BRIEF REPORT

Functional Polymorphisms in the Gene Encoding Macrophage Migration Inhibitory Factor Are Associated With Gram-Negative Bacteremia in Older Adults

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Macrophage migration inhibitory factor (MIF) is an immune mediator encoded in a functionally polymorphic locus. We found the genotype conferring low expression of *MIF* to be enriched in a cohort of 180 patients with gram-negative bacteremia, compared with 229 healthy controls (odds ratio [OR], 2.4; *P* = .04), an association that was more pronounced in older adults (OR, 4.6; *P* = .01). Among older subjects, those with low expression of *MIF* demonstrated 20% reduced MIF production from lipopolysaccharide-stimulated peripheral blood monocytes and 30% lower monocyte surface Toll-like receptor 4, compared with those with high expression. Our work suggests that older adults with low expression of MIF may be predisposed to hyporesponsiveness to lipopolysaccharide and gram-negative bacterial infection.

Keywords. MIF; macrophage; gram-negative infection; immune response.

Bacteremia and sepsis due to gram-negative organisms are important contributors to the burden of community- and healthcare-associated infections. The incidence of bacteremia, which has remained stable among younger people, is increasing in the older population [1]. Bloodstream infection with *Escherichia coli*, occurring predominantly from a urinary source, is an important risk for mortality among older adults [2]. Cellular recognition of lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4) is a key step in the innate inflammatory response to gram-negative bacteria. TLR4-deficient mice have decreased survival and increased bacterial dissemination during gram-negative infection. Genetic studies have shown polymorphisms in human *TLR4*, which blunt the response to LPS, to be associated with a risk for sepsis [3].

While older age is associated with an increase in resting cytokine levels, it also has been implicated in decreased leukocyte-stimulated responses to LPS. Decreased macrophage surface expression of microbial pattern-recognition receptors has been found in aged mice, compared with young mice [4]. In humans, macrophages and dendritic cells from older subjects have decreased expression of pattern-recognition receptors, leading to reduced stimulated cytokine production and poor response to influenza vaccination [5].

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine and upstream mediator of innate immunity that has been shown to regulate monocyte expression of TLR4 in mice [6]. There are functional polymorphisms in human *MIF* that separate the population into those with high and those with low expression of MIF. The most extensively studied polymorphisms are a tetranucleotide (5–8) repeat at position −794 position in the *MIF* promoter, where the 5-CATT variant is an allele associated with low expression and the >5-CATT variants are alleles associated with higher expression. The 8-CATT variant is rarely identified. A single-nucleotide polymorphism (SNP) at −173(G/C) of the *MIF* promoter also has been the focus of some studies, with the C allele associated with high expression and found in linkage disequilibrium with the high-expression −794 7-CATT allele [7].

A number of studies have shown levels of circulating MIF to be elevated in patients with sepsis and associated with poor clinical outcomes [8]. In early examinations, high-expression *MIF* alleles (−794 7-CATT and −173C) were associated with sepsis, acute lung injury, and mortality [9]. Conversely, the low-expression *MIF* genotype (−794 55-CATT) was implicated in increased mortality from meningococcal disease in a cohort of infants from the United Kingdom [10]. Additionally, in a large cohort of older adults with community-acquired pneumonia, Yende et al demonstrated increased risks of sepsis and mortality in patients with low-expression *MIF* genotypes [11].

Mouse models have demonstrated the importance of MIF in the balance between the immune response and inflammatory

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pathology. Although early studies showed MIF-deficient mice to be spared from the effects of inflammatory damage in overwhelming infection, more-recent work indicated that MIF is also essential for macrophage bacterial killing [12].

We explored *MIF* genotypes in patients with gram-negative bacteremia. We found the low-expression −794 55-CATT genotype to be enriched in the bacteremic population, compared with healthy controls. Moreover, this effect was most pronounced among older individuals. LPS-stimulated macrophage production of MIF, as well as surface TLR4 expression, were also significantly in older subjects with low-expression *MIF* alleles, compared with those with high-expression *MIF* alleles.

METHODS

Cohorts

The gram-negative bacteremia cohort consisted of adult inpatients (age, >18 years) at Duke University Medical Center who had at least 1 gram-negative organism isolated from a blood culture from March 2002 through July 2007. Patients who were in postoperative care, were active injection drug users, had human immunodeficiency virus infection, or were currently undergoing chemotherapy for a malignancy were excluded. Healthy patients presenting for primary care were recruited as controls. DNA was isolated from a peripheral blood sample, and patients' records were reviewed for demographic characteristics, disease details, and outcome. For in vitro studies, human peripheral blood mononuclear cells (PBMCs) were obtained from volunteers recruited at Yale University Health Services influenza vaccination clinics. Older participants (age, ≥ 50 years) and younger participants (age, 21–30 years) with no history of immunocompromise or illness in the 2 weeks before enrollment provided blood samples. We used an age of ≥ 50 years as the immunologic cutoff for older age in accordance with previous reports [13]. Studies were approved by the institutional review boards at the study sites.

