Low expression Macrophage Migration Inhibitory Factor (MIF) alleles and tuberculosis in HIV infected South Africans

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\textbf{A B S T R A C T}

Host immunity is crucial for controlling \textit{M. tuberculosis} infection. Functional polymorphisms in the cytokine macrophage migration inhibitory factor (MIF) show global population stratification, with the highest prevalence of low expression MIF alleles found in sub-Saharan Africans, which is a population with the greatest confluence of both TB and HIV infection and disease. We investigated the association between MIF alleles and tuberculosis (TB) and HIV in South Africa. We acquired clinical information and determined the frequency of two MIF promoter variants: a functional $-794\ CATT_5,6$ microsatellite and an associated $-173\ G/C$ SNP in two HIV-positive cohorts of patients with active laboratory-confirmed TB and in controls without active TB who were all HIV positive. We found a greater frequency of low expression MIF promoter variants ($-794\ CATT_5,6$) among TB disease cases compared to controls (OR = 2.03, \(p = 0.023\)), supporting a contribution of genetic low MIF expression to the high prevalence of TB in South Africa. Among those with HIV, circulating MIF levels also were associated with lower CD4 cell counts irrespective of TB status (\(p = 0.016\)), suggesting an influence of HIV immunosuppression on MIF expression.

\textbf{1. Introduction}

An estimated one quarter of the world’s population is infected by \textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}), with a significant proportion of these cases in Africa, where approximately 450,000 individuals died from tuberculosis (TB) in 2014 [1]. In South Africa, where TB is epidemic, most new cases occur in individuals co-infected with human immunodeficiency virus (HIV) [2]. These immune compromised individuals have a higher rate of TB disease, with both drug susceptible and drug resistant strains of \textit{M. tuberculosis}, and higher mortality. While there is an estimated 10% lifetime risk for developing active TB from reactivation in healthy individuals, the reactivation rate increases to 10% per year in those co-infected with HIV [3,4] and mortality in these patients is predicted by level of HIV immunosuppression [5–7].

A genetic component to TB infection and disease is recognized; for instance, increased susceptibility to infection occurs in children with rare defects in the IFN-\(\gamma\)/IL-12/IL-23 axis [8,9]. The contribution of genetics to adult infection has been more difficult to define. Candidate genes that affect macrophage handling of \textit{Mycobacteria}, including variants in the pattern recognition receptors DC-SIGN, TLRs, NOD2, and LTHA4H, or rare polymorphisms in \textit{SLC11A1} that influence phagosome function have been reported [9–11]. Macrophage migration inhibitory factor (MIF) is an upstream regulator of innate immunity that inhibits activation-induced apoptosis and sustains monocyte/macrophage responses [12]. Whether MIF contributes meaningfully to TB acquisition, reactivation, or severity of disease in different human populations

\textit{Abbreviations:} MIF, macrophage migration inhibitory factor gene; MIF, macrophage migration inhibitory factor protein; SNP, single nucleotide polymorphism

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https://doi.org/10.1016/j.cytox.2019.100004

Received 8 November 2018; Received in revised form 8 February 2019; Accepted 11 February 2019

Available online 16 February 2019

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remains understudied as is its relationship to HIV induced immunosuppression. Elevated serum levels of MIF have been observed in patients with TB disease [13,14] and MIF deficient mice are impaired in controlling mycobacterial infection and show both a higher pulmonary disease burden and decreased survival when compared with wild-type mice [13].

Two polymorphic sites have been identified in the human MIF promoter: a four-nucleotide microsatellite (−794 CATT<sub>5</sub>-8<sub>a</sub>) and a nearby single nucleotide polymorphism (−173 G/C) (15).

Fig. 1. Schematic diagram of the human MIF gene with its three exons and the two studied promoter polymorphisms: a four-nucleotide microsatellite (−794 CATT<sub>5</sub>-8<sub>a</sub>) and a nearby single nucleotide polymorphism (−173 G/C) (15).

