EXTENDED REPORT

The dinucleotide repeat polymorphism in the 3′UTR of the CD154 gene has a functional role on protein expression and is associated with systemic lupus erythematosus

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Objective: To investigate the association of the (CA)n dinucleotide repeat in the 3′ untranslated region (3′UTR) of the CD154 gene with systemic lupus erythematosus (SLE), and its functional role in protein expression.

Methods: The allelic and genotypic distributions of the polymorphism were compared in 80 patients with SLE and 80 controls. A complete clinical and analytical database was recorded in each patient in order to correlate the clinical manifestations in SLE with different alleles. To investigate the functional role of the polymorphism, the CD154 protein expression on activated lymphocytes from healthy homozygous controls was evaluated by flow cytometry.

Results: The 24 CA allele was the most represented in controls ($p = 0.029$), whereas the alleles containing >24 CA repeats were found in patients ($p = 0.0043$). Furthermore, when only homozygous women were considered, most controls carried two 24 CA alleles ($p = 0.041$), whereas most patients carried two alleles containing >24 CA repeats ($p = 0.032$). Also, patients carrying at least one 24 CA allele had less neurological involvement ($p = 0.034$), and carriers of at least one allele with fewer than 24 CA repeats presented more livedo reticularis ($p = 0.006$) and anti-Sm ($p = 0.01$) and anti-RNP ($p = 0.038$) autoantibodies. CD154 maximum expression in activated lymphocytes from all controls was reached after 54 hours, but it was more prolonged in controls carrying two alleles with >24 CA repeats ($p = 0.0068$).

Conclusion: The CD154 3′UTR microsatellite is associated with SLE, and the most represented alleles in patients were accompanied by a more prolonged protein expression in activated lymphocytes from controls.

CD154 is a 33 kDa membrane glycoprotein, belonging to the tumour necrosis factor superfamily, which is primarily and transiently expressed on activated mature CD4+ T lymphocytes.[1] It is also found on other leucocyte subpopulations but with lower expression levels.[2–5] CD154 is the ligand for CD40,[6] mainly expressed on B lymphocytes, but also on monocytes,[7] macrophages, dendritic cells,[8] activated platelets, epithelial and endothelial cells,[9 10] and fibroblasts. CD154/CD40 ligation induces activation of CD40 positive cells, bearing a pivotal role for both humoral and cellular immunity and inflammation.

T lymphocyte activation mediated by T cell receptor quickly induces a CD154 expression, which is regulated by its binding to CD40[11 12] and by other costimulatory molecules like CD28.[13 14] The normal interaction between T and B lymphocytes due to CD154 and CD40 ligation induces B cell activation, proliferation, differentiation, and immunoglobulin isotype switching in the presence of appropriate cytokines, regulating the B cell commitment to mature plasma or memory B cells.[15–17] The very brief expression of CD154 on T lymphocytes ensures that the interaction between these cells and CD40 positive target cells will be restricted to those that have recently encountered an antigen. A more prolonged expression of CD154 might be predicted to generalise the immune response to undesirable specifities, whereas a deficient expression might be associated with a poor or null response to antigens. So, it is not surprising that in the past few years an altered expression of CD154 has been associated with several diseases.[18] Mutations in the CD154 gene are the responsible for the X linked hyper-IgM syndrome,[19] whereas the CD154 protein is hyperexpressed in some autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis,[20] or multiple sclerosis.[21]

SLE, which is considered to be the prototype of human autoimmune diseases, is a multisystem autoimmune inflammatory disorder with a wide variety of presenting features and manifestations. It is characterised by a polyclonal B cell activation and a hyperactive T cell help, resulting in the production of antinuclear antibodies and the generation of circulating immune complexes. The aetiopathogenesis of SLE is not well elucidated, but it is generally agreed that it is characterised by a polygenetic control and by environmental influences. So, the interest of many investigators in recent years has been focused on the search for candidate genes in its susceptibility.

A CD154 hyperexpression in SLE has been further demonstrated. This protein is increased and shows prolonged expression on both T and B cells[22 23]; high levels of the soluble form have also been found.[24] So, in these patients, not only is

Abbreviations: ACR, American College of Rheumatology; BrdU, 5-bromo-2′-deoxyuridine; CI, confidence interval; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; OR, odds ratio; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PHA, phytohaemagglutinin; SLE, systemic lupus erythematosus; UTR, untranslated region

