Abstract

Background: Several lines of evidence suggest that chemokines and cytokines play an important role in the inflammatory development and progression of systemic lupus erythematosus. The aim of this study was to evaluate the relevance of functional genetic variations of RANTES, IL-8, IL-1α, and MCP-1 for systemic lupus erythematosus.

Methods: The study was conducted on 500 SLE patients and 481 ethnically matched healthy controls. Genotyping of polymorphisms in the RANTES, IL-8, IL-1α, and MCP-1 genes were performed using a real-time polymerase chain reaction (PCR) system with pre-developed TaqMan allelic discrimination assay.

Results: No significant differences between SLE patients and healthy controls were observed when comparing genotype, allele or haplotype frequencies of the RANTES, IL-8, IL-1α, and MCP-1 polymorphisms. In addition, no evidence for association with clinical sub-features of SLE was found.

Conclusion: These results suggest that the tested functional variation of RANTES, IL-8, IL-1α, and MCP-1 genes do not confer a relevant role in the susceptibility or severity of SLE in the Spanish population.

Background

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by autoantibody production, abnormalities of immune-inflammatory system function and inflammatory manifestation in several organs. Although the pathogenesis of SLE is unknown, the
Table 1: Taqman probes part number used for genotyping.

| Polymorphisms | Part number |
|---------------|-------------|
| RANTES -403 G/A (rs2107538) | C_15874407_10 |
| RANTES R3 C/T (rs2306630) | C_26625663_10 |
| IL-8 -353 A/T (rs4073) | C_11748116_10 |
| IL-8 +781 C/T (rs2227306) | C_11748169_10 |
| IL-1α -889 C/T (rs1800587) | C_9546481_20 |
| MCP-1 -2518 G/A (rs1024611) | C_2590362_10 |

In addition to these three genes, IL-1α also constitutes a strong candidate gene for SLE, since it is a proinflammatory cytokine that plays and important role in initiating and modulating the immune responses. There is a functional polymorphism in the promoter region of IL-1α gene at position -889 C/T (rs1800587), and the -889 C homozygous genotype has been associated with significantly lower transcriptional activity of the IL-1α gene and lower levels of IL-1α in plasma compared with the TT genotype [18].

This polymorphism was genotyped using TaqMan allelic discrimination assay. The PCR amplification was carried out with mixes consisting of 8 ng of genomic DNA, 2.5 µl of Taqman master mix, 0.125 µl of 20x assay mix and ddH2O up to 5 µl of final volume. The following amplification protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation...

Overall, the chemokines RANTES, IL-8, MCP-1 and cytokine IL-1α are strong candidate genes for which genetic association studies can shed light on the underlying mechanisms causing the immune dysregulation, such as inappropriate T cell activation or trafficking in SLE.

Methods

Patients

Peripheral blood samples were obtained after written informed consent from 500 SLE patients meeting the American College of Rheumatology (ACR) criteria for SLE [19]. These patients were recruited from five Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clinico (Granada), Hospital Virgen del Rocio (Seville) and Hospital Carlos-Haya and Hospital Virgen de la Victoria (Malaga). Similarly, blood was taken from 481 blood bank and bone marrow donors of the corresponding cities that were included as healthy individuals. Both patient and control groups were of Spanish Caucasian origin and were matched for age and sex. Eighty seven percent of the SLE patients were women, the mean age of SLE patients at diagnosis was 43 ± 13.3 years and the mean age at disease onset of SLE symptoms was 32 ± 15 years. The SLE clinical manifestations studied were articular involvement (76%), renal affection (37%), cutaneous lesions (62%), hematopoietic alterations (73%), photosensitivity (51%), neurological disease (17%) and serositis (28%). The study was approved by all local ethical committees from the corresponding hospitals.

