A common SNP in the CD40 region is associated with systemic lupus erythematosus and correlates with altered CD40 expression: implications for the pathogenesis

Vassilios M Vazgiourakis,1 Maria I Zervou,2 Christianna Choulaki,3 George Bertsias,3 Maria Melissourgaki,3 Neslihan Yilmaz,4 Prodromos Sidiropoulos,3 Darren Plant,5 Leendert A Trouw,6 Rene E Toes,6 Dimitris Kardassis,7 Sule Yavuz,4 Dimitrios T Boupamas,1,3 George N Goulielmos1

ABSTRACT

Background In systemic lupus erythematosus (SLE) sustained CD40L expression by T cells and platelets activates a variety of cells via its receptor CD40 contributing to disease pathogenesis. Although CD40 has recently been identified in genome-wide association study as a novel rheumatoid arthritis susceptibility gene such an association has not been documented for SLE.

Objective To investigate whether the rs4810485 CD40 single nucleotide polymorphism (SNP) is associated with increased risk for SLE and its impact on CD40 expression.

Materials and methods The primary sample set consisted of 351 patients with SLE and 670 matched healthy controls of Greek origin. 158 patients with SLE and 155 controls from Turkey were used as a replication sample. Genotyping of rs4810485 was performed by restriction fragment length polymorphism and the Sequenom MassArray technology. The expression of CD40 mRNA and protein was assessed in unstimulated and lipopolysaccharide-stimulated peripheral blood mononuclear cells by quantitative real time PCR and flow cytometry, respectively.

Results The minor allele T of CD40 rs4810485 SNP was significantly under-represented in Greek patients with SLE compared with healthy controls (OR=0.65, 95% CI 0.54 to 0.79). The association was replicated in the Turkish cohort (OR=0.57, 95% CI 0.41 to 0.80; meta-analysis of 509 patients with SLE and 825 healthy controls: OR=0.65, 95% CI 0.53 to 0.74, p = 2×10−8). In both cases and controls, the rs4810485 G/T and T/T genotypes were associated with significantly reduced CD40 mRNA and protein expression in peripheral blood CD14+ monocytes and CD19+ B cells compared with G/G genotype, both under basal conditions and following stimulation.

Conclusions CD40 has been identified as a new susceptibility locus in Greek and Turkish patients with SLE. The rs4810485 minor allele T is under-represented in SLE and correlates with reduced CD40 expression in peripheral blood monocytes and B cells, with potential implications for the regulation of aberrant immune responses in the disease.

INTRODUCTION

There is increasing evidence that different autoimmune diseases may share common pathogenic pathways. Systemic lupus erythematosus (SLE) is a multifactorial, systemic autoimmune disease characterised by production of autoantibodies directed against cell surface and nuclear components. The aetiology of the disease remains elusive even though it has been intensively studied.1,2 A number of genetic susceptibility loci, conferring low-to-moderate risk for SLE, have been recently identified through genome-wide association studies.3–5 The most important genes that confer susceptibility are located in the HLA locus,6 but non-HLA genes also operate such as IRF5, PTPN22, STAT4, CDKN1A and BLK.7

Recent genome-wide searches for susceptibility loci have provided new insights into autoimmune disease pathogenesis and have identified several loci showing significant linkage to the diseases, some of which have been confirmed by independent studies.8–10 Among them, the CD40 locus has been identified as a genetic risk factor for rheumatoid arthritis (RA),11 CD40 spans 11 kb, has nine exons,12 is constitutively and inducibly expressed on the surface of various immune and non-immune cell types, such as B cells, monocytes, dendritic cells, keratinocytes, epithelia, microglia and endothelial cells13 and is implicated in immune and non-immune responses.14 CD40-mediated cellular functions include T cell-dependent B-cell humoral responses,15 secretion of growth factors and cytokines from monocytes16 17 and expression of adhesion molecules on endothelial cells. CD40 signalling has been linked to pathogenic processes of chronic inflammatory and autoimmune diseases.18 A pathogenic role for CD40–CD40L interactions has been well established in lupus (reviewed by Koshy et al19) with sustained expression of CD40L on activated T cells19 and platelets20 in patients with active SLE. Importantly, interruption of this pathway results in disease attenuation both in animals21 and in humans.22