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the normal interaction between T and B lymphocytes due to CD154 and CD40 ligation possible but also between B lymphocytes by themselves or with soluble CD154. These interactions may have an important role in the immune deregulation found in this disease, and in fact this hyperexpression has been associated with raised levels of circulating autoantibodies.[25] On the other hand, immunohistological analysis has shown that the CD40 expression is up regulated in kidneys from lupus nephritis and other inflammatory renal diseases, and it is associated with the presence of mononuclear cells expressing CD154.[26]

The CD154 gene is located in the long arm of human X chromosome at the 26.3–27.1 position. It comprises five exons and four introns and contains a dinucleotide repeat of cytosine-adenine (CA) in the 3′ untranslated region (3′UTR).[27 28] It has been shown that the CD154 expression is post-transcriptionally regulated through mRNA stability.[29] Owing to its location, we suggest that different alleles (number of CA repeats) of the CD154 microsatellite may participate in the post-transcriptional CD154 regulation and contribute to the protein hyperexpression found in SLE.

We have previously reported allelic distribution of the CD154 microsatellite in a Spanish population,[30] demonstrating that it is a highly polymorphic marker in Spaniards. The present study aimed at determining if the CD154 polymorphism might be associated with SLE and its clinical manifestations. Therefore, we compared allelic frequencies in the normal population with those in a cohort of patients with SLE from the Canary Islands, Spain. The Canary Islands are an archipelago near the northwest Atlantic coast of Africa, and the present Canary Island population has a heterogeneous genetic background owing to the diverse origin of its founding populations. The Spanish, Berber, and African Black contribution has been estimated to be 70, 20, and 10% respectively.[31] Although it is an insular population, there is not an unusual level of inbreeding. Regarding the CD154 microsatellite, we have previously described heterozygosity in a woman of 0.72, and the genotype distribution in women did not deviate from the values expected under the Hardy-Weinberg equilibrium ($p = 0.23$).[30]

We have also analysed the clinical manifestations of SLE in each genotype in order to identify possible clinical associations. Finally, we have investigated the functional role of this microsatellite on protein expression by comparing the CD154 kinetic expression on T and B lymphocytes from healthy donors stimulated with phytohaemagglutinin (PHA) and anti-CD28.

MATERIAL AND METHODS

Patients and controls

The group of patients with SLE comprised 80 women, all attending the rheumatology service of the Hospital de Gran Canaria Doctor Negrín, Canary Islands, Spain, who presented with four or more of the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE.[32] A clinical and analytical database was recorded from each patient since the onset of the disease, considering not only the ACR criteria but also other clinical and analytical manifestations commonly found in SLE. All the items were listed for each patient during at least one clinical revision visit and were considered as positive when the patient presented it at least once at any time since the onset of SLE. Table 1 summarises the characteristics of the patients included.

Eighty women from Gran Canaria, non-selected and non-related healthy volunteers without any autoimmune history, were recruited as controls for a comparison of their allelic and genotypic distributions of the CD154 polymorphism with those of the patients with SLE. Because of the CD154 gene localisation in the X chromosome, only men previously genotyped[30] or homozygote women were considered when exploring the regulatory effect of the number of (CA)n repeats on CD154 protein expression in healthy controls.

This project received ethics approval from our institutional ethics committees.

Samples

Anticoagulated blood samples were collected from all patients with SLE and controls. High molecular weight DNA was extracted from whole blood according to a standard phenol-chloroform procedure.[33] Peripheral blood mononuclear cells (PBMC) from heparinised blood were obtained by Ficoll-Hypaque (Comercial Rafer) density gradient centrifugation. DNA was stored at 4°C and PBMC were cryopreserved at −80°C until use.

Genotype analysis

A segment of the 3′UTR of the CD154 gene containing the microsatellite was amplified by the polymerase chain reaction with forward 5′-CTCTTCCCTCCCCCAGTCTC-3′ and reverse 5′-AAGAAGAGAACTGACTAGCAACG-3′ primers (Roche Diagnostics),[34] as previously described.[30]

To avoid any error in allele assignment, an internal marker was added to an aliquot of polymerase chain reaction (PCR) products.[30] A loading buffer consisting of 0.1% bromophenol blue (Amersham Pharmacia Biotech AB) and 0.1% xylenecyanol (Bio-Rad Laboratories) in formamide (Amersham Pharmacia Biotech AB) was also added. Finally, samples were denatured at 95°C for 3 minutes and quickly placed in ice until use.

All samples were resolved over denaturing polyacrylamide gels (GeneGel Clean 15/24 Kit, Amersham Pharmacia Biotech AB) containing 5.6 M Urea and 32% formamide (Amersham Pharmacia Biotech AB).[35] Horizontal electrophoresis was performed in a Genephor Electrophoresis Unit (Amersham Pharmacia Biotech AB) at 600 V and 50°C and the gels were silver stained with the DNA Silver Staining Kit (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions.

