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Association of IL-6 gene alleles with systemic lupus erythematosus (SLE) and with elevated IL-6 expression

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To evaluate the association of alleles of regions having regulatory potential in the IL-6 gene, with SLE, the AT-rich minisatellite in the 3′ flanking region and the 5′ promoter-enhancer of the IL-6 gene were genotyped by PCR- and RFLP-based methods. The AT-rich minisatellite allele distribution pattern was significantly different in SLE (n = 146) as compared to 139 controls (χ^2_7 = 48.97, P = 0.001, Caucasians; and χ^2_7 =19.93, P = 0.006, African-Americans). In either race, short allele sizes (⩽792 bp) were seen exclusively in SLE patients (P = 0.001), whereas the 828-bp allele was over-represented in controls (P = 0.015 and 0.002). In contrast, there was no preferential association of SLE with G/C alleles in the 5′ region of the IL-6 gene. Furthermore, our results suggest that the 3′ minisatellite alleles have biological significance: (1) B lymphoblastoid cells of patients having one or two SLE-associated alleles secreted IL-6 in 3- to 4-fold higher levels than non-allelic cells (P < 0.05); (2) higher percentages (approximately 4-fold) of IL-6 positive monocytes were observed in individuals having SLE-associated IL-6 alleles; (3) in lupus patients having SLE-associated minisatellite alleles, IL-6 mRNA stability was significantly enhanced.

Keywords: *IL-6 alleles; SLE; lupus; minisatellites; cytokine alleles*

Introduction

SLE is a multifactorial disease that has a complex genetic and environmental etiology.[1] Studies of SLE patients from various ethnic backgrounds have shown strong associations between SLE and alleles of genes involved in lupus pathogenesis,[2–6] and a susceptibility gene with unknown function in the region of chromosome 1q41–42.[7] Thus, it is likely that susceptibility to SLE may be conferred by multiple genes.

B cell hyperactivity, elevated production of autoantibodies and over-expression of Type 2 cytokines IL-6 and IL-10 are characteristic of SLE.[8,9] IL-6 effects on lymphocyte differentiation and its over-expression in inflammatory and autoimmune conditions have been well documented, and the IL-6 gene locus has been cloned and sequenced.[10,11] We and others have shown that there are higher levels of IL-6 mRNA in SLE PBMC, and of IL-6 protein in serum and involved kidneys of patients with active disease, and that endogenous IL-6 is essential for SLE B cell hyperactivity.[9–16] In recent studies of murine SLE, treatment with anti-IL-6 antibody abrogated disease, supporting the concept that IL-6 has an important pathogenic role in lupus.[17]

IL-6 over-expression in SLE could result from an abundance of up-regulating factors and/or polymorphisms in regions having gene regulatory implications. Several reports are in support of the latter. A G/C polymorphism of the IL-6 promoter has been described recently;[18] it was shown to affect transcriptionally IL-6 expression, and a lower frequency of the CC genotype was found in patients with systemic-onset juvenile chronic arthritis.[19] Previously, by genotyping an AT-rich minisatellite in the 3′ region flanking the IL-6 gene,[20] we found association of SLE with increased size-variation of this region;[21] another group found that allelic variants of this region correlated with low bone mineral density,[22] suggesting that this region has biological relevance. The objective of the present study, was to evaluate the possible association of allelic regions of the IL-6 gene having gene-regulatory potential, with SLE and its dysregulated IL-6 expression.

Results

Size-alleles of the IL-6 3′ minisatellite are associated with SLE

The AT-rich minisatellite and respective flanking regions were PCR-amplified at high-stringency from genomic DNA of SLE and controls, using primers outside of the allelic region (Figure 1a). The one or two amplicons per individual were viewed by electrophoresis (PAGE), and sized in relation to the 100-bp ladder; we found eight

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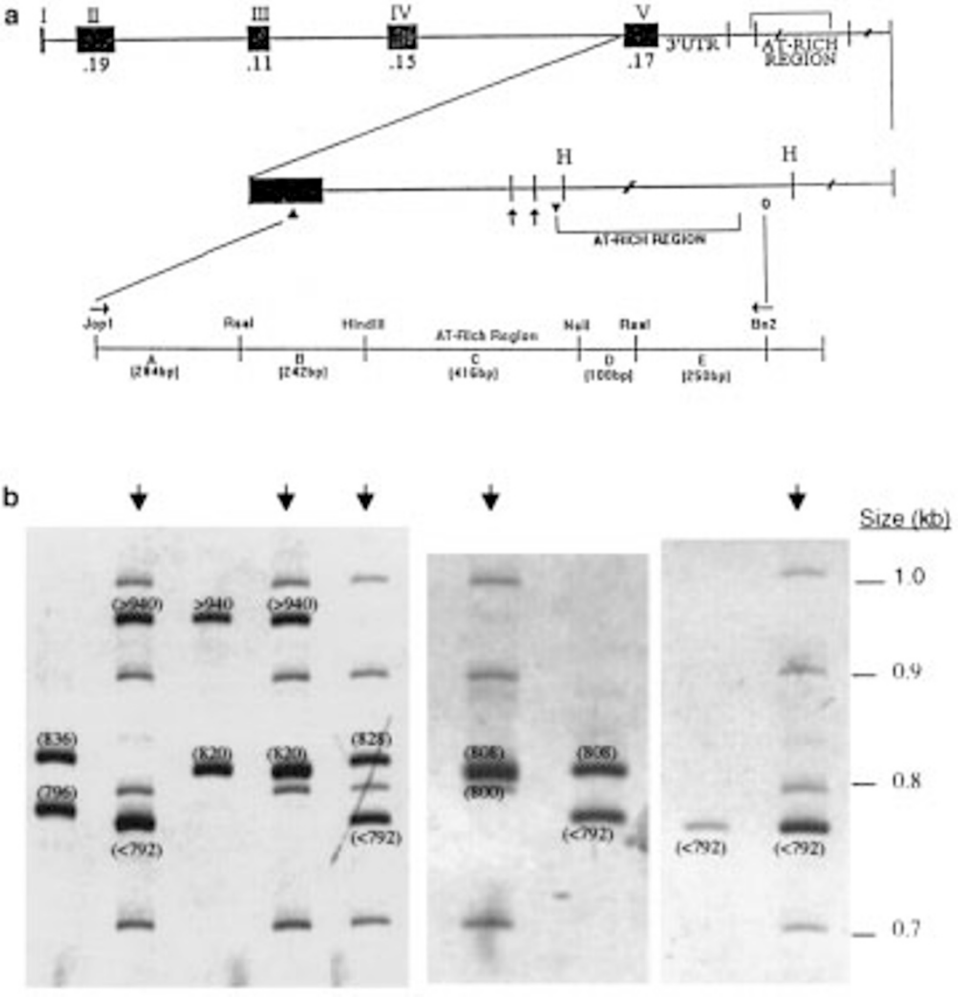

