Identification of a new putative functional IL18 gene variant through an association study in systemic lupus erythematosus

Elena Sánchez1,2,∗,†, Rogelio J. Palomino-Morales1,†, Norberto Ortego-Centeno3, Juan Jiménez-Alonso4, Miguel A. González-Gay2, Miguel A. López-Nevot5, Julio Sánchez-Román6, Enrique de Ramón7, M. Francisca González-Escribano8, Bernardo A. Pons-Estel9,‡, Sandra D’Alfonso10, Gian Domenico Sebastiani11, The Italian collaborative group, Marta E. Alarcón-Riquelme12 and Javier Martín1

1Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada 18100, Spain, 2Hospital Xeral-Calde, Servicio de Reumatología, Lugo 27004, Spain, 3Hospital Clínico San Cecilio, Unidad de enfermedades sistémicas autoinmunes, Granada 18012, Spain, 4Hospital Virgen de las Nieves, Servicio de Medicina Interna, Granada 18014, Spain, 5Hospital Virgen de las Nieves, Servicio de Inmunología, Granada 18014, Spain, 6Hospital Virgen del Rocío, Servicio de Medicina Interna, Sevilla 41013, Spain, 7Hospital Carlos Haya, Servicio de Medicina Interna, Málaga 29010, Spain, 8Hospital Virgen del Rocío, Servicio de Inmunología, Sevilla 41013, Spain, 9Sanatorio Parque, Rosario 860, Argentina, 10Department of Medical Sciences and IRCAD, University of Eastern Piedmont, Novara 28100, Italy, 11U.O.C. di Reumatologia Ospedale San Camillo, Roma 00151, Italy and 12Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala 75185, Sweden

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Interleukin-18 (IL-18) is a proinflammatory cytokine that plays an important role in chronic inflammation and autoimmune disorders. In this study, we aimed to determine the potential role of the IL18 gene in SLE. To define the genetic association of the IL18 and SLE, we have genotyped nine SNPs in an independent set of Spanish cases and controls. The IL18 polymorphisms were genotyped by PCR, using a predeveloped TaqMan allele discrimination assay. Two SNPs were still significant after fine mapping of the IL18 gene. The SNP (rs360719) surviving correction for multiple tests was genotyped in two replication cohorts from Italy and Argentina. After the analysis, a significance with rs360719 C-allele remained across the sets and after the meta-analysis (Pooled OR = 1.37, 95% CI 1.21–1.54, combined P = 3.8E-07, Pc = 1.16E-06). Quantitative real-time PCR was performed to assess IL18 mRNA expression in PBMC from subjects with different IL18 rs360719 genotypes. We tested the effect of the IL18 rs360719 polymorphism on the transcription of IL18 by electrophoretic mobility shift assay and western blot. We found a significant increase in the relative expression of IL18 mRNA in individuals carrying the rs360719 C-risk allele; in addition we show that the polymorphism creates a binding site for the transcriptional factor OCT-1. These findings suggest that the novel IL18 rs360719 variant may play an important role in determining the susceptibility to SLE and it could be a key factor in the expression of the IL18 gene.

∗To whom correspondence should be addressed at: Instituto de Parasitología y Biomedicina ‘López-Neyra’, CSIC, Parque Tecnológico de Ciencias de la Salud, Avenida del Conocimiento s/n 18100-Armilla, Granada, Spain. Tel: +34 958181621; Fax: +34 958181632; Email: elena@ipb.csic.es
†Both authors have contributed equally to this work.
‡B.A.P.-E. is coordinator of the Argentine collaborative group. The list of participants is given in Appendix section along with Italian collaborative group.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by enhanced autoantibody production, abnormalities of immune-inflammatory system function and inflammatory manifestation in several organs. Although the pathogenesis of SLE is unknown, a strong genetic component has been supported by studies on twins and families (1). Like most autoimmune diseases, the HLA genes make an important contribution, although other somewhat weaker but well established associations have been found with non-HLA genes (2).

