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# Relevance of Gamma Interferon, Tumor Necrosis Factor Alpha, and Interleukin-10 Gene Polymorphisms to Susceptibility to Mediterranean Spotted Fever▽

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**The acute phase of Mediterranean spotted fever (MSF) is characterized by dramatic changes in cytokine production patterns, clearly indicating their role in the immunomodulation of the response against the microorganism, and the differences in cytokine production seem to influence the extent and severity of the disease. In this study, the single nucleotide polymorphisms (SNPs) of tumor necrosis factor alpha (TNF-α) −308G/A (rs1800629) and interleukin-10 (IL-10) −1087G/A (rs1800896), −824C/T (rs1800871), and −597C/A (rs1800872) and the gamma interferon (IFN-γ) T/A SNP at position +874 (rs2430561) were typed in 80 Sicilian patients affected by MSF and in 288 control subjects matched for age, gender, and geographic origin. No significant differences in TNF-α −308G/A genotype frequencies were observed. The +874TT genotype, associated with an increased production of IFN-γ, was found to be significantly less frequent in MSF patients than in the control group (odds ratio [OR], 0.18; 95% confidence interval [95% CI], 0.06 to 0.51; *P* corrected for the number of genotypes [$P_c$], 0.0021). In addition, when evaluating the IFN-γ and IL-10 genotype interaction, a significant increase of +874AA/−597CA (OR, 5.31; 95% CI, 2.37 to 11.88; $P_c$, 0.0027) combined genotypes was observed. In conclusion, our data strongly suggest that finely genetically tuned cytokine production may play a crucial role in the regulation of the immune response against rickettsial infection, therefore influencing the disease outcomes, ranging from nonapparent or subclinical condition to overt or fatal disease.**

Several studies have demonstrated that cellular immunity plays a critical role in the protective immune response against *Rickettsia conorii*. Immune $CD4^+$ and $CD8^+$ T cells are both involved in the control of rickettsial infection (38). Perivascular infiltrated $CD4^+$ and $CD8^+$ T lymphocytes, macrophages, and natural killer cells produce chemokines and cytokines that activate endothelial rickettsicidal activities. Infected human cells, including endothelial cells, hepatocytes, and macrophages, activated by gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-1β (IL-1β), kill intracellular rickettsiae by one or a combination of three mechanisms, involving nitric oxide synthesis, hydrogen peroxide production, and tryptophan degradation (17). Moreover, clearance of rickettsiae requires activated cytotoxic $CD8^+$ T cells, which eliminate the remaining infected cells by inducing apoptosis (26, 41, 42).

The acute phase of Mediterranean spotted fever (MSF) is characterized by significantly increased serum levels of IFN-γ, TNF-α, IL-10, and IL-6 compared to the levels found during the convalescent phase of the disease or in healthy controls (10, 40). The IFN-γ levels sharply drop within the second week of the disease, whereas TNF-α, IL-10, and IL-6 gradually decrease, reaching normal levels after the third week of infection (40). The dramatic changes in the cytokine production pattern clearly indicate their role in the immunomodulation of the response against the microorganism, and differences in cytokine production seem to influence the extent and severity of the disease (26). On the other hand, the degree of cytokine expression depends not only on the type and intensity of the stimulation but also on host genetic factors, such as polymorphisms located in coding and regulatory regions of cytokine genes (6). Cytokine single nucleotide polymorphisms (SNPs) are important potential tools for genetic prediction of disease susceptibility (22). This genetic variability might be responsible for different rates of susceptibility to infectious diseases or rates of development of severe complications (28). Associations of TNF-α and/or IL-10 gene polymorphisms with cerebral malaria, mucocutaneous leishmaniasis, lepromatous leprosy, and increased mortality for meningococcus meningitides have been reported by different groups (1, 9, 19, 30, 39). Similar results have been obtained for IL-1 gene cluster (5, 21) and IFN-γ (25, 31) polymorphisms in studies of genetic susceptibility to tuberculosis.

