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# Novel Single Nucleotide Polymorphisms in the Distal IL-10 Promoter Affect IL-10 Production and Enhance the Risk of Systemic Lupus Erythematosus[1]

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# Novel Single Nucleotide Polymorphisms in the Distal IL-10 Promoter Affect IL-10 Production and Enhance the Risk of Systemic Lupus Erythematosus[1]

**Andrew W. Gibson,* Jeffrey C. Edberg,* Jianming Wu,* Rudi G. J. Westendorp,† Tom W. J. Huizinga,† and Robert P. Kimberly[2]***

**Family studies of first-degree relatives and analysis of twins indicate that as much as 75% of the differences in quantitative IL-10 production in man derive from heritable genetic factors. Studies of single nucleotide polymorphisms (SNP) in the proximal 1.0 kb of the IL-10 promoter have yielded inconsistent association with IL-10 production and variable results in promoter-reporter studies. However, in normal donors, an association of quantitative production with certain alleles of the IL-10.R short tandem repeat polymorphism at −4.0 kb suggested that SNPs in the more distal promoter might be informative. We have identified seven novel SNP sites in the genomic sequence of the first 4 kb of the IL-10 promoter region 5′ to the ATG start site from Caucasian individuals with either a high or a low IL-10 production phenotype. We have also identified eight SNP haplotypes in the distal promoter that segregate with significant differences in quantitative IL-10 production in normal donors. These SNPs are significantly associated with systemic lupus erythematosus in African-Americans and may define one component of the genetic susceptibility to systemic lupus erythematosus in this group.** ***The Journal of Immunology,*** **2001, 166: 3915–3922.**



Interleukin 10, which is produced primarily by monocytes and to a lesser extent by lymphocytes, has pleiotropic effects in immunoregulation and inflammation (1). IL-10 down-regulates the expression of Th1 cytokines, MHC class II Ags and costimulatory molecules on macrophages but stimulates Fc$\gamma$R expression on the same cells (1–3). IL-10 enhances B cell survival, proliferation, differentiation, and Ab production, and these effects appear to play a role in autoimmune diseases (1, 3–7).

Several studies have shown that unaffected family members of systemic lupus erythematosus (SLE)[3] patients produce high levels of IL-10 and that first-degree relatives of nonsurvivors of fatal meningococcal disease produce significantly lower levels of IL-10 than relatives of survivors (8–10). The implication of a heritable genetic basis for IL-10 production is supported by the concordance of IL-10 production in monozygotic twins, which suggests that genetics could account for up to 75% of IL-10 production (10). Indeed, the high IL-10 production associated with autoimmune diseases including rheumatoid arthritis (RA) and SLE may be a genetic risk factor for disease susceptibility (2, 8, 11–19).

The basis for heritable differences in IL-10 production is not known. The IL-10 gene promoter is polymorphic, and promoter-reporter studies have identified several positive and negative regulatory promoter sequences within the 1.3 kb region upstream of the transcription start site (20–23). Two CA-repeat microsatellites, IL-10.R and IL-10.G, −4 kb and −1.1 kb, respectively (24, 25), and three single nucleotide polymorphisms (SNP) at −1082 (G/A), −819 (C/T), −592 (C/A) upstream of the transcription start site have also been identified (26).

Variable associations between the proximal IL-10.G microsatellite alleles and IL-10 production and between SNPs or SNP haplotypes in the 1.4 kb IL-10 proximal promoter region and IL-10 production have been reported (27–33). Similarly, the associations between IL-10.G alleles and SLE and RA (28–31), between SNP haplotypes in the proximal IL-10 promoter and SLE, and between IL-10 SNP haplotypes and disease severity have been inconsistent (32–35). These results, coupled with the recent data indicating a relationship between alleles of the more distal IL-10.R microsatellite and IL-10 production suggested the possibility that other more distal promoter elements may be involved in IL-10 production. To identify additional SNPs that might affect IL-10 production, we have examined the −1.3 kb to −4 kb region of the IL-10 promoter from normal donors phenotyped for high or low levels of IL-10 production. Our results indicate that at least seven novel SNP sites exist in the distal region of the IL-10 promoter and that distal SNP haplotypes associate with quantitative IL-10 production. Our results also show that one of the novel SNP alleles associates with SLE in an African-American cohort and may be a risk factor for SLE in this group.

