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MAJOR ARTICLE

Intralesional Expression of mRNA of Interferon-γ, Tumor Necrosis Factor–α, Interleukin-10, Nitric Oxide Synthase, Indoleamine-2,3-Dioxygenase, and RANTES Is a Major Immune Effector in Mediterranean Spotted Fever Rickettsiosis

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Background. The mechanisms of immunity to *Rickettsia conorii* that have been elucidated in mouse models have not been evaluated in human tissues.

Methods. In this study, quantitative real-time polymerase chain reaction was used to determine the levels of expression of inflammatory and immune mediators in skin-biopsy samples collected from 23 untreated patients with Mediterranean spotted fever (MSF).

Results. In all 23 patients, the levels of intralesional expression of mRNA of tumor necrosis factor (TNF)–α, interferon (IFN)–γ, interleukin (IL)–10, RANTES, and indoleamine-2,3-dioxygenase (IDO), an enzyme involved in limiting rickettsial growth by tryptophan degradation, were higher than those in control subjects; 6 of the 23 patients had high levels of inducible nitric oxide synthase (iNOS), a source of microbicidal nitric oxide. Positive correlations between TNF-α, IFN-γ, iNOS, IDO, and mild/moderate MSF suggest that type 1 polarization plays a protective role. Significantly higher levels of intralesional expression of IL-10 mRNA were inversely correlated with levels of intralesional expression of IFN-γ mRNA and TNF-α mRNA. The mRNA-expression level of the chemokine RANTES was significantly higher in patients with severe MSF.

Conclusion. Mild/moderate MSF is associated with a strong and balanced intralesional proinflammatory and anti-inflammatory response, with a dominant type 1 immunity, whereas severe MSF is associated with increased expression of chemokine mRNA. Whether these factors are simply correlates of mild and severe MSF or contribute to antirickettsial immunity and pathogenesis remains to be determined.

Mediterranean spotted fever (MSF) is an acute, febrile, tick-transmitted rickettsiosis caused by *Rickettsia conorii* [1]. *Rickettsia* replicates in the cytoplasm of endothelial cells, eliciting widespread vasculitis, hypoperfusion, and multiple-organ injury associated with increased vascular permeability [2]. Although effectively treated with doxycycline, severe and fatal MSF occurs due to misdiagnosis or late initiation of treatment [3, 4].

Serum cytokines associated with T cell activation are observed in spotted-fever group (SFG) rickettsioses [5, 6]. Although local cutaneous immune responses to MSF have been studied by histopathological and immunohistochemical analysis, other immune and inflammatory mediators that could play an important role either

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Table 1. Clinical and laboratory data on patients with severe Mediterranean spotted fever.

Characteristic	Patient 4	Patient 7	Patient 15	Patient 16	Patient 17
Age, years	83	50	60	61	81
Sex	Male	Male	Male	Female	Female
Leukocyte count, no./μL	13,300	…	6900	29,300	21,000
Hemoglobin, g/dL	12.5	…	12.6	11.4	10.8
Lymphocytes, no./μL	7500	5300	4200	5500	11,200
Platelets, no./μL	127,000	74,000	103,000	193,000	94,000
Liver enzymes					
Alanine aminotransferase, U/L	38	262	207	191	213
Aspartate aminotransferase, U/L		227	196	149	377
Hepatomegaly/splenomegaly			Yes/no	No/no	
Renal-function tests					
Creatinine, mg/dL	2.7	1.3	3.8	0.8	1.2
Urea, mg/dL		48	47	8	
Admission to intensive-care unit?	No	No	Yes	Yes	No
Hypoxemia[a]	Yes	Yes	No	No	No
Arterial blood gases					
pH	7.45	7.47			
P_{CO_2}, mmHg	34.1	9.1			
P_{O_2}, mmHg	65	48			
HCO_3, mmol/dL	23	106			
Pneumonitis	Yes				
Neurological signs					
Confusion	Yes	Yes	Yes	Yes	Yes

[a] As determined by pulmonary-function test.

in protection against rickettsial infection or in the pathogenesis of MSF remain to be characterized [7].

In vitro studies have shown that intracellular killing of *R. conorii* within human endothelium, macrophages, and hepatocytes is mediated by either 1 or a combination of 3 mechanisms involving nitric oxide, hydrogen peroxide, and tryptophan degradation [8]. Human hepatocytes stimulated by interferon (IFN)–γ, tumor necrosis factor (TNF)–α, interleukin (IL)–β, and RANTES kill intracellular rickettsiae by a nitric oxide–dependent mechanism. Human macrophages and monocytes activated by cytokines kill intracellular rickettsiae by a hydrogen peroxide–dependent mechanism and, via expression of the tryptophan-degrading enzyme indoleamine-2,3-dioxygenase (IDO), by limitation of the availability of tryptophan. It is not known (1) whether these effector molecules are produced in humans and (2) whether the production of these molecules is dependent on a particular cytokine and chemokine environment.

IFN-γ and TNF-α have been associated with a favorable outcome in our in vitro and in vivo murine studies of rickettsial infection [9]. Th1 responses, characterized by the production of IFN-γ (which favors not only the activation of microbicidal mechanisms of macrophages and other infected target cells but also the differentiation of B cells for production of Th1 isotype antibody) together with the activation of antigen-specific cytotoxic $CD8^+$ T cells, appear to be protective [9, 10]. It is not known whether Th2 responses, characterized by the production of IL-4 and/or IL-10, play no role or have a detrimental effect. Also, it is not known whether immunopathological conditions play a role in human rickettsial infections. To determine the local immune and inflammatory responses at sites of rickettsial infection, we prospectively studied Portuguese patients with MSF. We examined the mRNA-expression levels of TNF-α, RANTES, IFN-γ, IL-10, IDO, and inducible nitric oxide synthase (iNOS), which are critical molecules in antirickettsial immune responses [8–12], in skin-biopsy samples from patients with MSF that were collected 3–14 days after onset of fever. The mRNA-expression levels of TNF-α, IFN-γ, RANTES, IDO, and iNOS in these patients were significantly greater than those in the control subjects.