In the present study, we aimed at genotyping the SNPs of the promoter regions of TNF-α −308G/A (rs1800629) and IL-10 −1087G/A (rs1800896), −824C/T (rs1800871), and −597C/A (rs1800872) and the IFN-γ T/A SNP at position +874 (rs2430561) in a group of Sicilian patients affected by MSF,

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TABLE 1. Distribution of IL-10 (−1087A/G and −597C/A), TNF-α (−308G/A), and IFN-γ (+874T/A) SNP genotype frequencies in 80 MSF patients and 288 healthy controls

| SNP | Genotypes | Patients | | Controls | | OR (95% CI) | P |
|---|---|---|---|---|---|---|---|
| | | n | Frequency (%) | n | Frequency (%) | | |
| IL-10 −1087G/A | GG | 17 | 21.2 | 67 | 23.3 | 0.89 (0.49–1.62) | 0.82 |
| | GA | 40 | 50.0 | 135 | 46.9 | 1.13 (0.69–1.86) | 0.71 |
| | AA | 23 | 28.8 | 86 | 29.9 | 0.95 (0.55–1.64) | 0.96 |
| IL-10 −597C/A | CC | 43 | 53.8 | 152 | 52.8 | 1.04 (0.63–1.71) | 0.98 |
| | CA | 31 | 38.7 | 106 | 36.8 | 1.09 (0.65–1.81) | 0.85 |
| | AA | 6 | 7.5 | 30 | 10.4 | 0.70 (0.28–1.74) | 0.57 |
| TNF-α −308G/A | GG | 60 | 75 | 209 | 72.6 | 1.13 (0.64–2.02) | 0.77 |
| | GA | 19 | 23.8 | 73 | 25.3 | 0.92 (0.51–1.64) | 0.88 |
| | AA | 1 | 1.2 | 6 | 2.1 | 0.60 (0.07–5.02) | 0.98 |
| IFN-γ +874T/A[a] | TT | 4 | 5.0 | 65 | 22.6 | 0.18 (0.06–0.51) | 0.0007 |
| | TA | 51 | 63.8 | 154 | 53.5 | 1.53 (0.92–2.55) | 0.13 |
| | AA | 25 | 31.2 | 69 | 23.9 | 1.44 (0.84–2.50) | 0.24 |

[a] The nonparametric Mantel-Haenszel procedure was applied for comparisons of IFNγ +874 genotype frequencies, as the MSF patient group did not fit to Hardy-Weinberg equilibrium. As multiple comparisons were made, Bonferroni's correction was applied to a significant *P* value that was multiplied by the number of genotypes (three) detected ($P_c$ = 0.0021).

taking into account the role that these cytokines might play in the immune response against *R. conorii* infection (26).

## MATERIALS AND METHODS

**Subjects.** Eighty Sicilian patients affected by *Rickettsia conorii* infection (43 females and 37 males, with ages ranging from 30 to 60 years [mean age, 47 ± 14]) were included in the study. The initial diagnosis of MSF was based on medical history (history of tick exposure, outdoor activities, and animal contact), clinical picture (fever, headache, maculopapular rash, and tache noire), and laboratory results (leukopenia, thrombocytopenia, and elevated levels of hepatic enzymes). The diagnosis was confirmed on the basis of detection of anti-*R. conorii* antibodies by enzyme-linked immunosorbent assay and indirect immunofluorescence assay and, when possible, by parasite isolation from the bone marrow aspirate and/or from peripheral blood, as previously described (40). Two hundred eighty-eight serum-negative healthy subjects (126 females and 162 males, with ages ranging from 29 to 58 years [mean age, 45 ± 9]) were included in the study. Written informed consent for enrolling in the study and for personal data management was obtained from all the subjects according to Italian laws. Blood specimens from patients and control subjects were collected in tripotassium EDTA sterile tubes, stored at −80°C, and then used for DNA extraction.

**Molecular analysis of alleles at the −308 nucleotide (−308G→A) of the TNF-α gene.** The −308G/A polymorphism (rs1800629) of TNF-α was identified using a modification of the PCR-restriction fragment length polymorphism assay described by Galbraith and Pandey (20). Briefly, 0.5 μM of forward and reverse primers (5′ AGG CAA TAG GTT TTG AGG GCC AT 3′ and 5′ GGC GGG GAT TTG GAA AGT T 3′) were mixed with 5 to 10 ng of DNA template, with a final concentration of 0.2 U *Taq* DNA polymerase (Perkin Elmer Biosystems, Rome, Italy), 200 μM of each deoxynucleotide, and 1× reaction buffer. PCR was performed for 35 cycles at 94°C, 58°C, and 72°C for 35 s each. Restriction enzyme digestion with NcoI (M-Medical, Milan, Italy) of the PCR-amplified product (159 bp) and subsequent electrophoresis on a 2% to 5% agarose gel discriminated between the two alleles: −308A showed two fragments of 146 bp and 13 bp, while −308G was undigested and resulted in a single band of 159 bp. Heterozygous individuals were detected by the presence of all three fragments.

