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Running title: IL-4 polymorphism in Brucella

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Keywords: Brucellosis, IL-4, IL-6, Macrophage migration inhibitory factor, VNTR

Abstract

This study aimed to investigate the association of IL-4, IL-6 and Macrophage migration inhibitory factor (MIF) polymorphisms and the susceptibility to Brucellosis. Consecutive adult patients with no known treatment against Brucellosis (n=120, Group I, ) who had no any other autoimmune and/or chronic disorders were included for the study. Age and sex matched controls who had no other autoimmune and/or chronic disorders were (n=120, healthy volunteers, Group II) selected as well. IL4_P2P2 genotype, IL4_P1 allele and IL4_VNTR-IL6_174CG compound genotype were frequent in the patient group compared to control subject and there were significant differences between the patients and the controls for the IL4_ P2P2 genotype (77.5% versus 87.5%; p<0.001; CI: 0.36 (0.21-0.62)) and IL4_P1 allele (12.1% versus 6.7%; p<0.030; CI: 0.92 (1.02-3.64)). The IL4-VNTR_IL6-174CG
compound genotype was also present at significantly higher frequency in the patient compared to control subjects (11.7% versus 4.2%; p<0.027, Cl:3.04, (1.06-8.68)). No statistically significant differences in the frequencies of the IL-6_174, MIF-173 or IL-4_P1P1 and IL4_P2P1 genotypes were observed between patients and control subjects. The IL4_VNTR P1 allele and P2P2 genotypes and IL4_VNTR-IL6_174CG P2P1-GG genotypes are common in southern part of Turkey and carriers of those polymorphisms are susceptible to Brucellosis.

Introduction

Brucellosis is the common bacterial zoonoses with a high rate of chronicity worldwide and no vaccine is available for the prevention of human Brucellosis. In humans, Brucellosis is mostly caused by Brucella melitensis. A Th1-mediated immune response plays a critical role in the host control of this pathogen. Cytokines play a critical role in the regulation of the immune response against Brucellosis infection, and mediate production of many pro- and anti-inflammatory signals. Transforming growth factor-beta 1 (TGFβ1), a powerful suppressive cytokine, inhibits macrophage activation and modulates T-cell function, and plays crucial roles in regulation of microbial replication and host responses to Brucella. Recent studies showed that TGFβ1 + 868 TT genotype and TT/GG diplotype may confer increased risk of Brucellosis in the infected population (1,2,3).

Serum IL-4 levels are increased in patients with symptomatic Brucellosis and an IL-4 gene polymorphism has been reported for its association with several diseases such as Behçet’s Disease (4,5,6,7). Several studies have investigated VNTR polymorphisms in different diseases (8,9,10,11). The analysis of differentially expressed genes demonstrated
activation of inflammatory and innate immune pathways in infected subjects. Brucella infection is resulted in upregulation of genes involved in phagocytosis and downregulation of protective host defense mechanisms, both of which may contribute to the chronicity of Brucellosis. Based on these findings, we decided to investigate the association of polymorphisms of IL4, IL6_174 variable number tandem repeats (VNTR) and Macrophage migration inhibitory factor (MIF) genes to the susceptibility of Brucellosis among cases from the Black Sea region of Turkey.

Methods

Study population

Consecutive patients with treatment naïve brucellosis (n=120, Group I, 120 patients of 74 male and 46 female) without any other autoimmune and/or chronic disorders, above the age of 18 years, managed by the Infectious Diseases outpatient clinics.

Age and sex matched controls were (n=120, healthy volunteers, Group II, 120 patients of 74 male and 46 female) diagnosed to have no autoimmune disorder or allergic disease and had no history of atopy, cardiac, liver, renal and pulmonary diseases attending the outpatient clinic of the same department and All subjects had no serious health problems (i.e. immunological diseases) and were taking no prescribed medications. As well as the appropriate clinic, the patients with positive blood cultures for Brucella spp., or patients with specific antibodies at significant titers and/or at least four-fold rise in antibody titer in serum specimens taken over 2 or 3 weeks, were diagnosed as brucellosis. Significant titers were those determined to be ≥1/160 in the standard tube agglutination test (STA)

Treatment protocol and patient control
Blood samples of patients were tested for IL-4 and IL-6 concentrations and MIF genes at baseline. One hundred twenty treatment naive patients with Brucellosis were treated with Rifampisin at a dose 600 mg/kg/day, (6 weeks) and doksisiklin at a dose, 200 mg/kg/day (6 weeks). All patients were treated successfully.