SUBJECTS AND METHODS

Human Subjects

Patients with a clinical diagnosis of MSF who were admitted to hospitals in Portugal were included in the study. The initial diagnosis of MSF was based on clinical signs, fever, rash, and/or eschar, with or without myalgias, headache, leukopenia, thrombocytopenia, and elevated levels of hepatic enzymes. In some cases, data on history of tick exposure, outdoor activities, and animal contact were obtained. The diagnosis was confirmed on the basis of detection of anti-*Rickettsia* antibodies, by immunofluorescence assay (IFA), and/or of *Rickettsia* organisms or DNA in blood, by culture or polymerase chain reaction

Table 2. Primers and probes used in real-time reverse transcriptase–polymerase chain reaction of cytokines, chemokine, and microbicidal enzyme genes, in patients with Mediterranean spotted fever.

Cytokines and chemokine (method)	Primer and probe sequences
RANTES (Quantitect Gene Expression Assay)	Hs_CCL5_1_FAM
iNOS (Quantitect Gene Expression Custom Assay)	Forward, 5′-GCACATTCAGATCCCCAA-3′
	Reverse, 5′-CATGCTGCTGAGGGCTTT-3′ (probe CACCACTACAGGCTCGT)
IDO (Quantitect Gene Expression Custom Assay)	Forward, 5′-TATATGCCACCAGCTCAC-3′
	Reverse, 5′-GGCCAGCATCACCTTTTG-3′ (probe GTCAAATCCCTCAGTCC)
TNF (Quantitect Gene Expression Assay)	Hs_TNF_1_FAM
IFN-γ (Quantitect Gene Expression Assay)	Hs_ IFN-γ _1_FAM
IL-10 (Quantitect Gene Expression Assay)	Hs_IL-10_1_FAM
GAPDH (Quantitect Gene Expression Assay)	Hs_GAPDH_1_FAM

NOTE. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDO, indoleamine-2,3-dioxygenase; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.

(PCR), or DNA in skin, by PCR. For each patient, epidemiological and clinical data were recorded on a standardized form.

The severity of MSF was scored on the basis of either admission to the intensive-care unit (ICU) or evidence of hepatic injury, respiratory or renal insufficiency, or hematological or neurological involvement. Hepatic injury was defined as elevated (>100 U/L) serum levels of alanine aminotransferase (ALT). Respiratory insufficiency was defined as hypoxemia (arterial Po_2 <70 mmHg), and renal insufficiency was defined as a serum level of creatinine that was ⩾1.8 mg/dL). Neurological involvement was characterized by confusion. Hematological involvement was defined as either mild/moderate (120,000–150,000 platelets/μL) or severe (<120,000 platelets/μL) thrombocytopenia with or without anemia and/or leukopenia. Of the 23 patients with MSF, 5 were considered to represent severe cases (table 1), on the basis of either involvement of ⩾3 organ systems or admission to the ICU (in the case of 2 patients), whereas 7 were considered to represent moderately severe cases, on the basis of involvement of 2 organ systems; the remaining 11 patients were considered to represent mild cases, on the basis of involvement of only 1 organ system.

Sample Collection

Blood samples were collected from the patients both at their initial visit to the clinic or admission to hospital (2–7 days after onset of symptoms; mean, 5 days) and at follow-up (14–21 days after onset of symptoms; mean, 15 days). Blood was centrifuged to separate serum or plasma, for IFA, and peripheral mononuclear cells (buffy coat), for culture. Samples were stored at −80°C until processed.

Skin-Biopsy Samples

At admission, skin punch–biopsy samples (diameter, 5 mm) were obtained from the 23 patients and 8 control subjects. Control skin-biopsy samples were obtained from discarded skin tissues from patients admitted for surgical treatment of inguinal hernia. In these latter patients, rickettsial infection was excluded by either serological analysis or PCR performed on the skin tissues. Informed consent was obtained from all subjects, and the experiments were performed with the approval of the ethical committees of the hospitals and the National Institute of Health. Before treatment, 20 skin-biopsy samples were obtained from eschars, and 3 were obtained maculopapular rashes.

Laboratory Confirmation of MSF

Serological analysis. Testing of acute and convalescent sera confirmed the diagnosis of *R. conorii* infection when (1) samples of acute serum contained IgM antibodies at a titer of ⩾32 and/or IgG antibodies at a titer of ⩾128 (on the basis of cutoff values established by the National Institute of Health for the Portuguese population) or (2) there was a 4-fold increase between the titer for acute serum and that for convalescent serum [13, 14].

Isolation. Isolation of *Rickettsia* from patients' blood, as well as strain identification, were performed as described elsewhere [15].

Detection of* Rickettsia *in skin-biopsy samples by PCR. DNA was extracted from the skin samples by use of a DNeasy Tissue Kit (Qiagen). PCR, nested-PCR, and rickettsial-strain characterization were performed as described elsewhere [16].

Detection of Immune-Response Markers in Skin Samples by Real-Time Quantitative Reverse Transcriptase (RT)–PCR

Total RNA was extracted from skin-biopsy samples by an organic extraction method using the RNaqueous Kit (Ambion). Samples were treated with DNase (DNA-free; Ambion). Extracted RNA was quantified, and the ratio of the absorbance values at wavelengths 260 nm and 280 nm was used to estimate the purity of the RNA.

Quantitative real-time RT-PCR assayed the mRNA expression of RANTES, TNF-α, IFN-γ, IL-10, IDO, iNOS, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the iCycler iQ Multicolor Real-Time Detec-

Table 3. Demographics, detection of *Rickettsia*-specific IgM and IgG antibodies in serum samples by immunofluorescence assay (IFA), and results of rickettsial isolation from blood of skin biopsy based on polymerase chain reaction (PCR), in patients with Mediterranean spotted fever.