Genotyping

For all the considered SNPs, samples were genotyped using a pre-developed TaqMan allelic discrimination assay. Table 1 shows the part number and reference of each SNP (Applied Biosystems, Foster City, CA, USA). PCR was carried out with mixes consisting of 8 ng of genomic DNA, 2.5 µl of Taqman master mix, 0.125 µl of 20x assay mix and ddH2O up to 5 µl of final volume. The following amplification protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation...
at 92°C for 15 sec and annealing and extension at 60°C for 1 min. After PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRISM 7900 Sequence Detection Systems using the SDS 2.2.2 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA).

Statistical analysis
Allele and genotype frequencies were obtained by direct counting. Hardy-Weinberg equilibrium and statistical analysis to compare allelic and genotypic distributions were performed using the chi-square test. Odds ratio (OR) with 95% confidence intervals (95%CI) were calculated according to Woolf's method. The software used was StatCalc program (Epi Info 2002; Centers of Disease Control and Prevention, Atlanta, GA, USA). For the haplotype analysis, pair-wise linkage disequilibrium measures were investigated and haplotypes were constructed using the expectation-maximization (EM) algorithm implemented in UNPHASED software [20]. P values below 0.05 were considered statistically significant. The power of the study to detect an effect of a polymorphism in disease susceptibility was estimated using the Quanto v 0.5 software (Department of Preventive Medicine University of Southern California, CA, USA).

Results
Table 2 shows the allele and genotype distribution of the RANTES, IL-8, IL-1α, and MCP-1 polymorphisms. For all polymorphisms, the distribution of genotypes did not deviate from that expected from populations in Hardy-Weinberg equilibrium.

RANTES typing
Genotyping of RANTES -403 G/A and R3 T/C was performed in 500 and 442 SLE patients and 481 and 438 healthy controls, respectively (table 2). No statistically significant differences were observed when the allele and genotype distribution was compared between SLE patients and healthy controls. Also, we found no association for the two marker haplotypes (table 3).

IL-8 typing
IL-8 -353 T/A and +781 C/T was genotyping in 439 and 467 SLE patients and 412 and 429 healthy controls, respectively for each polymorphism. We found a similar distribution in the allele and genotype frequencies between SLE patients and controls for both genetic variants. The haplotype estimation for the -353 T/A and +781 C/T IL-8 polymorphisms revealed a strong degree of linkage disequilibrium between the two variants (D' = 0.9) and showed a slight but non-significant increase of the -353T/+781C haplotype in SLE patients (8.5% vs 6.2%, P = 0.08, OR = 1.41, 95%CI = 0.94–2.10) (Table 3).

IL-1α typing
IL-1α -889 was typing in 417 SLE patients and 420 healthy controls. We did not find any significant difference when allele and genotype frequencies were compared between SLE patients and healthy controls.

MCP-1 typing
Table 2 show the allele and genotype distribution of the MCP-1 -2518 A/G polymorphism in 450 SLE patients and 427 controls. No significant differences in the allele and genotype frequencies of the MCP-1 -2518 A/G polymorphism were found between SLE patients and controls.

In addition, available clinical features of patients with SLE were analysed for possible association with the different alleles or genotypes of these polymorphisms. When we stratified SLE patients according to the presence of renal involvement, no statistically significant differences were observed in the distribution of RANTES -403, RANTES R3, IL-1α -889 and MCP-1 -2518 polymorphisms between SLE patients with and without lupus nephritis (table 4). Regarding IL-8 polymorphisms, the AT -353 genotype and the -353T/+781C haplotype showed an increased among lupus patients without nephritis compared with patients with nephritis (39.2% vs 49.4%, P = 0.03, OR = 0.66, 95%CI = 0.44–0.99 for AT -353 genotype) (5.7% vs 10%, P = 0.05, OR = 0.55, 95%CI = 0.28–1.05 for -353T/+781C haplotype) (table 4). Similarly, no significant differences were observed between all these genetic variants and the following variables: sex, age at onset, articular involvement, cutaneous lesions, photosensitivity, hematological alterations, neurological disorders and serositis (data not shown).