Allele assignment was performed by densitometry using the Molecular Analyst Software (Bio-Rad Laboratories). The length of the amplified fragment was estimated by reference to the standards used as internal ladder, and the number of repeats was calculated.

Functional analysis of the microsatellite

PBMC culture and activation

PBMC from controls were cultured at 500 000 cells/ml in 24 well culture plates (Costar), in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) (Gibco).

PHA 1/600 (Difco laboratories) and 1/800 soluble anti-CD28 monoclonal antibody (Kolt-2, Menarini) or anti-CD28 alone were added for PBMC activation. PBMC without stimuli were also cultured in complete medium in order to determine the CD154 basal expression. All cultures mentioned were maintained at 37°C and 5% CO_2 in a humidified atmosphere.

Flow cytometry

Activated PBMC were washed twice in phosphate buffered saline (PBS) and stained with several monoclonal antibodies (mAbs) in order to evaluate the CD154 expression on T and B lymphocytes. The following mAbs, all of them purchased by Becton Dickinson, were used: mouse IgG1 irrelevant antibodies, FITC, PE, or PerCP conjugated, were used as a negative control of staining; mouse anti-human CD45-FITC,

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Table 1 Clinical and analytical features of patients with SLE

Clinical feature	No (%)	Analytical feature	No (%)
Malar rash	43 (54)	Proteinuria	35 (44)
Discoid rash	13 (16)	Urinary cellular casts	7 (9)
Photosensitivity	45 (56)	Haemolytic anaemia	6 (8)
Oral ulcers	32 (40)	Thrombocytopenia	20 (25)
Alopecia	60 (75)	Leucopenia	55 (69)
Vasculitis	20 (25)	Lymphopenia	59 (74)
Raynaud's phenomenon	37 (46)	Low complement levels	62 (78)
Livedo reticularis	16 (20)	Anti-DNA	71 (89)
Arthritis	66 (83)	Anti-Sm antigen	20 (27)
Serositis	23 (29)	Anti-RNP	31 (41)
Neurological disorder	13 (16)	Anti-SSA/anti-SSB	36 (49)
Thrombosis	9 (11)	Anticardiolipin	36 (49)

All data were evaluated in 80 patients except anti-RNP (n=75) and anti-Sm, anti-SSA/anti-SSB, and anticardiolipin (n=73).

CD14-PE, CD4-PerCP, and CD19-PerCP were used to analyse cell subpopulations; CD69-FITC and CD25-PE were used for checking activation levels; and CD154-PE was used to analyse the CD154 expression. Cultured cells were incubated with the appropriate combinations of antibodies for 30 minutes at 4°C and washed twice in PBS to remove the unbound antibodies. Labelled cells were then analysed in a Becton Dickinson FACSort flow cytometer with the CELLQuest software. The percentage of T and B lymphocytes expressing CD154 was calculated and the mean fluorescence intensity (MFI) on a lineal scale was quantified as the ratio of the geometric means of the CD154-PE and the irrelevant anti-mouse-IgG-PE antibodies. All these values are reported as mean (SD).

Proliferation assay

After 72 hours of stimulation, cell cultures were pulsed with 5-bromo-2′-deoxyuridine (BrdU) at a final concentration of 110 µmol/l for 18 hours. BrdU incorporation into cellular DNA was measured with the BrdU labelling and detection kit III (Roche Diagnostics) according to the manufacturer's instructions. Absorbance was measured with an enzyme linked immunosorbent assay (ELISA) reader (Anthos 2010, Anthos Labtec Instruments) at 405 nm with a reference filter at 492 nm.

Statistical analysis

Allelic and genotypic frequencies were calculated by simple counting. The χ^2 test for statistical significance was performed to compare the allelic distribution between controls and patients, and probability values were corrected by Bonferroni's method. 2×2 contingency tables for each allele were constructed in order to compare frequencies in both groups, considering the p values of the Yates's continuity correction for the χ^2 test. The odds ratio (OR) and the exact limits for the 95% confidence intervals (CI) were calculated for each significant p value ($p<0.05$) to assess the relative disease risk conferred by a particular allele. To investigate further the association between clinical features and different alleles of the CD154 polymorphism, 2×2 contingency tables with absence/presence of one allele and absence/presence of a clinical feature were constructed in patients with SLE, and probability values of the Yates's continuity correction for the χ^2 test were taken into account. All statistical tests mentioned above were applied with the Epi Info version 6 software.[36]

The two tailed Student's *t* test was performed to compare the CD154 expression levels and cell proliferation rates between controls carrying two different genotypes. The Kolmogorov-Smirnov normality test was applied with the SPSS statistical software.