Patient (age, years [sex])	Blood isolation	Days between onset of fever and skin biopsy	Skin PCR	IFA results: Acute phase, Day	IFA results: Acute phase, Titer IgM/IgG	IFA results: Convalescent phase, Day	IFA results: Convalescent phase, Titer IgM/IgG
1 (20 [male])	Neg	7	Pos	7	Neg/Neg		NA
2 (54 [female])	Neg	2	Pos	5	Neg/Neg	8	128/1024
3 (44 [female])	Neg	5	Pos	6	Neg/Neg		NA
4 (83 [male])	Neg	NA	Pos	NA	Neg/Neg		NA
5 (49 [female])	Neg	6	Pos	6	Neg/Neg		NA
6 (67 [female])	Neg	3	Pos	3	128/1024		NA
7 (50 [male])	Pos	6	Pos	6	Neg/Neg	14	1024/4096
8 (30 [male])	Neg	8	NA	8	32/256	24	64/2048
9 (55 [female])	NA	8	NA	14	1024/4096		NA
10 (49 [male])	Neg	2	NA	6	32/Neg		NA
11 (42 [male])	Neg	10	NA	10	Neg/512		NA
12 (29 [male])	Neg	10	NA	10	512/512		NA
13 (48 [male])	Pos	8	NA	8	Neg/Neg		NA
14 (67 [female])	Neg	5	NA	5	Neg/Neg	14	1024/4096
15 (60 [male])	Neg	3	Pos	3	32/Neg	11	1024/4096
16 (61 [female])	Pos	3	Pos	3	Neg/Neg	17	1024/4096
17 (81 [female])	Pos	NA	Pos	NA	128/256		NA
18 (42 [female])	NA	4	NA	4	Neg/Neg	20	128/2048
19 (81 [female])	Neg	5	Pos	5	Neg/Neg	7	32/Neg
20 (81 [female])	Neg	NA	Pos	NA	Neg/Neg		NA
21 (75 [female])	Neg	NA	Pos	NA	32/4096		NA
22 (63 [female])	Neg	5	NA	5	Neg/Neg	17	64/1024
23 (69 [female])	Neg	12	NA	13	128/1024		NA

NA, not available; Neg, negative; Pos, positive.

tion system (Bio-Rad) in combination with the QuantiTect Probe RT-PCR Gene Expression kit (Qiagen)

The primers and probes used in the present study (Qiagen) are shown in table 2. Amplifications and the thermal-cycler parameters used followed the manufacturer's recommendations. Each sample was analyzed in duplicate, and the average was used to calculate relative mRNA expression. We used the delta-delta method (i.e., comparative C_T [ΔC_T] method) for quantitative analysis of gene expression [17]. The results were normalized to those for GAPDH in the same sample and were expressed as copy number per 10,000 GAPDH copies.

Statistical Analysis

Data analysis was performed by use of frequency distributions and central-tendency measures. Comparison of frequencies between groups of patients was evaluated by χ^2 test or Fisher's exact test. The Mann-Whitney and Kruskal-Wallis tests were used to compare distributions, allowing us to examine the relation between the mRNA levels of different cytokines and their association with the severity of MSF. Correlations between cytokines were analyzed on the basis of Spearman and Pearson's correlation coefficients, allowing us to examine the relation between the mRNA levels of different cytokines and their correlation with the severity of MSF. $P < .05$ was regarded as being statistically significant.

RESULTS

Study Population and Clinical Data

The clinical diagnosis of MSF in the 23 patients in the present study was confirmed by serological analysis and/or rickettsial isolation and/or PCR-based detection of rickettsial DNA in skin-biopsy samples (table 3); 2 of these patients (9%) were treated as outpatients, and the remaining 21 patients (91%) were admitted to the hospital, where 2 of them (9%) subsequently were transferred to the ICU. Epidemiological, clinical, and laboratory data are presented in tables 4 and 5.

The levels of aspartate aminotransferase (AST) in patients with severe MSF were significantly higher than those in patients with mild/moderate MSF (mean levels of AST were 211 U/L and 58 U/L, respectively; $P = .01$). Although ALT levels in pa-

Table 4. Epidemiological and clinical features of patients with Mediterranean spotted fever.

Epidemiological and clinical features	Men (n = 9)	Women (n = 14)
Age, mean (95% CI), years	45.7 (31.2–60.1)	63.5 (55.8–71.2)
Animal contact	5 (56)	11 (79)
Tick-bite history	7 (78)	12 (86)
Month at onset		
May	1	2
June	0	3
July	4	4
August	3	5
September	1	0
Time between first symptoms and admission, mean (95% CI), days	6.6 (4.2–8.9)	5.3 (3.4–7.2)
Duration of hospitalization, mean (95% CI), days	7.8 (3.4–12.3)	5.6 (3.9–7.4)
Rash	9 (100)	13 (93)
Fever	9 (100)	14 (100)
Eschar	6 (67)	14 (100)

NOTE. Data are no. (%) of patients, unless otherwise specified. CI, confidence interval.

tients with severe MSF were higher than those in patients with mild/moderate MSF, the difference was not statistically significant (mean levels of ALT were 207 U/L and 82 U/L, respectively; $P = .17$)

Risk factors that could potentially be associated with moderate/severe MSF included diabetes mellitus (1 patient), alcoholism (2 patients), age ⩾65 years (8 patients), and coronary artery disease (1 patient). Also, 2 patients had HIV and hepatitis C virus (HCV) infection; both of them were receiving antiretroviral and anti-HCV therapy and had very low viral loads.

