cycles of denaturation for 15 seconds at 92°C and annealing/extension for 1 minute at 60 °C. The alleles were analyzed with SDS v2.2 software.

Disease type classification: Pneumonia was classified based on clinical diagnosis as documented in the patient's medical record, which included physical exam and chest radiography findings. Classification of a case as meningitis was based either on positive pneumococcal cultures grown from the CSF or from charted clinical signs and symptoms consistent with meningitis plus positive blood cultures and a lumbar puncture demonstrating pleocytosis with predominance of neutrophils, high protein, low glucose, and high opening pressure.

Disease severity classification: The diagnosis of the severity of infection was based on the consensus criteria for the systemic inflammatory response syndrome and sepsis published by the American College of Chest Physicians (151). Sepsis was defined if the subjects with documented invasive *S. pneumoniae* had at least two of the following abnormalities: temperature > 38 $^{\circ}$C or < 36 $^{\circ}$C; heart-rate > 90 beats/minute; respiratory rate > 20 breaths/minute or $PaCO_2$ < 32 mmHg; white blood-cell count > 12,000 cells/mm^3, < 4,000 cells/mm^3, or > 10% immature band forms. Severe sepsis involved sepsis plus evidence of end-organ damage. Because only clinical data from time 0 was collected, it was not possible to identify those with septic shock refractory to resuscitation efforts, which requires ongoing data. Those individuals with shock at time 0 were placed into the severe sepsis category. For this study, the definition of end-organ damage included but was not limited to: systolic blood pressure (SBP) < 90 or mean-arterial pressure (MAP) < 70 or need for pressors for > 60 minutes; urine output < 0.5 cc/kg body weight for > 60 minutes; PaO_2/FiO_2 < 250; platelets < 80,000 cells/mm^3 or decrease by more than 1/3; pH < 7.30 and lactate > 1.5x upper limit of normal. Evidence of meningitis was included in the definition of end-organ damage, and these individuals were placed in the severe sepsis category. The author of this study was blinded to *MIF* genotype when assignments of disease severity were made.

Statistical analysis: The data were analyzed using GraphPad Prism software (GraphPad Software) and Microsoft Excel (Microsoft). Data are presented as mean ± SD for all experiments.

Differences between two means were calculated using independent two-tailed t-tests. Distributions were presumed to be normal unless noted in the text. For the comparison of mean MIF levels in subjects with pneumococcal infections, a normal distribution was defined as a skewness that was not more than twice the standard error for skewness. Differences between more than two means were tested using a one-way analysis of variance (ANOVA) test with post-test analysis by Tukey test. The relationship between genotype and disease type or severity was examined using the Fishers' exact test for contingency tables containing small numbers. A p-value less than 0.05 was taken as significant. Because this was an exploratory study, no assumptions about power were made prior to completion.

RESULTS

MIF release from THP-1 monocyte cells stimulated with LPS and PGN

Previous studies have shown that macrophage cell lines release MIF when stimulated with LPS (152). To confirm these results, THP-1 cells, a monocytic cell line population, were incubated with varying concentrations of LPS for 6-24 hours, and MIF release was determined by ELISA assay. Data are not shown for various time points because the 6-hour incubation yielded the most MIF release with the least cell death as seen under the microscope. As shown in Fig. 1, the optimal release of MIF occurred with an LPS concentration of 10 μg/ml for 6 hours. Lower concentrations of LPS did not yield MIF release. Figs. 2 and 4 show further experiments confirming MIF release from THP-1 cells treated with 10 μg/ml LPS for 6 hours. This LPS concentration was significantly higher than the dilutions used in the first report of LPS-stimulated MIF release by Calandra and colleagues. In that study, MIF release was measured by Western blot from RAW 264.7 macrophages with as little as 10 pg/ml LPS, peaked at 1 ng/ml, and was not observed at LPS concentrations greater than 1 μg/ml. The results from that study may have differed from those observed in the experiments discussed above because MIF release was measured in a different, less objective manner and because a different cell line was used. In this study, MIF was released in significant amounts into the supernatants of cells treated with high concentrations of LPS when compared to untreated cells.

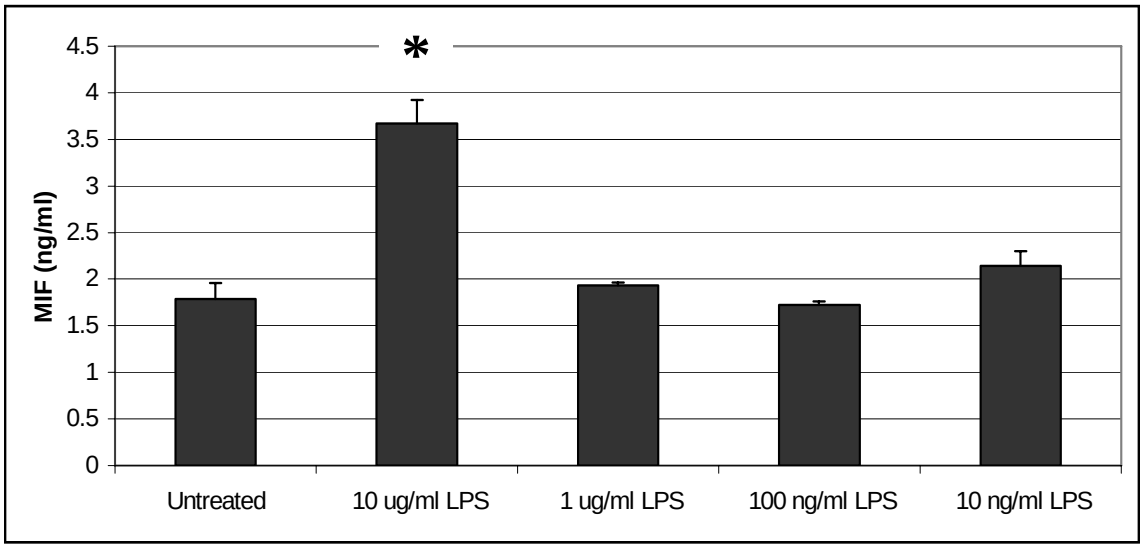

Figure 1. THP1 cells treated with varying concentrations of LPS. Each column represents the mean ± SD of three wells. *One-way ANOVA, $p = 0.007$. Significant differences ($p < 0.05$) were seen for untreated vs. 10 μg/ml LPS-treated cells; 1 μg/ml vs. 10 μg/ml LPS-treated cells; and 10 ng/ml vs. 10 μg/ml LPS-treated cells; and between 100 ng/ml vs. 10 μg/ml LPS-treated cells at a significance of $p < 0.01$.

To assess cell membrane integrity, lactate dehydrogenase (LDH) assays were performed (Figs. 3, 5). Because this enzyme is constitutively expressed in all cells, and cell death induces enzyme release, the presence of LDH indicates cell death. The THP-1 cells treated with LPS showed significantly higher levels of cell death when compared with the untreated cells in some, though not all, experiments (Figs. 3, 5). The increased MIF measured in the supernatants of those cells treated with LPS, thus, may have been partially due to cell death. Since MIF is stored in preformed pools in cells, cell death will lead to elevated MIF in cell supernatants. However, even in the experiments where LPS-treatment did not lead to increased cell death (Figs. 4 and 5), there was still significant MIF found in the supernatants, indicating that MIF release from pre-formed pools in living cells is also likely a contributor to increased cytokine levels.

To explore the effect of gram-positive cell walls on monocyte MIF release, PGN at concentrations of 100 ng/ml to 100 μg/ml was used to stimulate THP-1 cells. PGN-treated cells did not release significantly higher amounts of MIF than untreated cells at any concentration (Fig. 4). The LDH assays for this experiment demonstrate that PGN did not induce cell death (Fig. 5). The concentration of PGN was similar to that used in previously published monocyte stimulation

experiments, so likely these results represent a biological lack of effect of this particular molecule (153,154).