Ethics statement

Informed written consent was obtained from all patients and subjects before enrollment to the study, according to the ethical guidelines of the 2008 Declaration of Helsinki and the investigation was approved by the ethical, investigation and biosecurity committee of Gaziosmanpasa University Medical Faculty. Both the study group and control group were recruited from the Turkish population. All studies were approved by the Ethics Committee. All participants provided witnessed written informed consent prior entering the study.

Collection of blood samples and biochemical assays

a: Genotype determination. In this study, MIF gene -173 GC MIF polymorphism was genotyped by the PCR-Restriction Fragment Length Polymorphism (RFLP) technique. Amplification of the polymorphic fragment the PCR primers based as forward primer was 5’-ACT-AAG-AAA-GAC CCG-AGG-C-3’ and the reverse primer was 5’-GGG-GCA-CGT-TGG-TGT-TTA-C-3’ (X5). Fifty ng genomic DNA was amplified in a 25-μl final reaction. PCR cycles were 94°C (5 minutes) for 1 cycle, followed by 94°C (45 seconds), 60°C (45 seconds), and 72°C (45 seconds) for 32 cycles. A final cycle of 72°C for 10 minutes completed the reaction. Amplified PCR product was digested in a 25μl final reaction volume using 2 μl of Reaction Buffer and 1 μl of Alu I restriction enzyme at 37°C 12 hour. Controls of known genotype were included for every set of digestions carried out. The digested
products were resolved on 2% agarose gel stained with ethidium bromide and visualized using UV transillumination.

**b:** The IL-6 174GC polymorphism were analyzed by polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) analysis. The PCR amplifications were carried out in a total volume of 25 μl reaction containing 100 ng of genomic DNA, 2.5 μl of 10x PCR buffer, 200 mM dNTP, 10 pM each primers, and one unit of Taq DNA polymerase. The IL-6 -174GC polymorphism was analyzed as previously described by Tseng et al. (12) using forward (f) 50-TTGTCA AGA CAT GCC AAA GTG CGG AG-30 and reverse (r) 5’-GTG CAA TGT GAC GTC CCT TAG CAT-3’ primers. The amplification condition consisted of an initial melting step of 5 min at 94 °C; followed by 40 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. After the amplification, the 156 bp PCR product was digested with FastDigest BseL-I restriction endonuclease (Fermentas) at 37 °C for 30 min and analyzed on a 3 % agarose gel stained with ethidium bromide. Two fragments (139 and 17 bp) for G allele and three fragments (117, 22 and 17 bp) for C allele were observed. Second PCR was performed to confirm samples whose results were not clear.

**c:** For IL4 gene VNTR polymorphism, PCR was performed with a 25 μl reaction mixture containing 50 ng DNA, 20 pM of each primer, 200 mM of deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl2, 0.5 U Taq polymerase, 10 mM KCl buffer (Fermentas, Shenzhen, China). Amplification was carried out using primers F5’ AGG CTG AAA GGG GGA AAG C-3’, R5’-CTG TTC ACC TCA ACT GCT CC-3’, with initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. P1P1 genotype was homozygous wild type, P1P2 genotype heterozygous mutant, P2P2 genotype homozygous mutation type, wild type allele was P1 and mutant type allele was P2, respectively. PCR product was of 183 bp for P1 allele and 253 bp for P2 allele. The second PCR was performed to confirm samples whose
results were not clear. The polymerase chain reaction (PCR) assay as described by Mout et al. was used (13). In order to validate the accuracy and reproducibility of alls method, each PCR reaction included negative and positive controls.

**Statistical Analysis.**

All statistical analyses were performed using computer SPSS Statistical Program Version 20.0 and Openepi 3.01 software package program. Continuous data was given as mean±SD (standart deviation) and (minmax). Chi² test was used to significance of differences in the allele frequency and genotype distribution between the two study groups. Hardy-Weinberg equilibrium test was performed for both study groups. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated. P value less than 0.05 was considered statistically significant. A multivariate correlation/regression analysis was applied to the data, using the survival as the dependent variable.