High mRNA-Expression Levels of TNF-α, IL-10, IFN-γ, RANTES, IDO, and iNOS, in Skin from Patients with MSF

The expression of TNF-α mRNA in the patients with MSF was significantly higher ($P < .0007$) than that in the control subjects. The median TNF-α mRNA-expression in the patients with MSF was ~10,000 copies, compared with 3300 copies in the control subjects (figure 1*A*). The mRNA-expression levels of IL-10, IFN-γ, RANTES, and IDO showed similar patterns: levels in the patients with MSF were significantly higher ($P < .0001$) than those in the control subjects; the median copy numbers of these different cytokines' mRNA in the patients with MSF versus the healthy control subjects were as follows: IL-10, 3434 versus 287; IFN-γ, 860 versus 5; RANTES, 13,736 versus 1187; and IDO, 2888 versus 43 (figure 1*B*–1*E*). In 17 of the patients with MSF, the mean mRNA-expression levels of iNOS were comparable to those in the control subjects (copy numbers 687 and 760, respectively) (figure 1*F*); in the remaining 6 patients, the levels were significantly higher than those in the control subjects. Taken together, these data suggest that rickettsial infection is associated with increased expression of several immune and inflammatory mediators in the skin of patients with MSF, with a trend toward mixed type 1, proinflammatory, and anti-inflammatory responses.

Correlations between mRNA-Expression Level of TNF-α, IFN-γ, and IL-10

The critical factors responsible for immunoregulation in particular infections, as well as the variation in response and outcome between different patients, are determined by the balance between pro- and anti-inflammatory mediators [18–21]. To determine whether such an immunoregulatory mechanism existed in the patients in the present study, we compared the mRNA-expression levels of pro- and anti-inflammatory cytokines. We observed nonlinear relationships between the mRNA-expression levels of TNF-α and IL-10 (figure 2*A*), as well as between those of IFN-γ and IL-10 (figure 2*B*). In 67% of the patients with MSF, a significantly ($P < .05$) positive correlation was detected between the ⩽3800-copies expression level of IL-10 mRNA and an elevated level of TNF-α mRNA; in contrast, in 33% of the patients with MSF, a significantly negative correlation was observed between a high (>3800 copies) level of IL-10 mRNA expression and a low level of TNF-α mRNA expression. Similarly, in 73% of the patients with MSF, the ⩽5000-copies level of IL-10 mRNA expression correlated with elevated IFN-γ mRNA expression (figure 2*B*), whereas an elevated (>5000 copies) level of IL-10 mRNA expression correlated with down-regulation of IFN-γ mRNA ($P = .06$). These data suggest the presence of an immunoregulatory mechanism, mediated by anti-inflammatory IL-10, that can help to avoid

Table 5. Laboratory findings on the day of admission, in patients with Mediterranean spotted fever.

Abnormal laboratory results	Males	Females
Anemia <11 g/dL	0	2 (15)
Leukopenia <4400 cells/μL	1 (11)	4 (29)
Leukocytosis >11,300 cells/μL	1 (11)	1 (8)
Thrombocytopenia		
Mild/moderate (120,000–159,000 PLTs/μL for males; 120,000–149,000 PLTs/μL for females)	1 (11)	5 (36)
Severe (⩽120,000 PLTs/μL)	5 (56)	4 (29)
Renal failure (creatinine ⩾1.8 mg/dL)	2 (9)	0
Hepatocellular injury		
Alanine aminotransferase (ALT)		
Mild/moderate (41–100 U ALT/L for males; 31–100 U ALT/L for females)	3 (38)	5 (36)
Severe (ALT >100 U/L)	3 (38)	7 (50)
Aspartate aminotransferase (AST)		
Mild/moderate (37–100 U AST/L for males; 31–100 U AST/L for females)	2 (29)	8 (62)
Severe (>100 U AST/L)	4 (57)	4 (31)

NOTE. Data are no. (%) of patients for whom data were available. PLTs, platelets.

severe tissue damage due to excess intralesional production of proinflammatory and type 1 cytokines.

Local Production of High mRNA-Expression Levels of TNF-α and IFN-γ: Frequent Association with High mRNA-Expression Levels of iNOS and IDO

In the patients with MSF, a significant ($P = .028$) positive correlation was observed between the level of IFN-γ mRNA expression and that of iNOS mRNA, as well as between the level of IFN-γ mRNA expression and that of IDO mRNA (figure 2*C* and 2*D*), correlations that were not observed in the control subjects (data not shown). In most of the patients with mild or severe MSF, higher levels of IFN-γ mRNA expression correlated with higher levels of iNOS mRNA expression and of IDO mRNA expression. In the patients with MSF, a similarly significant ($P = .002$) positive correlation was observed between the mRNA-expression levels of TNF-α and those of iNOS and of IDO (figure 2*E* and 2*F*), a correlation that was not observed in the control subjects (data not shown). These data suggest that TNF-α and IFN-γ play a protective role in rickettsial diseases in humans, via activation of intracellular bactericidal mechanisms.

Local Production of High Levels of TNF-α mRNA: Association with High Levels of IFN-γ mRNA

In the patients with MSF, a significant ($P = .0001$) positive correlation was observed between the level of TNF-α mRNA expression and that of IFN-γ mRNA expression (figure 3*A*), a correlation that was not observed in the control subjects (figure 3*B*). These results suggest that TNF-α and IFN-γ have potentially synergistic effects with respect to the activation of intracellular bactericidal effector mechanisms.