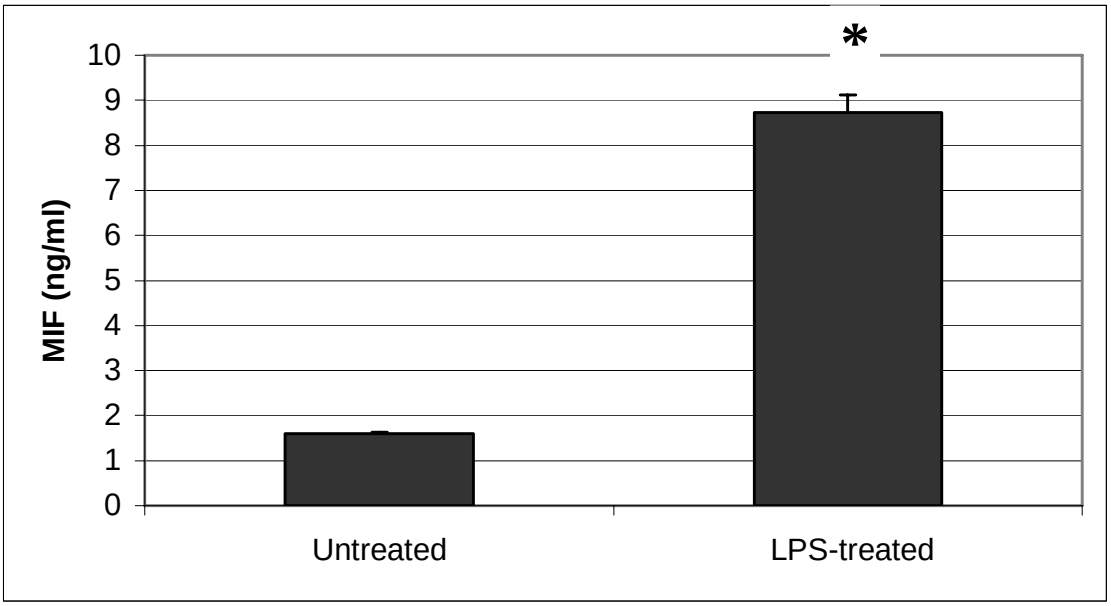

Figure 2. MIF release from THP-1 cells treated with LPS. 10 μg/ml LPS was applied for 6 hours. Each column represents the mean ± SD of three wells. Each experiment was repeated at least three times. *T-test, p = 0.0032 for MIF release from untreated vs. treated cells.

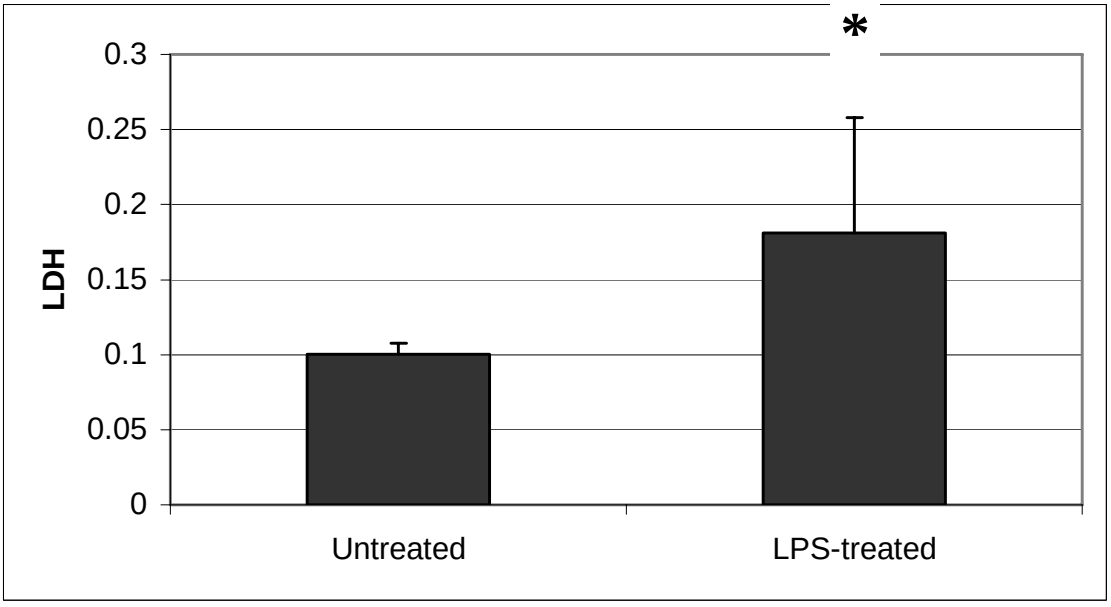

Figure 3. Cell integrity of THP-1 cells treated with LPS. Cell integrity was measured by LDH release. Each column represents the mean ± SD of three wells. Each experiment was repeated at least three times. *T-test, p = 0.0005 for LDH release from LPS-untreated vs. treated cells.

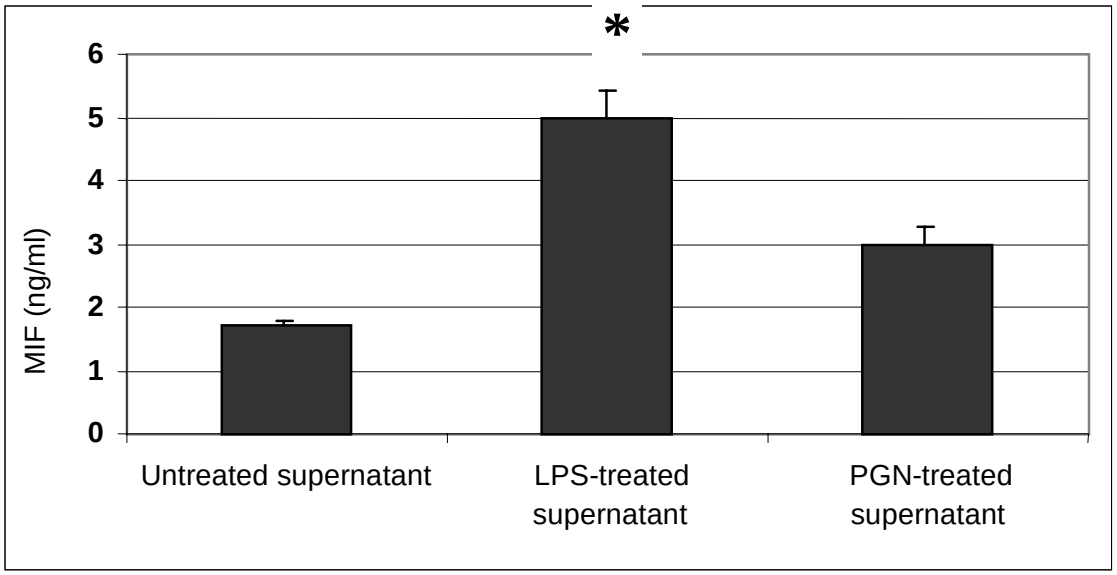

Figure 4. MIF content and release from THP-1 cells treated with LPS or PGN. Cells were stimulated for 6 hours with LPS (10 μg/ml) or PGN (100 μg/ml) Each column represents the mean ± SD of three wells. Each experiment was repeated at least three times. *T-test, p = 0.0120 for MIF release from LPS-untreated vs. treated cells. T-test, p = 0.8632 for PGN-treated vs. untreated cells.

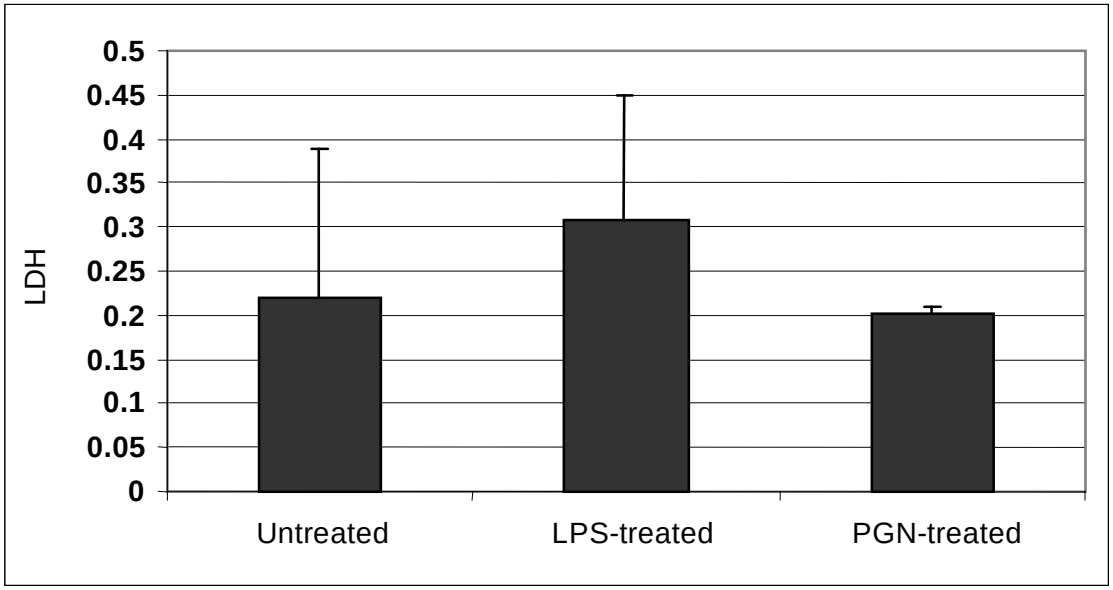

Figure 5. Cell integrity of THP-1 cells. Cell death measured by LDH release. Each column represents the mean ± SD of three wells. Each experiment was repeated at least three times. T-test, p = 0.5332 for LDH release from untreated vs. LPS-treated cells. T-test, p = 0.8490 for PGN-treated vs. untreated cells.