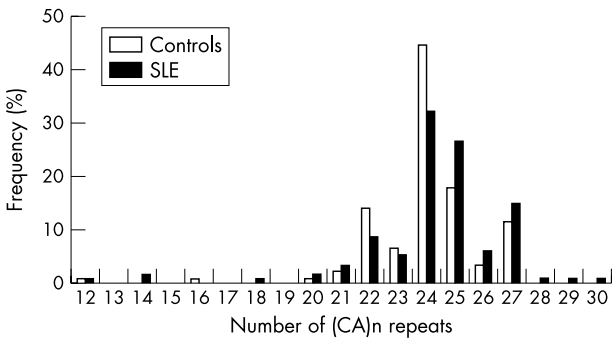

Figure 1 Frequency distribution of the number of (CA)n repeats in patients with SLE and controls.

RESULTS

Allele frequencies at the polymorphic locus in controls and patients

As a first approach to exploring the association of the CD154 polymorphism with SLE, we compared the allelic distribution of this microsatellite in 80 controls and 80 patients with SLE. Seven alleles ranging from 21 to 27 CA repeats, corresponding to PCR products from 116 to 128 bp, were represented more than three times in controls and patients. Allelic distributions in both groups fitted with normal distributions, the alleles containing 24 CA repeats being the central or median allele and the most represented one in both groups (fig 1). We found no differences between the allelic distributions in controls and patients when applying the Bonferroni correction for the χ^2 test, but when we compared each allele frequency with all the others by the Yates's continuity correction, the allele containing 24 CA repeats was more commonly represented in controls than in patients with SLE ($p = 0.029$, OR = 0.59, 95% CI 0.36 to 0.95), suggesting that it might be considered as a protective factor for SLE. Another interesting finding was that the alleles containing >24 CA repeats were more represented in patients with SLE

Table 2 Allelic distributions of the CD154 polymorphism in 80 controls and 80 patients with SLE (160 alleles in each group)

(CA)n	Controls No (%)	SLE No (%)	p Value	OR (95% CI)
<24 CA	38 (24)	32 (20)	0.5	NS
24 CA	71 (44)	51 (32)	0.029	0.59 (0.36 to 0.95)
>24 CA	51 (32)	77 (48)	0.0043	1.98 (1.23 to 3.21)

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Table 3 Genotypic distribution of CD154 in 36 homozygous controls and 34 patients with SLE

Genotype	Controls No (%)	SLE No (%)	p Value	OR (95% CI)
<24/<24	6 (17)	5 (15)	0.92	NS
24/24	18 (50)	8 (24)	0.041	0.31 (0.1 to 0.95)
>24/>24	12 (33)	21 (62)	0.032	3.23 (1.09 to 9.67)

than in controls. Therefore, to explore the possibility that the longest alleles tended to be associated with SLE, we compared the allelic distributions of alleles carrying 24 CA repeats, and less than or more than 24 CA repeats. The Bonferroni correction of the χ^2 test for a 3×2 contingency table showed that the allelic distributions in controls and patients were different ($p = 0.02$). To investigate which alleles contributed to this difference, we then constructed 2×2 contingency tables comparing the presence/absence of one allele in controls and patients. Besides the protective role of the 24 CA allele, the OR for the alleles containing >24 CA repeats suggested a possible risk factor of these longest alleles for SLE ($p = 0.0043$) (table 2).

The CD154 gene is located in the X chromosome and all our controls and patients were women. To avoid any deviation of the random X inactivation[37] we compared the genotypic distributions in homozygous women only, with the homozygote status defined as a woman carrying two 24 CA alleles, two alleles containing >24 CA repeats, or two alleles containing <24 CA repeats. The Yates's correction for the χ^2 test was calculated for the 2×2 contingency tables considering the absence/presence of one genotype in controls and patients. Table 3 shows that 50% of controls carried two 24 CA alleles, whereas only 24% of patients with SLE did ($p = 0.041$, OR = 0.31, 95% CI 0.1 to 0.95). On the other hand, most patients carried two alleles containing >24 CA repeats (62%), compared with only 33% of controls ($p = 0.032$, OR = 3.23, 95% CI 1.09 to 9.67).

CD154 polymorphism and clinical and analytical manifestations of SLE

We next explored if the CD154 polymorphism was not only associated with the disease but also with any clinical or analytical manifestation. We found some clinical associations in patients carrying at least one 24 CA allele or one allele containing <24 CA repeats (table 4). The most striking association with shortest alleles was found with livedo reticularis because 38% of patients carrying at least one allele containing <24 CA repeats presented this clinical feature, whereas only 10% without these alleles did ($p = 0.006$). When we compared the autoantibody specificity in patients carrying different alleles, we found that subjects carrying any short allele presented more susceptibility to produce anti-Sm ($p = 0.01$) and anti-RNP ($p = 0.038$) antibodies.