Figure 1 (**a**) The IL-6 gene: 3′ flanking region. Shown are Exon V (boxed), the 3′ UTR, the AATAAA polyadenylation signals (underlined), and the 3′ flanking region, the latter containing the allelic AT-rich element. Primers for PCR-amplification are underlined, with arrows below. The restriction endonucleases used to generate the 3′ probes for the mobility shift assays are indicated by downward arrows: H: *Hin*dIII, R: *Rsa*I, N: *Nsi*I. (**b**) Sizing the IL-6 minisatellite alleles. The 3′ flanking region of the IL-6 gene, including the IL-6 minisatellite, was PCR-amplified from genomic DNA samples. PCR products were electrophoresed (6% PAGE) and gels were stained with ethidium bromide. Mixtures of PCR samples and the 100-bp DNA ladder were run in lanes marked with an arrow.

Table 1 Association of IL-6 3′ minisatellite size-alleles with SLE: allele frequency distribution in Caucasian and African-American population samples

Allele size (bp)	*Caucasians (n = 178)*					*African-Americans (n = 108)*				
	SLE Freq. (%)		*Control Freq. (%)*		χ^2 *(P value)*	*SLE Freq. (%)*		*Control Freq. (%)*		χ^2 *(P value)*
⩽792[a]	23	13.53	0	0	26.9 (0.001)[b]	11	8.87	0	0	8.60 (0.001)[b]
796	49	28.82	79	42.48	7.19 (0.007)[b]	9	7.26	7	7.61	0.01 (0.92)[b]
800	29	17.06	36	19.36	0.31 (0.58)[b]	21	16.94	8	8.69	3.09 (0.08)[b]
808	14	8.24	3	1.61	8.57 (0.003)[b]	28	22.58	17	18.48	0.54 (0.46)[b]
820	16	9.41	8	4.30	3.69 (0.04)[b]	14	11.29	10	10.87	0.009 (0.9)[b]
828	18	10.59	37	19.89	5.89 (0.015)[b]	16	12.90	28	30.43	10.0 (0.002)[b]
836	13	7.65	19	10.22	0.72 (0.40)[b]	5	4.03	3	3.26	0.09 (0.77)[b]
⩽940	8	4.71	4	2.15	1.78 (0.39)[b]	20	16.13	19	20.65	0.73 (0.39)[b]
Total	170	100	186	100	58.97 (0.001)[c]	124	100	92	100	19.93 (0.006)[c]

[a]Allele sizes ranged from 780–792 bp. [b]Frequency of alleles at each observation point was compared, SLE *vs* controls by Fisher's exact test. [c]Frequency distribution of all the alleles in SLE samples was compared to controls by a χ^2 test, and was significantly different also with Bonferroni's correction.

allele sizes (Figure 1b). As seen in Table 1, overall allele distribution was statistically significantly different in SLE, as compared to controls ($P = 0.001$ and $P = 0.006$ in Caucasians and African-Americans respectively). The ⩽792-bp allele was associated with SLE patients, of either Caucasian or African-American descent, but not with controls ($P = 1.80E-08$ and 2.86E-03). In Caucasians, there was also SLE-association of the 808-bp allele (8.24% *vs* 1.61%, $\chi^2 = 0.003$) and the 820-bp allele (9.41% *vs* 4.3%, $\chi^2 = 0.05$); the 796-bp and 828-bp alleles were preferentially associated with controls (42.48% and 19.89% *vs* 28.82% and 10.59%, $\chi^2 = 0.007$ and 0.015 respectively). In African-Americans, the 828-bp allele was also preferentially associated with controls (30.43% *vs* 12.9%, $\chi^2 = 0.002$). Allele sizing was performed three times on selected DNA samples, and size differences between individuals were consistently reproduced. Allele sizes were confirmed in selected DNA samples, using an ABI DNA Sequencer (not shown).