Discussion
In this work, we have tested six functional polymorphisms of four strong candidate genes for association with SLE. No evidence of association was detected for RANTES (-403 G/A, R3 T/C), IL-8 (-353 A/T, +781 C/T), IL-1α (-889 C/T), and MCP-1 (-2518 G/A) polymorphisms. However, a significant association was observed for the IL-8 haplotype with SLE nephritis, which cannot be considered as significant after correction for multiple comparisons.

All these genes have been previously associated with susceptibility and development to several autoimmune disorders, included SLE [16,21–27]. For example, recent studies in Asian populations found another RANTES polymorphism (-28C/G) to be associated with increased risk of developing SLE, but failed to detect any association of RANTES -403 polymorphisms with SLE [22,23]. We did not test the -28C/G variant as -28G allele is relatively uncommon in Caucasians [28].
Table 2: Allele and genotype frequencies of RANTES, IL-8, MCP-1 and IL-1α polymorphisms in SLE patients and healthy controls.

| Allele/Genotype   | SLE patients | Controls | P  | OR (95%CI) |
|-------------------|--------------|----------|----|------------|
| **RANTES -403**   |              |          |    |            |
| Genotypes         |              |          |    |            |
| GG                | 369          | 73.8     | 333| 69.3       | 0.1 |
| GA                | 113          | 22.6     | 135| 28         | 0.04| 0.75 (0.55–1.01) |
| AA                | 18           | 3.6      | 13 | 2.7        | 0.4 |
| Alleles           |              |          |    |            |
| G                 | 851          | 85       | 801| 83.3       |    |
| A                 | 149          | 15       | 161| 16.7       | 0.2 |
| **RANTES R3**     |              |          |    |            |
| Genotypes         |              |          |    |            |
| CC                | 326          | 73.8     | 340| 77.6       | 0.06|
| CT                | 104          | 23.5     | 90 | 20.6       | 0.3 |
| TT                | 12           | 2.7      | 8  | 1.8        | 0.4 |
| Alleles           |              |          |    |            |
| C                 | 756          | 85.5     | 770| 88         |    |
| T                 | 128          | 14.5     | 106| 12         | 0.1 |
| **IL-8 -353**     |              |          |    |            |
| Genotypes         |              |          |    |            |
| AA                | 126          | 28.7     | 125| 30.3       | 0.6 |
| AT                | 215          | 49       | 194| 47.1       | 0.5 |
| TT                | 98           | 22.3     | 93 | 22.6       | 0.9 |
| Alleles           |              |          |    |            |
| A                 | 467          | 53.2     | 444| 53.8       |    |
| T                 | 411          | 46.8     | 380| 46.2       | 0.7 |
| **IL-8 +781**     |              |          |    |            |
| Genotypes         |              |          |    |            |
| CC                | 164          | 35       | 143| 33.3       | 0.6 |
| CT                | 238          | 51       | 221| 51.5       | 0.8 |
| TT                | 65           | 14       | 65 | 15.2       | 0.6 |
| Alleles           |              |          |    |            |
| C                 | 566          | 60.6     | 507| 59.1       |    |
| T                 | 368          | 39.4     | 351| 40.9       | 0.5 |
| **IL-1α -889**    |              |          |    |            |
| Genotypes         |              |          |    |            |
| CC                | 220          | 52.7     | 209| 49.7       | 0.4 |
| CT                | 164          | 39.3     | 166| 39.5       | 0.9 |
| TT                | 33           | 7.9      | 45 | 10.7       | 0.2 |
| Alleles           |              |          |    |            |
| C                 | 604          | 72.4     | 584| 69.5       |    |
| T                 | 230          | 27.6     | 256| 30.5       | 0.2 |
| **MCP-1 -2518**   |              |          |    |            |
| Genotypes         |              |          |    |            |
| AA                | 238          | 57.2     | 250| 58.5       | 0.6 |
| AG                | 173          | 35       | 154| 36         | 0.7 |
| GG                | 39           | 7.8      | 23 | 5.4        | 0.1 |
| Alleles           |              |          |    |            |
| A                 | 739          | 74.6     | 654| 76.6       |    |
| G                 | 251          | 25.4     | 200| 23.4       | 0.3 |
The genetic variant IL-8 -845C showed a high association to severe lupus nephritis (LN) in an African American population [16], but also this allele has a very low frequency in Caucasian populations [16,29]. The trend of association that we have found between the haplotypes and LN and the reported association of other IL-8 variants this African American population, shows that variants in this chemokine may have a minor influence on the risk of developing nephritis in SLE patients.