Comparison of mRNA-Expression Levels of TNF-α, IL-10, IFN-γ, RANTES, iNOS, and IDO, in Mild, Moderate, and Severe MSF

To assess the contribution that pro- and anti-inflammatory mediators make to the development of mild and severe MSF, we examined the mRNA-expression levels of IFN-γ, TNF-α, IL-10, RANTES, iNOS, and IDO in patients with mild, moderate, or severe MSF. Although the median mRNA-expression levels of IFN-γ, TNF-α, IL-10, iNOS, and IDO were not statistically significantly different between patients with these 3 differing severities of illness (table 6), our data demonstrated a trend toward expression of substantially high levels of certain biomolecules in either mild or severe MSF (table 7): TNF-α mRNA-expression levels of ⩾6500 copies were detected in 64%, 86%, and 100% of patients with mild, moderate, and severe MSF, respectively; in contrast, IFN-γ mRNA-expression levels of ⩾500 copies were detected in 45%, 57%, and 20% of patients with mild, moderate, and severe MSF, respectively. All patients with severe MSF had very high IDO mRNA-expression levels (⩾1500 copies), compared with only 45% of patients with mild MSF; in contrast, ⩾800 copies of iNOS mRNA were expressed in 45% of patients with mild MSF, whereas similar levels were expressed in only 14% and 20% of patients with moderate and severe MSF, respectively. The level of IL-10 mRNA expression was comparable in all 3 groups of patients: levels of ⩾3000 copies were detected in 64%, 43%, and 60% of patients with mild, moderate, and severe MSF, respectively. All of the patients with severe MSF had high RANTES mRNA-expression levels

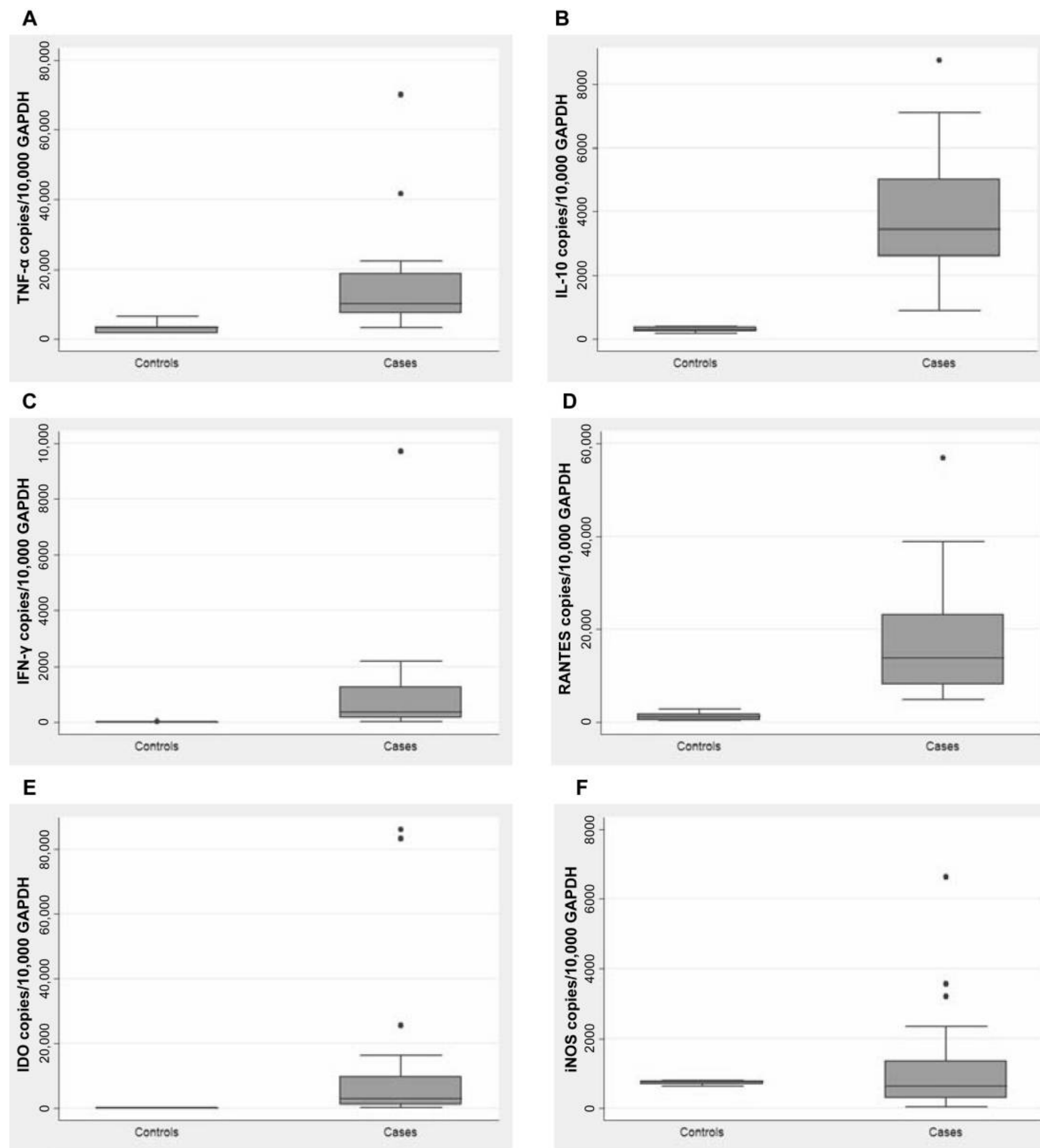

Figure 1. mRNA levels of cytokines and a chemokine: tumor necrosis factor (TNF)–α *(A),* interleukin (IL)–10 *(B),* interferon (IFN)–γ *(C),* RANTES *(D),* indoleamine-2,3-dioxygenase (IDO) *(E),* and inducible nitric oxide synthase (iNOS) *(F),* in patients with Mediterranean spotted fever (Cases) and in control subjects (Controls), quantified by real-time reverse-transcriptase polymerase chain reaction. Cycle threshold values for genes of interest were normalized to those for glyceraldehyde-3-phosphate dehydrogenase and were used to calculate the relative extent of mRNA expression. The tops and bottoms of the shaded boxes denote the 25th and 75th percentiles, respectively, and the horizontal line within each of the boxes denotes the median value; dots denote outliers.