The promoter-enhancer of the IL-6 gene contains the sequences necessary for gene regulation. In preliminary studies, the entire IL-6 promoter-enhancer region was sequenced using DNA of 21 Caucasian SLE patients and 30 controls, but we could not find a preferential association of promoter–enhancer alleles with SLE (not shown).

Since a G/C polymorphism in the IL-6 promoter (at –174) could affect IL-6 expression,[19] we investigated whether it would be associated with SLE. A cohort of Caucasian patients ($n = 89$) and controls ($n = 76$) was typed by a PCR + RFLP-based technique (Figure 2). As seen in Table 2, G/C allele and genotype frequencies in the SLE group were similar to those of controls.

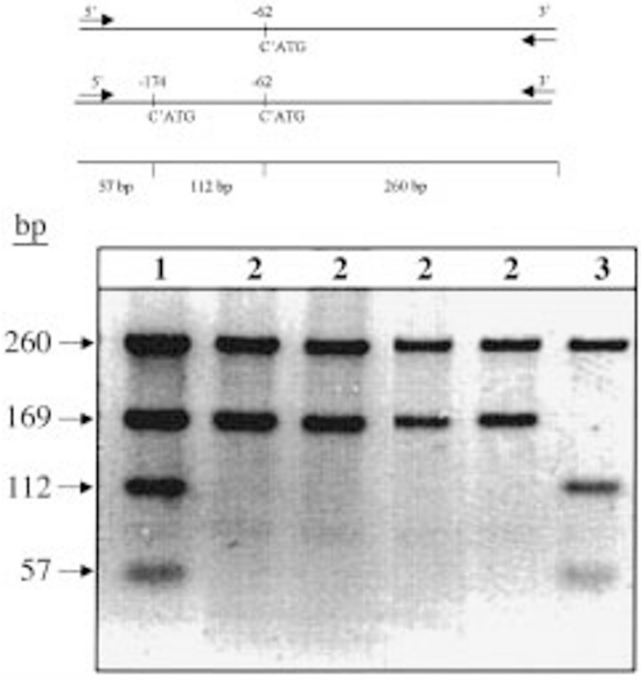

Figure 2 The IL-6 gene: 5′ flanking region. Shown are: part of the promoter-enhancer region having the G-C point mutation, the two *Nla*III restriction sites in the C allele at –174 and –62, and the PCR primer sites. IL-6 promoter-enhancer (G/C) alleles. The 5′ promoter-enhancer region, including the –174 G-C polymorphism, was PCR-amplified and PCR products were incubated with *Nla*III, electrophoresed (8% PAGE) and silver-stained. The three genotypes: GC (1), GG (2) and CC (3).

SLE-associated alleles of the IL-6 3′ minisatellite correlate with increased IL-6 production

To evaluate a possible correlation between the SLE-associated IL-6 3′ alleles and IL-6 expression, we measured IL-6 production by EBV-B lymphoblastoid cells derived from SLE patients having different minisatellite alleles. Equal amounts of EBV-B cells were incubated with medium only, LPS (10 μg/ml), or ATRA (10^{-6} m). The latter has been shown to specifically trigger IL-6 production by EBV-B cells. After a 24-h incubation, supernatants were harvested and measured for IL-6 content by ELISA. Only supernatants of cell cultures having >85% viability were processed. As seen in Table 3, cell lines having one or two SLE-associated alleles secreted 3- to 6-fold higher constitutive and induced IL-6 levels ($P < 0.05$).

In addition, the number of IL-6- secreting monocytes derived from unaffected individuals having SLE-associated alleles was compared by flow cytometry to that of other controls. A representative experiment is seen in Figure 3. PBMC derived from a heterozygous individual having an SLE-associated allele (820 bp) had an increase in the number of IL-6+ monocytes (CD69–, TNF∀–) as compared to a (homozygous) matched control having the non-SLE-associated allele 828 bp. Similar data were obtained from three pairs of unaffected donors, matched for age, sex and ethnic background (6.93 ∀ 2.8% IL-6 + monocytes in allelic donors, *vs* 1.8 ∀ 1.0 % in others; $P = 0.03$). No co-expression of the early activation marker CD69 and of TNF-∀ was observed (Figure 3), thus ruling out the possibility that the increase in IL-6+ monocytes was due to non-specific *ex vivo* stimulation.

SLE-associated alleles of the IL-6 3′ minisatellite correlate with increased IL-6 mRNA stability

Recently, we reported that IL-6 mRNA stability is significantly increased in SLE, and even more so, in patients with active disease.[23] In the present study, we analyzed the individual correlation between stability of mRNA with 3′ minisatellite allele sizes and with activity of disease. PBMC from SLE patients and unaffected controls matched for age, sex, and ethnic background (Caucasian), were stimulated with 5 μg/ml PWM for 5 h (T_0), and incubated for 2 additional h with 5 μg/ml Actinomycin D (AD). Cytoplasmic RNA was extracted from cell aliquots harvested at T_0, 30 min, 60 min, 90 min and 120 min (T_{120}). RNA samples were slot-blotted, and sequentially hybridized to ^{32}P-labeled cDNA probes for IL-6 and gamma actin. Laser densitometry IL-6 readings were normalized to actin. As seen in Figure 4, T_{120} mRNA levels of control PBMC ($n = 11$) dropped below 50% of T_0 levels (43% ± 19.74%). In contrast, significantly delayed mRNA decay occurred in 12/12 SLE with active disease, regardless of their IL-6 minisatellite alleles ($P < 0.05$), and T_{120} mRNA levels in PBMC of patients with inactive disease having 1–2 SLE-associated alleles were significantly different from those of patients with inactive disease who had no SLE-associated alleles (68.3% ± 10.6% *vs* 41.14% ± 8.65%, $P = 0.0018$). Interestingly, the three controls having the SLE-associated allele 808 bp also had delayed mRNA decay (70.5% ± 11.3% *vs* 32.7% in the seven controls not having SLE-associated alleles, $P = 0.0014$).