Our group has focused on the overlap between the genetic determinants for SLE, RA, type-1 diabetes mellitus (T1D), psoriasis and other autoimmune diseases. Through analysis of well-characterised case–control studies, we have previously documented significant associations between T1D and psoriasis with two SLE and RA susceptibility genes—namely, TRAF1/C5 and STAT4, respectively.23–26 Assuming that genes involved in
regulation of inflammatory responses may confer risk for different immune-mediated diseases, and based on the pathogenic role of the CD40-CD40L pathway in lupus, herein we demonstrate, for the first time, that the CD40 single nucleotide polymorphism (SNP) rs4810485 is associated with SLE in individuals of Greek and Turkish origin. Importantly, our in vitro studies demonstrate that rs4810485 may affect CD40 expression in primary mononuclear cells, with potential implications for the disease pathogenesis.

MATERIALS AND METHODS

Study population
The primary sample set included 351 patients with SLE from Greece who were followed up at the Department of Rheumatology, Clinical Immunology, and Allergy, University of Crete. All patients met the 1982 American College of Rheumatology revised classification criteria. Six hundred and seventy age- and gender-matched healthy individuals were recruited from the Department of Transfusion Medicine. The study was approved by the ethics committee of the University Hospital of Crete. The replication sample set consisted of 158 patients with SLE and 155 age- and gender-matched healthy controls from Turkey (Marmara University Medical School). Sixteen patients with SLE and 24 controls were selected for the CD40 expression experiments presented in detail elsewhere (supplementary text).

Analysis of the rs4810485 CD40 polymorphism
Whole blood was collected in EDTA-containing tubes and genomic DNA was extracted using the QIamp DNA Blood Mini kit (QIAGEN Inc, Valencia, California, USA). Genotyping for CD40 rs4810485 was performed by PCR-restriction fragment length polymorphism or the Sequenom MassArray technology according to the manufacturer’s instructions (supplementary text).

Results

Preparation of mononuclear cells, cell culture and stimulation
Peripheral blood mononuclear cells (PBMCs) from patients with SLE and healthy controls were isolated, cultured and stimulated as described in the supplementary text.

RNA extraction and quantitative real-time RT-PCR
Total RNA was extracted from fresh or cultured PBMCs using the RNeasy RNA Isolation kit (Qiagen) and incubated with RNase-free DNase I (Qiagen) according to the manufacturer’s protocol. Reverse transcription reactions were performed with 500 ng total RNA using random hexamers and the Thermoscript RT system (Invitrogen, Carlsbad, California) and stored at −20°C until analysed. PCR conditions and the quantification of the mRNAs levels of CD40 and GAPDH are described in the supplementary text.

Immunophenotyping of PBMCs
A direct immunofluorescence technique was used to evaluate CD40 protein expression within subsets of PBMCs (supplementary text). Normalised mean fluorescence intensity (MFI) was expressed against the IgG isotypic control according to the following equation: normalised MFI = (MFI_{CD40} − MFI_{IgG})/MFI_{IgG}.

Statistical analysis
Statistical analysis was performed with GraphPad Prism statistical program (GraphPad Software, San Diego, California, USA). In the case–control comparisons, only unrelated samples were used (supplementary text).