Basal serum MIF levels from healthy individuals with different MIF genotypes

Afternoon serum MIF levels were measured by ELISA from healthy control pBMC donors with known *MIF* CATT-polymorphism genotypes. For each individual, alleles are numbered 5-8, depending on the number of CATT repeats, and genotypes are listed as two

numbers, indicating the repeats present on each allele. The SNP–173 genotypes for these individuals was not known. As shown in Fig. 6, the mean serum MIF levels between individuals did not show a perfectly reliable pattern. However, a one-way ANOVA test revealed that there were significant differences between several of the samples (see Fig. 6), indicating that the 5-allele containing genotypes in general secreted lower levels of MIF than the non-5 containing genotypes. Though the sample size was very small, this suggestive trend is consistent with published data indicating increasing serum MIF levels with increasing numbers of CATT repeats (155). The CATT-5,5 #1 sample had slightly higher MIF release than would be expected if there were a completely linear relationship between CATT repeats and MIF release. Yet, since each column represents only one person, variation between individuals may have accounted for this deviation. The fact that CATT-5,5 #2 sample did not show this elevation supports this idea of individual differences. The existence of individual variation in MIF release based on genotype is interesting and could support a mechanism for any observed differences.

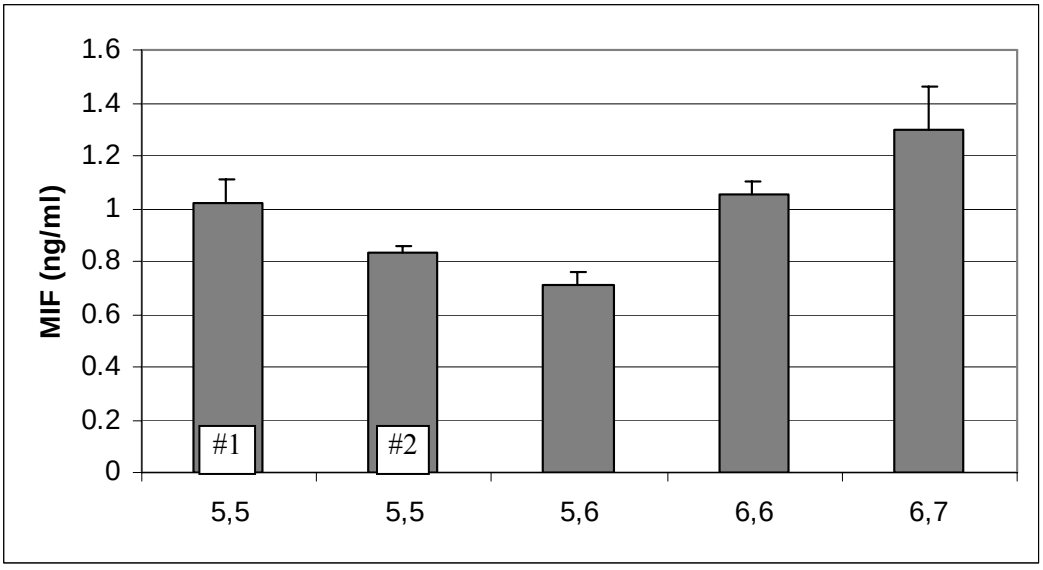

Figure 6. Basal afternoon serum MIF levels from individuals with known CATT genotypes. Each column represents an individual with two CATT alleles (5-8) separated by commas and is the mean ± SD of three wells. One-way ANOVA, $p < 0.0001$. Differences are significant ($p < 0.05$) for: 5,5 #1 vs. 5,6; 5,5 #1 vs. 6,7; 5,5 #2 vs. 6,6; 5,5 #2 vs. 6,7; 5,6 vs. 6,6; 5,6 vs. 6,7; 6,6 vs. 6,7.

MIF release and storage in unstimulated pBMCs from individuals with different genotypes

Blood was drawn from healthy controls with known –794 CATT genotypes, and pBMCs were purified by Ficoll gradient. For each experiment, blood was drawn from all subjects at the same time of day to avoid circadian variation in MIF expression and release. After purification, the pBMCs were lysed, and MIF content was determined by ELISA. To standardize for overall cellular transcription activity, MIF content was normalized to total protein content as determined by Bradford assay. As shown in Fig. 7, there were no significant differences in MIF-to-total protein content ratios in pBMCs from individuals with different CATT genotypes (one-way ANOVA, $p = 0.7839$). These experiments were repeated multiple times with significant intra-individual variation.

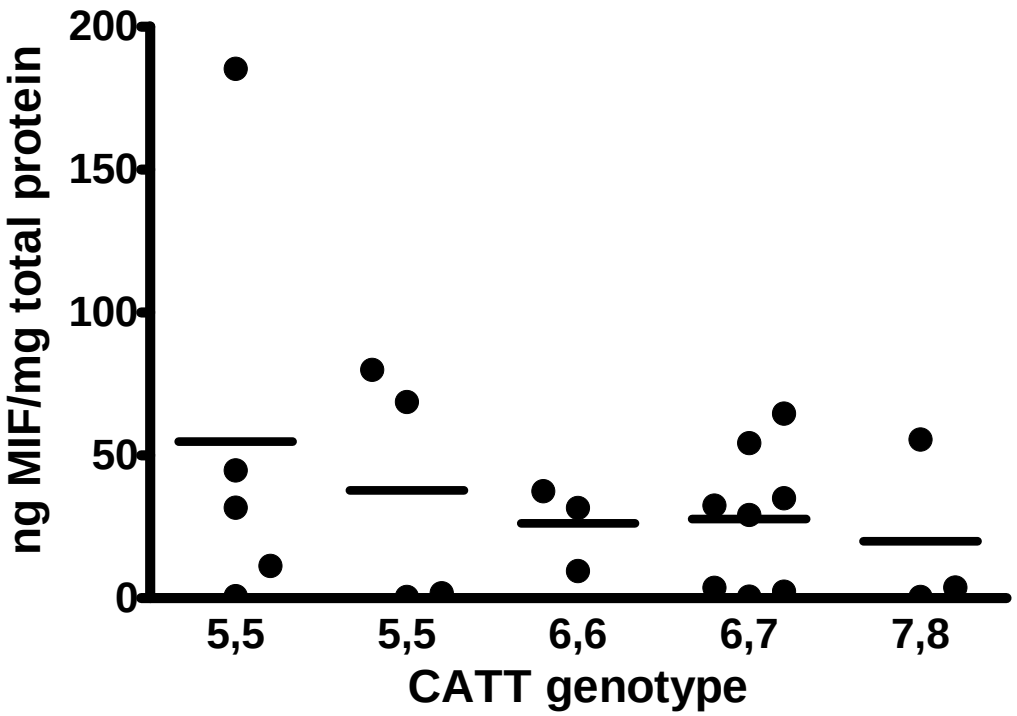

Figure 7. Basal MIF stored in pBMCs from individuals with different CATT genotypes. MIF protein levels are normalized to total cell protein levels. Each column represents one individual with two CATT-alleles separated by commas (5-8). Each point signifies one experiment and represents the mean of three wells. The horizontal lines represent means of all points in a column. One-way ANOVA $p = 0.7839$.

Basal MIF release was determined from supernatants of the adherent pBMCs from healthy individuals cultured overnight. As shown in Fig. 8, mean basal MIF release did not differ

between individuals with various CATT genotypes (one-way ANOVA, p = 0.8460). There was great variation in basal MIF release for all individuals between experiments, which may have masked any differences. LDH assays were not completed, so it is not known whether the differences between individual MIF supernatant levels is due to variation in cell death rates or variation in secretion.