**Haplotype molecular analysis of alleles at the −1082 nucleotide of the IL-10 gene.** Three different biallelic polymorphisms, rs1800896 (−1087G/A), rs1800871 (−824C/T), and rs1800872 (−597C/A), of the IL-10 gene were identified using a −1082/−819/−592 haplotype-specific typing method (24), as previously described (18). Briefly, 12 couples of 3′ and 5′ allele-specific sequence primer pairs were separately mixed in a 13-μl total volume containing 5 to 10 ng of DNA template, 2.00 mM $MgCl_2$, 9.8 mM ammonium sulfate, 39.6 mM Tris-HCl, 200 μM deoxyribonucleotide triphosphates (dNTPs), and 0.2 U Taq-DNA polymerase (Perkin Elmer Biosystems, Rome, Italy). Cycling was performed at 96°C for 1 min followed by 5 cycles at 96°C for 25 s, 70°C for 45 s, and 72°C for 45 s; 20 cycles at 96°C for 25 s, 65°C for 50 s, and 72°C for 45 s; and finally 5 cycles at 96°C for 25 s, 55°C for 60 s, and 72°C for 120 s. PCR products, potentially containing the −592/−819, −592/−1082, or −819/−1082 possible allele combinations, were detected by electrophoresis on 2% agarose.

**Molecular analysis of alleles at the +874 nucleotide of the IFN-γ gene.** The +874T/A polymorphism of IFN-γ (rs2430561) was typed using the amplification refractory mutational system methodology described by Pravica et al. (32), as previously described (25). Briefly, PCR products were obtained after amplification in a 10-μl total volume containing 1× reaction buffer, 200 μM (each) dNTP, 0.5 μM each specific primer, 0.2 U of TaqGold-DNA polymerase (Perkin Elmer Biosystems, Rome, Italy), and 5 to 10 ng of DNA template. Cycling was performed at 95°C for 6 min, followed by 10 cycles at 96°C for 30 s, 60°C for 40 s, and 72°C for 40 s, and finally 20 cycles at 96°C for 30 s, 56°C for 40 s, and 72°C for 50 s. The amplified products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 μg/ml).

**Statistical analysis.** Cytokine polymorphisms and genotype frequencies were evaluated by gene counts. The data were tested for the goodness of fit between the observed and expected genotype frequencies ($\chi^2$ test). When the observed genotype frequencies fit to Hardy-Weinberg equilibrium, $\chi^2$ tests (3-by-2 or 2-by-2 tables; Graphpad Instat Package, Graphpad Software Inc., San Diego, CA) were performed to calculate significantly different genotype distributions between patients and controls. A nonparametric Mantel-Haenszel procedure was applied for comparisons in which genotype frequencies of at least one group did not fit to Hardy-Weinberg equilibrium. As multiple comparisons were made, Bonferroni's correction was applied to significant *P* values that were multiplied for the number of genotypes detected ($P_c$).

## RESULTS

In this study, the SNPs of TNF-α −308G/A, of IL-10 −1087G/A, −824C/T, and −597C/A, and of IFN-γ +874T/A were typed in 80 patients affected by MSF and in 288 control subjects matched for age, gender, and geographic origin. The observed genotype frequencies were within the Hardy-Weinberg equilibrium, with the exception of IFN-γ genotype distribution in the MSF patient group. No statistical differences were observed for IL-10 −1087G/A, −824C/T, or −592C/A or for TNF-α −308G/A between patients and controls (Table 1). Since IL-10 −824C/T and −597C/A SNPs are in complete linkage disequilibrium (13), only data from the SNP at position −597 were included in the table. The analysis of IFN-γ +874T/A genotype frequencies by the nonparametric Mantel-Haenszel procedure showed that significant differences existed

TABLE 2. Distribution of IFN-γ (+874T/A) and IL-10 (−1087A/G or −597C/A) SNP combined genotypes in 80 MSF patients and 288 healthy controls