RESULTS

The CD40 rs4810485 SNP is associated with SLE in two independent ethnic cohorts
Allele and genotype frequencies for rs4810485 in the primary cohort of Greek patients with SLE and healthy controls are

### Table 1: Genotypes and allele frequencies of the CD40 rs4810485 polymorphism analysed in 351 patients with SLE and 670 controls from Greece and 158 patients with SLE and 155 controls from Turkey

<table>
<thead>
<tr>
<th>Genotype</th>
<th>rs4810485 Position</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>GG</td>
<td>GT</td>
<td>TT</td>
</tr>
<tr>
<td>Patients with SLE (n=351)</td>
<td>139 (39.6)</td>
<td>190 (54.1)</td>
</tr>
<tr>
<td>Healthy controls (n=670)</td>
<td>216 (32.2)</td>
<td>326 (48.7)</td>
</tr>
<tr>
<td>*OR (95% CI)</td>
<td>1.00</td>
<td>0.91 (0.69 to 1.20)</td>
</tr>
<tr>
<td>*p Value (df 1)</td>
<td>–</td>
<td>5.0 × 10⁻¹</td>
</tr>
<tr>
<td>†p Value (df 2)</td>
<td>–</td>
<td>2.1 × 10⁻⁷</td>
</tr>
<tr>
<td>Turkish cohort, N (%)</td>
<td>69 (43.7)</td>
<td>82 (51.9)</td>
</tr>
<tr>
<td>Healthy controls (n=155)</td>
<td>52 (33.5)</td>
<td>72 (46.5)</td>
</tr>
<tr>
<td>*OR (95% CI)</td>
<td>1.00</td>
<td>0.86 (0.53 to 1.39)</td>
</tr>
<tr>
<td>*p Value (df 1)</td>
<td>–</td>
<td>5.0 × 10⁻¹</td>
</tr>
<tr>
<td>†p Value (df 2)</td>
<td>–</td>
<td>1.0 × 10⁻⁵</td>
</tr>
<tr>
<td>Combined cohort, N (%)</td>
<td>208 (40.9)</td>
<td>272 (53.4)</td>
</tr>
<tr>
<td>Healthy controls (n=825)</td>
<td>268 (32.5)</td>
<td>398 (48.2)</td>
</tr>
<tr>
<td>*OR (95% CI)</td>
<td>1.00</td>
<td>0.88 (0.69 to 1.12)</td>
</tr>
<tr>
<td>*p Value (df 1)</td>
<td>–</td>
<td>3.0 × 10⁻¹</td>
</tr>
<tr>
<td>†p Value (df 2)</td>
<td>–</td>
<td>2.2 × 10⁻¹¹</td>
</tr>
</tbody>
</table>

A meta-analysis of the two cohorts is also presented. Reduced frequency of the rs4810485 minor allele T and the T/T genotype in patients with SLE compared with healthy controls.

*p Values with df 1 and OR (95% CI) were calculated taking as reference the G/G genotype or the major (G) allele.

†p Values with df 2 were calculated with a 2×3 χ² test of independence and account for the overall difference between the three genotypes.

df, degrees of freedom; SLE, systemic lupus erythematosus.
The rs4810485 T allele is associated with reduced CD40 mRNA in freshly isolated and lipopolysaccharide-stimulated PBMCs from patients with SLE and healthy controls

Expression of CD40 was evaluated by real-time PCR in freshly isolated PBMCs from patients with SLE and healthy controls with different rs4810485 genotypes. CD40 mRNA levels were almost twofold lower in patients with SLE who had the T/T or G/T genotype than those with the G/G genotype (figure 1A).

To further verify our results, we examined the CD40 mRNA in freshly isolated PBMCs from healthy controls, and we also found significantly reduced expression in individuals with T/T and G/T compared with the G/G genotype (figure 1A).

We next examined CD40 mRNA expression after stimulation with lipopolysaccharide (LPS) 100 ng/ml for 24 h. As shown in figure 1B, LPS treatment induced CD40 expression both in patients with SLE and in healthy controls. In both groups, stimulated PBMCs with the G/T and T/T genotype had significantly reduced CD40 mRNA compared with those with the G/G genotype. PBMCs from patients with SLE with the G/G genotype showed the strongest induction of CD40 after LPS stimulation (figure 1B).

Collectively, these results indicate that the rs4810485 T allele is associated with reduced CD40 mRNA expression both in the basal, non-stimulated and the stimulated state.