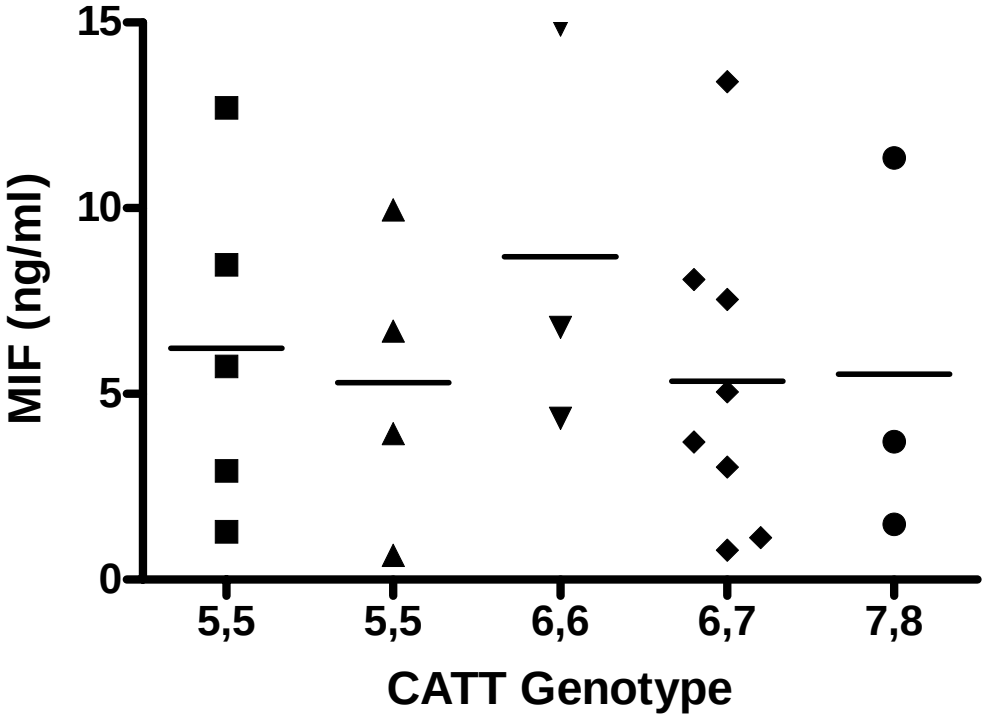

Figure 8. Basal MIF released from pBMCs from individuals with different CATT genotypes. Each column represents one individual with two CATT-alleles separated by commas (5-8). Each point signifies one experiment and represents the mean of three wells. The horizontal lines represent means of all points in a column. One-way ANOVA p = 0.8460.

MIF release from pBMCs stimulated with LPS

Adherent pBMCs were stimulated with 10 μg/ml LPS for 6 hours, and MIF release was measured from cell supernatants by ELISA. As shown in Fig. 9, there were no significant differences in stimulated MIF release according to genotype (ANOVA, p = 0.3409). Again, MIF release varied dramatically between experiments, indicating that intrinsic variations may mask any effect of CATT genotypes. Because these studies relied on pBMCs taken from individuals

over several different time points, it is likely that many other host factors contributed to the level of response of the cells. Such factors could include the level of stress, which would alter endogenous corticosteroids, or the possibility of sub-clinical infections, which could affect the responsiveness of cells. In addition, LDH assays were not performed for these pBMC experiments, so disparities in levels of cell death in response to LPS may have contributed to the wide deviations. Furthermore, because the sample size was small, individual variation in LPS-stimulated MIF release could have be due to multiple other factors, such as TLR4 polymorphisms or concomitant stress leading to differing corticosteroid levels.

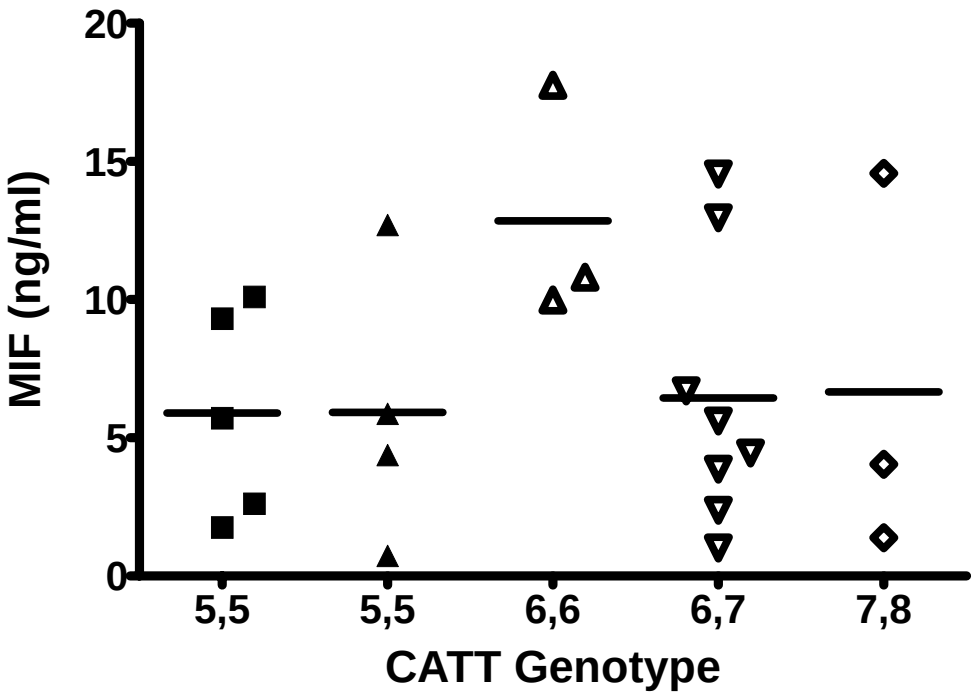

Figure 9. MIF released from pBMCs of individuals with different genotypes stimulated with LPS. Cells were stimulated by 10 μg/ml LPS for 24 hours. Each column represents one individual with two CATT-alleles separated by commas (5-8). Each point signifies one experiment and represents the mean of three wells. The horizontal lines represent means of all points in a column. One-way ANOVA p = 0.3409.

Patient enrollment and baseline characteristics

Between September 2004, and September 2005, 68 patients over the age of 18 with invasive *S. pneumoniae* infections were admitted to the three participating hospitals and were eligible for enrollment based on age and microbiological data (Fig. 10). Patients were required to

be in the hospital at the time of consent. Of these 68 patients, 36 individuals died or were discharged home before their microbiological cultures grew colonies and, thus, they could not be identified and enrolled while still hospitalized. Thirty-two eligible patients or their representatives were approached, and 30 individuals consented to be a part of this observational study.

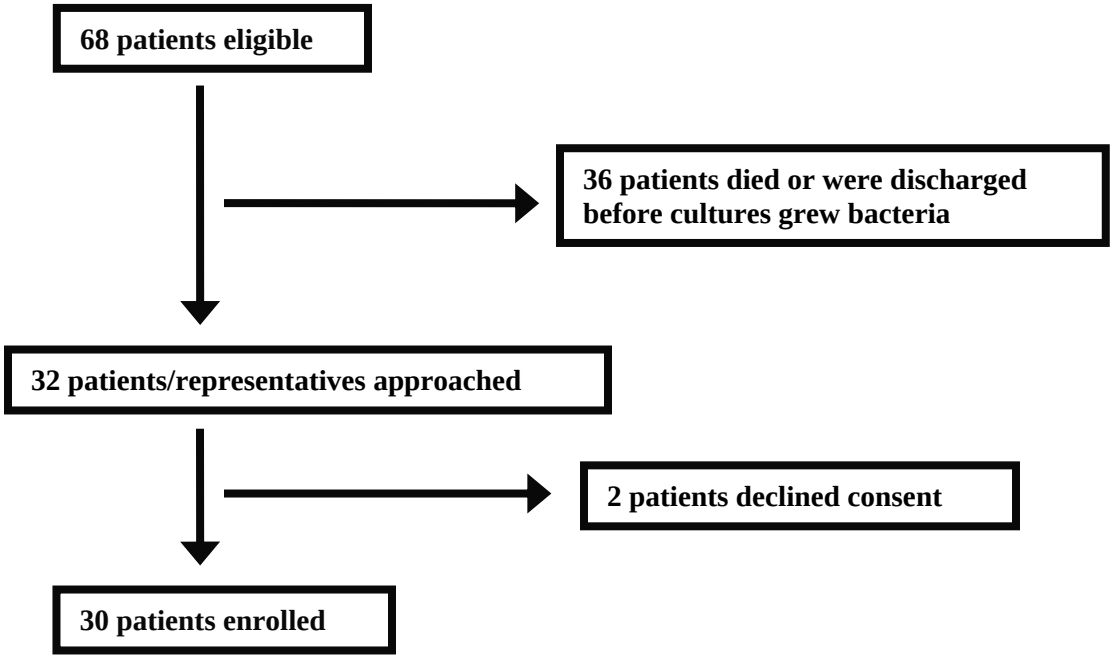

Figure 10. Patient enrollment. Eligibility was determined if individual was at least 18 years old, had microbiological cultures from a normally sterile site that grew *S. pneumoniae*, and were in the hospital at time of consent.

Baseline characteristics for enrolled subjects are shown in Table 1 and Fig. 11. The high mean heart rate, respiratory rate, and white blood cell count indicate the systemic response to the invasive bacterial infection. The ethnic/racial distribution reflects the diversity of the communities serviced by the participating hospitals in New Haven and Hartford. Clinical characteristics were recorded as the value closest to the time of antibiotic administration (defined as time 0). Sources of infection based on culture results and clinical data (signs, symptoms, laboratory values, imaging) are shown in Table 2. Not surprisingly, pneumonia was the most common source of infection, occurring in 80% of subjects. All patients with pneumonia also had positive blood cultures, and 3/30 (10%) had both meningitis and pneumonia. Presenting signs and symptoms are shown in Table 3. Clinical data were recorded closest to

time 0 and were assumed normal if not recorded in the medical record. The most common presenting features were consistent with the frequent diagnosis of pneumonia and included cough/sputum production (73%), dyspnea/shortness of breath (63%), abnormal lung exam (63%), and new chest radiograph abnormalities (77%). Non-specific characteristics, such as fevers/chills (73%) and fatigue (53%), were also common.