| Genotypes | | Patients | | Controls | | OR (95% CI) | $P_c$[a] |
|---|---|---|---|---|---|---|---|
| IFN-γ | IL-10 | *n* | Frequency (%) | *n* | Frequency (%) | | |
| +874TT | −1087GG | 1 | 1.25 | 17 | 5.90 | 0.20 (0.03–1.54) | >1.00 |
| | −1087GA | 2 | 2.50 | 37 | 12.84 | 0.17 (0.04–0.74) | 0.32 |
| | −1087AA | 1 | 1.25 | 11 | 3.80 | 0.32 (0.04–2.54) | >1.00 |
| | −597CC | 1 | 1.25 | 41 | 14.20 | 0.08 (0.01–0.57) | 0.060 |
| | −597CA | 2 | 2.50 | 22 | 7.60 | 0.31 (0.07–1.35) | >1.00 |
| | −597AA | 1 | 1.25 | 2 | 0.70 | 1.83 (0.16–20.50) | >1.00 |
| +874TA | −1087GG | 12 | 15.00 | 32 | 11.10 | 1.41 (0.69–2.89) | >1.00 |
| | −1087GA | 24 | 30.00 | 62 | 21.53 | 1.562 (0.90–2.72) | >1.00 |
| | −1087AA | 15 | 18.75 | 60 | 20.80 | 0.88 (0.47–1.65) | >1.00 |
| | −597CC | 34 | 42.50 | 70 | 24.30 | 2.30 (1.37–3.87) | 0.051 |
| | −597CA | 14 | 17.50 | 76 | 26.40 | 0.59 (0.31–1.12) | >1.00 |
| | −597AA | 3 | 3.75 | 12 | 4.20 | 0.90 (0.25–3.26) | >1.00 |
| +874AA | −1087GG | 4 | 5.00 | 18 | 6.25 | 0.79 (0.26–2.40) | >1.00 |
| | −1087GA | 14 | 17.50 | 36 | 12.50 | 1.49 (0.76–2.91) | >1.00 |
| | −1087AA | 7 | 8.75 | 15 | 5.20 | 1.75 (0.69–4.44) | >1.00 |
| | −597CC | 8 | 10.00 | 41 | 14.20 | 0.67 (0.30–1.49) | >1.00 |
| | −597CA | 15 | 18.75 | 12 | 4.20 | 5.31 (2.37–11.88) | 0.0027 |
| | −597AA | 2 | 2.50 | 16 | 5.50 | 0.44 (0.10 to 1.94) | >1.00 |

[a] Comparisons among the different combined genotype frequencies were performed by applying the nonparametric Mantel-Haenszel procedure. As multiple comparisons were made, Bonferroni's correction was applied to *P* values that were multiplied by the number of genotypes (27) involved in the analyses.

in genotype frequencies between MSF patients and controls. The +874TT genotype, associated with increased production of IFN-γ (32, 33), was significantly less frequent in MSF patient than in the control group, and the *P* value after Bonferroni's correction ($P_c$) for the three genotypes detected was 0.0021 with an odds ratio (OR) of 0.18 and a 95% confidence interval (95% CI) ranging between 0.06 and 0.51, suggesting that the presence of the homozygous TT genotype might be protective against MSF.

As previously reported (26, 40), IL-10 production during *R. conorii* infection might counteract the IFN-γ-mediated activation of intracellular microbicidal mechanisms. To evaluate if interactions among genotypes that are able to influence IFN-γ and IL-10 production are involved in MSF susceptibility or protection, the distribution in patients and controls of the 27 possible IFN-γ and IL-10 (−1087G/A, −824C/T, and −597C/A) SNP combined genotypes were evaluated (Table 2). Again, only data for SNPs at position −597 were included in the table. No significant differences were observed among +874T/A/−1087A/G combined genotype frequencies of patients and controls, whereas a significant increase of +874AA/−597CA (OR, 5.31; 95% CI, 2.37 to 11.88; $P_c$, 0.0027) combined genotypes was observed. In addition, a roughly nonsignificant reduction of +874TT/−597CC (OR, 0.08; 95% CI, 0.01 to 0.57; $P_c$, 0.060) combined genotypes and an increase of +874TA/−597CC (OR, 2.30; 95% CI, 1.37 to 3.87; $P_c$, 0.051) combined genotypes were observed. Altogether, these data indicate that interaction among the +874T/A IFN-γ SNP and −597C/A, as well −824C/T, IL-10 genotypes might have a role in susceptibility to *R. conorii* infection.