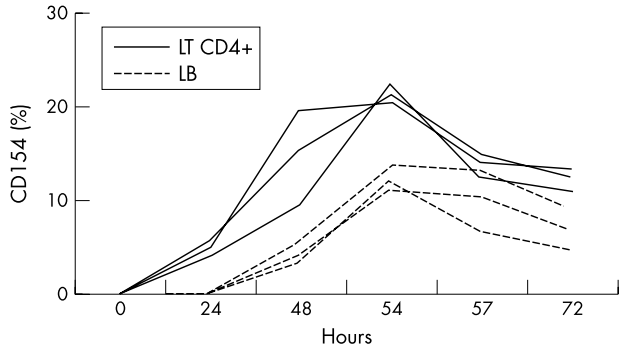

Figure 2 CD154 kinetic expression on PBMC from controls stimulated with PHA and anti-CD28. PBMC from three homozygote controls carrying two 24 CA alleles were stimulated with PHA and anti-CD28 antibody and were harvested after 24, 48, 54, 57, and 72 hours. CD154 expression was measured by flow cytometry on CD4+ T lymphocytes (continuous line) and B cells (dotted lines).

On the other hand, only three patients from a total of 43 carrying at least one allele with 24 CA repeats presented a neurological disorder (considering seizures and/or psychosis) compared with 10 patients of a total of 37 patients without any allele of 24 CA repeats ($p = 0.034$).

We did not found any association with the other clinical features evaluated (mentioned in table 1).

Functional significance of the polymorphic (CA)n repeats for CD154 expression

To investigate the possible role of the 3'UTR polymorphism on CD154 expression, we evaluated the protein expression on PBMC activated with PHA and soluble anti-CD28 mAb from 30 healthy controls. We only considered healthy donors and not patients in order to assign the differences in the protein expression to the genetic polymorphism, and avoid any altered protein expression associated with the patients with SLE. Furthermore, only men or homozygous women were considered in order to correctly assign a protein expression level to a concrete allele, which would not be possible in heterozygous cases.

Firstly, we studied the CD154 kinetic expression in PBMC from controls carrying the 24 CA alleles as being the most common in our population. Maximum CD154 protein levels on T and B lymphocytes were reached after 54 hours of activation and they had fallen at 72 hours (fig 2). Then, we compared the CD154 kinetic expression by flow cytometry in controls with different alleles after 54 and 72 hours.

As PBMC were cultured for a long time, the activation rates were measured by flow cytometry as CD69 and CD25 expression, considering those cells were activated when positive for just one or both activation markers. About 80% and more than 90% of T and B lymphocytes were activated at 54 or 72 hours respectively and no differences were found among different alleles.

Table 4 Clinical and analytical features of patients with SLE associated with CD154 polymorphism

Genotype*	Clinical feature	With allele‡ n/N (%)†	Without allele§ n/N (%)†	p Value	OR (95% CI)
<24 CA/X	Anti-Sm	12/25 (48)	8/48 (17)	0.01	4.62 (1.36 to 15.93)
	Anti-RNP	15/25 (60)	16/50 (32)	0.038	3.19 (1.05 to 9.76)
	Livedo reticularis	11/29(38)	5/51(10)	0.006	6.75 (1.51 to 34.08)
24 CA/X	Neurological disorder	3/43 (7)	10/37 (27)	0.034	0.2 (0.03 to 0.9)

*X represents any allele; †n = number of patients with‡ or without§ at least one corresponding allele presenting the indicated clinical or analytical feature; N = number of patients with‡ or without§ at least one corresponding allele.

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Table 5 CD154 expression on CD4+ T and B lymphocytes from controls after PHA and anti-CD28 activation. Data are presented as the mean (SD) of 10 controls from each genotype. The ratio between the MFI of CD154-PE and an isotype control antimouse IgG1-PE antibody is indicated in square brackets

	CD4+ T lymphocytes		B lymphocytes	
Genotype	54 Hours % [MFI]	72 Hours % [MFI]	54 Hours % [MFI]	72 Hours % [MFI]
<24/<24	15 (10), [1.8 (0.4)]	15 (7), [1.8 (0.3)]	15 (9), [1.8 (0.5)]	16 (11), [1.7 (0.5)]
24/24	20 (10), [2.5 (1)]	16 (11), [2.1 (0.9)]	16 (11), [2.2 (0.8)]	11 (6), [1.6 (0.3)]
>24/>24	23 (7), [2.6 (0.6)]	27 (12), [2.5 (0.7)]	20 (11), [2.2 (0.8)]	24 (12), [2.3 (0.6)]

The percentage of the CD4+CD154+ and CD19+CD154+ from the total of CD4+ and CD19+ cells respectively are indicated.