Using the American College of Chest Physicians/Society for Critical Care consensus definitions, subjects were grouped according to severity of infection at time 0 (156). Five subjects (17%) with bacteremia did not meet the systemic inflammatory response syndrome criteria for sepsis. Because the numbers in this study were small, these subjects were grouped with simple sepsis subjects for most analyses. Ten subjects (33%) mounted a simple septic response without end-organ damage, and 15 (50%) had severe sepsis with evidence of end-organ damage. Individuals with septic shock were recorded as severe sepsis because data was recorded from one point in time (time 0), so the determination of whether the hypotension was responsive to fluids over time could not be made. Two of the subjects (7%) died during their hospitalization.

Table 1. Baseline Characteristics. *

Female gender—no. (%)	14 (47)
Age—years	62 ± 17
Mean arterial pressure—mmHg	79 ± 17
Heart rate—beats/min	102 ± 20
Respiratory rate—breaths/min	24 ± 8
White blood cell count—cells/mm^3	27 ± 48

*Plus-minus values are means ± standard deviations.

Er

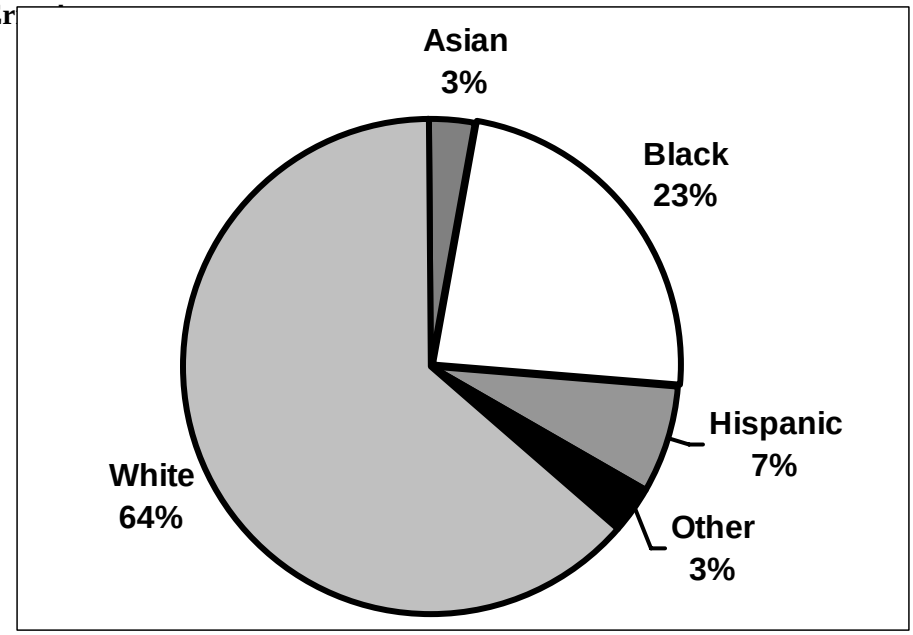

Figure 11. Race/ethnicity of enrolled subjects.

Table 2. Source of infection.	
Bacteremia without a focus—number (%)	2 (7)
Pneumonia	21 (70)
Meningitis	2 (7)
Pneumonia + meningitis	3 (10)
Other focus	2 (7)

Table 3. Presenting signs and symptoms.	
Subjective fevers/chills—no. (%) with sign/symptom	22 (73)
Night sweats	2 (7)
Fatigue	16 (53)
Weakness	14 (47)
Dizziness	3 (10)

Myalgias	10 (33)
Headache	6 (20)
Neck stiffness	3 (10)
Neurologic symptoms	1 (3)
Photophobia	0
Chest pain/pressure/tightness	13 (43)
Cough/sputum production	22 (73)
Dyspnea/shortness of breath	19 (63)
Nausea/vomiting/abdominal pain	13 (43)
Urinary symptoms	3 (10)
Altered Mental status	7 (23)
Lethargy	6 (20)
Disorientation	3 (10)
Abnormal lung exam	19 (63)
New chest radiograph abnormality	23 (77)
New head CT abnormality	5 (17)

MIF levels in patients with invasive *Streptococcus pneumoniae* infection

Serum MIF levels collected upon patient enrollment were measured by ELISA. The mean MIF level for all subjects was 20.4 ± 13.13 ng/ml (range = 5-48 ng/ml). The distribution of the MIF levels of this population was confirmed normal with a test for skewness (skewness = 0.533, standard error of skewness = 0.434). This mean is significantly higher than the normal reported values of 1.8-4.5 ng/ml (T-test of the mean normal MIF value versus the mean MIF value for subjects with invasive pneumococcal disease, $p < 0.0001$) (157). Individuals with bacteremia were grouped with individuals with simple sepsis for analysis. As discussed above, septic shock

patients were categorized as severe sepsis. MIF levels did not differ significantly between disease severity categories ($p = 0.8977$), CNS invasion ($p = 0.5201$), SNP-173 genotype ($p = 0.9370$), CATT-5 allele carriage ($p = 0.3017$), or CATT-7 allele carriage ($p = 0.2723$), as illustrated in Figs. 12-16.

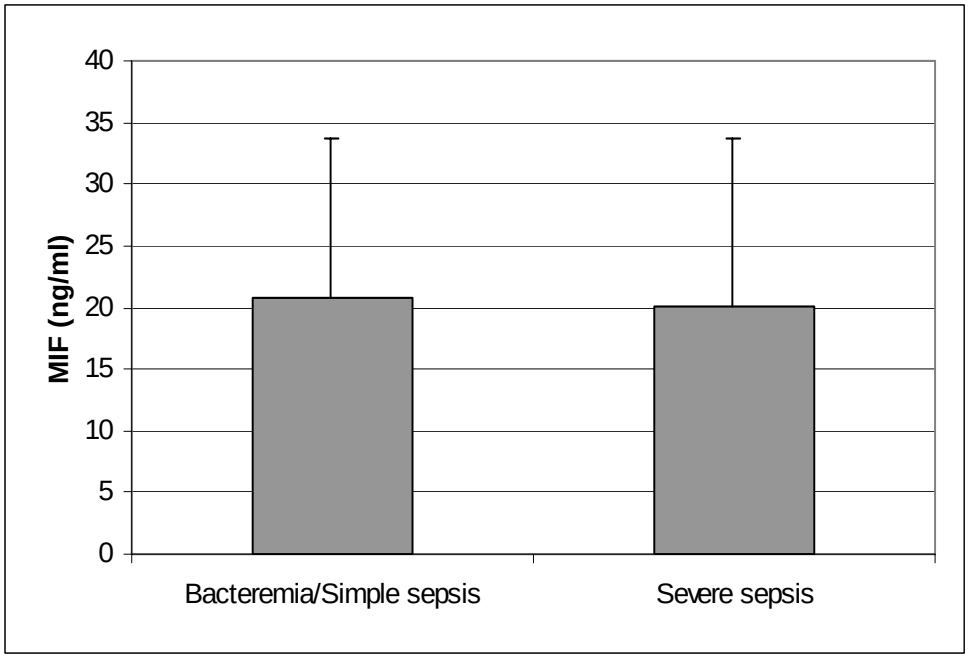

Figure 12. MIF levels and severity of infection. Serum MIF levels were measured from individuals with invasive pneumococcal infections by ELISA. Individuals were grouped by severity of disease. Columns represent means ± SD of 3 measurements. T-test, $p = 0.8977$ for differences in MIF level with disease severity.