Interleukin-18 (IL-18) is an important proinflammatory cytokine, member of the IL-1 cytokine family, which has been shown to exert innate and acquired immune responses (3–5). IL-18 is expressed by a wide range of immune cells (6) and has been found to have multiple biological functions. The IL-18 has recently been shown to be a pleiotropic cytokine that can mediate both Th1 and Th2 driven immune responses (7,8). In combination with IL-12, IL-18 induces IFN-γ production in Th1 cells, B cells and natural killer cells, promoting Th1-type immune responses (9,10), but it can also stimulate Th2 immune responses in the absence of IL-12 (11,12). Abnormalities in the production of Th1 and Th2 cytokines have been shown in SLE patients (13). In addition, IL-18 can accelerate spontaneous autoimmune disease in MRL/lpr mice, characterized by glomerulonephritis, vasculitis and symmetrical malar rash, suggesting that it is an important mediator of lupus-like disease (14). Likewise, elevated serum levels of IL-18 have been described in SLE patients compared with controls (15–17) and the elevation of IL-18 was positively correlated to SLE disease activity index (15). Because of its multiple functions in inflammation and immunological responses, a potential pathological role in the development of chronic inflammation has been suggested for IL-18, including autoimmune diseases such as SLE.

The IL18 gene is located on chromosome 11q22.2–22.3 (18), a close linkage region with SLE in European populations (19,20). In addition, several polymorphisms within the IL18 promoter gene have been associated with different inflammatory and autoimmune diseases (21–28). These findings suggest that IL18 is a candidate proinflammatory cytokine gene involved in the susceptibility to autoimmune diseases, such as SLE. The aim of this study was to determine the relationship between the IL18 gene and susceptibility to SLE.

RESULTS

Selection of markers for analysis

To investigate whether the IL18 gene on Chromosome 11q22.2–22.3 associates with SLE, we selected a total of nine polymorphisms spanning this candidate gene for genotyping. Tagging SNPs as well as random SNPs with minor allele frequencies above 0.01 in Caucasian populations were included to ascertain maximum haplotype information for the gene and to ensure coverage of intergenic regions which may harbour regulatory polymorphisms.

IL18 is associated with susceptibility to SLE

In all populations, genotype frequencies were in Hardy–Weinberg equilibrium in patients and controls for all the polymorphisms analysed. The success rate of genotyping (that is, the percentage of samples that could be analysed) was >95% for all polymorphisms in both SLE cases and controls.

Single SNP analysis performed in the first set of SLE patients (n = 752) and controls (n = 595) from Spain revealed significant association between rs360719 and rs1946518 SNPs in the IL18 gene and SLE (allele-P = 7.8E-07 and allele-P = 0.03, respectively) (Table 1). However, only the rs360719 surviving after correction for multiple test (PBonferroni correction = 0.01, P FDR correction = 0.01), and this variant also remains significantly associated after permutation analysis with 10 000 permutations (P10000 < 0.00001).

To delineate the haplotypic architecture of the whole gene, we estimated the underlying haplotype block structure of 19 SNPs with minor allele frequency >5% in the Caucasian population. We used imputation to fill in missing genotypes and to test ungenotyped variants within the gene of our existing Spanish genotyping. Figure 1 shows a plot of –log10(P-value) for all the SNPs used in the imputed case–control association study against chromosomes position.

To seek replication of the Spanish association in IL18, we genotyped the rs360719 variant in two independent populations from Italy and Argentina (Table 2). Risk allele frequencies were similar in the three populations. Interestingly, in Argentinean and Italian populations, we found that the frequencies of the rs360719 CC genotype were slightly increased in SLE patients compared with healthy controls (P = 0.02, OR = 1.88 95% CI 1.07–3.31 in Italians and P = 0.09, OR = 1.70 95% CI 0.89–3.23 in Argentineans), although these differences did not reach statistical significance in the allele frequencies (P = 0.1, OR = 1.20 95% CI 0.95–1.51 in Italians and P = 0.2, OR = 1.18 95% CI 0.90–1.53 in Argentineans).

Using homogeneity and combinability test according to the Breslow–Day method, we carried out a meta-analysis comprising 2579 individuals to maximize the number of samples for the association analysis (Breslow–Day P-value = 0.2). We then used the Mantel–Haenszel test to calculate pooled OR for IL18 rs360719 polymorphism (Table 2), and corroborated the genetic association with SLE for this polymorphism (pooled OR = 1.37, 95% CI 1.21–1.54, P = 3.8E-07, Pcorr = 1.16E-06). In addition, the clinical and demographic features of patients with SLE were analysed for possible association with the different alleles or genotypes of all the IL18 polymorphisms. No statistically significant differences were observed in the distribution of these variants (data not shown).

In view of these interesting results, we decided to perform functional experiments in order to confirm the potential role of the IL18 rs360719 polymorphism in the pathogenesis of SLE.