Table 5 shows the CD154 expression on T and B lymphocytes stimulated with PHA and anti-CD28 for 54 or 72 hours. We found that after 72 hours of activation, the CD154 protein expression had only decreased on lymphocytes from controls carrying 24 CA alleles. On the other hand, lymphocytes from controls carrying alleles with <24 CA repeats maintained the same expression levels as after 54 hours, and lymphocytes from controls carrying alleles with >24 CA repeats still increased the CD154 protein expression. Less than 0.5 % of T and B lymphocytes expressed CD154 in all analysed samples when no stimuli were added to cultures. Figure 3 shows the flow cytometric analysis of the percentage of activated CD4+ T lymphocytes expressing CD154 from representative samples with different alleles.

Considering that the CD154 expression on lymphocytes from controls carrying alleles with >24 CA repeats were the most different after 72 hours of PBMC stimulation, we performed a two tailed Student's *t* test to compare the protein expression on lymphocytes from carriers or non-carriers of two alleles containing >24 CA repeats (table 6). Although the percentage of T lymphocytes expressing CD154 after 54 hours of PBMC activation was higher in controls with >24 CA repeats ($p<0.05$), the MFI showed no differences and the protein expression on B cells was similar in both groups. However, when we compared the CD154 expression after 72 hours, both T and B lymphocytes from controls carrying two alleles with >24 CA repeats showed a significative higher protein expression, not only in the percentage of cells expressing CD154 but also the MFI.

To exclude the possibility that the differences found in the CD154 expression might be due to differences in proliferation and/or apoptotic rates, we measured the PBMC proliferation by BrdU uptake as described in ''Material and methods'', and apoptosis was estimated by flow cytometry by forward and size scattered criteria. No differences were found in proliferation or apoptosis rates among the different alleles (data not shown).

Finally, PBMC from controls were also stimulated only with soluble anti-CD28 to test if the differences found in the CD154 expression might be attributed to different effects of anti-CD28 on these cells. Only about 3% and 15% of the CD4+ T and B lymphocytes, respectively, were activated and <0.5% were CD154+ in all samples, which were the same activation and CD154 levels found in PBMC cultured without stimuli.

DISCUSSION

The aetiology of autoimmune diseases is not well elucidated, but it is commonly agreed that their development may be the consequence of predisposing genes involved in the intrincate network necessary to mount mature and regulated immune reactions against foreign and altered self antigens. For SLE the interaction among several genes and certain environmental factors is likely to be associated with disease predisposition.[38] The genetics of SLE are complex, being associated not only with certain major histocompatibility complex (MHC) class II alleles but also with many polymorphic non-MHC genes coding for proteins involved in the immune system regulation.[39]

Growing evidence suggests that CD154 protein hyperexpression has an important role in autoimmunity,[40] leading to excessive B cell activation, accelerated immunoglobulin class switching, and autoantibody production.[41 42] A CD154 prolonged expression after in vitro activation of T lymphocytes from patients with SLE, and an increased baseline expression in some patients with clinically active disease have been reported.[43] Lupus B cells also hyperexpress CD154,[44] so these cells might not only be activated and costimulated by the CD154 on T cells but also they might be able to amplify and sustain the pathogenic autoimmune response by themselves. Finally, raised levels of soluble CD154 have been also found in sera from patients with SLE.[24 45] Both raised surface and soluble levels of CD154 are functional and correlate with anti-dsDNA antibody titres and the clinical disease activity of SLE.[22] Besides the CD154 protein hyperexpression in peripheral blood, it has been also found in T lymphocytes infiltrating renal tissue. Direct interaction of these T cells with renal parenchymal and non-parenchymal CD40 positive target cells, may have a role in the immunopathogenesis of glomerulonephritis present in some patients with SLE.[26] When all these data are taken into account CD154-CD40 interaction is suggested to play central part in the induction and maintenance of the immune and inflammatory responses in SLE.

The CD154 hyperexpression found in SLE might be an intrinsic defect and not merely due to autoantigenic activation, because raised levels of CD154 are present in lupus even during remission of the disease. However, all reported studies about CD154 in this disease have been directed towards mRNA or protein levels, and not to a possible genetic involvement.