MIF Genotyping

DNA was isolated from peripheral blood samples by using a commercially available kit (Invitrogen). Analysis of the *MIF* promoter −794 microsatellite (rs5844572) and SNP (rs755622) was performed as previously described [14].

MIF Promoter Experiments

A Dual-Luciferase reporter assay system was used (Promega), in which 10^6 human THP-1 monocytes (ATCC) were cultured in Roswell Park Memorial Institute 1640 medium/10% fetal bovine serum and cotransfected with 1 mg each of the −794 5-8-CATT *MIF* promoter ligated to a luciferase gene and the β-actin promoter luciferase control, using the Amaxa nucleofactor platform (Lonza) [15]. The SNP status of the *MIF* constructs, in accordance with the most commonly occurring genotypes, were 5G, 6G, 7C, and 8G. The cells were incubated at 37°C for 24 hours and then stimulated by LPS (*E. coli* 0111: B4, 100 ng/mL) for 8 hours.

Human Monocyte Studies

PBMCs were isolated using a Ficoll-Histopaque (Sigma-Aldrich) gradient. For flow cytometry, cells were stained for CD11b-APC, CD14-APC-Cy7, and TLR4-PacBlue (eBioscience). Data were acquired using the LSR II cytometer (BD Biosciences) and were analyzed using FlowJo (Tree Star). For stimulation assays, 10^6 PBMCs were stimulated with LPS for 6 hours, and supernatants were collected and assayed for MIF by enzyme-linked immunosorbent assay or were permeabilized and stained for intracellular MIF (mouse monoclonal immunoglobulin G1, R & D Systems) and TLR4.

Statistical Analyses

Differences between the demographic characteristics of the bacteremia cohort and those of controls were analyzed using the Student *t* test. The proportions of case and controls with a low-expression *MIF* genotype were compared by χ^2 analysis. TLR4 expression and stimulated MIF responses were compared by the Student *t* test.

RESULTS

We investigated *MIF* polymorphisms in a cohort of 180 patients with gram-negative bacteremia and 229 locally recruited healthy controls (Table 1). Because of ethnicity-dependent stratification at the −794 locus, our analysis was limited to white subjects [14]. The gram-negative bacteremia and control groups were approximately 60% male, and as expected, case patients were older than controls. Of the cases, 9% were undergoing hemodialysis, 25% had diabetes, and 30% had a history of malignancy. Two-thirds of the case patients had community-acquired bacteremia, predominantly from a urinary source; *E. coli* was the most frequent pathogen. Fever was present in 86%, 40% had associated hypotension, and survival at discharge was 80%.

Genotyping of the −794 CATT polymorphism was successful in 96% of samples. Values for neither group deviated from Hardy-Weinberg equilibrium, and allelic distributions were consistent with prior observations [14]. Five patients had 8-CATT alleles and were excluded. The 55-CATT genotype was more frequently identified in cases, compared with controls (10% vs 4%; odds ratio [OR], 2.4; $P = .04$). This difference was more pronounced among older patients (11% vs 3%; OR, 4.6; $P = .01$). Genotyping of the −173 SNP did not demonstrate any differences in allelic distribution and was not pursued further.

Examining the functional significance of the *MIF* promoter CATT length in transfected THP-1 monocytes, we found no differences in unstimulated transcription among the different fragment lengths, but LPS-stimulated transcription was significantly lower in the 5-CATT transfected cells, compared with

Table 1. Characteristics and *MIF* Genotype Distributions in Case Patients With Culture-Confirmed Gram-Negative Bacteremia and Locally Recruited Healthy Controls

Characteristic	Cases	Controls	OR (95% CI)	*P*
Demographic[a]				
Age, y, mean ± SD	60.8 ± 14.4	48.8 ± 17.91	. . .	<.001
Male sex	115 (63.9)	141 (61.6)	. . .	
Infection route				
Community acquired	96/153 (62.7)	. . .	. . .	
Hospital acquired	57/153 (37.2)	. . .	. . .	
Infection source				
Urinary	41/109 (37.6)	. . .	. . .	
Line related	23/109 (21.1)	. . .	. . .	
Biliary	12/109 (11.1)	. . .	. . .	
Infecting organism				
Enterobacteriaceae	134/151 (88.7)	. . .	. . .	
E. coli	44/151 (32.8)	. . .	. . .	
Klebsiella species	35/151 (26.2)	. . .	. . .	
Enterobacter species	27/151 (20.1)	. . .	. . .	
Infection outcome				
Alive at discharge	139/175 (79.4)	. . .	. . .	
MIF genetics				
Whole population				
−794 55-CATT	17/179 (9.5)	9/215 (4.2)	2.4 (1.043–5.530)	.04
Subjects aged ≥ 50 y				
−794 55-CATT	16/140 (11.4)	3/110 (2.7)	4.6 (1.305–16.23)	.01