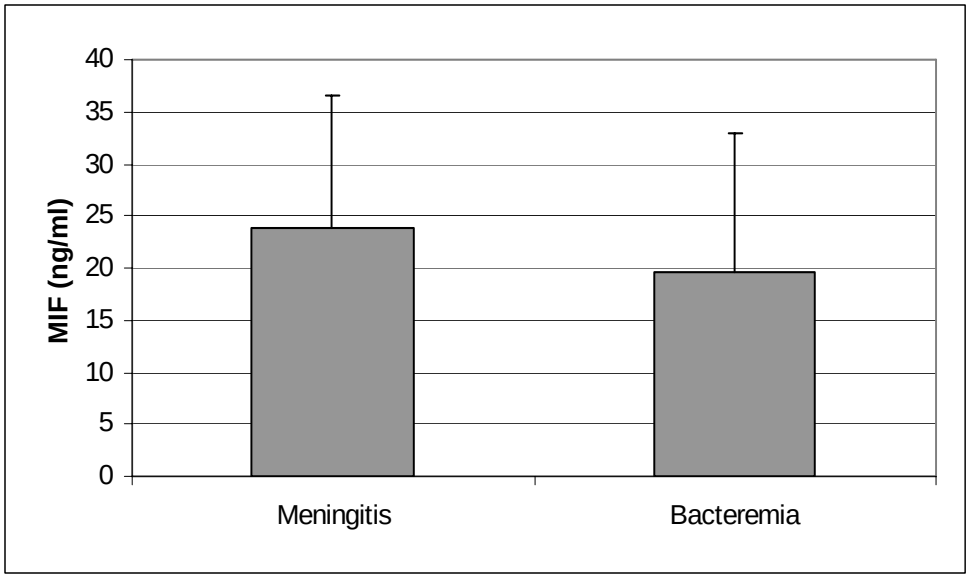

Figure 13. MIF levels and type of infection. Serum MIF levels were measured from individuals with invasive pneumococcal infections by ELISA. Individuals were grouped by whether the infection invaded the CNS space. Columns represent means ± SD of 3 measurements. T-test, p = 0.5201 for differences in MIF level with CNS invasion.

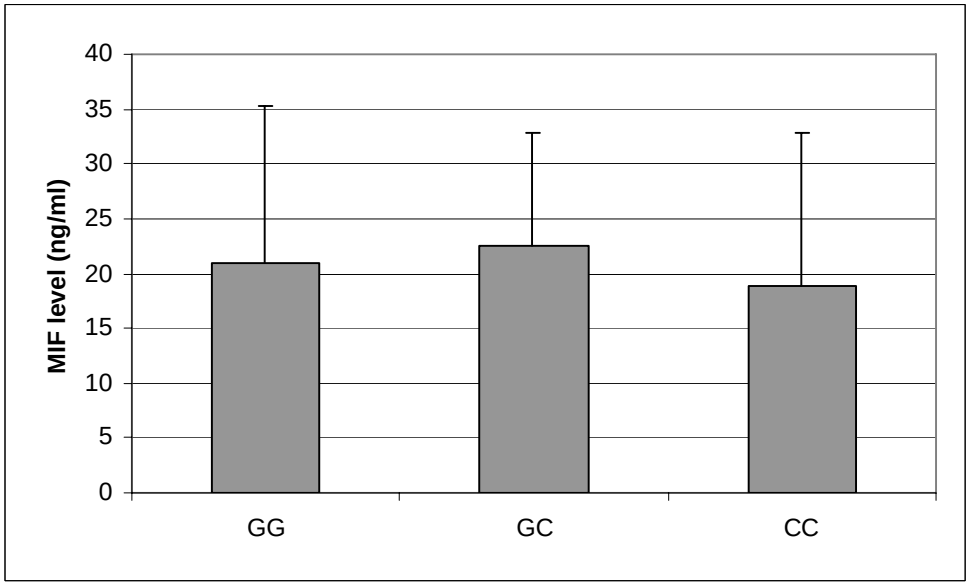

Figure 14. Serum MIF levels and SNP–173 genotype. Each column contains genotypes with two alleles (G or C). Columns represent means ± SD of 3 measurements. ANOVA, p = 0.9370 for differences in MIF level with SNP–173 genotype.

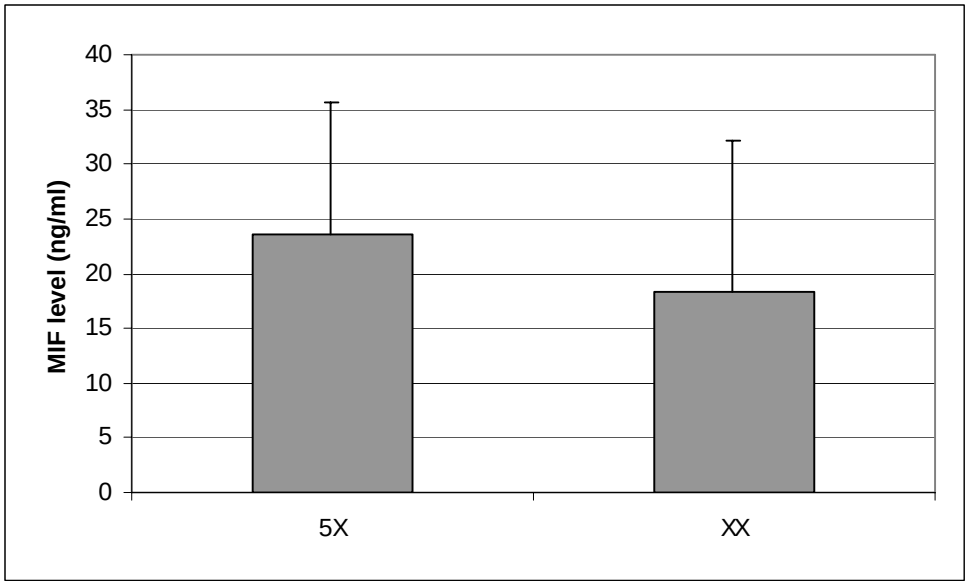

Figure 15. Serum MIF levels and 5-CATT genotype. Each column contains genotypes with two alleles, either 5 or X. X = 6, 7, or 8-CATT repeats. Columns represent means ± SD of 3 measurements. T-test, p = 0.3017 for differences in MIF level with CATT-5 allele.

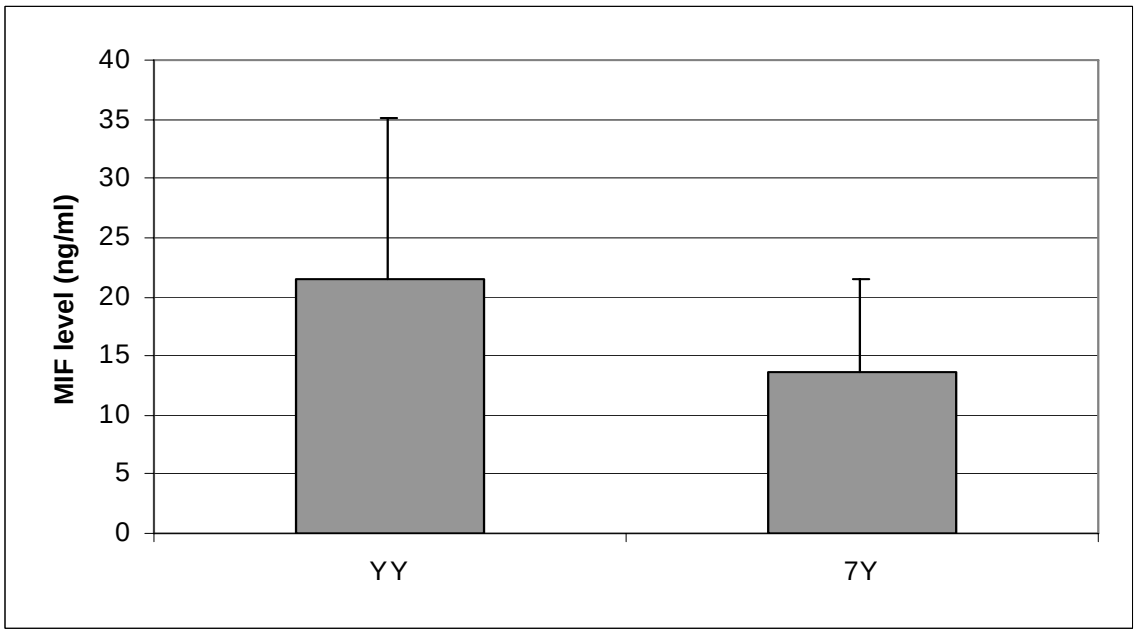

Figure 16. Serum MIF levels and 7-CATT genotype. Each column contains genotypes with two alleles, either 7 or Y. Y = 5 or 6-CATT repeats. Columns represent means ± SD of 3 measurements. T-test, p = 0.2723 for differences in MIF level with CATT-7 allele.

SNP genotypes and disease severity

The distribution of SNP–173 genotype and disease severity is shown in Table 4. For the –173 polymorphism, each allele is listed as a G or a C. The genotype is listed as two letters, indicating the two alleles for that individual. The wild-type GG genotype accounted for 65% of

individuals with bacteremia/simple sepsis and for 91% of individuals with severe sepsis. Though these numbers suggest that the variant C allele, which has been associated with higher MIF levels, may offer some protection against severe response to pneumococcal infection, statistical tests did not bear out this result (Fishers' exact test, $p = 0.1914$).