Expression analysis

A relative quantification of mRNA was performed in total RNA from 23 healthy individuals carrying different genotypes for IL18 rs360719 polymorphism (Fig. 2). A statistically
significant deviation was observed when we compared the relative expression of the \textit{IL18} in samples from healthy subjects stratified according to their \textit{IL18} rs360719 genotypes, showing an increased expression in individual carriers of the C allele in each of the reference genes (CC + CT: \( n = 11 \), versus TT: \( n = 12 \); \( P = 0.012 \) for \( \beta\text{-actin} \), \( P = 0.03 \) for \textit{GADPH} and \( P = 0.016 \) for \textit{ABL}.

**Electrophoretic mobility shift assay**

Our \textit{in silico} analysis of the wild-type and variant sequences (http://www.cbrc.jp/research/db/TFSEARCH.html) indicates that transcription factor OCT-1 binds to the protective allele (T) but not to the risk allele (C) at position -1297 of \textit{IL18} gene. To investigate the effect of this polymorphism on transcription factor binding, we performed an electrophoretic mobility shift assay (EMSA) assay. We observed a higher level of binding of protein to the \textit{IL18} -1297T allele than to the -1297C allele (Fig 3), which support the sequence-based prediction of OCT-1 binding to the rs360719 alleles of \textit{IL18} gene. Three protein complexes formed on the OCT-1 probe (complexes 1–3) were detected consistently (Fig 3). To confirm the specificity of OCT-1 T or C-allele oligonucleotides, a competition assay was carried out using excess amounts of cold oligonucleotides as competitors (Fig 3). This indicates that all three DNA–protein complexes represent a specific interaction between Jurkat nuclear protein and the OCT-1 sequence. To further confirm the results, we performed a supershift assay with anti-human OCT-1 antibody. As show in Figure 3, when anti-human OCT-1 antibody was added into the reaction mixture, the super-shifted band due to the antibody binding to complex appeared.

**Immunooassays**

To test the interaction between IL-18 and OCT-1, we analysed the expression patterns of both proteins in unstimulated and stimulated Jurkat cells. Western blot analysis showed an increased expression of the IL-18 after activation of Jurkat cells with a proinflammatory stimulus such as phorbol myristate acetate (PMA) and ionomycin (Supplementary Material, Fig S1). Conversely, we observed that the OCT-1 expression is decreased after stimulation. Similar results were obtained after analysis by flow cytometry (Supplementary Material, Fig S2). We found an increased expression of IL-18 in Jurkat cells after activation with PMA-ionomycin (12.73 ± 1.58) with respect to the IL-18 expression in unstimulated Jurkat cells (7.03 ± 0.83) \( (P = 0.01) \). In addition, a decreased expression of OCT-1 was observed in stimulated Jurkat cell (5.42 ± 0.005) with respect to unstimulated cells (5.42 ± 1.45) \( (P = 0.02) \).

**DISCUSSION**

Although the role of IL-18 in inflammatory and autoimmune processes has been well established (14), the available genetic data are largely contradictory, reflecting the small samples used, the different diseases analysed and the ethnic groups investigated (29). The aim of this study was to investigate the role of \textit{IL18} gene variations in SLE, for this purpose we performed the most powerful genetic study to date. Regarding the \textit{IL18} rs187238 and rs1946518 polymorphisms, which have been found associated with susceptibility to SLE and different phenotypes of the disease in Asiatic populations (30–32), we could not confirm these previously reported associations. These contradictory data could be due to genetic or environmental ethnical heterogeneity, which is clearly present, since allele and genotype frequencies are significantly different between Chinese and Spanish populations. Similarly, these ethnic differences in allele frequency of autoimmune disease-associated polymorphisms have been found in other susceptibility genes to SLE, such as \textit{PDCD1} and \textit{PTPN22} (33).

We have identified a novel variation which seems to affect the expression of the \textit{IL18} gene (\textit{IL18} rs360719), which could be a key genetic variant in the role of the \textit{IL18} gene in autoimmune diseases. Our results have shown that, in the combined analysis of three Caucasians populations, including 1356 SLE patients and 1223 healthy controls, the \textit{IL18} rs360719 C-allele was associated with an increased risk of SLE (OR 1.37). Interestingly, we observed an increased expression of IL-18 levels in correlation with the \textit{IL18} rs360719 polymorphism. Although these data support an evidence of association between \textit{IL18} rs360719 polymorphism and SLE, the full inclusion of \textit{IL18} gene as a candidate gene to SLE will require further independent studies in different