Table 2 The distribution of IL-6 promoter (G/C) alleles in SLE patients is similar to controls

DNA donors[a]	*G/C*[b] *phenotype frequency*			*G/C allele frequency*	
	GG	*GC*	*CC*	*G*	*C*
SLE (n = 91)	45 (0.495)	37 (0.406)	9 (0.099)	127 (0.691)	55 (0.309)
Controls (n = 76)	35 (0.461)	34 (0.448)	7 (0.091)	104 (0.684)	48 (0.316)

[a]Caucasian cohort, n = 167. [b]Genotyped by PCR + RFLP. The promoter region, including the –174 G-C polymorphism, was PCR-amplified. PCR products were incubated with *Nla*III, electrophoresed (8% PAGE) and silver-stained (Figure 2).

Table 3 EBV-B cell lines derived from patients having SLE-associated 3′ minisatellites secrete higher levels of constitutive and induced IL-6

Minisatellite alleles (bp)	*IL-6 secreted (pg/ml)*[a]		
	Medium only	*LPS (10 μg/ml)*	*ATRA (10^{-4} M)*
790 808	169.3	231.0	356.5
790 820	63.2	229.8	88.1
790 836	39.7	221.4	193.8
792 940	75.6	257.5	169.9
790	131.6	300.9	180.9
Group 1,[b] mean ± s.d.	99.8 ± 48.4[d]	248.13 ± 32.4[e]	197.84 ± 97.8[f]
828 836	27.2	37.6	42.5
828	18.3	30.9	35.6
836 970	22.4	40.1	40.7
836 940	30.9	51.3	104.0
Group 2,[c] mean ± s.d.	23.45 ± 5.3	39.98 ± 8.5	55.7 ± 32.3

[a]EBV-B cells were stimulated as indicated. Cell supernatants were harvested at 24 h, and measured for IL-6 content by ELISA. [b]One or two SLE-associated alleles. [c]Non-SLE-associated alleles. Statistical analysis was performed using a 2-tailed, unpaired Student's t-test. P-values were: [d]P = 0.02 (Gr 1[b] *vs* Gr 2). [e]P = 5.9E-05 (Gr 1[b] *vs* Gr 2). [f]P = 0.03 (Gr 1[b] *vs* Gr 2).

Discussion

The present study demonstrates that alleles of the AT-rich minisatellite in the 3′ region flanking the IL-6 gene are associated with SLE of either Caucasian or African American background. Although the overall distribution of alleles was not identical in the two ethnic groups studied, the short alleles (≤792 bp) were strongly associated with SLE patients of either genetic background.

Reports from several groups have shown that cytokine gene alleles could be associated with magnitude of gene expression and with autoimmunity. Several studies have shown association of TNF alleles with SLE in Caucasian, but not in a Chinese population.[24–26] The association of TNF alleles with MHC haplotypes and with SLE could stem from the proximity of TNF genes to the MHC locus rather than direct involvement with disease, and size/sequence alterations in non-MHC-associated cytokine genes having a pathogenic role in lupus may bear more relevance to disease.

In a diallelic polymorphism of the IL-1-β gene, one of the homozygous combinations was associated with higher secretion of IL-1β, and there was strong association between an IL-1-α RFLP in the 5′ promoter region and a subset of juvenile rheumatoid arthritis patients. Indeed specific IL-1Ra alleles were associated with SLE in a Japanese population.[9,27]

The magnitude of IL-10 secretion varied with the haplotype of the IL-10 promoter microsatellite, and the G alleles were associated with SLE of Caucasian descent;[28,29] moreover, higher incidence of certain IL-10 and Bcl-2 genotypes was found in Caucasian and Hispanic American SLE.[30]

Most of these are point mutation polymorphisms in promoter regions of the genes. However, an RFLP due to variable numbers of repeats in the 6th intron of the IL-1α gene, in a region similar in structure to the AT-rich element studied by us was recently described,[31] and a rare variable repeat polymorphism of the IL1Ra gene was associated with increased IL-1Ra expression.[32]

Our studies of EBV-B cells and freshly excised PBMC clearly indicate that the 3′ alleles that were over-represented in SLE correlate with IL-6 up-regulation. The mechanism/s underlying this correlation are not clear yet and we cannot exclude the possibility that the 3′ minisatellite alleles are mere markers for other genes.