Table 4. SNP –173 genotype and disease severity

	GG—no. (%)	GC or CC
Bacteremia/Simple sepsis	11 (65)	6 (35)
Severe sepsis	10 (91)	1 (9)

SNP genotypes and CNS disease

The distribution of SNP-173 genotype and pneumococcal CNS invasion is shown in Table 5. The wild-type GG genotype made up 78% of individuals without meningitis and 60% of individuals with meningitis. There were no statistical associations between genotype and meningeal infection (Fishers' exact test, $p = 0.5737$).

Table 5. SNP-173 Genotype and CNS infection.

	GG—no. (%)	GC or CC
No meningitis	18 (78)	5 (22)
Meningitis	3 (60)	2 (40)

CATT genotypes and disease severity

The distribution of the CATT repeat genotypes and disease severity is displayed in Table 6. Each allele is labeled from 5-8, depending on the number of CATT repeats. Genotypes are expressed as two alleles, separated by commas. There were a variety of genotypes represented.

The most common genotype in both groups was the 6,6-CATT repeat genotype, representing 47% of the bacteremia/simple sepsis group and 60% of the severe sepsis group. These can be compared with a series of control genotypes that have previously been completed in our laboratory in which the two most common groups are the 6,6-repeat (32%) and the 5,6-repeat (36%) genotypes (Fig. 17). Though the distributions of the genotypes of the pneumococcal samples differ from the control samples, it is difficult to draw any conclusions since the sampled populations are ethnically different.

As shown in Table 7, there was no association found between genotypes containing a 5-CATT allele, which is a lower MIF-expressing allele, and disease severity. In the healthy control group, 50% of individuals had a 5-CATT allele whereas 40% of those with pneumococcal bacteremia/simple sepsis and 33% of those with severe sepsis had this allele. There was also a lack of association between genotypes containing the 7-CATT allele, which has been associated in many prior studies with disease severity or susceptibility, and disease severity in this study (Table 8). In the healthy control group, 24% of individuals had a 7-CATT allele in their genotypes while 13% of both groups of sepsis severity had this allele.

Table 6. MIF CATT genotypes and severity of infection.

Studied Group	5,5	5,6	5,7	5,8	6,6	6,7	6,8	7,7	7,8
Bacteremia/simple sepsis—no. (%)	3(20)	3(20)	0	0	7(47)	1(7)	0	1(7)	0
Severe sepsis	2(13)	2(13)	1(7)	0	9(60)	0	0	1(7)	0

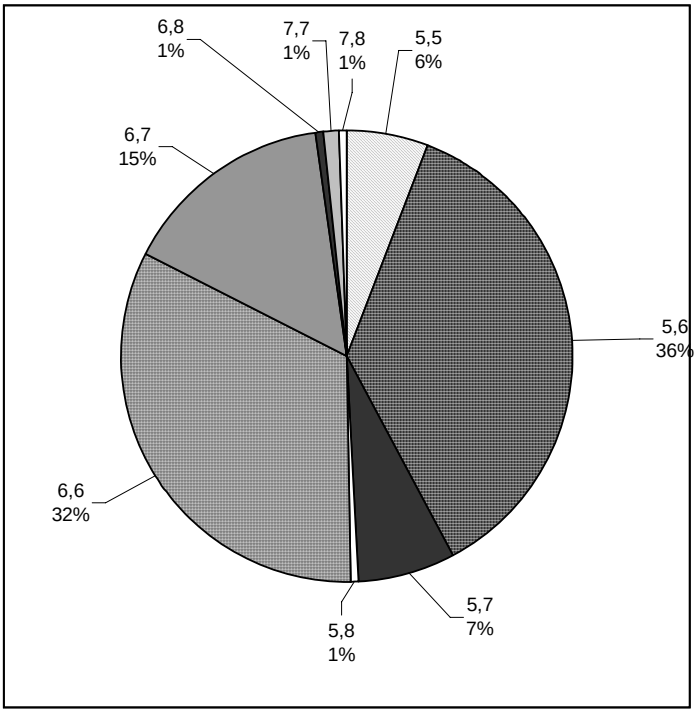

Figure 17. Distribution of a series of 168 control genotypes. Genotypes come from Caucasian individuals chosen as healthy controls for genotyping studies.

Table 7. Lack of association between 5-CATT alleles and disease severity. X = 6, 7, or 8 allele. p = 1.000.

	55 or 5X—no. (%)	XX
Bacteremia/Simple sepsis	6 (40)	9 (60)
Severe sepsis	5 (33)	10 (67)

Table 8. Lack of association between 7-CATT allele and disease severity. Y = 5, 6, or 8 allele. p = 1.000.

	YY—no. (%)	77 or 7Y
Bacteremia/Simple sepsis	13 (87)	2 (13)
Severe sepsis	13 (87)	2 (13)

CATT genotypes and CNS disease

The distribution of CATT genotypes in CNS infection is shown in Table 9. As shown in Table 10, there was no association found between the CATT-5 allele and bacterial invasion of the CNS by the pneumococci (p = 0.3268). There was a trend suggestive of a possible association between the 7-CATT allele and CNS infection, but this did not reach statistical significance (Table 11, p = 0.1188). Using these numbers, there was a relative risk of meningitis in genotypes containing the 7-CATT allele of 1.5 (95% CI, 0.74-3.2).

Table 9. MIF CATT genotypes and type of infection.

Studied Group	5,5	5,6	5,7	5,8	6,6	6,7	6,8	7,7	7,8
No meningitis—no. (%)	4(16)	4(16)	0	0	15(60)	1(4)	0	1(4)	0
Meningitis	1(20)	1(20)	1(20)	0	1(20)	0	0	1(20)	0

Table 10. Lack of association between 5-CATT alleles and disease type. X = 6, 7, or 8 allele. p = 0.3268.

	55 or 5X—no. (%)	XX
No meningitis	8 (32)	17 (68)
Meningitis	3 (60)	2 (40)

Table 11. Trend towards association between 7-CATT allele and disease type. Y = 5, 6, or 8 allele. p = 0.1188.

	YY—no. (%)	77 or 7Y

No meningitis	23 (92)	2 (8)
Meningitis	3 (60)	2 (40)

DISCUSSION

This project explored the relationship between infectious stimuli, especially gram-positive infections, and MIF expression using both *in vitro* and *in vivo* approaches. The data presented herein support a role for MIF in the innate immune response to infection, although the details of this cytokine's actions need to be elucidated. Key findings of our studies were as follows. Stimulation of THP-1 monocytes with LPS confirmed induction of MIF expression, but this occurred at much higher levels than previously published (158). The reason for this difference may be related to the state of activity of the monocytic cells prior to stimulation. In this study, the confluence of the cells before LPS addition affected the amount of MIF released (data not shown), and it is possible that this contributed to the incongruity with prior reports. Part of the LPS-induced release of MIF in this study may have been due to increased cell death in the THP-1 cells at such high concentrations, which was seen inconsistently with LDH assays. Cell death was not measured in prior reports of LPS stimulation of MIF release, so it is unclear what role this played in prior studies. Regardless, MIF surely is involved in the response to gram-negative bacteria, as shown in this study and many previous reports. The function of MIF in gram-positive infections is less apparent.

Despite earlier data supporting a role for MIF in gram-positive infections, PGN stimulation of THP-1 cells did not lead to MIF release in this investigation. The absence of an MIF response may be due in part to the lack of cell death in PGN-treated cells but may also indicate that gram-positive bacterial products act through different mechanisms than gram-negative organisms and, thus, result in different cytokine expression profiles. PGN has been shown to act via TLR2, but the role of MIF in TLR2 activation has not been well documented (159). It is possible that MIF release occurs with some gram-positive bacterial products, such as

exotoxins, but not in response to others, such as PGN. Further work with PGN, teichoic acid, and whole gram-positive bacteria needs to be completed both in cell cultures and in mouse models in order to fully elucidate the role of MIF in gram-positive infections.

Much remains to be investigated concerning *MIF* promoter polymorphisms as well. In the studies of healthy controls with various *MIF* CATT genotypes, there were no differences found between genotypes and basal or stimulated pBMC MIF content or release. Reporter assays have shown sequential increases in MIF release with increasing CATT repeats, and serum samples from individuals with known CATT genotypes in this study showed lower MIF expression with 5-CATT allele-containing genotypes (160). However, these results have not been reproduced with *ex vivo* cells, and the pBMC experiments described above did not confirm the findings of prior studies correlating repeat number and MIF levels. There was significant variation between experiments, and this variation may have masked any trend in *MIF* expression based on genotype. In addition, the series of experiments with pBMCs was based on a small number of healthy control individuals, and more individuals may be required to see true differences between genotypes.