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**Table 1. Fine mapping of the \textit{IL18} gene in the Spanish patients with SLE and matched controls**

<table>
<thead>
<tr>
<th>Chr.</th>
<th>Position</th>
<th>SNP</th>
<th>Cases (aa/Aa/AA)</th>
<th>Controls (aa/Aa/AA)</th>
<th>Frequency cases</th>
<th>Frequency controls</th>
<th>Genotypic test</th>
<th>Allele test</th>
<th>ORa</th>
<th>L95</th>
<th>U95</th>
<th>Multiple test correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>111541359</td>
<td>rs360719</td>
<td>97/314/325</td>
<td>34/235/324</td>
<td>0.34</td>
<td>0.25</td>
<td>7.9E–06</td>
<td>7.8E–07</td>
<td>1.53</td>
<td>1.29</td>
<td>1.81</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>111540668</td>
<td>rs1946518</td>
<td>146/375/215</td>
<td>91/304/197</td>
<td>0.45</td>
<td>0.41</td>
<td>0.03</td>
<td>0.03</td>
<td>1.19</td>
<td>1.02</td>
<td>1.38</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
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<td>rs87238</td>
<td>55/317/365</td>
<td>34/256/302</td>
<td>0.29</td>
<td>0.27</td>
<td>0.2</td>
<td>0.4</td>
<td>1.08</td>
<td>0.91</td>
<td>1.28</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>111536290</td>
<td>rs1946518</td>
<td>56/301/380</td>
<td>41/229/321</td>
<td>0.28</td>
<td>0.26</td>
<td>0.6</td>
<td>0.3</td>
<td>1.09</td>
<td>0.91</td>
<td>1.29</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>111534931</td>
<td>rs4937113</td>
<td>81/375/215</td>
<td>97/283/211</td>
<td>0.43</td>
<td>0.40</td>
<td>0.1</td>
<td>0.1</td>
<td>1.13</td>
<td>0.96</td>
<td>1.32</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>111531913</td>
<td>rs156022</td>
<td>102/346/215</td>
<td>14/128/450</td>
<td>0.13</td>
<td>0.13</td>
<td>0.2</td>
<td>0.9</td>
<td>1.01</td>
<td>0.80</td>
<td>1.26</td>
<td>1</td>
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<tr>
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<td>10/45/705</td>
<td>13/35/558</td>
<td>0.03</td>
<td>0.03</td>
<td>0.2</td>
<td>0.9</td>
<td>1.02</td>
<td>0.65</td>
<td>1.58</td>
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<td>111522081</td>
<td>rs5744276</td>
<td>21/249/456</td>
<td>21/321/434</td>
<td>0.22</td>
<td>0.23</td>
<td>0.6</td>
<td>0.4</td>
<td>0.93</td>
<td>0.77</td>
<td>1.11</td>
<td>1</td>
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<tr>
<td>11</td>
<td>111521724</td>
<td>rs5744280</td>
<td>103/306/301</td>
<td>75/256/262</td>
<td>0.36</td>
<td>0.34</td>
<td>0.3</td>
<td>0.3</td>
<td>1.08</td>
<td>0.91</td>
<td>1.27</td>
<td>1</td>
</tr>
</tbody>
</table>

\*Allele-OR of the minor allele.

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populations. An important issue is how this \( \text{IL18} \) variant affects the expression of the gene. Several transcription factor binding sites that may be involved in the gene regulation of \( \text{IL18} \) were identified by Kalina et al. (34). The rs360719 polymorphism in \( \text{IL18} \) leads to loss of the OCT-1 transcription factor binding site. OCT-1 is known as a ubiquitously expressed factor and
is involved in the regulation of several genes. It can also repress the expression of certain genes, including some cytokines (35–39). We have shown that the presence of the T allele at position -1297 (rs360719) may play a key role in the transcription of the \( \text{IL18} \) gene and its role could be mediated through OCT-1 binding. This suppression would result in reduced IL-18 production and potential protection against IL-18 overexpression in disorders such as SLE, in which a persistent inflammatory response appears to be an underlying pathogenic process. Such a functional explanation would be consistent with the observation that the rs360719 C-allele is associated with SLE, in which IL-18 overexpression is generally observed (15–17). Thus, the functional experiments that we describe indicate differences in the

### Table 2. Individual and pooled genetic association analysis of \( \text{IL18} \) rs360719 polymorphism in three sets of SLE cases and controls