## Materials and Methods

### *Normal donor and patient populations*

Fifty-two normal Caucasian donors, characterized for quantitative IL-10 production (36), provided genomic DNA for genotyping. An additional 128 normal Caucasian donors from the Leiden University Medical Center (Leiden, The Netherlands) blood donor service provided genomic DNA to confirm genotype frequencies. Sixty-four African-American normal donors and 60 African-American SLE patients from the University of Alabama (Birmingham, AL) also provided DNA for analysis. All studies were approved by the respective Institutional Review Boards, and

*Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama, Birmingham, AL 35294, and †Department of Rheumatology and General Internal Medicine, University of Leiden Medical Center, Leiden, The Netherlands

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[2] Address correspondence and reprint requests to Dr. Robert P. Kimberly, Division of Clinical Immunology and Rheumatology, University of Alabama, 1530 3rd Avenue South, Birmingham, AL 35294-0006. E-mail address: rpk@uab.edu

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; RA, rheumatoid arthritis; UTR, untranslated region.



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Table I. *Primers used to PCR-amplify and sequence the IL-10 promoter*[a]

| IL-10 Promoter Primers | |
|---|---|
| UPP2 | TCCATAGGTCACACAGCAGGCATCCA |
| LWR2 | AGTCAGGAGGACCAGGCAACACAGC[a] |
| UPP4 | CTGTAGAATGCACCCTCCAAAATCT |
| S1F | GACTATAGAGTGGCAGGGCC |
| S2F | ATTTCAACTTCTTCCACCCCATCT |
| S3F | CAGCTGAAGAGGTCCAAACAT |
| S4F | ACAGCTGAGGGCCTCTGCTCA |
| UPP3 | CTGCTTTGGGCTTCTTGATGAGTGAG |
| S5F | CCTCTCTTCATGGGCTCTT |
| S6F | ACACATTTCAGAACAAATAAAGAA |
| S7F | AGATCCTAACCTCAACCCTATTTA |
| S8F | AACAAAGGAAGGAGGGGTTCACAGC |
| S4Ra | GCGCAGAGGCCCTCAGCTGT[a] |
| S3R | ATGTTTCCACCTCTTCAGCTG[a] |
| S4Rb | CTACTGTACACCATCTCCAGCACATA |
| S5R | CTACTGTACTCCATCTCCAGC[a] |
| S6R | GTAGAAGGTAAGGGGAGC[a] |
| S7R | CCTTGGCCCTGCCACTCTATAGTC[a] |
| S8R | TCTGTGTTAGCCTGCAGGTCTAACC[a] |
| S9R | TTTGAGACAGAGTCTCGCTCTG[a] |
| S10R | AGATACCTGTTCATCTGAAAAGTCA[a] |
| S11R | CCCAAGCCCATAGGTCATGTC |
| UP3R | CACTCATCAAGAAGCCCAAAGCAG |

[a] Reverse primers were labeled with the M13 sequence for dye-primer sequencing to confirm heterozygosity.

donors provided written informed consent. All SLE patient donors met the revised American College of Rheumatology criteria for systemic lupus (37).

### *IL-10 production*

Determination of IL-10 production in LPS-stimulated whole blood assays is described elsewhere (36). Briefly, whole blood samples were diluted 1:1 with RPMI 1640 (Life Technologies, Paisley, U.K.). LPS (*Escherichia coli* 0111; B4, Boivin method; Difco, Detroit, MI) was added to a final concentration of 1 $\mu$g/ml and cells were stimulated for 24 h at 37°C under 5% $CO_2$ atmosphere. Determination of IL-10 concentrations by ELISA (BioSource, Fleurus, Belgium) was performed according to the manufacturer's guidelines.

### *PCR amplification and sequencing*

DNA was extracted from whole blood using standard molecular techniques. Forward and reverse primers used to PCR-amplify and sequence the 4.1-kb region of the IL-10 promoter (GenBank accession number AF295024) are given in Table I. Primers were purchased from Life Technologies or from the Oligonucleotide Synthesis Core Facility at the University of Alabama. PCR amplifications were performed using the Expand Long Template PCR System and the manufacturer's suggestions (Roche Diagnostic Systems, Indianapolis, IN), or recombinant *Taq* polymerase and deoxynucleoside triphosphates from Life Technologies (Grand Island, NY). Primers IL-10UPP and IL-10LWR (Table I) were used to PCR amplify a 4.1-kb fragment of the IL-10 gene containing 4.057 kb of the 5′-untranslated region (UTR) and 43 bases of the open reading frame. Typically, 125 ng of genomic DNA template were used in a 50-$\mu$l PCR performed in a Perkin-Elmer (Norwalk, CT) 9600 Thermal Cycler.