2. Materials and methods

2.1. Study population

Our study populations comprised cohorts in KwaZuluNatal, South Africa: an initial cohort from Durban and a cohort based in Tugela Ferry and Greytown. The second cohort was recruited after the initial cohort and comprises patients with more severe disease. TB disease cases for the Greytown cohort were obtained from inpatient hospital wards and patients had more advanced immunosuppression as evidenced by lower CD4 counts. The greater immunosuppression offered the advantage of amplifying TB severity risk, which may not have been as apparent in subjects with less advanced disease in the Durban cohort. Informed consent was obtained from all subjects in conformity with human investigation protocols approved by the South African Medical Association Research Ethics Committee (SAMAREC, #1423) and Yale University (HIC# 1412015086).

2.2. Durban cohort

All subjects were participants in the iThimba (n = 126) [20] or TB String Study (n = 70) [21]. The iThimba study enrolled subjects with TB and controls from McCord Hospital in Durban. The TB String Study enrolled subjects from King Dinizulu Specialized TB Hospital. All subjects were screened for HIV with ELISA and included if HIV positive. Subjects then were screened for TB and designated as having one of three conditions: (1) TB disease (2), latent TB, or (3) no latent TB or disease. All subjects included in the study were HIV infected but antiretroviral therapy (ART), TB therapy, and isoniazid prophylaxis therapy (IPT) naïve at the time of assessment. Bacillus Calmette Guérin (BCG) vaccination was not documented, but all subjects were likely vaccinated in infancy per South African standards. Subjects with TB disease were defined as having symptoms of pulmonary TB and a culture positive sputum (spontaneous or induced). Subjects from the String Study had a positive intra-gastric string test. Subjects from the iThimba cohort with latent TB were defined as having no symptoms of TB disease, having a tuberculin skin test (TST) with induration greater than 5 mm, a positive ESAT-6 and/or CFP-10 (RD-1) specific IFNγ ELISpot, an induced sputum that was culture-negative for <i>M. tuberculosis</i>, and normal lung parenchyma on chest radiograph (CXR). Subjects from the iThimba cohort classified as controls were defined as having no TB symptoms, a negative TST, a negative RD-1 ELISpot, an induced sputum that was culture-negative for <i>M. tuberculosis</i>, and normal lung parenchyma on CXR.

2.3. Tugela Ferry/Greytown cohort

TB subjects were enrolled from the in-patient wards of the Church of Scotland Hospital (n = 46) in Tugela Ferry and the Specialized TB Hospital (n = 54) in Greytown, South Africa. Control subjects were enrolled from the HIV antiretroviral outpatient clinic at the Church of Scotland Hospital (n = 65). All subjects were screened for HIV with an ELISA test and included if HIV positive, in addition to screening for TB disease. Subjects with TB disease were defined as having symptoms of pulmonary TB and a culture positive sputum (spontaneous or induced) or positive GeneXpert MTB/RIF test. Controls were defined as having a negative symptom screen for TB disease and no documented prior history of TB or treatment. Latent TB testing was not conducted on subjects in this cohort, and this control group is presumed to contain both subjects with and without latent TB. Both cohorts included subjects who were ART naïve as well as those actively undergoing ART therapy. Bacillus Calmette Guérin (BCG) vaccination was not documented, but all subjects were likely vaccinated in infancy per South African standards. Subjects with no evidence of TB infection were defined as having no TB symptoms and no documented history of prior treatment for active TB. All subjects included in the study were HIV infected.