<table>
<thead>
<tr>
<th>Population</th>
<th>TT, n (%)</th>
<th>TC, n (%)</th>
<th>CC, n (%)</th>
<th>P-value</th>
<th>Odds ratio (95% CI)</th>
<th>Allele T, n (%)</th>
<th>Allele C, n (%)</th>
<th>P-value</th>
<th>Odds ratio (95% CI)</th>
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<tbody>
<tr>
<td>Spain SLE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (735)</td>
<td>325 (44.2)</td>
<td>314 (42.7)</td>
<td>96 (13.1)</td>
<td>0.3</td>
<td>TC: 1.14 (0.91–1.41)</td>
<td>964 (65.6)</td>
<td>506 (34.4)</td>
<td>7.8E–07</td>
<td>1.53 (1.29–1.81)</td>
</tr>
<tr>
<td>Controls (593)</td>
<td>324 (54.6)</td>
<td>235 (39.6)</td>
<td>34 (5.7)</td>
<td>7.9E–06</td>
<td>CC: 2.43 (1.61–3.63)</td>
<td>883 (74.5)</td>
<td>303 (25.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy SLE</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cases (348)</td>
<td>182 (52.3)</td>
<td>131 (37.6)</td>
<td>35 (10.1)</td>
<td>0.4</td>
<td>TC: 0.88 (0.65–1.19)</td>
<td>495 (71.1)</td>
<td>201 (28.9)</td>
<td></td>
<td></td>
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<tr>
<td>Controls (364)</td>
<td>200 (55)</td>
<td>144 (39.5)</td>
<td>20 (5.5)</td>
<td>0.02</td>
<td>CC: 1.88 (1.07–3.31)</td>
<td>544 (74.7)</td>
<td>184 (25.3)</td>
<td>0.1</td>
<td>1.20 (0.95–1.51)</td>
</tr>
<tr>
<td>Argentina SLE</td>
<td></td>
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</tr>
<tr>
<td>Cases (275)</td>
<td>128 (47.1)</td>
<td>119 (42.7)</td>
<td>28 (10.2)</td>
<td>0.4</td>
<td>TC: 0.86 (0.61–1.22)</td>
<td>375 (68.4)</td>
<td>173 (31.6)</td>
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<tr>
<td>Controls (245)</td>
<td>122 (49.8)</td>
<td>108 (44.1)</td>
<td>15 (6.1)</td>
<td>0.09</td>
<td>CC: 1.70 (0.89–3.23)</td>
<td>352 (71.8)</td>
<td>138 (28.2)</td>
<td>0.2</td>
<td>1.18 (0.90–1.53)</td>
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<tr>
<td>Pooled</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (1358)</td>
<td>635 (46.8)</td>
<td>564 (41.5)</td>
<td>159 (11.7)</td>
<td>0.6</td>
<td>TC: 1.04 (0.89–1.22)</td>
<td>1834 (67.5)</td>
<td>882 (32.5)</td>
<td>3.8E–07</td>
<td>1.37 (1.21–1.54)</td>
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<tr>
<td>Controls (1202)</td>
<td>646 (53.7)</td>
<td>487 (40.5)</td>
<td>69 (5.7)</td>
<td>1.2E–07</td>
<td>CC: 2.16 (1.61–2.89)</td>
<td>1779 (74)</td>
<td>625 (26)</td>
<td></td>
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</tbody>
</table>

*\( P_c = 1.16E–06 \).
transcriptional activity of the rs360719 T and rs360719 C-alleles and also identify a possible major repressor site in the IL18 promoter (the rs360719 T-allele). On this basis the results from the present study suggest that the IL18 rs360719 gene polymorphism may play a major role in SLE; however, complete re-sequencing in the area to examine all possible functional variants and perhaps additional functional data in different cell types, such as antigen presenting cells or endothelial cells, would be needed to confirm this hypothesis.