For SNP discovery, two independent 4.1-kb PCR amplicons were amplified from each of the initial 12 donors and were sequenced in both the forward direction (direct dye terminator sequencing) and the reverse direction (M13-based dye primer sequencing) in a series of overlapping sequencing reactions. PCR-amplified products were electrophoresed in a 2% agarose gel and DNA fragments were purified from excised gel slices using the QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). DNA sequencing was performed using 10 ng of gel-purified DNA and the ABI PRISM BigDye Terminator and Dye Primer Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, CA) followed by electrophoresis on the ABI 377 automated sequencer. All heterozygotes identified in the forward sequence were confirmed in a second PCR amplicon sequenced in the opposite direction using a different (M13-based) sequencing chemistry.

For genotyping of populations at the more common SNP sites, primers IL-10UPP and IL-10S9R were used to amplify a 1.443-kb IL-10 promoter fragment containing the −3575, −2849, and −2763 SNP sites. Gel-purified DNA fragments were sequenced using S6F and S9R primers (Table I).

### *Cloning of IL-10 promoter segments*

For extended SNP haplotype determination the 4.1-kb IL-10 promoter fragments were cloned into pGEM-T vector (Promega, Madison, WI). Plasmids were propagated in *E. coli* DH5$\alpha$ and plasmid DNA purified from bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen).

All DNA sequence comparison alignments were performed using DNAstar SeqMan or MegAlign programs (DNAstar, Madison, WI). DNA sequence motif searches for putative transcription factor binding sites were performed using TESS, MatInspector V2.2 and TFSEARCH web-based search programs (www.cbil.upenn.edu/tess/).

### *IL-10 mRNA determination*

mRNA was isolated from pelleted whole blood cultures after stimulation with LPS for 18 h. mRNA (15–20 ng) was separated in 1% agarose, blotted onto Hybond N membrane and probed for 18 h at 42°C using a 0.7-kb *Bgl*II-*Hin*dIII, $^{32}$P-labeled DNA fragment of IL-10 (pH15C, no. 68192; American Type Culture Collection, Manassas, VA). Washed blots were exposed to Biomax film (Eastman Kodak, Rochester, NY) and band intensities quantified relative to GAPDH mRNA using a phosphor-imaging system (Molecular Dynamics, Sunnyvale, CA). For mRNA half-life studies, semiquantitative PCR was used. For this method, mRNA was isolated from cultures treated with actinomycin D and cDNA was synthesized. mRNA levels were quantified relative to an internal $\beta$-actin standard as described (38). PCR products were separated on agarose gel and quantified by ethidium bromide staining using an Eagle Eye II (Stratagene, La Jolla, CA).

### *Statistical analysis*

The $\chi^2$ test was used to compare differences in the distributions of phenotypes and allele frequencies. A $p$ value of 0.05 was used to reject the null hypothesis.

## Results

### *Phenotypic characterization of normal donors*

The production of IL-10 by peripheral blood leukocytes from disease-free, normal volunteers in response to an LPS stimulus was determined by the standardized assay, previously described (36).

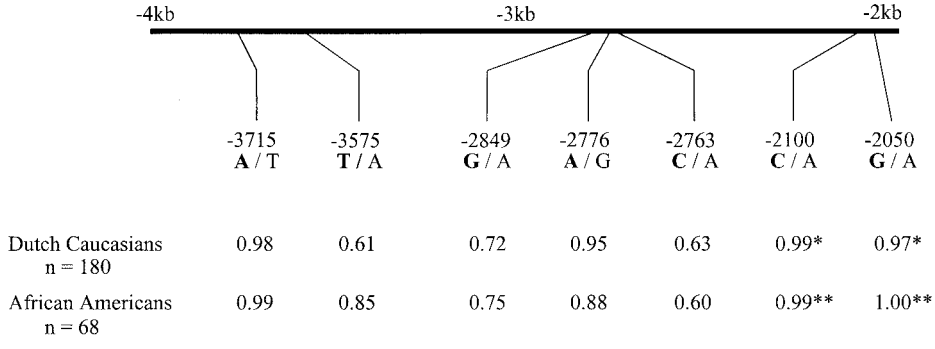


**FIGURE 1.** Identification of novel SNPs in the distal IL-10 promoter. A schematic representation of the IL-10 promoter is shown with seven novel SNPs found in Caucasian donors. *, $n = 62$; **, $n = 14$.