The 3'UTR of mRNA is strongly implicated in the post-transcriptional regulation of gene expression, and it has been suggested that some diseases arise from anomalies in this region.[46] The CD154 protein expression is post-transcriptionally regulated,[47 48] and some proteins have been shown to bind to the CD154 3'UTR, regulating the mRNA stability.[49 50] Furthermore, the binding region of these proteins is located just upstream from the CA microsatellite. In this study we postulated that this microsatellite might have a role in the mRNA stability and so in the CD154 protein expression.

As a first approach for testing our hypothesis, we explored whether the CD154 microsatellite was associated with SLE, and we found that alleles carrying 24 CA repeats were negatively associated. On the other hand, the alleles containing >24 CA repeats were not only the most represented alleles in the SLE group but also in homozygous patients. Therefore these data suggest that the microsatellite located in the mRNA 3'UTR is associated with SLE predisposition.

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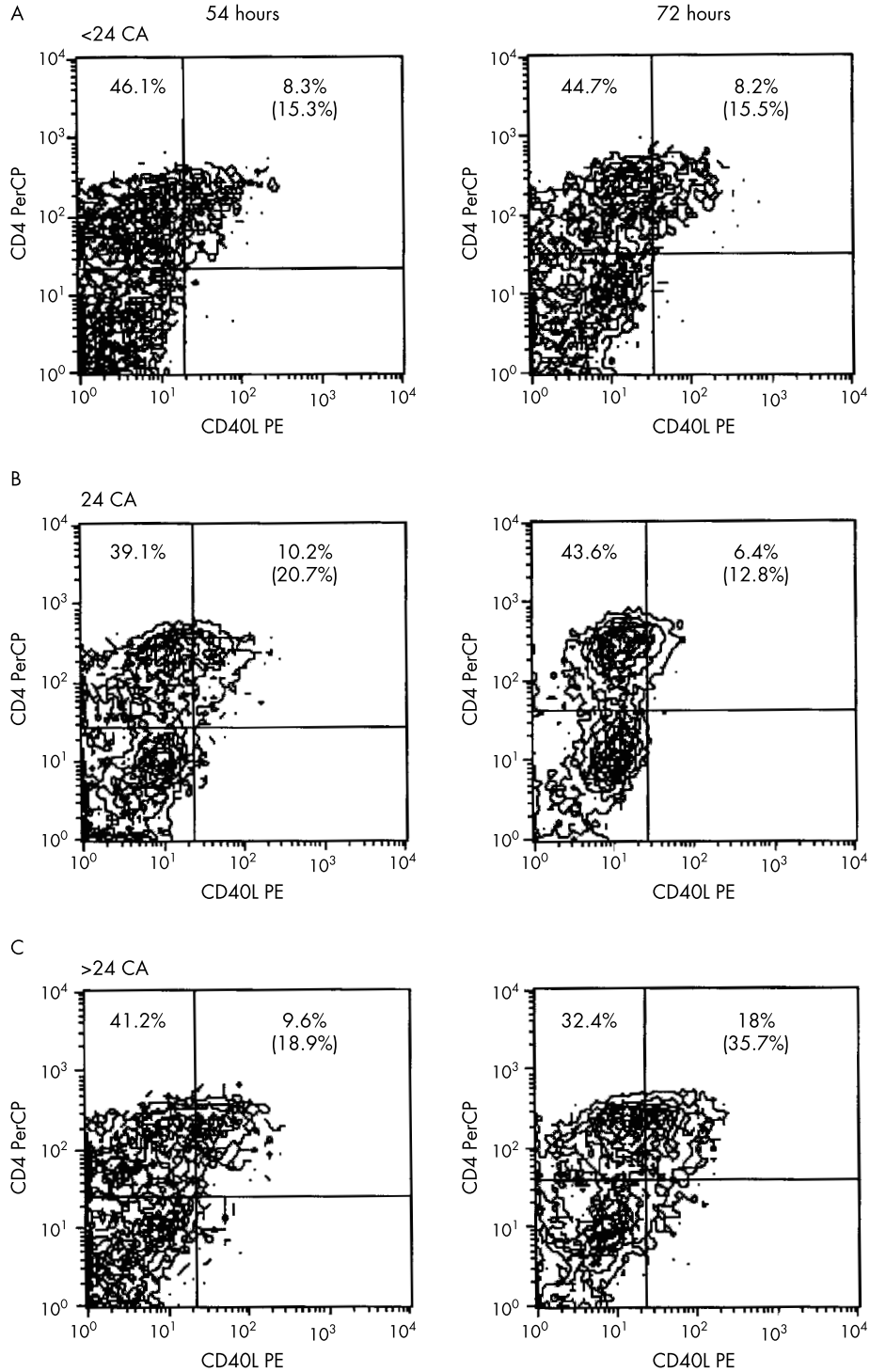

Figure 3 Flow cytometry plots showing the CD154 expression on T lymphocytes from representative controls. PBMC from controls with different genotypes were activated with PHA and anti-CD28 for 54 (first column) or 72 hours (last column). Percentages of CD4+CD154− and CD4+CD154+ are indicated (left and right of each plot, respectively), and the percentage of the CD4+CD154+ cells from the total of CD4+ cells is shown in parenthesis. All the CD4+ cells were CD14−, all of them being CD4+ T lymphocytes.