Figure 3. Action of OCT-1 in the preferential binding of the rs360719 T-allele and repression of transcription. (A) Electrophoretic mobility shift assay for the two alleles of the rs360719 polymorphism showing stronger protein binding to the T-allele compared with the C-allele. Lanes 1–3 contain labelled probe alone (consensus: lane 1; wild-type: lane 2 and mutant: lane 3). Lanes 4–6 contain labelled probe (consensus: lane 4; wild-type: lane 5 and mutant: lane 6) and nuclear extract. Lanes 7–9 shows supershifts experiments using an antibody specific to OCT-1 (consensus: lane 7; wild-type: lane 8 and mutant: lane 9). Competition experiments were performed using 100-fold molar excess of the cold probe consensus sequence (lanes 10–12). (B) Proposed model of interaction of the polymorphic site with the OCT-1 transcription factor in the IL18 gene.
Studies using animal models that develop spontaneous lupus-like autoimmune disease have provided further evidence that IL-18 is involved in the pathology (14,40). Interestingly, MRL/lpr mice have significantly elevated serum levels of IL-18 compared with MLR/+ + controls, and MRL/lpr mice treated with IL-18 or IL-18 plus IL-12 resulted in accelerated proteinuria, glomerulonephritis, vasculitis and increased levels of proinflammatory cytokines. These data together with our findings suggest that IL-18 is a possible novel therapeutic target in the treatment of autoimmune SLE.

In conclusion, we have identified a putative functional variant within the IL18 promoter region that seems to have an important role in IL-18 expression associated with susceptibility to SLE.

MATERIALS AND METHODS

Patients

Three independent case–control cohorts from Spain, Italy and Argentina were analysed. The study includes, 750 SLE patients and 595 controls from Spain, 330 SLE patients and 366 controls from Italy and 276 SLE patients and 262 controls from Argentina. The Spanish, Italian and Argentinean SLE cases have all been previously described (41–43). Both patient and control groups were matched for age and sex in each geographic region. All cases fulfil the American College of Rheumatology (ACR) criteria for the classification of SLE (44). The samples were collected according to the Helsinki Declaration. All subjects provided informed consent for this study. The study was approved by the various institutional review boards and Ethical Committees at each of the participating locations.

IL18 polymorphisms selection

SNPs spanning a 27 kb region from 2.9 kb upstream to 3.3 kb downstream of IL18 transcribed sequence were surveyed in the NCBI-dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html) and the International HapMap (http://www.hapmap.org) websites. We used the HapMap database of the CEU population (Utah residents with ancestry from northern and western Europe). Tag SNPs were selected using the pairwise method under a restriction of minor allele frequency > 0.01 and $r^2$ threshold > 0.8, aiming to identify a set of tag SNPs that efficiently captures all known common variants and is likely to tag most unknown variants. In all, seven tag SNPs were identified that capture all 26 alleles with a mean $r^2$ of 0.945. Six tag SNPs were located in an intron region and only one tag SNP was in the promoter region. No non-synonymous variations or polymorphisms that may interfere with mRNA splicing have been described in the IL18 gene. In addition, variants in IL18 with potential functional effects or locations in putative transcription factor binding sites were chosen for genotyping. They consisted of two promoter SNPs, the rs187238 polymorphism was selected because it was previously associated with SLE and have been suggested that this variant could alter the IL18 promoter activity. The rs360719 was selected based on the minor allele frequency and its ability to bind the transcription factor OCT-1. The SNPs associated in the Spanish fine mapping, after quality control and correction for multiple testing, were typed in the Italian and Argentinean samples.

IL18 genotyping

DNA was obtained from peripheral blood mononuclear cells (PBMC), using standard methods. The genotyping of all IL18 polymorphisms was performed using a pre-development TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in a total reaction volume of 5 µl, containing 50 ng genomic DNA as template, 2 µl of TaqMan genotyping master mix, 0.1 µl of 20× assay mix and ddH2O up to 5 µl of final volume. The amplification protocol used was initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. After PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7900 Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems).

All samples were genotyped in the same centre to avoid genotyping inconsistencies and to verify the genotyping consistency; random samples were genotyped twice showing 99% identical genotypes.

Imputation analysis

Imputation was performed on the cases and controls using IMPUTE, using a method described by Marchini et al. (45). We imputed all SNPs in the HAPMAP within the range of our data plus 3 kb either side, giving us 28 SNPs (19 imputed) for our case–control analysis.

The output from IMPUTE gives probabilities for each genotype, rather than point estimates. The use of probabilities allowed us to account for the uncertainty in imputation within the case–control analysis using SNPTEST.