2.4. MIF analyses

Peripheral blood samples were used for genomic DNA extraction. The Easy-DNA Kit (Invitrogen, catalog number K1800-01) was used according to the manufacturer’s instructions. The concentration of extracted genomic DNA was quantified on a spectrophotometer (NanoDrop 2000, Thermo Scientific) and standardized to 10 ng/μl. Genomic DNA was stored at −20 °C until further use. For analysis of the MIF −794 CATT<sub>5</sub>-8<sub>a</sub> microsatellite (rs5844572), DNA fragments between 340 and 352 base pairs in length containing the MIF promoter microsatellite were amplified by PCR. The forward primer was 5’-TGCAGGAACCAA TACCCATAGG-3’, and the reverse primer was 5’-X-AATGGTAACTG GGGAC-3’ (X = 6-FAM fluorescent label). A 25 μl reaction mixture containing Invitrogen Supermix, primers, and 0.5 μl of DNA at 10 ng/μl concentration was amplified by PCR in the following conditions: denaturation at 95 °C for 12 min, followed by 40 amplification cycles of
95°C for 30 s, 54°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. The PCR products were purified, diluted to 1:10 in ddH₂O, and sequenced on an ABI 3730 sequencer [13]. For analysis of the MIF −173 G/C SNP (rs755622), a TaqMan reaction was used with a real-time PCR machine (TaqMan Lightcycler 480) [13]. A 5 μl reaction mixture containing TaqMan Universal PCR Master Mix, No AmpErase UNG, 20X Assays-On-Demand SNP Genotyping Assay Mix, and 1.0 μl of 10 ng/μl DNA. DNA was amplified in a TaqMan Lightcycler 480 with a hold of 10 min at 95°C, denaturing for 15 s at 92°C, and annealing for 1 min at 60°C. The denaturing and annealing steps were repeated for 50 cycles. The G allele is tagged with FAM (465–510 nm) while the C allele is tagged with VIC (533–580 nm). Four negative controls and four positive controls were included on each plate run.

Serum MIF levels were measured by sandwich ELISA as previously [13] using specific antibodies and a native sequence human MIF standard following our originally developed protocol [22]. The capture and detector anti-MIF monoclonal antibody clones were 3H2F and 10G8D, respectively. The sensitivity of the ELISA is ≥0.834 ng/ml (CV% = 3.7) and the range is 0–100 ng/ml.

CD4+ lymphocyte counts were measured with a Becton Dickinson LSR II flow cytometer using antibodies from Biolegend). The reference CD4+ T cell value in HIV+ South Africans without evidence of latent TB was 283 ± 113 [23].

2.5. Statistical and genetic analysis

The sequencing results were analyzed by GeneMapper software. Data were analyzed by the JMP software version 12.1.0 (JMP, NC, USA) and Genepop genetics software [24,25]. Allelic frequencies were calculated in Genepop and an exact test was performed to identify departures from Hardy-Weinberg proportions. Odds ratios (OR) with 95% confidence interval (CI) were calculated for the different MIF alleles. In the analysis of the MIF −173G/C SNP, the homozygous GG genotype was used as the reference group (i.e. OR = 1), and a dominant genetic model employed as in previous studies [26]. To disentangle the independent effects of each MIF polymorphism, we used haplotypic analysis. Arlequin suite software (version 3.5.2.2) was used for estimation of gametic phase based on Gibbs sampling strategy and the Excoffier-Laval-Balding (ELB) method [27,28]. P-values reported are for Mann Whitney U or Kruskal-Wallis tests cases of non-parametric data, or Student’s t-test or one-way analysis of variance (ANOVA) for parametric data.

3. Results

3.1. Study population

Overall, the study population totalled 361 HIV infected patients. The Durban cohort consisted of 196 patients of whom 101 had TB disease and 95 who were controls (latent TB or negative for latent TB). The Tugela Ferry/Greytown cohort consisted of 165 patients of whom 100 had TB disease and 65 were controls without TB disease (presumed to contain subjects both with and without latent TB). Tables 1a and 1b provides the clinical and demographic characteristics of the studied subjects. Both cohorts were demographically similar, but the Tugela Ferry/Greytown patients with and without active TB disease had significantly lower mean and median CD4 cell counts.