($\geqslant$10,000 copies), an expression level that was close to being significantly higher in patients with severe MSF than in patients with mild or moderate MSF (table 7).

DISCUSSION

Most of our understanding of immune responses in rickettsial diseases stems from murine models of disseminated spotted-fever rickettsiosis. Few studies in patients with rickettsial diseases have examined the levels of pro- and anti-inflammatory and immune mediators in the sera of such patients. However, no previous human studies directly examined either the local mediators of inflammation or the immune response at the site of infection (e.g., skin) in rickettsial diseases. The present study is, to our knowledge, the first comprehensive study of intralesional expression of cytokine and chemokine transcripts in patients with MSF. Certain patterns are evident in the study's results. First, the data revealed mixed type 1, proinflammatory, and anti-inflammatory responses, as reflected by elevated levels of mRNA expression of IFN-γ, TNF-α, and IL-10 in patients with MSF. The correlation between elevated intralesional ex-

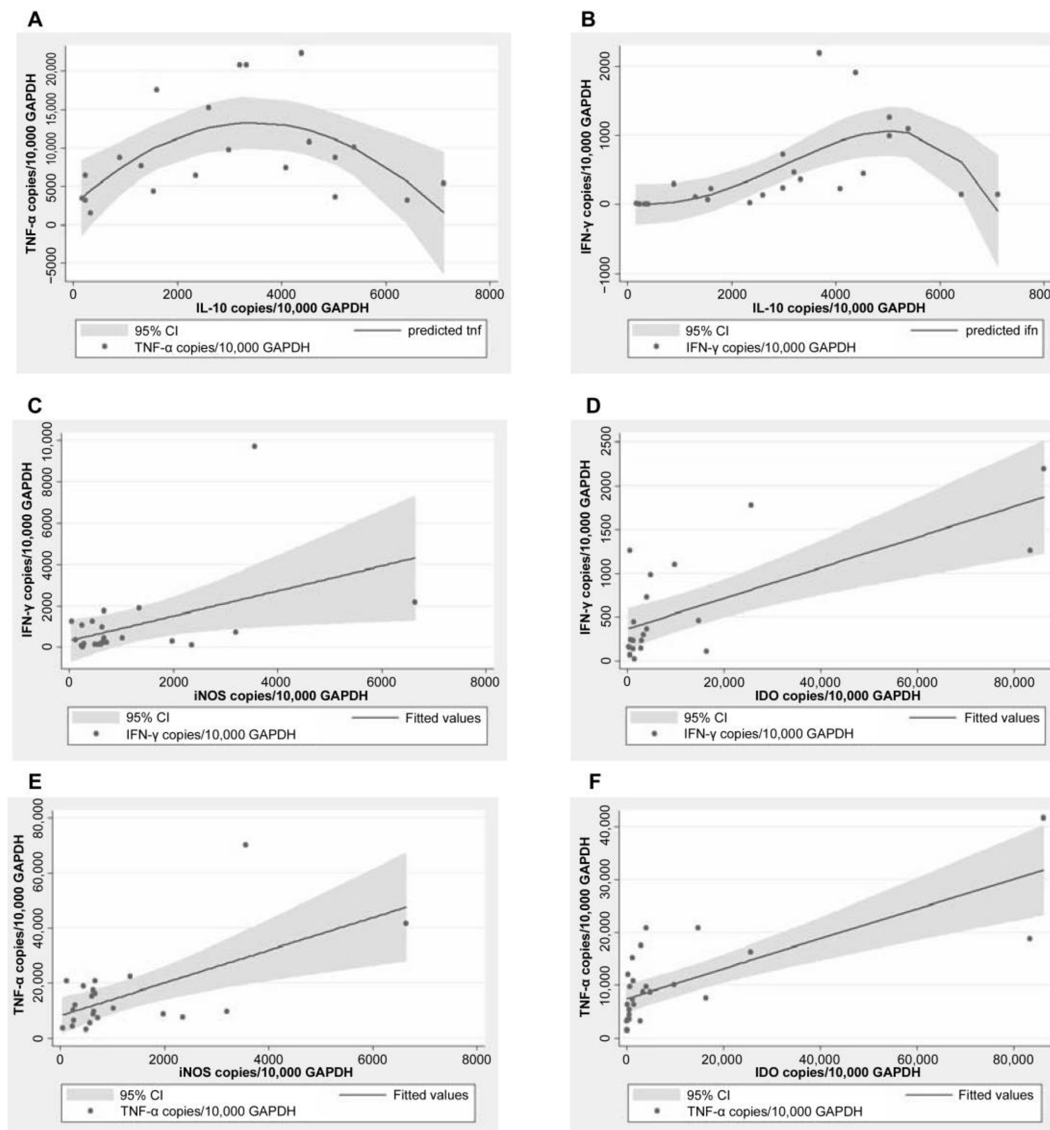

Figure 2. Correlation between mRNA-expression levels of tumor necrosis factor (TNF)–α and interleukin (IL)–10 *(A),* interferon (IFN)–γ and IL-10 *(B),* IFN-γ and inducible nitric oxide synthase (iNOS) *(C),* IFN-γ and indoleamine-2,3-dioxygenase (IDO) *(D),* TNF-α and iNOS *(E),* and TNF-α and IDO *(F).*

pression of IFN-γ mRNA, particularly in patients with mild/moderate MSF, and the absence of rickettsiae in the blood suggests that there is an IFN-γ–mediated reduction in bacterial load in these patients. Second, the data demonstrate a positive correlation between high levels of IFN-γ mRNA and TNF-α mRNA and the production of enzymes involved in limiting microbial growth—enzymes such as iNOS [22–24] and IDO [8, 25]—in the patients with MSF but not in the control subjects. Both iNOS and IDO are produced by many types of innate immune cells, in response to either bacterial endotoxins or proinflammatory and Th1 cytokines such as TNF-α and IFN-γ [25–32]. Thus, the correlation, in these patients with MSF, between elevated mRNA expression of IFN-γ and TNF-α and mRNA expression of iNOS and IDO suggests that IFN-γ and TNF-α play a synergistic role in the induction of iNOS and IDO.