Similar observation could be made for the reported association of the IL-1α -889C/T variant to SLE in a White and African American populations from United States, which we failed to replicate [30]. With regard to the MCP-1 -2518 polymorphism, an American study showed that an A/G or G/G genotype may predispose to the development of SLE and further indicated that SLE patients with these genotypes may be at higher risk of developing LN [3].

The fact that we do not observe an association and fail to confirm some previous studies may be caused by a Type II error (false-negative). This is however unlikely because our sample has more than 80% power to detect the relative risk similar to the other studies at the 5% significance level. Furthermore, the genotype frequencies did not differ from Hardy-Weinberg expectations, and allele and genotype frequencies in our Spanish population are similar to those reported previously in other Caucasian populations [16,26,31,32]. The failure to replicate reported associations is a common event in the search for genetic determinants of complex diseases, due either to genuine population heterogeneity or a different sort of bias [33]. The lack of replication in our population may alternatively be explained by a different racial composition of that study from ours, or that presence of environmental factors to which the Asian, American, and African populations, but not the Spanish population, are exposed. In addition, genetic differences are known to exist between the different ethnic groups, such as, African American and Caucasians.

### Conclusion
In conclusion, our results suggest that functional genetics variation in RANTES, IL-8, IL-1α, and MCP-1 do not play a major role in SLE susceptibility in the Spanish population.

### Competing interests
The author(s) declare that they have no competing interests.

### Authors’ contributions
ES carried out the genotyping and statistical analysis and drafted the manuscript, JMS collected the samples, JLC collected the samples, EDR collected the samples, RGP collected the samples, FJGH collected the samples, JJA collected the samples, MFGE collected the samples, JM participated in the manuscript design and coordination and helped to draft the manuscript, BK participated in the manuscript design, reviewed the statistical analysis and helped to draft the manuscript.

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### Table 3: Haplotype frequencies for RANTES and IL-8 polymorphisms in SLE patients and controls.

| Gene  | Haplotype | SLE patients | Healthy controls | P_value | OR (95%CI) |
|-------|-----------|--------------|------------------|---------|------------|
| RANTES | -403A/R3C | 25 (5.7)     | 25 (5.8)         | ns      |            |
|       | -403A/R3T | 50 (11.3)    | 40 (9.3)         | ns      |            |
|       | -403G/R3C | 355 (80.7)   | 356 (83.4)       | ns      |            |
|       | -403G/R3T | 10 (2.3)     | 6 (1.5)          | ns      |            |
| IL-8  | -353T/+781C | 69 (8.6)    | 48 (6.2)         | 0.08    | 1.41 (0.94–2.10) |
|       | -353T/+781T | 316 (39.2)  | 303 (39.4)       | ns      |            |
|       | -353A/+781C | 403 (50)    | 406 (52.7)       | ns      |            |
|       | -353A/+781T | 18 (2.2)    | 13 (1.7)         | ns      |            |
Table 4: Relationship between RANTES, IL-8, MCP-1 and IL-1α polymorphisms and the presence of nephritis in SLE Spanish patients.