### Table 1b

| Demographics and CD4 cell levels of the Tugela Ferry/Greytown Cohort. |
|---------------------------|---------------------------|---------------------------|
|                          | TB Disease, n (%)         | No TB, n (%)               |
|                          | n = 100 (51.5)            | n = 95 (48.5)              |
| Age, median (IQR)        | 36 (29-46)                | 37 (28.5-46)               |
| Female sex               | 40 (40%)                  | 39 (60%)                  |
| CD4 T-cell count, median (IQR) | 78 (30.75-163.8) | 207 (73.0-480.3) |
|                          | Total n = 165             |                          |
|                          | p value                   |                          |
|                          |                           | 0.88                      |
|                          |                           | < 0.01                   |

### Table 1a

| Demographics and CD4 cell levels of the Durban cohorts (iThimba and String Study). |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
|                               | TB Disease, n (%) | No TB, n (%)    | Total n = 196   |
| Age, median (IQR)             | 34 (29-38)       | 34 (29-42)      | 34 (29-39)     |
| Female sex                    | 55 (54%)         | 79 (83%)        | 134 (68%)      |
| CD4 T-cell count, median (IQR)| 131.5 (79.5-259.5)| 431.5 (330.75-576.75)| 302.0 (130.75-470.75)|

### 3.2. MIF allele, genotype, and haplotype frequencies.

We first investigated two commonly occurring MIF promoter polymorphisms in subjects from Durban cohorts (iThimba and String Study): a −173 G/C SNP and a −794 CATT5.8 promoter microsatellite. The MIF allele and genotype frequencies of subjects with TB disease, latent TB, and negative latent TB were not found to be significantly different from those predicted by the Hardy-Weinberg equilibrium. Genetic stratification was not observed in the studied groups as measured by the fixation index (Fst = 0.004 for both loci), indicating that the patients and controls were from a similar genetic background. Statistically significant levels of linkage disequilibrium were found between the two studied MIF loci across all study groups (D’ = 0.76), as reported previously in a southern African population [16].

The MIF genotype distributions at the −173 G/C and −794 CATT5.8 promoter polymorphisms did not demonstrate statistically significant differences in the frequencies of these allelic variants for the latent TB versus negative latent TB groups (data not shown). The latent TB and negative latent TB groups were combined as a single control group for comparison with TB disease. The frequency of the low expression MIF ′ −794 CATT5.8 (5/5 + 5/6 + 6/6) genotypes were significantly higher in subjects with TB disease (82.7%) when compared to controls (68.1%) (OR = 2.23, 95% CI = 1.08–4.71, p = 0.019; Table 2a). We also observed a similar increase in low expression MIF −794 CATT5.8 (5 + 6) alleles in subjects with TB disease compared with controls (90.8% vs. 83.0%, OR = 2.03, 95% CI = 1.05–3.99, p = 0.023; Table 2a).

MIF promoter haplotypes were re-constructed computationally and their estimated frequencies and relative odds ratios were calculated. The G6 haplotype was the most prevalent, constituting 30.3% of all haplotypes across the TB subject and control groups; this is in accord with the high prevalence of these two allelic variants and the known linkage disequilibrium between these two promoter polymorphisms [16]. The most significant comparison was observed in active TB subjects versus in controls for the C7 haplotype - which incorporates the functional high expression MIF allele that has been associated with high MIF production in prior studies of pneumococcal infection [31]. A greater proportion of C7 haplotypes were found in the control group compared to the TB disease group (C7-active TB: 8.9% vs. controls: 16.0%; OR = 0.51, 95% CI = 0.27–0.96, p = 0.038).

MIF genotype distributions for the combined Tugela Ferry and Greytown cohorts are shown in Table 2b. In contrast to the observation in the Durban patients, an association between low expression MIF
Table 2a
Durban Cohorts. Genotypic and allelic frequencies of MIF CATT −794 subjects with TB vs controls, with uncorrected OR. “X” represents any allele, so 7/X is equivalent to “7-containing”. 5/5 + 5/6 + 6/6 is equivalent to all non-7 containing.

| MIF −794 | TB Disease, n (%) | No TB, n (%) | OR (95% CI) | p value |
|-----------|-------------------|--------------|-------------|---------|
| n = 98    | n = 94            |              |             |         |
| 5/5 + 5/6 + 6/6 | 81 (82.7) | 64 (68.4) | 2.23 (1.08–4.71) | < 0.02 |
| 7/X       | 17 (17.3)        | 30 (31.9)   | 1           |         |
| 5 + 6     | 178 (90.8)       | 156 (83.0)  | 2.03 (1.05–3.99) | < 0.02 |
| 7         | 18 (9.2)         | 32 (11.1)   | 1           |         |

Genotypes or alleles and TB disease was not observed. These patients comprised a more immunosuppressed population than the Durban patients (Table 2a) and included patients on ART therapy. Latent TB testing also was not conducted on subjects in this cohort, and this control group potentially included subjects with latent TB.