Interestingly, a strong correlation between the SLE-associated 3′ alleles and increased IL-6 mRNA stability was seen in patients with inactive disease. Previously, other groups have reported, in SLE, on increased expression and mRNA stability of other short-lived transcripts: c-myc, c-myb, c-raf, and the CD40L.[33–35] In the present study, IL-6 mRNA levels were expressed as IL-6 : actin ratios, suggesting that delayed mRNA decay is not a generalized trait in SLE. Not surprisingly, patients with active lupus had all increased mRNA stability, regardless of IL-6 minisatellite size, suggesting that additional disease-related factors contribute to the IL-6 over-expression of SLE. Thus, we have shown that IL-1∃ and TNF-∀, whose serum levels increase with disease activity in SLE,[9] affect IL-6 levels posttranscriptionally,

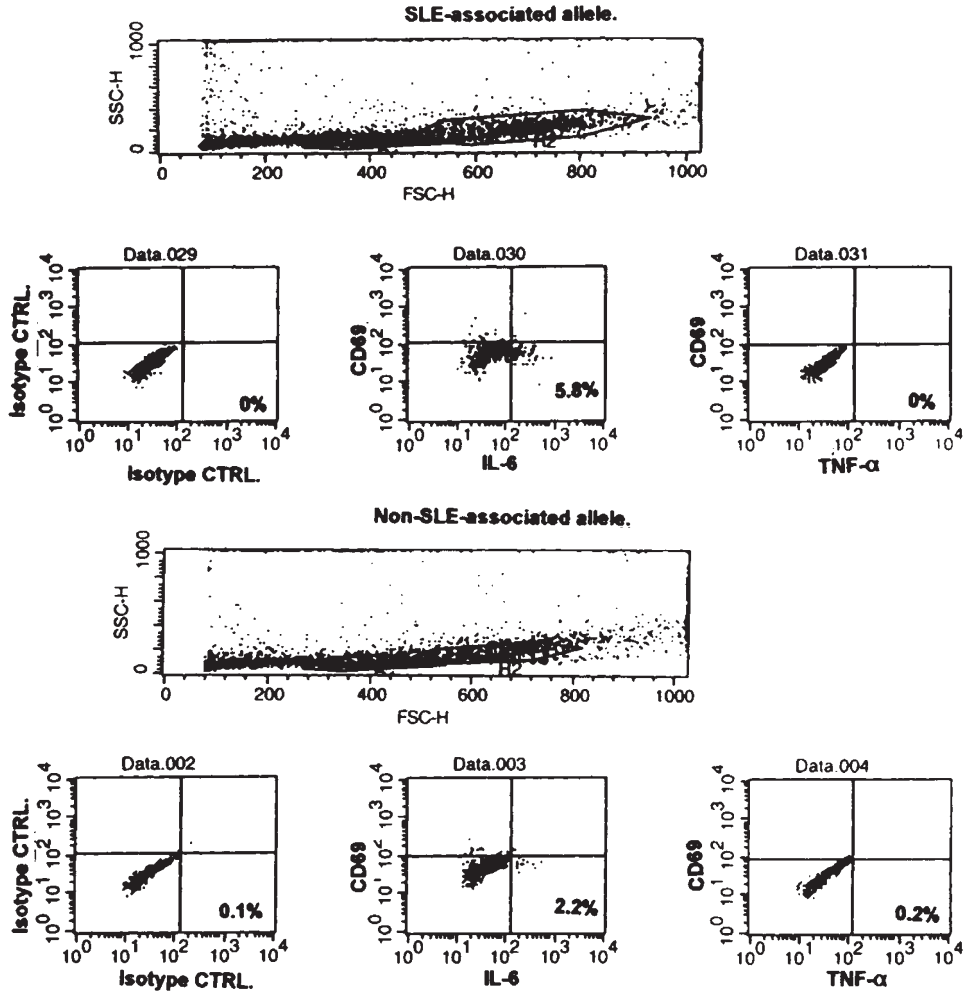

Figure 3 SLE-associated IL-6 minisatellite alleles correlate with increased number of monocytes constitutively expressing IL-6. Monocytes were evaluated by flow cytometry for intracellular IL-6 or TNF-a (FL1) and CD69 (FL2). Monocytes were gated by forward/side light scatter, and 10^4 events were acquired and analyzed. Quadrant markers for analysis of fluorescence intensity were set with PE- and FITC-conjugated isotype (negative) controls.

by stabilizing IL-6 mRNA.[23] Preliminary studies from this group suggest that the 3′ minisatellite and its flanking regions have factor-binding activity. Indeed, downstream of the IL-6 minisatellite, there are consensus binding sites for AP-1 and GATA family factors, and preliminary footprinting experiments done by our group showed binding of PBMC nuclear extracts to one of the GATA sites (unpublished). These and reporter gene construct experiments will be the focus of our future studies.

Although AT-rich sequences are common throughout the genome, the pattern of the AT-rich IL-6 minisatellite occurs only in the 3′ flanking region of the IL-6 gene (Genbank search); it was originally sequenced and identified as a VNTR (variable number of tandem repeats) by Bowcock and colleagues,[20] but was not evaluated for association with disease or function. Murray and colleagues[22] recently showed a strong association between shorter alleles of this minisatellite and low bone mineral density in post menopausal women, but did not study correlation with IL-6 production.