## DISCUSSION

In this study, the analysis of some relevant polymorphisms for three cytokine genes profoundly involved in the immune response against *R. conorii* allowed us some insight into the protective or predisposing genotypes for this infection. Concerning the TNF-α locus, there is evidence supporting the fact that a genetically determined production excess is associated with the predisposition to infectious and autoimmune pathologies (22). In particular, an association between the −308A allele and some infectious diseases, such as mucocutaneous leishmaniasis, lepromatous leprosy, trachoma, hepatitis B, meningococcal sepsis (30), and cerebral malaria (27), as well as susceptibility to infectious pathogens of aged patients has been demonstrated (11). We were unable to demonstrate an association between this TNF-α polymorphism and MSF. These findings do not exclude the possibility that other polymorphisms of TNF-α genes might be involved in the predisposing background for the clinical manifestations of MSF. In the MSF patient group, we observed the reduction of the frequency of the IFN-γ +874TT genotype. As previously demonstrated, TH1 responses (which lead to the production of IFN-γ), responsible for the activation of the antimicrobial activity of phagocytes as well as the intracellular killing of *Rickettsia* organisms by the endothelial cells and the TH1-dependent immunoglobulin G subclass production, are known to play a central role in the anti-*R. conorii* response (7, 10, 15–17, 26, 29). The SNP at position +874 of the IFN-γ gene maps to a putative nuclear factor-κB (NF-κB) binding site. The presence of a T allele improves the NF-κB binding efficiency and is associated with raised IFN-γ expression in vitro (32, 33). In this view, our data seem to suggest that a genetic predisposition to produce high IFN-γ levels might have a protective effect for the clinical outcome of the disease. Data from studies of intracellular infectious diseases have demonstrated that a genetically mediated reduction of the IFN-γ response is associated with an increased susceptibility to a large arrays of pathogens (2, 8, 36, 43). Recently, we reported, for the first time, that +874T allele frequency is reduced in subjects affected by

chronic tuberculosis (25). This finding has been confirmed by other groups (2, 35), and in a recent meta-analysis, the high IFN-γ-producing +874T allele has been shown to be negatively associated with tuberculosis (31). Present data seem to confirm the protective role of a genetically determined high IFN-γ production against bacterial infections characterized by an intracellular parasitism.

On the other hand, we have previously demonstrated that the serum cytokine profile in MSF patients is characterized not only by increasing proinflammatory cytokine levels (IFN-γ, IL-6, and TNF-α) but also by a significant increase of IL-10 levels (10, 26). In addition, de Sousa et al. (14) have recently reported that the intralesional cytokine expression profile is characterized by a mixed, proinflammatory and anti-inflammatory response, as reflected by elevated levels of mRNA expression of IFN-γ, TNF-α, and IL-10, in patients with MSF. Higher intralesional expression of IFN-γ mRNA correlates with mild/moderate MSF, suggesting that there is an IFN-γ-mediated reduction in bacterial load in these patients and that the simultaneous expression of higher levels of immunosuppressive IL-10 mRNA may decrease responsiveness to IFN-γ (36).

The cytokine profile described above has been observed in other intracellular bacteria-mediated infectious diseases, such as brucellosis (3): IL-10 is associated with disease progression and is involved in counteracting the IFN-γ- and TNF-α-mediated activation of intracellular microbicidal mechanisms.

Accordingly, the results of the present study, indicating different distributions of IL-10 −597C/A and IFN-γ +874T/A combined genotypes in MSF patients and healthy controls, suggest that a complex genetic background, i.e., IL-10 and IFN-γ functional interaction, might be involved in susceptibility to MSF. In particular, the present data seem to suggest that a genetic cytokine profile characterized by the combination of +874A- and −597C-positive genotypes might increase susceptibility to MSF.

Actually, several investigators have shown that the presence of the C allele in the −597 IL-10 promoter sequence, resulting in an increased promoter activity, is associated with high levels of IL-10 production (12, 34). More recently it has been reported that IL-10 production levels depend on both the cells and the antigenic stimulus involved (37). In particular, whole-cell blood cultures, or partially or totally purified peripheral blood mononuclear cells from −592C-positive subjects, stimulated with lipopolysaccharides of gram-negative bacteria, are able to produce higher levels of IL-10 (12, 34, 37). Lipopolysaccharides act through interaction with a complex receptor machinery in which the Toll-like receptor 4 (TLR-4) is an essential component.

As demonstrated in an animal experimental model (23), dendritic cells expressing TLR-4 play a crucial role in stimulating IFN-γ production and natural killer activity and are able to limit rickettsial growth (23). In this view, it is intriguing that we have recently reported that the +896A/G polymorphism at the TLR-4 gene might be associated with MSF susceptibility (4). Thus, our present and previous results indicate that a potentially large number of genetic variants, with subtle effects on the functions of specific immunological mediators, must be taken into account in the regulation of the immune response against *R. conorii* as well against other pathogens.

In conclusion, our data strongly suggest that a finely genetically tuned cytokine production balance might play a crucial role in regulating the interactions among pathogens and hosts, probably influencing different outcomes, ranging from nonapparent or subclinical conditions to overt disease or fatal consequences. In this view, the evaluation of cytokine genotypes may be a useful tool for the identification of MSF patient subgroups that may need a differently tailored antibiotic therapy schedule.

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