|                           | SLE with nephritis | SLE without nephritis | P       | OR (95%CI) |
|---------------------------|--------------------|-----------------------|---------|------------|
| **RANTES -403**           |                    |                       |         |            |
| Genotypes                 |                    |                       |         |            |
| GG                        | 136                | 73.5                  | 230     | 73         | 0.9       |
| GA                        | 44                 | 23.8                  | 71      | 22.5       | 0.7       |
| AA                        | 5                  | 2.7                   | 14      | 4.4        | 0.3       |
| Alleles                   |                    |                       |         |            |
| G                         | 54                 | 14.6                  | 99      | 15.7       |           |
| A                         | 316                | 85.4                  | 531     | 84.3       | 0.6       |
| **RANTES R3**             |                    |                       |         |            |
| Genotypes                 |                    |                       |         |            |
| CC                        | 89                 | 77.4                  | 225     | 68.8       | 0.08      |
| CT                        | 23                 | 20                    | 92      | 28.1       | 0.1       |
| TT                        | 3                  | 2.6                   | 10      | 3          | 0.8       |
| Alleles                   |                    |                       |         |            |
| C                         | 201                | 87.4                  | 542     | 82.9       |           |
| T                         | 29                 | 12.6                  | 112     | 12.1       | 0.1       |
| **IL-8 -353**             |                    |                       |         |            |
| Genotypes                 |                    |                       |         |            |
| AA                        | 47                 | 26.7                  | 59      | 22.4       | 0.3       |
| AT                        | 69                 | 39.2                  | 130     | 49.4       | 0.03      |
| TT                        | 60                 | 34.1                  | 74      | 28.2       | 0.2       |
| Alleles                   |                    |                       |         |            |
| A                         | 163                | 46.3                  | 248     | 47.2       |           |
| T                         | 189                | 53.7                  | 278     | 52.8       | 0.8       |
| **IL-8 +781**             |                    |                       |         |            |
| Genotypes                 |                    |                       |         |            |
| CC                        | 74                 | 39.6                  | 99      | 35.3       | 0.3       |
| CT                        | 85                 | 45.4                  | 151     | 54         | 0.07      |
| TT                        | 28                 | 15                    | 30      | 10.7       | 0.2       |
| Alleles                   |                    |                       |         |            |
| C                         | 233                | 62.3                  | 349     | 62.3       |           |
| T                         | 141                | 37.7                  | 211     | 37.7       | 0.9       |
| **IL8 -353T/+781C**       |                    |                       |         |            |
| Haplotypes                |                    |                       |         |            |
| -353T/+781C               | 15                 | 5.7                   | 39      | 10         | 0.05      |
| -353T/+781T               | 104                | 39.7                  | 149     | 38.2       | 0.7       |
| -353A/+781C               | 140                | 53.4                  | 193     | 49.5       | 0.3       |
| -353A/+781T               | 3                  | 1.2                   | 9       | 2.3        | 0.3       |
| **IL-1α -889**            |                    |                       |         |            |
| Genotypes                 |                    |                       |         |            |
| CC                        | 72                 | 49.3                  | 138     | 50.9       | 0.7       |
| CT                        | 59                 | 40.4                  | 115     | 42.4       | 0.7       |
| TT                        | 15                 | 10.3                  | 18      | 6.7        | 0.2       |
| Alleles                   |                    |                       |         |            |
| C                         | 203                | 69.5                  | 391     | 72.1       |           |
| T                         | 89                 | 30.5                  | 151     | 27.9       | 0.4       |
Table 4: Relationship between RANTES, IL-8, MCP-1 and IL-1α polymorphisms and the presence of nephritis in SLE Spanish patients.

| MCP-1 -2518       |
|-------------------|
| Genotypes         | Alleles | A  | G  |
| AA                | 86      | 233| 83 |
| AG                | 61      | 73.7| 26.3|
| GG                | 11      | 7  | 22 |
| Genotype frequency|         |    |    |

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