3.3. MIF serum levels and relationship with MIF genotype and disease and HIV immunosuppression

We observed significant differences in MIF serum values between the active TB group and control group in the Durban cohorts, with a median serum MIF concentration of 17.3 ng/ml (IQR 8.1–35.1) in the active TB group compared to a median concentration of 10.5 (IQR 6.7–19.7) in the control group (p < 0.015, X²). We found increased circulating MIF concentrations in patients without TB disease who had a high expresser MIF haplotype (C7) in the Durban cohorts, with a median serum MIF concentration of 14.2 (IQR 9.3–22.2) in individuals with a C7 haplotype compared to a serum concentration of 9.4 (IQR 6.6–19.1) in individuals without the C7 haplotype (p < 0.05, X²). However, MIF haplotype (or genotype) association with circulating MIF levels was not observed in subjects with TB disease (data not shown). A more detailed analysis of MIF concentrations in relationship to CD4 count levels, however, combining data from both Durban and Tugela Ferry and Greytown cohorts revealed a negative correlation between circulating MIF levels and CD4 count levels, i.e., significantly higher levels of MIF as CD4 cell levels decreased. (Fig. 2). This correlation between lower CD4 cell counts and higher levels of MIF was observed irrespective of TB status and independent of host MIF genotype.

4. Discussion

To further define innate immunity to tuberculosis, particularly in the context of HIV, we explored the relationship between MIF gene polymorphisms and MIF levels in adults with and without TB disease with co-occurring HIV disease in KwaZulu Natal, South Africa. Accruing evidence supports a role for commonly occurring promoter polymorphisms in MIF in TB susceptibility or clinical severity across different populations [13,29–32]. Variant alleles at both the functional −794 CATT5.8 and the closely associated −173 G/C MIF promoter sites occur commonly in the population (minor allele frequency > 5%) [16], and the observation that the highest global prevalence of low expression MIF alleles is in South Africa, where TB is highly endemic, prompted the present investigation. We were especially interested in studying subjects with HIV co-infection given the critical role of CD4 T cell immunity in controlling TB latency and influencing TB disease progression. Experimental studies support an important role for MIF in the innate control of experimental mycobacterial infection [13,33] and we hypothesized that a significant effect of variant MIF alleles would be uncovered by examining TB patients co-infected with HIV and in whom CD4 immunity may be impaired.

In the present case-control study, we investigated the association between MIF promoter polymorphisms and TB in HIV co-infected individuals in a Durban population and a Tugela Ferry/Greytown validation cohort, both from KwaZulu Natal province, South Africa. In the Durban patients, we observed that MIF promoter microsatellites associated with lower expression (i.e., −794 CATT5.8) were significantly overrepresented in the TB disease group compared to the control group. Conversely, the high expression C7 MIF promoter haplotype was observed to be significantly overrepresented in the control group, which is consistent with MIF being protective of TB disease.

The functional MIF promoter microsatellite was not found to be associated with active TB in the Tugela Ferry/Greytown cohort, however this cohort included cases that were more immunosuppressed than in the Durban cohort, and immunosuppression may have dominated over the protective effect of the high expression MIF polymorphisms. Additionally, less rigorous criteria were employed for defining the control group in this cohort. A negative symptom screen and patient-provided history were the basis for inclusion of controls; chest x-ray radiographs were obtained for all subjects only in the Durban population leading to the possibility of including subjects with TB disease in the control group.

Elevated levels of MIF in serum were found in the TB disease group when compared to controls in both the Durban and Tugela Ferry/Greytown populations, which is likely due to an inflammatory response
to TB disease [13]. However, an association between circulating MIF and genotype was not observed in subjects with active TB. Conceivably, active pulmonary disease stimulates tissue inflammatory responses that suprave the influence of MIF genotype on MIF levels as measured in the serum compartment. Yende et al. [34] for instance, observed protection of high MIF expresser genotypes in community-acquired pneumonia but no relationship with circulating MIF levels.