**Results**

The study group included 120 patients, 18 to 77 years old (Mean±SD, 38.18±15.34). The control group included 120 subjects, 20 to 75 years old (Mean±SD, 46.79± 12.25 ). No statistically significant differences in the frequencies of the MIF-173, IL-6_174 or IL-4_P1P1 and IL4_P2P1 genotypes were observed between patients and control subjects. When we examined the IL-4 genotype frequencies according to the presence of Brucellosis, we found a statistically significant association between the P2P2 genotype (p=0.001; CI: 0.36 (0.21-0.62) (Table-1). Table-1 presents the distribution of IL-4 VNTR genotypes in the patient group and the control group. The frequencies of the P1P1, P2P1, and P2P2 genotypes of the VNTR polymorphism in the patient group were 1.7%, 20.8%, and 77.5%, respectively, and 0.8%, 11.7%, and 87.5% in the control group, respectively. Our results showed that there was a higher IL-4 P1 allele compared to control group (p<0.030; CI: 1.92 (1.02-3.64)). Our study
also showed a significantly higher frequency of IL4-VNTR_IL6-174CG compound genotype when compared to controls ((11.7% vs. 4.2%, p<0.027, Cl:3.04, (1.06-8.68)) (Table-2). Multivariate correlation/regression analysis was insignificant.

Discussion

*Brucella* is a facultative intracellular bacterial parasite. The pathogenesis of Brucellosis and the nature of the protective immune response are closely related to this property (14). Macrophages are an early barrier for defense against *Brucella*. They phagocytize and degrade invading microorganisms and thus actively participate in innate immunity. Additionally, after processing microorganisms within intracellular compartments, they promote the adaptive immune response by presenting peptides with major histocompatibility complex (MHC) to T lymphocytes (15,16,17). Studies showed that *Brucella* increases expression of chemokines, interleukin-6 and adhesion molecules (16,17,18). Acute human brucellosis also raises interleukin-8 (IL-8) (19). Significantly increased levels of IL-4, IL-6 were found for untreated Brucellosis patients (20).

While most patients recover with a standard antibiotic treatment, 5-40% of patient suffers relapsing Brucellosis (21). Acute and relapse patients demonstrated consistently elevated cytokine gene expression and secretion levels compared to controls. Notably, these include basal secretion of IL-6, IL-8, IL-1β, IL-2, and TNF-α secretion in response to LPS and heat-killed B. melitensis (HKBM), and IFN-γ secretion in response to HKBM (21). One of the animal study found upregulated macrophage migration inhibition factor (MIF) in infected rams (22). On the subject of Brucellosis, it seems that host factors play an important role on the fate of disease progress. Studies have shown that the production and level of IL-6 and IL-8 cytokines are associated with the polymorphism of the encoding genes (19). In this respect, cytokines, their level or the polymorphism of genes that produce cytokines may
confer resistance or predispose Brucellosis. One study suggested that IL-10 modulates macrophage function and contributes to an initial balance between pro-inflammatory and anti-inflammatory cytokines, thereby promoting enhanced bacterial survival and persistent infection (23). While one study showed the lower frequency of -251 IL-8 AA genotype in the controls compared with that of the patients (23), the other study revealed higher frequency of IL-12B rs3212227 AA genotype in patients (23,24). Furthermore, the latter study suggested that rs3212227 A variant could contribute to an inherited predisposition to Brucellosis. Another study considered IL-17 rs4711998, rs8193038, rs3748067 AA genotypes and AAGAA haplotype as susceptibility factors for Brucellosis while the inheritance of IL-17 rs3819024GG and rs3819025AA genotypes might be resistance factors against the disease (25). The distributions of alleles for IL-18 polymorphisms at positions -137G/+113T/+127C/codon 35/3A found higher in healthy controls, that individuals who inherited the aforementioned genotypes/alleles are able to produce higher levels of IL-18 at the onset of infection, and it leads to more IFN-gamma production and control Brucella infection before the emerging brucellosis (26).

In this study, the distribution of the IL-4 gene polymorphic genotypes was analyzed in patients with Brucellosis in Turk population to assess the possible role of these genotypes in the pathogenesis of Brucellosis. The present study indicates that the percentage of the IL-4 polymorphism allele and the distribution of genotypes differed significantly between the patient group and the control group. When we examined IL-4 genotype frequencies compared to control, we found a statistically significant association between the P2P2 genotype and P1 allele of Brucellosis.