The data in the present study show that some patients developed moderate/severe MSF in spite of significantly higher mRNA levels of dermal TNF-α and, to a lesser extent, of dermal IFN-γ. On the basis of these data, we propose 2 potential mechanisms that could account for the development of severe MSF. First, the simultaneous expression of higher levels of immunosuppressive IL-10 mRNA may decrease responsiveness to IFN-γ. IL-10 is associated with disease progression or with counteracting the IFN-γ– and TNF-α–mediated activation of intracellular microbicidal mechanisms [29, 30]. Analysis of the serum cytokine profile in Sicilian patients with MSF has also suggested that IL-10 plays a Th1-inhibitory role [6]. In addition,

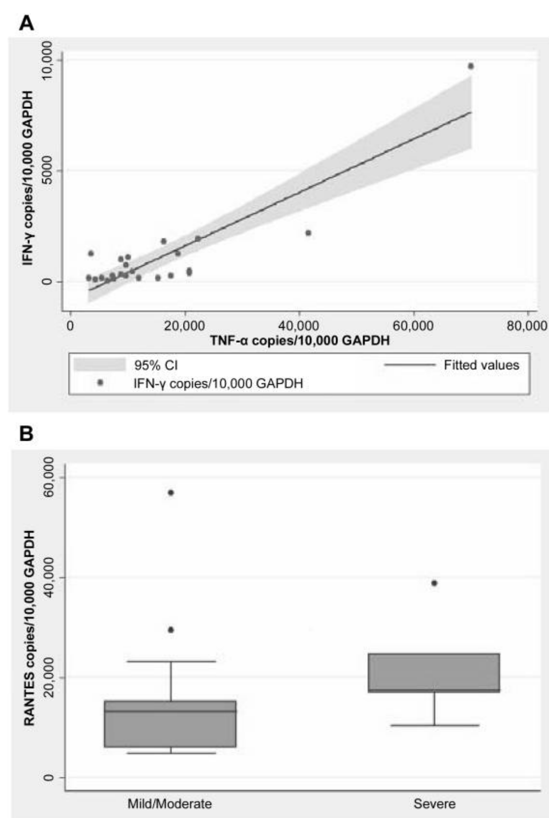

Figure 3. *A,* Correlation between mRNA-expression levels of tumor necrosis factor (TNF)–α and interferon (IFN)–γ, in patients with Mediterranean spotted fever (MSF). In these patients, there was a significant positive ($P = .0001$) correlation between the level of TNF-α mRNA expression and that of IFN-γ mRNA expression, a correlation that was not observed in the control subjects (data not shown). *B,* Comparison between the level of RANTES mRNA expression in patients with mild/moderate MSF and that in patients with severe MSF. The level of expression of RANTES mRNA is close ($P = .073$) to being significantly higher in patients with severe MSF than in patients with mild/moderate MSF. The tops and bottoms of the shaded boxes denote the 25th and 75th percentiles, respectively, and the horizontal line within each of the boxes denotes the median value; dots denote outliers.

the production of Th1-promoting cytokines, such as IL-12, by peripheral blood mononuclear cells from patients with MSF was enhanced in vitro by the addition of either recombinant IFN-γ or anti–IL-10 monoclonal antibodies [33]. Second, unbalanced pro- and anti-inflammatory cytokine responses with higher levels of TNF-α were observed in patients with severe MSF. This immunopathological condition represents disease manifestations that are caused by the immune system while the latter is attempting to control infection. The present study does not provide direct evidence for immunopathological condition in rickettsial disease. However, previous immunohistochemical and histopathological analyses of eschars either from fatal cases of Rocky Mountain spotted fever (RMSF) or from patients with MSF suggest the possibility that immune-mediated pathological mechanisms are present in MSF [7, 34, 35]. This hypothesis is based on 3 main features: (1) the eschars from patients with RMSF contained a massive quantity of rickettsiae in the endothelium and vascular smooth-muscle cells of the blood vessels, whereas very few organisms were present in the eschars from patients with MSF; (2) abundant lymphohistiocytic infiltrates, mainly T lymphocytes, were observed in lesions from patients with MSF; and (3), compared with the cutaneous necrosis in eschars from patients with RMSF and the association between such necrosis and occlusive thrombosis, the eschar lesion in patients with MSF was associated with dermal edema, edema of the vascular wall, and endothelial swelling [7, 34, 35]. Thus, the occurrence of moderate/severe vascular injury and cutaneous necrosis in MSF, in the absence of occlusive thrombosis and overwhelming infection but in the presence of abundant lymphohistiocytic infiltration, suggests the possibility of an immunopathological mechanism of the lesion. Moreover, the absence of rickettsiae in the blood of patients with severe MSF (table 3) further supports the hypothesis that severe MSF could be due to an immunopathological condition rather than to an overwhelming infection. On the basis of the aforementioned data, we postulate that balanced pro- and anti-inflammatory cytokines mediate protective type 1 immunity against *Rickettsia* while avoiding an immunopathological condition.