The current and previously published findings collectively suggest that low genotypic MIF expression is a risk factor for the development of TB disease in HIV infected hosts, presumably due an inadequate level of constitutive or induced MIF production. MIF facilitates macrophage clearance of Mycobacterium by enhancing downstream innate cytokine and reactive oxygen production, but it also promotes neutrophil survival and metalloproteinase production, which may enhance pulmonary tissue damage [13]. Indeed, these later mechanisms have been invoked to explain the contribution of high genotypic MIF expression to invasive pneumococcal infection [35,36], increased mortality from meningococcal disease [37], and cystic fibrosis severity [38].

Acute HIV infection elevates circulating MIF levels, which decrease after initiation of anti-retroviral therapy [39,40]. This response is in accord with the down-regulation of systemic inflammation and promotion of immune recovery that occurs with effective anti-retroviral treatment. Among all subjects, and irrespective of TB status, we observed a previously undescibed negative correlation between circulating MIF levels and CD4 lymphocyte counts. MIF is produced by several cell types [41] and progressive CD4 cell decline and immunosuppression may remove immunologic restraints on MIF production or prompt increased MIF production from diverse sites affected by advancing disease and different opportunistic infections. Serial measurement of circulating MIF levels may thereby offer prognostic information in HIV infection and consideration may be given for its potential value as a clinical biomarker in high risk and TB co-infected cohorts. Additional parasitic or other infectious diseases as well as significant malnutrition were not evident in this studied cohort, however follow-up investigations of the relationship between MIF expression and CD4 cell counts will need to control for these potential confounders. At the clinical level, high levels of MIF production and pro-inflammatory cytokines may contribute to organ failure and the high mortality rate associated with active TB in patients with very low CD4 cells [5-7,42].

This first examination of MIF promoter variants in TB cases in South Africa supports the hypothesis that the high frequency of low expression MIF alleles in South Africans: > 70% for the −794 CATT allele in this study and higher than that reported in any other region globally [16], is an important contributor to the prevalence of active TB in this population. Closer study of MIF promoter variants and their interaction with HIV infection and HIV induced immunosuppression, may contribute to better understanding of severe TB disease pathogenesis in HIV co-infected patients and offer prognostic biomarker information for TB progression and measures of therapeutic response and support more aggressive or targeted therapies in those carrying MIF low expression risk alleles.

Conflicts of interest

R Bucala is listed as a co-inventor on patents describing the clinical utility of MIF genotype determination.

Acknowledgements

This work was supported by grants from the National Institutes of Allergy and Infectious Disease at the National Institutes of Health [Grant Numbers F32 AI085712-01A1, K08-AI-097223-02 to RD], [Grant Number RO1AI042310 to RB], the Wilbur Downs Fellowship [DR, MW], the William Wirt Winchester Foundation [RD], and a Fogarty Global Health Equity Scholars grant [DR]. RB is a co-inventor on a Yale patent describing the utility of MIF genotype determination. We are grateful to Nothembia Nontala, Nasreen Ismail, Zenele Mncube, Pamela Govender, Hakeem Ngomu, Shalom Govender, and Duran Ramsuran for laboratory and sample collection assistance, and Tarynn Leslie, Ntombizodumo (Zoe) Hlubi, and Thulani Khumalo for administrative assistance at the HIV Pathogenesis Programme. We also thank Ansuri Singh, Marlon Govender, Karen Moodley for assistance with recruitment and cohort information at McCord Hospital, Durban, and The Africa Centre for use of their plate reader. Thanks to Dr. Kabwe, Sibyl Bhengu, Phume Mhlongo, Sister Mathebula, and Edna Waryawa at Church of Scotland Hospital. Special thanks to Sister Lee-Magan Larkan at the Greytown TB Hospital. Gratitude also is expressed to Marta Piecychna and Kerry Grrendel for laboratory assistance at Yale School of Medicine. Finally, we thank the participating students at all sites for their participation.

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