When we compared the clinical features among patients carrying different alleles, we found that the 24 CA allele, suggested to be protective for SLE, was associated with a low risk of neurological disorder. On the other hand, we found that anti-RNP and anti-Sm antibodies, and livedo reticularis were significantly more common in patients carrying at least one allele containing <24 CA repeats. CD154 induces immunoglobuline isotype switching and its hyperexpression described in SLE is associated with autoantibody production,[22] but surprisingly we have found that the least represented alleles are associated with concrete autoantibody specifities. Nevertheless, in this study we have not considered antibody titres because they are dependent on disease activity, nor autoantibody isotypes because almost all our patients carried anti-IgG autoantibodies. It has been suggested by other authors that anti-Sm antibodies, which are

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Table 6 CD154 expression on CD4+ T and B lymphocytes from controls, in carriers of longer alleles compared with the others. Data are presented as mean (SD). p Values were calculated by the two tailed Student's *t* test comparing the percentage (and the MFI in square brackets) between the indicated genotypes

	CD4+ T lymphocytes		B lymphocytes	
Genotype	54 Hours % [MFI]	72 Hours % [MFI]	54 Hours % [MFI]	72 Hours % [MFI]
Non >24 CA/>24 CA	18 (9), [2.2 (0.8)]	16 (9), [1.9 (0.6)]	16 (9), [2 (0.6)]	14 (9), [1.7 (0.5)]
>24 CA/>24 CA	23 (7), [2.6 (0.6)]	27 (12), [2.5 (0.7)]	20 (11), [2.2 (0.8)]	24 (12), [2.3 (0.6)]
p Value	0.048 [NS]	0.0068 [0.029]	NS [NS]	0.024 [0.0073]

almost invariably accompanied by anti-RNP antibodies in SLE, might be associated with a relative milder disease.[51 52]

Once we had shown that the CD154 genetic polymorphism is associated with SLE, we next explored whether this microsatellite might have any functional consequence on CD154 protein expression. When PBMC from healthy men or homozygous women were activated in vitro with a T specific stimulus such as PHA and anti-CD28 mAb, we found lower CD154 levels on CD4+ lymphocytes than the expression reported by others using PMA and ionomycin, probably owing to the more specific stimuli we used in our system. On the other hand, although PHA and anti-CD28 are T specific stimuli, we observed activation and CD154 expression on B lymphocytes, probably induced by the contact with activated T lymphocytes.

We found that different alleles were associated with different CD154 kinetic expressions. Of note, T and B lymphocytes from controls carrying two alleles containing >24 CA repeats maintained a longer protein expression, whereas the homozygotes for 24 CA repeats were those that first down regulated the CD154 protein expression.

Taking into account our data from the genetic and expression approaches, we interestingly found that the most represented alleles in SLE were the alleles associated with a prolonged CD154 protein expression. Furthermore, the alleles associated with a reduced risk for SLE were the alleles associated with the shortest protein expression. T and B lymphocytes from patients with SLE have been described as presenting a CD154 basal expression, and as reaching higher levels when they are activated in vitro, than lymphocytes from healthy controls. We postulate from our results that the CD154 hyperexpression reported in patients with SLE may be caused by both a continuous antigenic stimulation and a genetic predisposition for maintaining the CD154 expression on T and B lymphocytes in response to these antigens.

In conclusion, to the best of our knowledge, we demonstrated here for the first time that the CD154 3'UTR microsatellite may have a functional role on CD154 protein expression and it is associated with SLE. Nevertheless, it would be very interesting to confirm the results presented here in longer series of patients and to evaluate this association in a second cohort of the same population as well as in other ethnic groups. Future studies to confirm the direct role of the microsatellite on mRNA stability suggested in the present work are also needed. Additionally, because CD154 may have an important role in autoimmunity, the analysis of this polymorphism in other autoimmune diseases where a CD154 hyperexpression has been described may provide us with important information to help in the understanding of autoimmune diseases.

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