Since the AT-rich repeats are palindromic and could form cruciform structures, size/sequence changes in this region may result in altered secondary structures. DNA segments containing similar repeated sequence motifs, and being also located in gene flanking regions, were identified as Matrix-Associated Regions (MARs). These are thought to have an important role in DNA replication, and modulate gene expression by enhancer or silencer activity.[36,37] The close proximity of such a region may increase accessibility to the IL-6 gene, a fact that may be relevant to SLE, a disease characterized by increased endogenous cell activation. Although the 5′ flanking region of IL-6 contains all the necessary gene-regulating sequences, the nuclear factor-binding activity of the 3′ minisatellite/flanking regions and the correlation between minisatellite alleles and IL-6 expression imply that, besides the MARS-like potential of this region that may confer easy accessibility of factors to the IL-6 gene, the minisatellite may have more direct effects on gene expression, and that size/sequence alterations of this element may have functional implications, like the IDDM-linked and the HRASI minisatellites that are also located 3′ of the polyadenylation signal.[38–40]

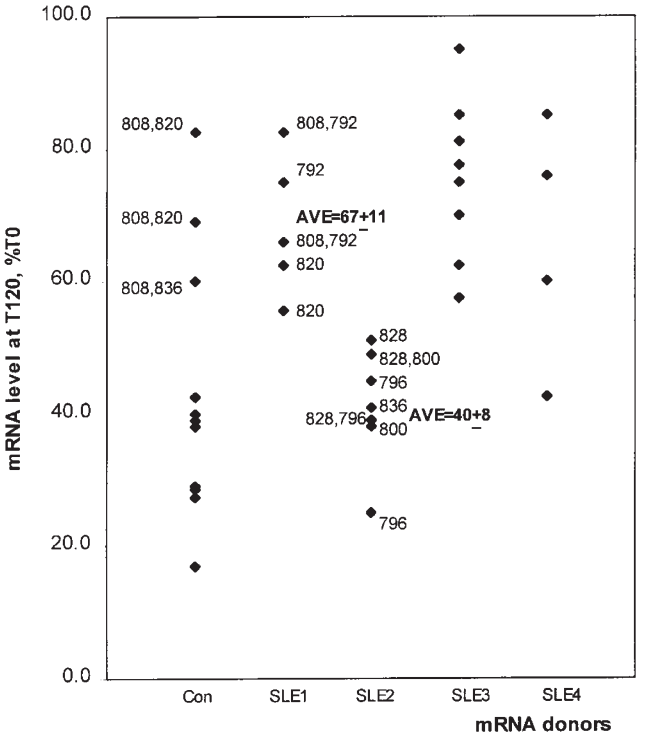

Figure 4 SLE-associated IL-6 minisatellite alleles correlate with delayed decay of IL-6 mRNA. T_{120} levels of IL-6 mRNA were expressed as percent T_0 levels, and were compared between RNA samples from controls (Con), patients with quiescent disease (SLE 1 and 2) and with active lupus (SLE 3 and 4). Individuals in groups SLE 1 and SLE 3 had SLE-associated minisatellite alleles.

Materials and methods

Patients and controls

Female patients fulfilled the American College of Rheumatology criteria for SLE.[41] Disease activity was assessed on a 0–20 scale, using a modified SLAM (Systemic Lupus Activity Measure) index.[42] A 4–10 score was considered as low activity, and ⩾11 as high activity of disease. Controls consisted of unaffected, healthy women, unrelated to the patients and unrelated to each other. The Caucasian controls ($n = 93$) were recruited from the same ethnic background as the SLE (Ashkenazi Jews and non-Jews of Eastern and Western European descent) and were represented in similar proportions to those observed in the SLE population studied ($n = 85$). The background of the Caucasian controls who had SLE-associated alleles was European non-Jewish, ($n = 2$) and Ashkenazi Jewish ($n = 1$). The African-American group consisted of 62 SLE patients and 46 unrelated controls. In the IL-6 expression studies, all subjects were 24 to 50-year-old non-pregnant Caucasian females, who were free of viral/bacterial infections at the time their blood was drawn.

PBMC isolation and cell cultures

Forty-five ml heparinized venous blood was drawn from each patient. PBMC were isolated on Ficoll Hypaque density gradients (Sigma, St Louis, MO, USA) as described previously,[12] and aliquots were EBV-transformed by the Cedars-Sinai Cell Culture Lab, and early passages of established cell lines were cryopreserved (–70°C). To measure IL-6 expression, PBMC and B lymphoblastoid cells were incubated in RPMI medium containing 10% FCS, amino acids and antibiotics,[12] ± Pokeweed Mitogen (PWM), Lipopolysaccharide (LPS) or All Trans Retinoic Acid (ATRA), as indicated.

Genomic DNA purification and PCR amplification

Genomic DNA was extracted with Tri reagent (Mol Res Center Inc, Cincinnati, OH, USA), and stored in TE buffer at –20°C, as described previously.[18] The 3′ AT-rich minisatellite (Figure 1a) and the promoter region containing the –174 G/C mutation (Figure 2) were amplified with gene specific primers (Promega, WI, USA) that were designed outside of the allelic regions:

5′GCAACTTTGAGTGTCACG,
3′TCCCTCAGTTCCTGGGTAGG (minisatellite);
5′CTTAGCGCTAGCCTCAATGACGAC,
3′GAGCGAGCGCAGGGGTGACTGACA (promoter).

The PCR was performed in a Stratagene Robocycler 40 thermal cycler, using standard methods. The amplified promoter fragment was restricted with *Nla*III (New England Biolabs, MA, USA).

Allele sizing

PCR product samples (15 μl) were electrophoresed on modified (6%, minisatellite, and 8%, promoter) polyacrylamide gels (Hydrolink Long Ranger, JT Baker, Phillipsburg, NJ, USA), running in selected lanes PCR products mixed with the 100-bp ladder (Gibco/BRL) as MW standards. The gels were ethidium bromide- or silver-stained (Bio-Rad).