Real-time quantitative PCR

To analyse constitutive IL18 mRNA expression, PBMCs from 23 selected healthy individuals were isolated by Ficoll density gradient centrifugation. Total RNA was isolated with Trizol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). RNA integrity was verified both electrophoretically and by the 260/280 nm absorption ratio. Reverse-transcription was performed in a total volume of 20 µl with Superscript™ First-Strand Synthesis System for RT–PCR (Invitrogen) as recommended by the manufacturer. Real-time quantitative PCR was performed on an ABI PRISM 7500 Fast SDS (Applied Biosystems) using a TaqMan gene expression assay (Applied Biosystems) in a total volume of 20 µl using 10 µl of TaqMan Fast Universal PCR Master Mix, 1 µl of each probe and 200 ng of cDNA. Cycle conditions were 95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Each sample was tested in triplicate and a sample without template was included as a negative control. Relative expression levels of IL18 mRNA were normalized according to β-actin, GAPDH and ABL expression using the $2^{-\Delta\Delta CT}$ method (46). We have used three different
endogenous controls in order to minimize the potential variability characteristic of each single housekeeping gene.

Cell culture
Jurkat cells were purchased from American Type Culture Collection (ATCC) and were cultured in RPMI (Rosweli Park Memorial Institute) 1640 medium (PAA Laboratories GmbH) supplemented with 10% fetal bovine serum (FBS, Gibco), 1 mM glutamine and 1% penicillin/streptomycin. They were kept at 37°C in a humidified 5% CO2/95% air incubator.

Electrophoretic mobility-shift assay
Nuclear extract from Jurkat cells was prepared by the mini-extraction procedure as described previously (47). The double-stranded oligonucleotides (50 ng) spanning the IL18 rs360719 polymorphism were as follows: top-strand T allele oligonucleotide 5'-CACCTCGTGTTCATAGTTGGGCAAA T-3' and top-strand C allele oligonucleotide 5'-CACCTCTG TCTTTCACTATTTGGCCCAAT-3'. A pair of oligonucleotides corresponding to the OCT-1 consensus binding sequence (48) (5'-GGTCACTGCAAGCTTCTAGAA-3' and 3'-TTCTAGTGATTCCATTGCCA-5') was end-labelled with (γ-32P)adenosine 5'-triphosphate (ATP) by using T4 polynucleotide kinase (Promega corporation, Madison, WI). For EMSAs with nuclear extract, 20 000–50 000 cpm double-stranded oligonucleotides corresponding to 0.5 ng were used for each reaction. The binding-reaction mixtures were set up containing 15 pmol DNA probe, 5 μg nuclear extract, 2 μg poly(dI-dC).poly(dI-dC) and binding buffer 2× (40 mM HEPES pH 7.5, 200 mM NaCl, 4 mM MgCl2, 4 mM DTT, 10% glycerol, 200 μg/ml BSA) up to 20 μl. The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. In the competition assay 100-fold excess amounts of cold oligonucleotides against the probe used were incubated with the Jurkat nuclear extracts. In the super-shift assay a mouse monoclonal antibody for OCT-1 antibody for OCT-1 (Abnova). Fifty micrograms of nuclear proteins was separated on a 7.5% (for OCT-1) or 12% (for IL-18) SDS–PAGE and transferred to PVDF membrane (Immobilon P, Millipore). The membrane was blocked in washing solution (0.01 M Tris, 0.1 M NaCl, 0.1% Tween 20; pH 7.5) with 5% non-fat dried milk, for 1 h at room temperature. It was first incubated overnight with 1 μg/ml (for IL-18) or 10 μg/ml (for OCT-1) of primary antibody at 4°C and then with a peroxidise-conjugated secondary antibody for 1 h at room temperature. The bands were detected with a chemiluminescent system (ECL, Amershams, Arlington Heights, IL) and exposed to X-ray film.

Flow cytometry analysis
The expression of IL-18 and OCT-1 was determined by flow cytometry. Jurkat cells were cultured for 18 h with 5 ng/ml phorbol myristate acetate (PMA) and 1 μM ionomycin to induce IL-18 and OCT-1 expression, followed by 6 h incubation with monensin in order to trap cytokine production within the cells. The cultured cells were fixed and permeabilized according to manufacturer’s recommendations with CytoFix/CytoPerm Kit (BD Biosciences Inc, Franklin Lakes, NJ). Afterwards, cells were incubated with anti-human IL-18 (10 μg/ml) or with anti-human OCT-1 (10 μg/ml) (Santa Cruz and ABNova, respectively), followed by incubation with phycocerythrin (PE)-anti-rabbit IgG or fluorescein isothiocyanate (FITC)-anti-mouse IgG secondary antibodies, respectively. Samples were analysed in a FACSCalibur flow cytometer (BD Biosciences, Inc) using the CellQuest Software (BD Biosciences, Inc.).