In the present study, we have also analyzed the mRNA-expression levels of IFN-γ, TNF-α, iNOS, IDO, RANTES, and IL-10 in 3 groups of patients—those with mild, moderate, or severe MSF. The data do not show statistically significant differences, in median levels of expression of these biomolecules, between these 3 groups of patients; however, this could be due to (1) the multifunctionality of many biomolecules, (2) widespread interactions between them, differences in the timing of when samples were obtained, and (3) the fact that local immune responses in the skin of patients with systemic rickettsial diseases may not exactly reflect the severity of overall illness. Despite these confounding factors, certain patterns are evident. First, IFN-γ is critical for protection against severe MSF, and that protection is dependent on expression of iNOS mRNA (table 7). The data in the present study are consistent with the protective role played by IFN-γ in animal models of SFG rickettsiosis [10, 36]; however, they are in disagreement with the results of a previous study [5], which showed that IFN-γ was absent in acute and convalescent plasma samples from patients with African tick-bite fever (ATBF), an SFG rickettsiosis caused by *R. africae.* These discordant results could be due to the possibility that cutaneous, rather than systemic, production of IFN-γ is the better indicator of protection against *Rickettsia.* Second, in the present study, high ($\geqslant$6500 copies) levels of TNF-α mRNA were detected in only 64% of patients with mild MSF, whereas they were detected in 100% of patients with severe MSF. These data suggest that TNF-α plays a dual role,

Table 6. Median levels of mRNA expression of cytokines, in patients with mild, moderate and severe Mediterranean spotted fever (MSF).

Cytokines and chemokine	Mild MSF (n = 11)	Moderate MSF (n = 7)	Severe MSF (n = 5)	P^a
IFN-γ				.381
Median	238.1	721.9	230.0	
Interquartile range	132.1–1905.1	293.2–1256.9	230.0–361.0	
Total range	23.4–9712.9	157.1–1777.6	103.7–1094.2	
TNF-α				.930
Median	9712.9	11,958	10,055.4	
Interquartile range	5388.6–22,314.3	8753.7–18,764.0	7620.6–17,507.8	
Total range	3204.0–70,029.9	3555.1–20,820.0	7360.9–20,820.0	
iNOS				.879
Median	628.5	673.6	628.5	
Interquartile range	493.1–1347.1	283.2–1972.3	246.5–721.9	
Total range	238.1–6634.0	43.6–3204.0	123.3–2345.5	
IDO				.423
Median	1301.3	3944.7	3944.7	
Interquartile range	586.4–2789	460.1–25,632	2888.0–9712.9	
Total range	528.5–86,217	274–83,280	1214.1–16,335	
IL-10				.785
Median	3680.5	3204.1	3317	
Interquartile range	2605.0–5027.7	2989.5–5027.7	1602.0–4083.8	
Total range	1547.5–7110.2	888.8–8753.7	1301.3–5388.6	
RANTES				.073
Median	14,722.0	8753.8	17,507.5	
Interquartile range	8167.5–23,101.3	5578.6–13,268.2	16,911.1–24,759.3	
Total range	4856.4–56,881.9	5388.6–23,101.3	10,410.0–38,851.5	

NOTE. IDO, indoleamine-2,3-dioxygenase; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.

[a] Kruskal-Wallis test

being involved in both protective antirickettsial immunity and the pathogenesis of severe MSF. Supporting this conclusion are the results of a previous study [6], which has demonstrated high plasma levels of TNF-α in acute serum from patients with ATBF, levels that, on follow-up, subsequently decreased to levels that were comparable to those in control subjects. Third, the present study revealed expression of high levels of RANTES mRNA in all patients with MSF, in contrast to what was observed in the control subjects, with significantly higher expression in patients with severe MSF than in those with mild/moderate MSF (figure 3*B*). RANTES is a chemokine that mediates the trafficking and homing of several lymphoid and myeloid cells, such as T cells, monocytes, and dendritic cells, toward the site of infection [37]. The expression of RANTES mRNA in the skin of patients with MSF could therefore explain the presence of prominent perivascular cellular infiltration in SFG rickettsiosis [7, 10, 34, 38]. The data in the present study also suggest that, like TNF-α, RANTES plays a dual role, being involved in both antirickettsial immunity and the pathogenesis of severe MSF. The protective role of RANTES could be due to RANTES-mediated chemotaxis of IFN-γ–producing CD4 Th1 lymphocytes toward the cutaneous site of infection. This conclusion is supported both by histological observation of an abundance of CD4 T cells in eschars from patients with MSF and by studies suggesting that RANTES plays a microbicidal role in certain viral and bacterial infections, including rickettsioses [8, 39]. On the other hand, the pathological role of RANTES could be attributed to expression of a very high level of the latter, which could substantially increase T cell infiltration at the site of infection, with subsequent sustained production of excessive amounts of proinflammatory cytokines. The latter

Table 7. Levels of mRNA expression of cytokines, above an established cutoff value, in patients with mild, moderate, and severe Mediterranean spotted fever (MSF).

Cutoff values of cytokines and chemokine	Mild MSF	Moderate MSF	Severe MSF	P^a
IFN-γ ⩾500 copies	5 (45)	4 (57)	1 (20)	.556
TNF-α ⩾6500 copies	7 (64)	6 (86)	5 (100)	.439
iNOS ⩾800 copies	5 (45)	1 (14)	1 (20)	.1
IDO ⩾1500 copies	5 (45)	4 (57)	5 (100)	.277
IL-10 ⩾3000 copies	7 (64)	3 (43)	3 (60)	.146
RANTES ⩾10,000 copies	7 (64)	3 (43)	5 (100)	.137

NOTE. Data are no. (%) of cases, in indicated group. IDO, indoleamine-2,3-dioxygenase; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.

[a] By Fisher's exact test.

can lead to indirect damage to endothelial cells, leading to the vascular dysregulation and leakage observed in SFG rickettsial infections. In support of this hypothesis, it has been shown that a high concentration of RANTES induces the activation of T cells in a manner that is usually followed by diverse effects, including substantial T cell proliferation or apoptosis and the release of proinflammatory cytokines [40]. A closer examination of the production of each of these mediators in a time-course study, as well as identification of the cellular source of these mediators in large groups of patients with MSF of different degrees of severity, is warranted, to attain a better understanding of MSF in humans.

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