Abbreviations: CI, confidence interval; *E. coli, Escherichia coli*; OR, odds ratio.

[a] Data are for 180 cases and 229 controls.

6-CATT, 7-CATT, and 8-CATT (Figure 1*A*). Stimulated transcription appeared to increase with CATT length, but the differences between the 6-, 7-, and 8-CATT plasmids were not statistically significant.

We next investigated the relationship between *MIF* genotype and LPS-stimulated MIF release in PBMCs from 146 healthy volunteers. No effect of *MIF* genotype on MIF release was observed in the whole population (Figure 1*B*). Examining the older subjects separately, PBMCs from those with the low-expression genotype had lower MIF production, compared with subjects with the high-expression genotype (5.9 ng/mL for subjects with 55-CATT vs 7.4 ng/mL for those with >55-CATT; $P = .03$).

We then investigated the relationship between *MIF* genotype and monocyte surface TLR4 expression (Figure 1*C*). Among older subjects, those with the low-expression *MIF* genotype (55-CATT) showed 30% lower surface TLR4 expression than those with the high-expression genotype (>55-CATT; $P = .02$). A *MIF* genotype–dependent influence on TLR4 expression was not observed in the younger group or in the population as whole.

Finally, we sought to examine more directly the influence of *MIF* genotype on TLR4 expression by selecting 3 subjects with the low-expression *MIF* genotype (55-CATT) and 3 with the high-expression *MIF* genotype (>55-CATT) and simultaneously assessing LPS-stimulated MIF production and TLR4 expression from their PBMCs. Monocytes from subjects with the high-expression *MIF* genotype had more intracellular MIF and expressed higher levels of TLR4 in response to LPS than those from subjects with the low-expression *MIF* genotype (Figure 1*D*).

DISCUSSION

We found that the low-expression 55-CATT *MIF* genotype was associated with gram-negative bacteremia, particularly in older patients, and that this genotype confers reduced LPS-stimulated MIF production and surface TLR4 expression in monocytes from older adults. Our study extends the unique relationship between MIF and TLR4 reported in mouse models [6, 12] to human infection and suggests that this locus may be of special importance in the context of an aging immune system. Although the differences we detected were modest, they were demonstrable in small, clinically relevant cohorts, indicating that the role of MIF in gram-negative infections is an important area for study.

We focused on culture-documented bloodstream infections with gram-negative pathogens. By studying a microbiologically well-characterized group, we aimed to provide evidence for a direct effect of MIF on LPS-stimulated immune responses. We