Data analysis
Allele and genotype frequencies were obtained by direct counting. We used the χ2 test for statistical analysis to compare allelic and genotypic distributions. We assessed the quality of the genotype data by testing for Hardy–Weinberg equilibrium for all samples using Fisher’s exact test and found no differences. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated according to Woolf’s method. All statistics described earlier were performed with PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). P-values below 0.05 were regarded as statistically significant. The Breslow–Day test of combinability and the Mantel–Haenszel test were carried out using the StatsDirect software v2.4.6. The pooled OR was calculated according to a fixed-effects model (Mantel–Haenszel meta-analysis). Genotypic ORs were calculated using the Unphased software with homozygosity for non-associated allele as reference with OR = 1.

Results relative to mRNA expression are shown as mean ± standard deviation. Because the variances were homogeneous (Bartlett’s test P > 0.05), a statistical analysis of the mean of relative expression of the IL-18 and OCT-1 was performed using the ANOVA test included in GraphPad Prism 4 software (GraphPad Software; Inc, La Jolla, CA).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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APPENDIX: THE LIST OF PARTICIPANTS

The Argentine collaborative participants: Hugo R. Scherbarth MD, Pilar C. Marino MD, Estela L. Motta MD—Servicio de Reumatologia, Hospital Interzonal General de Agudos ‘Dr. Oscar Alende’, Mar del Plata, Argentina; Susana Gamron MD, Cristina Drenkard MD, Emilia Menso MD—Servicio de Reumatología de la UHMI 1, Hospital Nacional de Clínicas, Universidad Nacional de Córdoba, Córdoba, Argentina; Alberto Alliev MD, Guillermo A. Tate MD—Organización Médica de Investigación, Buenos Aires, Argentina; Jose L. Presas MD—Hospital General de Agudos Dr. Juán A. Fernandez, Buenos Aires, Argentina; Simon A. Palatnik MD, Marcelo Abdala MD, Mariela Bearzotti PhD—Facultad de Ciencias Medicas, Universidad Nacional de Rosario y Hospital Provincial del Centenario, Rosario, Argentina; Alejandro Alvarellos MD, Francisco Caeiro MD, Ana Bertoli MD—Servicio de Reumatología, Hospital Privado, Centro Medico de Córdoba, Córdoba, Argentina; Sergio Paira MD, Susana Roverano MD—Hospital José M. Cullen, Santa Fe, Argentina; Cesar E. Graf MD, Estela Bertero PhD—Hospital San Martín, Paraná; Cesar Capraru MD, Griselda Buchanan PhD—Hospital Felipe Heras, Concordia, Entre Ríos, Argentina; Carolina Guillérón MD, Sebastían Grimaudo PhD, Jorge Manni MD—Departamento de Inmunología, Instituto de Investigaciones Médicas ‘Alfredo Lanari’, Buenos Aires, Argentina; Luis J. Catoggio MD, Enrique R. Soriano MD, Carlos D. Santos MD—Sección Reumatología, Servicio de Clínica Médica, Hospital Italiano de Buenos Aires y Fundación Dr. Pedro M. Catoggio para el Progreso de la Reumatología, Buenos Aires, Argentina; Cristina Prigione MD, Fernando A. Ramos MD, Sandra M. Navarro MD—Servicio de Reumatología, Hospital Provincial de Rosario, Rosario, Argentina; Guillermo A. Berbotto MD, Marisa Jorfen MD, Elisa J. Romero PhD—Servicio de Reumatología Hospital Escuela Eva Perón. Granadero Baigorria, Rosario, Argentina; Mercedes A. García MD, Juan C Marcos MD, Ana I. Marcos MD—Servicio de Reumatología, Hospital Interzonal General de Agudos General San Martín, La Plata; Carlos E. Perandones MD, Alicía Eimon MD—Centro de Educación Médica e Investigaciones Clínicas (CEMIC), Buenos Aires, Argentina; Cristina G. Battaglotti MD—Hospital de Niños Dr. Orlando Alassia, Santa Fe, Argentina.

The Italian collaborative participants: Nadia Barizzone (University of Eastern Piedmont, Novara, Italy), Mauro Galeazzi (Siena University, Siena, Italy), Maria Giovanna Danieli (Clinica Medica di Scienze Mediche e Chirurgiche, Università Politecnica delle Marche), Sergio Migliaresi (Rheumatology Unit Second University of Naples, Naples, Italy), Enrica Bozzolo (IRCCS San Raffaele Hospital, Milan, Italy).