RNA extraction; measurement of IL-6 mRNA steady-state levels and stability

PBMC 2×10^6/ml, were incubated ± PWM, 5 μg/ml, at 37°C for 5 h (T_0), when new transcription was inhibited with Actinomycin D, 2 μg/ml. Aliquots of $7–10 \times 10^6$ PBMC were harvested at indicated times. Total cytoplasmic RNA samples were slot-blotted in duplicates onto Nytran filters, 1 μg/slot, UV-cross-linked, and hybridized with ^{32}PcDNA probes (a 1.2-kb *Eco*RI fragment of IL-6 cDNA and a 2.2-kb *Bam*HI fragment of γ-actin cDNA labeled by random priming), as described previously.[21]

Autoradiograms were processed by integrating densitometry, and intensity readings of bands probed with IL-6 cDNA were normalized relative to actin. Results are expressed as % normalized T_0 value.

ELISA

EBV-B cells were incubated at 5×10^5/ml for 24 h ± LPS (10 μg/ml) or ATRA (10^{-6} m), and supernatants were harvested, spun, filtered, to remove cells and cellular debris, and were stored at –70°C until used. IL-6 content was measured with a Medgenix Easia kit (Biosource Int, CA, USA) using the kit standards and following manufacturers' indications.

Flow cytometry sample preparation and analysis

PBMC were processed as described previously,[22] using Brefeldin-A (Pharmingen CA) to inhibit protein transport. Following cell-fixation with 4% paraformaldehyde (Electron Microscopy Sciences, PA, USA) and permeabilization (Perm-Fix solution Pharmingen, CA, USA), the cells were pre-incubated with 10% rat serum, and incu-

bated with FITC-anti-human IL-6 rat mAb (Pharmingen) or FITC-conjugated isotype control (rat IgG), washed (Perm-Wash Pharmingen), and fixed in 1% paraformaldehyde. Flow analysis of monocyte-size cells gated by forward scatter/side scatter and confirmed by CD 14 staining was done on a FACSCalibur flow cytometer, using the CELLQUEST software (Becton Dickinson, CA, USA).

Statistical analysis

Frequency distribution of the eight size-alleles in SLE patients was compared to that of controls by a Chi-square test for homogeneity (2-tailed *P* value at 7 degrees of freedom), using PC SAS Software Release 6.01 (Statistical Applications Software, Cary, NC, USA). *P* values that were $\leqslant 0.05$ after the Bonferroni correction were considered significant. At each observation point ($n = 8$), allele frequency of SLE was compared to controls by a 2-tailed Fisher's Exact Test. The statistical analysis of IL-6 values was calculated using a 2-tailed, non-paired Student's *t*-test (Microsoft Excel).