In the present study, we are not able to show a significant difference compared to controls for MIF-173GC, IL4-VNTR_MIF-173GC, IL6-174CG_MIF-173GC. Studies on IL-4, IL-6
and MIF are limited. The result of one study searching IL-6 promoter polymorphism at position -174 among Spanish Brucellosis were the same as in our study: No differences were found in the IL-6 variants between the patients and the controls (27). On the other hand two studies from our country found that IL-6 (-174) GC genotype was more frequent in the patients (28). They claimed that the IL-6 (-174) GC genotype may be a risk factor for the development of brucellosis.

When we examined IL-4, IL-6 and MIF genotype frequencies according to Brucellosis, this study showed a significantly increased IL4_P2P2 genotype, IL4_P1 allele and IL4_VNTR-IL6_174CG compound genotype in Brucellosis in a Turk population in northern part of Turkey and we conclude that the possession of the P1 allele and P2P1 genotype of the IL-4 gene VNTR polymorphism may constitute a risk for developing Brucellosis. We claim that genetic polymorphisms are population specific. In this respect, we suggest that genetic profiles of cytokine expression together with the anthropological ascription of local people may guide Brucellosis treatment.

CONFLICTS OF INTEREST STATEMENT
Authors declare that they have no conflict of interest.

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TABLE LEGENDS

Table 1: Genotypes-Alleles

| Genotype/allele | Patients n=120 (%) | Control n=120 (%) | p   | OR          |
|-----------------|-------------------|------------------|-----|-------------|
| **IL4-VNTR**    |                   |                  |     |             |
| P1P1 genotypes  | 2 (1.67)          | 1 (0.83)         | NA  | NA          |
| P2P2 genotypes  | 93 (77.50)        | 105 (87.50)      | 0.001 | 0.36 (0.21-0.62) |
| P1 allele       | 29 (12.08)        | 16 (6.67)        | 0.030 | 1.92 (1.02-3.64) |
| P2 allele       | 211 (87.92)       | 224 (93.33)      | NA  | NA          |
| IL6-174CG       |                   |                  |     |             |
| GG genotypes    | 67 (55.83)        | 65 (54.17)       | NA  | NA          |
| GC genotypes    | 43 (35.83)        | 42 (35.00)       | NA  | NA          |
| CC genotypes    | 10 (8.34)         | 13 (10.83)       | NA  | NA          |
| G allele        | 177 (73.75)       | 172 (71.67)      | NA  | NA          |
| C allele        | 63 (26.25)        | 68 (28.33)       | NA  | NA          |
| **MIF-173GC**   |                   |                  |     |             |
| GG genotypes    | 81 (67.50)        | 86 (71.67)       | NA  | NA          |
| GC genotypes    | 30 (25.00)        | 29 (24.17)       | NA  | NA          |
| CC genotypes    | 9 (07.50)         | 5 (4.16)         | NA  | NA          |
| G allele        | 192 (80.00)       | 201 (83.75)      | NA  | NA          |
| C allele        | 48 (20.00)        | 39 (16.25)       | NA  | NA          |

VNTR: variable number tandem repeats
MIF: Macrophage migration inhibitory factor
NA: Not available
Table 2: Compound Genotypes