Basal membrane protein expression of CD40 in PBMCs is reduced in patients with SLE and controls with the rs4810485 T allele

We next used flow cytometry to examine the effect of rs4810485 on CD40 membrane protein expression (measured by CD40 MFI or the percentage of CD40+ cells) on freshly isolated CD19+ B cells and CD14+ monocytes. To minimise any possible interfering factors, we included patients with inactive disease and without significant B-cell lymphopenia (<3% of total lymphocytes). Figure 2A shows representative flow cytometry histograms for CD40 expression on unstimulated B cells from patients with SLE (upper panel) and healthy controls (lower panel) with different rs4810485 genotypes. In both patients and controls, rs4810485 T/T and G/T were associated with significantly reduced CD40+ B cells compared with G/G (p<0.05 for T/T vs G/G and G/T vs G/G pairwise comparisons) (figure 2B).

The rs4810485 T allele also correlated with decreased proportion of peripheral blood CD40+ CD19+ B cells both in patients with SLE and in healthy controls (supplementary figure S1). We performed a similar analysis in CD14+ monocytes, and found that rs4810485 T/T and G/T patients and controls had reduced CD40 MFI in comparison with their G/G counterparts (figure 2C, D). In contrast, we found no significant variation in the proportion of
CD40+ monocytes according to rs4810485 genotype (data not shown). Overall, these results suggest that under basal, non-induced, conditions, the rs4810485 allele T is associated with lower CD40 surface expression on B cells and monocytes from patients with SLE and healthy controls.

Stimulation of PBMCs induces lower membrane expression of CD40 in patients with SLE and controls with the rs4810485 T allele

Although CD40 is constitutively expressed on the surface of mononuclear cells, its expression is further upregulated upon cell stimulation. To examine the effect of rs4810485 SNP on inducible CD40 expression, PBMCs were cultured for 24 h in the presence or absence of LPS 100 ng/ml, and CD40 expression was evaluated by flow cytometry. In LPS-stimulated CD19+ B cells, CD40 membrane expression (MFI) was significantly lower in patients with the T/T or G/T genotype than in those with the G/G genotype (figure 3A, B); the effect was more pronounced in patients with SLE than in healthy controls (figure 3B). Similar results were obtained with the percentage of CD40+ CD19+ B cells (supplementary figures S2A,B).

With regard to CD14+ monocytes, LPS-induced CD40 MFI levels were lower in both patients and controls with the rs4810485 T/T and G/T rather than the G/G genotype (figure 3C, D). The proportion of CD40+ monocytes also correlated inversely with presence of the minor T allele (supplementary figure S2C, D).

DISCUSSION

Identification of shared genetic determinants for clinically distinct disorders is the emerging premise underlying the results of recent genome-wide scans.\(^{28, 29}\) In this study, we explored one such example of overlap between disease susceptibility loci by investigating the association of the RA-associated SNP rs4810485 of the CD40 gene with SLE. We report for the first time that rs4810485 is associated with SLE in the Greek and Turkish Mediterranean population, and correlates with differential CD40 expression in healthy controls and more profoundly in patients with SLE, with the T/T genotype associated with lower induction than the G/G genotype.

In view of the role of CD40, a tumour necrosis factor superfamily membrane member, in generating effective immune responses through interaction with its ligand CD40L, we reasoned that it may represent an excellent candidate susceptibility gene for various autoimmune diseases.\(^{30}\) The rs4810485 SNP has recently been associated with RA in individuals of
European but not Korean ancestry. A replication study in a Greek population confirmed the association of rs4810485 with RA. Importantly, the \(CD40\) locus resides in a region (20q11–13) that has been previously linked with SLE in European-Caucasians, Mexican-Americans and African-Americans. This suggests an underlying pathogenic mechanism involving a risk allele of a shared disease locus that may account for increased susceptibility in two distinct diseases. Other studies have also suggested the \(CD40\)–\(CD40L\) pair as strong SLE candidate genes because of their ability to induce T-cell mediated humoral responses. However, in a previous family-based genetic study of \(CD40\) in SLE no association was found; probably, the SNP data available at the time of the previous study did not allow for detection of the effect from rs4810485. Of note, several other SNPs in genes involved in the \(CD40\) signalling pathway have been identified, including TNFAIP3 (A20, an E3-ubiquitin ligase) and TRAF1–C5. TRAF1 is an adaptor protein that cooperates with TRAF2 to enhance CD40 signals.