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References

1 Hahn BH. An overview of the pathogenesis of SLE. In: Walla DJ, Hahn BH (eds). *Dubois' Lupus Erythematosus*. 4th edn. Lea & Febiger: Philadelphia, London, 1993, pp 67–70.
2 Fielder AHL, Walport MJ, Batchelor JR. Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility. *Br Med J* 1983; **286**: 425–428.
3 Green JR, Montasser M, Woodrow JC. The association of HLA-linked genes with systemic lupus erythematosus. *Am Hum Genet 1986;* **50**: 93–96.
4 Salmon J, Millard S, Schachter LA *et al*. Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans. *J Clin Invest* 1996; **97**: 1348–1354.
5 Dits AJ, Bootsma H, Derksen RH *et al. Arthritis Rheum* 1995; **38**: 1832–1836.
6 Davies EJ, Teh LS, Ordi-Ros J *et al.* A dysfunctional allele of the mannose binding protein gene associates with systemic lupus erythematosus in a Spanish population. *J Rheumatol* 1997; **24**: 485–488.
7 Tsao BP, Cantor RM, Kalunian KC *et al*. Evidence for linkage of a candidate chromosome 1 region to human systemic lupus erythematosus. *J Clin Invest* 1997; **99**: 725–731.
8 Tsokos GC, Kovacs B, Liossis SN. Lymphocytes, cytokines and immune trafficking. *Curr Opin Rheumatol* 1997; **9**: 380–386.
9 Honda M, Linker-Israeli M. Cytokine gene expression in human SLE. In: Tsokos G, Kamer G (eds). *Lupus: Molecular Pathways of Pathogenesis.* Humana Press: Totowa, NJ, USA, 1998.
10 Hirano T, Yasukawa K, Harada H *et al*. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 1986; **324**: 73–76.
11 Hirano T, Akira S, Taga T, Kishimoto T. Biological and clinical aspects of IL-6. *Immunol Today* 1990; **11**: 199–206.
12 Linker-Israeli M, Deans RJ, Wallace DJ *et al*. Elevated levels of endogenous IL-6 in SLE: a putative role in pathogenesis. *J Immunol* 1991; **147**: 117–123.
13 Linker-Israeli M. Cytokine abnormalities in human lupus. *Clin Immunol Immunopath* 1992; **63**: 10–12.
14 Kitani A, Hara M, Hirose T *et al*. Autostimulatory effects of IL-6 on excessive B cell differentiation in patients with SLE: analysis of IL-6 production and IL-6R expression. *Clin Exp Immunol* 1992; **88**: 75–83.
15 Teppo AM, Metsarime K, Fyrhrquist F. Radioimmunoassay of IL-6 in plasma. *Clin Chem* 1991; **37**: 1691–1695.
16 Pelton BK, Hylton W, Denman AM. Activation of IL-6 production by UV irradiation of blood mononuclear cells from patients with SLE. *Clin Exp Immunol* 1992; **89**: 251–254.
17 Finck BK, Chan B, Wofsy D. IL-6 promotes murine lupus in NZB/ NZW F_1 mice. *J Clin Invest* 1994; **94**: 585–591.
18 Olomolaiye O, Wood NA, Bidwell JL. A novel NlaIII polymorphism in the human IL-6 promoter. *Eur J Immunogenet* 1998; **25**: 267.
19 Fishman D, Faulds G, Jeffrey R *et al*. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma levels, and on association with systemic-onset juvenile chronic arthritis. *J Clin Invest* 1998; **102**: 1369–1376.
20 Bowcock AM, Ray A, Erlich H, Sehgal PB. Rapid detection and sequencing of alleles in the 3′ flanking region of the IL-6 gene. *Nucleic Acids Res* 1989; **17**: 6855–6864.
21 Linker-Israeli M, Wallace DJ, Prehn J *et al*. A greater variability in the 3′ flanking region of the IL-6 gene in patients with systemic lupus erythematosus (SLE). *Autoimmunity* 1996; **23**: 199–209.
22 Murray RF, McGuigan F, Grant S *et al*. Polymorphisms of the IL-6 gene are associated with bone mineral density. *Bone* 1997; **21**: 89–92.
23 Linker-Israeli M, Honda M, Nand R *et al*. Exogenous IL-10 and IL-4 down regulate IL-6 expression of SLE-derived monocytes. *Clin Immunol* 1999; **91**: 6–16.
24 Sturfelt G, Hellmer G, Trudesson L. TNF microsatellites in SLE—a high frequency of the TNF abc 2–3-1 haplotype in multicase SLE families. *Lupus* 1996; **5**: 618–622.
25 D'Alfonso S, Colombo G, Della Bella S *et al*. Association between polymorphisms in the TNF region and SLE in the Italian population. *Tissue Antigens* 1996; **47**: 551–555.
26 Fong KY, Howe HS, Tin SK *et al*. Polymorphism of the regulatory region of the gene in patients with SLE. *Ann Acad Med Singapore* 1996; **25**: 90–93.
27 Suzuki H, Matsui Y, Yashiwagi H. IL-1Ra poymorphisms in Japanese patients with SLE. *Arthritis Rheum* 1997; **40**: 389–390.
28 Eskdale J, Wordsworth P, Bowman S *et al*. Association between polymorphisms at the human IL-10 locus and SLE. *Tissue Antigens* 1997; **49**: 635–639.
29 Eskdale J, Gallagher G, Verveij CL, Keisjers V, Westendorp RG, Huizinga TW. Interleukin-10 secretion in relation to human IL-10 locus haplotypes. *Proc Natl Acad Sci USA* 1998; **95**: 9465–9470.
30 Merian R, Quismorio FP, Strassman G *et al*. Synergistic effect between IL-10 and Bcl-2 genotypes in determining susceptibility to SLE. *Arthritis Rheum* 1997; **40**: 9S 316.
31 Bailly S, diGiovinne FS, Blakemore AL, Duff GW. Genetic polymorphism of human IL-1 alpha. *Eur J Immunol* 1993; **23**: 1240–1245.
32 Danis VA, Millington M, Hyland VJ, Grennan D. Cytokine production by normal human monocytes: intersubject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. *Clin Exp Immunol* 1995; **99**: 303–310.
33 Boumpas DT, Eleftheriades EG, Barez S, Tsokos GC. Oncogene expression and regulation in normal lymphocytes and lymphocytes from patients with autoimmune diseases. *Anticancer Res* 1988; **8**: 977–984.
34 Koshy M, Berger D, Crow MK. Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes. *J Clin Invest* 1996; **98**: 826–837.
35 Vakalanka RK, Kirou KA, Raman C, Crow MK. Kinetics of CD40 ligand (CD40L) mRNA expression in systemic lupus erythematosus (SLE) patients and healthy controls. *Arthritis Rheum* 1998; **41**(S): S235.

36 Boulikas T. Nature of DNA sequences at the attachment regions of genes to the nuclear matrix. *J Cell Biochem* 1993; **52**: 14–22.
37 Forrester WC, van Genderen C, Jenuwein T, Grosschedl R. Dependance of enhancer mediated transcription of the immunoglobulin: gene on nuclear matrix attachment regions. *Science* 1994; **4**: 1221–1225.
38 Kennedy GC, German MS, Rutter WJ. The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription. *Nature Genet* 1995; **9**: 293–298.
39 Krontiris DG, Devlin D, Karp DD, Robert NJ, Rish N. An association between the risk for cancer and mutations in the HRAS1 minisatellite locus. *N Engl J Med* 1993; **329**: 517–523.
40 Kiaris H, Ergazaki M, Spandidos DA. Instability at the H-ras minisatellite is associated with the spontaneous abortion of the embryo. *Biochem Biophys Res Comm* 1995; **214**: 788–792.
41 Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–1276.
42 Liang MH, Socher SA, Larson MG, Schur PH. Reliability and validity of six systems for the clinical assessment of disease activity in SLE. *Arthritis Rheum* 1989; **32**: 1107–1110.