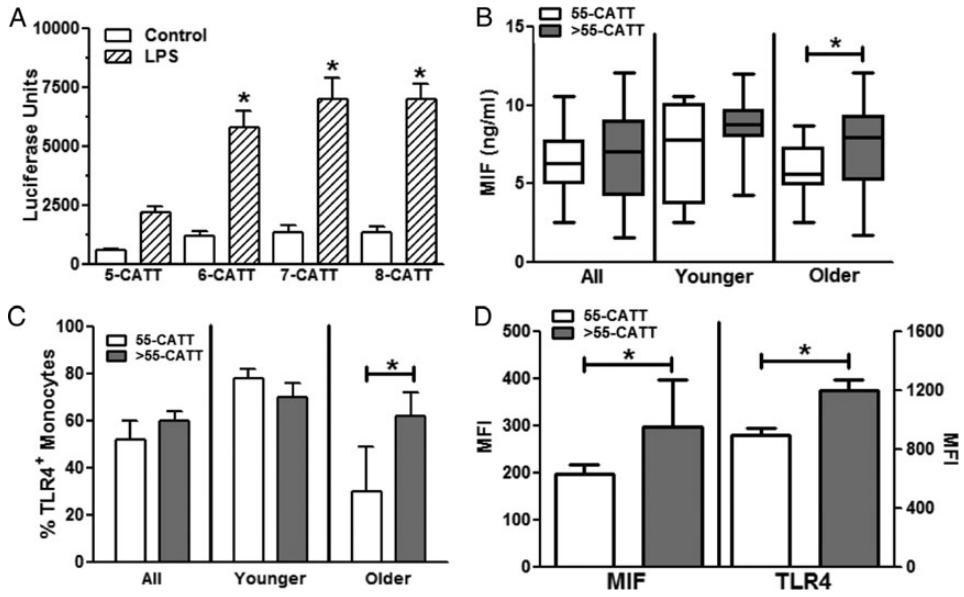

Figure 1. Macrophage migration inhibitory factor (MIF) polymorphisms regulate stimulated MIF production and surface Toll-like receptor 4 (TLR4) expression in monocytes. *A*, Human THP-1 monocytes transfected with *MIF* promoter/luciferase reporter fusion plasmids bearing 0, 5, 6, 7, and 8 CATT repeats show CATT-dependent enhancement of transcription upon stimulation with lipopolysaccharide (LPS). Data are mean ± SD. **P* < .05, for comparisons to 5-CATT. *B*, Primary monocytes from older healthy subjects (age, ≥ 50 y; 11 with 55-CATT, and 89 with >55-CATT) stimulated with LPS demonstrate MIF production that is dependent on the subject's *MIF* genotype. This difference was not detectable in younger subjects (age, 21–30 y; 7 with 55-CATT, and 39 with >55-CATT). The box indicates 25th and 75th percentiles, and the whiskers denote maximum and minimum values. **P* < .05. *C*, Monocyte surface expression of TLR4, detected by flow cytometry, is also higher only in older subjects with high-expression *MIF* genotypes, compared with those with low-expression *MIF* genotypes. Data are mean ± SD. **P* < .05. *D*, When a more detailed examination of LPS stimulation was pursued in human $CD14^+$ monocytes from 6 subjects (3 with 55-CATT, and 3 with >55-CATT), the intensity of intracellular MIF and TLR4 staining were observed to be dependent on the *MIF* genotype. Data are mean ± SD. **P* < .05.

did not find any differences in *MIF* genotype distributions among patients with different gram-negative pathogens. Previous studies of *MIF* genetics examined sepsis as a clinical rather than microbiologic phenotype and focused on patients who were more critically ill than those in our cohort [9]. Although we did not observe any effect of *MIF* genotype on the severity of illness or mortality, our ability to ascertain this may have been limited by low patient numbers.

Serum samples were not available from our study, but we anticipate that levels of circulating MIF will be elevated in response to gram-negative bacteremia [8]. Given that MIF may be either beneficial or detrimental to host outcome, depending on the nature of the underlying infection or host comorbidities, prospective sample collections will be necessary to better explore the role of MIF in bacteremia in older adults. Further evidence of the multifactorial action of MIF is suggested by the results of Renner et al, who reported low-expression *MIF* genotypes to be associated with mortality from meningococcal disease but protective from the occurrence of disease [10].

Work demonstrating that high-expression *MIF* genotypes are associated with sepsis mortality in younger patients but protective against sepsis and mortality in older patients suggests that age is an important modulator of the role of MIF [9, 11]. It has been proposed that the ability to generate interleukin 10 (IL-10) to downregulate pathogenic inflammation decreases in elderly individuals [1]. It is notable that, of the cytokines measured at study entry, only levels of IL-10 correlated with *MIF* genotype in patients with community-acquired pneumonia who progressed to lethal septic shock [11]. Reduced levels of IL-10 have been observed in MIF-deficient mice in models of mycobacterial infection and are postulated to increase the pathology of the disease [15]. Although a functional link between MIF and IL-10 was not examined in the current study, a MIF-dependent effect on IL-10 production might predispose individual with low expression of *MIF* to adverse outcomes during gram-negative infection and should be examined further. Additionally, older individuals have been reported to show reduced monocyte TLR4 expression that is mediated by an age-related defect in the expression of the PRAT4A chaperone (A. Shaw, personal communication), and it would be of interest to assess MIF's direct effect on PRAT4 function. Given MIF's upstream role in the innate response, there may be additional *MIF*-dependent actions during the systemic inflammatory responses that are yet to be discovered.

Several studies have highlighted age-related impairments in macrophage pathogen recognition and various effector functions [4], and a role for MIF in macrophage handling of gram-

negative pathogens also has been demonstrated recently [12]. The upstream effects of MIF on TLR4 expression and macrophage responsiveness may contribute to these phenomena and, additionally, may contribute to an age-associated dysfunction in adaptive immunity [1] because of the importance of macrophages in antigen presentation to initiate T-cell and antibody-mediated adaptive responses. Future mechanistic studies should be directed at elucidating the primacy of these responses in human infection and identifying tractable points of therapeutic intervention.

Notes

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