The rs4810485 SNP resides in the second intron of the \(CD40\) gene and its functional consequence remains to be identified. As a first attempt to study this issue, we looked for a possible association between rs4810485 and \(CD40\) expression in peripheral blood B cells and monocytes. In both patients with SLE and healthy controls, individuals with the T/T or G/T genotype had significantly reduced basal and induced \(CD40\) mRNA and protein levels compared with those with the G/G genotype, suggesting that rs4810485 could be a functional polymorphism. This result is biologically plausible since the minor allele T associated with reduced \(CD40\) expression was found to be underrepresented in patients with SLE (OR=0.63 in the meta-analysis of the Greek and Turkish cohorts). Of interest, in the presence of the major allele G, both basal and induced \(CD40\) levels were significantly higher in patients with SLE than in healthy controls, indicating that rs4810485 might also influence \(CD40\) expression in a disease-specific manner.

To explore whether rs4810485 might modify any transcription factor-binding site, we performed sequence analysis...
using the Genomatix Software (Genomatix Software GmbH, “Gene2Promoter” program, Muenchen, Germany). Allele G disrupted the putative binding of the transcription factor Pax6, which was predicted to bind to allele T. Although no information exists about the expression and function of Pax6 in SLE immune cells, another member of the Pax family transcription factors, Pax7 is implicated in mature B-cell programming and its repression is required for plasma cell differentiation.40 Moreover, rs4810485 allele G is predicted to enhance the DNA binding of a member of the ETS1 transcription factors family. ETS1 is a negative regulator of B- and Th17-cell differentiation that has been associated with the development of SLE.41 Of interest, lower levels of ETS1 mRNA have been detected in PBMCs of patients with SLE.42

Increased CD40 expression in antigen presenting cells might contribute to enhanced signalling upon interaction with CD40L,43 which is upregulated on activated T cells in patients with SLE and RA. Interactions between CD40 and CD40L might also influence the function of non-immune cells such as endothelial cells, leading to endothelial dysfunction44 and vascular inflammation,45 which are common in these patients.

In conclusion, we have demonstrated that the rs4810485 SNP of the CD40 locus is associated with SLE in a genetically homogeneous population. We also provide evidence that rs4810485 may affect CD40 mRNA and protein expression on B cells and monocytes, with potential implications for regulation of the inflammatory responses in the disease. The CD40/CD40L pathway has an established pathogenic role and represents a valid therapeutic target in lupus. Our data corroborate these observations and offer a plausible explanation for the increased susceptibility to SLE conferred by this genetic variant of CD40. Sustained expression of CD40L coupled with increased expression of CD40 observed in over half of lupus patients amplify cellular and humoral responses in a variety of immune and non-immune cellular targets, thus contributing to disease pathogenesis. Intriguingly, polymorphisms in the CD40 gene may affect expression and identify a subgroup of patients with SLE with a higher efficacy of anti-CD40 therapeutic intervention.

Acknowledgements The authors thank Dr Katerina Pyrovolaki and Mrs Eleni Koutala (Medical School of Crete) for their technical assistance in FACS analysis and Dr Panos Verginis (IMBB-FORTH, Heraklion) for critical review of the manuscript.

Funding This work was supported in part by grants from the European BTCure IMI project, the University of Crete (Greece) and the University of Marmara (Instanbul, Turkey). The study was approved by the ethics committee of University of Crete. Ethics approval

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES
A common SNP in the **CD40** region is associated with systemic lupus erythematosus and correlates with altered **CD40** expression: implications for the pathogenesis

Vassilios M Vazgiourakis, Maria I Zervou, Christianna Choulaki, et al.

*Ann Rheum Dis* published online September 12, 2011
doi: 10.1136/ard.2010.146530