To examine the role of *MIF* polymorphisms *in vivo*, samples were collected from individuals with invasive *S. pneumoniae* infections. Morbidity and mortality from pneumococcal infections are due in part to cytokine activation, and the role for MIF in this infection has not been explored (161). Because it is not clear why some individuals experience fulminant infections with this bacterium while others are asymptomatic carriers and because it is a cytokine-mediated disease, pneumococcal infection is a good model in which to study *MIF* polymorphisms. In addition, since vaccines for prevention of invasive pneumococcal infection exist, identification of another target population at high risk for infection could help to prevent infections (162). As previously reported in the literature with general sepsis and inflammatory states, MIF levels in pneumococcal infections were elevated across all genotypes in this study (163). MIF levels did not differ by genotype in this study, though. This may be due to the fact

that serum was collected from patients at different time points of their hospital stays, depending on when their microbiological cultures grew bacteria. Because those subjects who were in the more acute stages of illness likely had higher MIF levels, the discrepancy in dates of blood draw may have masked any differences between genotypes. The blood was also drawn at different times of day, though the importance of circadian release of MIF in acute stress situations is not well studied.

There have not been previously published research examining MIF levels in various genotypes during infection, so the differences in protein level that occur at baseline may be minimized during the acute increase in MIF across all genotypes. However, the increase in susceptibility to infections in cystic fibrosis and malaria patients based on *MIF* polymorphisms argues for an influence of genotype on MIF release or function (164, 165). If all genotypes truly did secrete the same amount of MIF during infection, presumably the polymorphisms should not have shown an association with infections. However, the lack of differences in protein levels despite a positive association could also indicate that the polymorphism is linked to another locus that plays a functional role. Yet, studies of the *MIF* polymorphisms in rheumatologic diseases also support the idea that even during active inflammatory states, there is variation in *MIF* expression based on genotype (166, 167). These differences may only be seen at certain time points in the infection or may only be evident at the outset of infection.

Neither of the *MIF* polymorphic sites had an effect on severity of pneumococcal infection in this study. However, because the sample size was so small, this lack of influence is not certain. A larger study will need to be carried out in order to state definitively that there are no differences between *MIF* genotypes and severity of pneumococcal disease. In addition, because enrollment in this study required both positive microbiological proof of infection and inpatient status, it selected for individuals with moderately severe infections. Those with fulminant infections often died before their cultures grew out pneumococcus, and those with mild infections were often discharged on oral medications. As a result, the spread of severity of disease was likely limited,

which possibly masked differences in disease severity seen with *MIF* genotype. Because there are so many differences between various ethnic and racial groups, the diversity of the enrolled subjects likely restricted the ability for this study to elucidate differences based on genotype alone rather than other factors. A future study controlling for these limitations may help to elucidate the role of *MIF* polymorphisms in severity of pneumococcal disease.

There was a suggestive trend towards a higher proportion of pro-inflammatory 7-CATT alleles in the meningitis population, although the sample size with CNS infection was very small. Because the number of meningitis cases in this study was limited, a larger population will need to be studied before any conclusions can be made about the pro-inflammatory CATT allele and meningitis. A sample size of 118 (98 bacteremia, 20 meningitis) would be needed to detect a 30% difference in high-expressing 7-CATT allele-containing genotypes with a power of 80% at a significance of 0.05. This trend would have interesting implications for meningitis pathogenesis if it proves to be true. It may be that the more inflammatory genotypes allow for increased expression of adhesion molecules in the cerebral vascular endothelium or allow for more leakiness in the blood-brain barrier. MIF has been shown to upregulate intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in animal models of inflammatory disease (168, 169). Those with higher amounts of MIF may have higher levels of adhesion molecule expression, which potentially could allow for more bacteria and inflammatory cells to pass into the CSF space. Pneumococci are thought to traverse the blood-brain barrier by adhering to and then traversing the endothelial cells (170). In addition to allowing bacteria through, increased adhesion molecules allow for more leukocytes to cross the blood-brain barrier and contribute to the inflammatory response.

The argument for a role of cytokine polymorphisms in cerebral infectious diseases is not a new one. For instance, homozygosity for the TNF2 allele, the promoter polymorphism associated with higher TNF-α expression, has been associated with a 4-fold risk of cerebral malaria and a greater than 7-fold risk of death or neurological sequelae from the cerebral

infection in Gambian children (171). The authors of this study postulate that the high levels of TNF-α expression may increase cerebral disease by increasing endothelial adhesion molecules, thus allowing parasites to cross the blood-brain barrier, or by increasing nitric oxide, which could inhibit neuronal transmission (172, 173).

There appears to be a role for adhesion molecules in bacterial meningitis as well as parasitic infections. For instance, one study of the CSF of children with and without bacterial meningitis demonstrated elevated levels of soluble ICAM-1 in the infected group (174). Though the study does not prove pathogenesis, the association is intriguing because meningitis is an inflammatory disease where cellular mediators cross the blood-brain barrier. Several animal studies also support the idea that adhesion molecules contribute to meningeal inflammation. In a rabbit model of meningitis, animals treated with anti-CD18 monoclonal antibodies, which prevent leukocyte adhesion, were injected intra-cisternally with whole bacteria or cell walls and were found to have less cerebral edema and blood brain barrier injury (175). Specifically, this antibody prevented cerebral edema and mortality after injection of a normally lethal dose of *Streptococcus pneumoniae* bacteria. In a mouse model of cytokine-induced meningitis, deficiency of selectins, which are important for leukocyte rolling and extravasation, led to decreased CSF leukocytosis and blood brain barrier permeability (176). The results from these animal studies suggest that adhesion molecule expression is an integral part of the development of bacterial meningitis. The activation of such molecules by MIF could provide a mechanism by which increased MIF levels contribute to increased risk of meningeal disease.

If further studies confirm that genotypes leading to elevated MIF levels do indeed contribute to susceptibility to meningitis, there could be many other additional reasons why this is the case. As discussed above, MIF plays many upstream pro-inflammatory and steroid-regulatory roles. The alteration of any or all of these factors could be the contributing cause of meningitis. For example, MIF increases expression of matrix metalloproteinases, and these molecules have been linked to blood-brain barrier disruption and neuronal injury in animal models of meningitis

(177). Nearly any of the pro-inflammatory effects of MIF could be the contributors to meningitis susceptibility, and it may be that this cytokine has multiple effects leading to vulnerability. In order to elucidate this pathogenesis, animal knockout models will be required to unravel which aspects of the inflammatory cascade are important.

There were several flaws in the design of the *in vivo* portion of this study that may have contributed to the non-significant findings. First, there was no contemporaneous control group. An ideal control population may have been racially/ethnically-matched individuals who were admitted to the hospital with fever but no positive blood cultures or racially/ethnically-matched healthy control individuals. A second weakness was that the population for this study was ethnically and racially heterogeneous, which is in contrast to other studies examining differences in expression of genotypes discussed in the Introduction. These studies generally have shown that different ethnic groups have distinct genotype distributions (178). Third, because this was a pilot study, the population for this project was small. In order to power this study to find a difference between genotypes, more than 100 subjects would need to be enrolled. The effect of sample size was especially important for the population with meningitis because there were only 5 subjects enrolled during the study period. Fourth, as discussed above, the design of this study selected for individuals with moderately severe disease and excluded those with fulminant or mild disease. Allowing posthumous collection of patient samples as well as enrollment of discharged patients would have helped to eliminate this type of bias. Alternatively, enrolling patients upon admission prior to getting positive culture results would allow for sample collection from a group of patients with a broader spectrum of disease severity. Another limitation was the assumption that values that were not recorded in the medical record were normal. This supposition about missing data could create both false positive or false negative associations. Finally, as discussed above, the fact that patients were enrolled at different points in their hospital stays likely affected the measured MIF protein levels and may have concealed any differences between genotypes.

In summary, this pilot study of *MIF* promoter polymorphisms and *S. pneumoniae* infections points to a potentially intriguing link between MIF and CNS infections. These results need to be expanded upon with further research. In addition to enrolling additional patients with pneumococcal infections, animal models will likely also continue to prove a useful tool for exploring the role of MIF in gram-positive CNS infections. Moreover, in diseases that have a complex genetic basis, it is likely that multiple polymorphisms come together to cause susceptibility for a particular individual. For instance, the TLR2 gene contains a polymorphism that results in an amino acid substitution and renders cells more responsive to bacterial peptides (179). Individuals with this polymorphism may be more susceptible to gram-positive infections. If an individual with this TLR2 polymorphism also had polymorphisms causing increased expression of TNF-α and MIF, one could imagine a scenario where this unique genetic combination of would result in more widespread inflammation and organ damage in the setting of a gram-positive infection. This is a simplified example of the multitude of polymorphisms that have been associated with disease. In the future, individuals with certain genetic profiles will be candidates for earlier preventative interventions and more specific targeted therapies. While association studies can be useful in elucidating which genes play a role, eventually the combinations of many different genes will need to be examined in the context of disease. As technology advances, this type of research into subtle inter-individual genetic differences will likely become integral to the way in which medicine is practiced.

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