| Compound Genotype (IL4-VNTR_IL6-174CG_MIF-173GC) | Patients n=120 (%) | Control n=120 (%) | p | OR |
|------------------------------------------------|--------------------|------------------|---|----|
| P2P2-GG-GG | 35 (0.2917) | 44 (0.3667) | NA | NA |
| P2P2-GG-GC | 12 (0.1000) | 12 (0.1000) | NA | NA |
| P2P2-GG-CC | 4 (0.0333) | 3 (0.0250) | NA | NA |
| P2P2-GC-GG | 23 (0.1917) | 22 (0.1833) | NA | NA |
| P2P2-GC-GC | 7 (0.0583) | 11 (0.0917) | NA | NA |
| P2P2-GC-CC | 3 (0.0250) | 1 (0.0083) | NA | NA |
| P2P2-CC-GG | 4 (0.0333) | 9 (0.075) | NA | NA |
| P2P2-CC-GC | 5 (0.0416) | 3 (0.0250) | NA | NA |
| P2P1-GG-GG | 10 (0.0834) | 4 (0.0333) | NA | NA |
| P2P1-GG-GC | 4 (0.0333) | 1 (0.0083) | NA | NA |
| P2P1-GC-GG | 7 (0.0583) | 7 (0.0583) | NA | NA |
| P2P1-GC-GC | 1 (0.0083) | 0 | NA | NA |
| P2P1-GC-CC | 2 (0.0167) | 1 (0.0083) | NA | NA |
| P2P1-CC-GG | 7 (0.0583) | 7 (0.0583) | NA | NA |
| P2P1-CC-GC | 1 (0.0083) | 1 (0.0083) | NA | NA |
| P2P1-GG-GG | 51 (0.4250) | 59 (0.4917) | NA | NA |
| P2P2-GG-GC | 33 (0.2750) | 34 (0.2833) | NA | NA |
| P2P2-GG-CC | 9 (0.075) | 12 (0.1000) | NA | NA |
| P2P1-GG-GG | 14 (0.1167) | 5 (0.0416) | **0.027** | 3.04, (1.06-8.68) |
| P2P1-GG-GC | 10 (0.0834) | 8 (0.0667) | NA | NA |
| P2P1-GG-CC | 1 (0.0083) | 1 (0.0083) | NA | NA |
| P2P1-CC-GG | 2 (0.0167) | 1 (0.0083) | NA | NA |
| P2P1-CC-GC | 2 (0.0167) | 0 | NA | NA |
| P2P2-GG | 62 (0.5167) | 75 (0.6250) | NA | NA |
| P2P2-GC | 24 (0.2000) | 26 (0.2167) | NA | NA |
| P2P2-CC | 17 (0.1417) | 11 (0.0917) | NA | NA |
| P2P1-GG | 6 (0.0500) | 2 (0.0167) | NA | NA |
| P2P1-GC | 2 (0.0167) | 1 (0.0083) | NA | NA |
| P2P1-CC | 2 (0.0167) | 0 | NA | NA |
| P1P1-GG | 20 (0.1667) | 1 (0.0083) | NA | NA |

IL4-VNTR_IL6-174CG

| Compound Genotype | Patients n=120 (%) | Control n=120 (%) | p | OR |
|-------------------|--------------------|------------------|---|----|
| P2P2-GG | 51 (0.4250) | 59 (0.4917) | NA | NA |
| P2P2-GC | 33 (0.2750) | 34 (0.2833) | NA | NA |
| P2P2-CC | 9 (0.075) | 12 (0.1000) | NA | NA |
| P2P1-GG | 14 (0.1167) | 5 (0.0416) | **0.027** | 3.04, (1.06-8.68) |
| P2P1-GC | 10 (0.0834) | 8 (0.0667) | NA | NA |
| P2P1-CC | 1 (0.0083) | 1 (0.0083) | NA | NA |
| P1P1-GG | 2 (0.0167) | 1 (0.0083) | NA | NA |

IL4-VNTR_MIF-173GC

| Compound Genotype | Patients n=120 (%) | Control n=120 (%) | p | OR |
|-------------------|--------------------|------------------|---|----|
| P2P2-GG | 62 (0.5167) | 75 (0.6250) | NA | NA |
| P2P2-GC | 24 (0.2000) | 26 (0.2167) | NA | NA |
| P2P2-CC | 17 (0.1417) | 11 (0.0917) | NA | NA |
| P2P1-GG | 6 (0.0500) | 2 (0.0167) | NA | NA |
| P2P1-GC | 2 (0.0167) | 1 (0.0083) | NA | NA |
| P2P1-CC | 2 (0.0167) | 0 | NA | NA |
| P1P1-GG | 20 (0.1667) | 1 (0.0083) | NA | NA |

IL-174CG_MIF-173GC

| Compound Genotype | Patients n=120 (%) | Control n=120 (%) | p | OR |
|-------------------|--------------------|------------------|---|----|
| GG-GG | 47 (0.3917) | 48 (0.4000) | NA | NA |
| GG-GC | 16 (0.1333) | 14 (0.1167) | NA | NA |
| GG-CC | 4 (0.0333) | 3 (0.0250) | NA | NA |
| GC-GG | 30 (0.2500) | 29 (0.2417) | NA | NA |
| GC-GC | 8 (0.0667) | 11 (0.0917) | NA | NA |
| GC-CC | 5 (0.0416) | 2 (0.0167) | NA | NA |
| CC-GG | 4 (0.0333) | 9 (0.0750) | NA | NA |
| CC-GC | 6 (0.0500) | 4 (0.0333) | NA | NA |