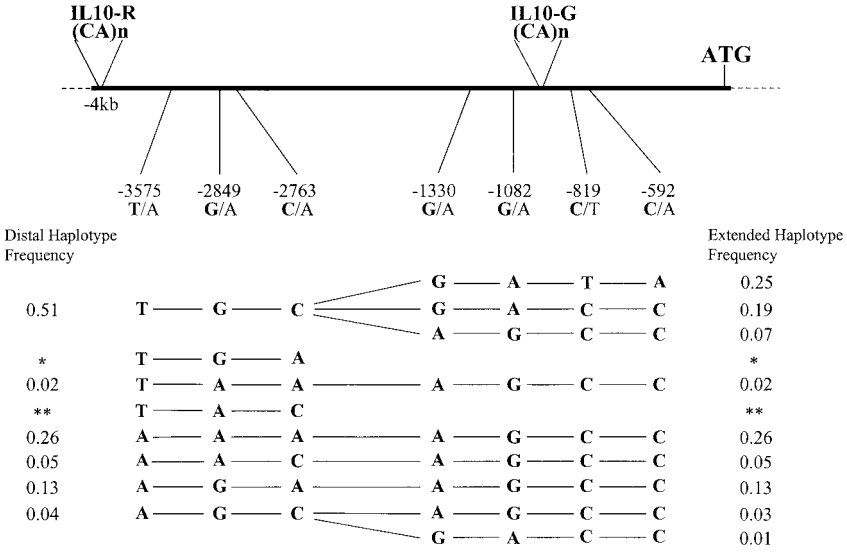


**FIGURE 2.** Identification of SNP haplotypes in the IL-10 promoter. Using the three distal common SNPs all eight potential haplotypes were either observed unambiguously or established by cloning and direct sequencing. The TGA haplotype (∗) was identified unambiguously in the independent populations of 128 normal Caucasian and 64 normal African-American donors with an approximate haplotype frequency of 0.02 and 0.08, respectively. The TAC haplotype (∗∗) was identified unambiguously in normal African-American donors with an approximate haplotype frequency of 0.02.

Twenty-six donors with low IL-10 production and twenty-six donors with high IL-10 production were chosen for further study from the upper and lower quartiles of a population of 163 phenotyped normal donors. Each was genotyped for SNPs in the proximal 1.3-kb promoter (−1330G/A, −1082G/A, −819C/T, −592C/A). To explore the possibility of additional novel SNPs within the proximal 1.3-kb promoter in these phenotyped donors, genomic DNA from 12 donors, 6 chosen randomly from each group, was sequenced in both directions. No additional SNPs were identified.

*Identification of novel IL-10 promoter polymorphisms*

To identify SNPs in the more distal promoter region that might correlate with IL-10 production, we PCR amplified a 4.1-kb DNA fragment from the IL-10 promoter, which included the 5′-UTR and 43 bases of the open reading frame. Eight low and six high IL-10-producing Caucasian donors were selected and the promoter region between −1.3 kb and −4 kb sequenced. DNA sequences obtained using the BigDye Terminator Cycle Sequencing system were confirmed by sequencing the reverse strand using the M13-based Dye Primer system. Seven novel genetic SNPs were identified within the −1.3 kb to −4 kb promoter region of IL-10 (Fig. 1) and the frequencies of these SNPs were characterized in two independent populations (Fig. 1, see below). At three of these sites (−3575T/A, −2849G/A, −2763C/A) both alleles are commonly represented in the populations studied. At each of the other four sites (−3715 **A**/T, −2776 **A**/G, −2100**C**/A, −2050**G**/A) one allele (in bold) predominates while the other rare allele has a frequency <5%.

Our sequencing data from the region between −1.3 kb and −4 kb (GenBank accession number AF295024) also showed several sequence differences compared with the published IL-10 promoter sequences (GenBank entries U16720 and X78437).[4] Except for differences at the SNP sites, the initial 14 Caucasian donor sequences were all otherwise identical, suggesting that the differences between the GenBank sequences and our data are unlikely to be the result of PCR-induced mutations. To address the possibility that differences in the ethnicity of the donors could explain sequence differences seen in the GenBank entries for the IL-10 promoters, we PCR amplified and sequenced the 4-kb IL-10 promoter region from 14 African-American normal donors. In our African-American cohort, we identified two novel SNP sites [−1466 (**C**/T) and −429 (**G**/T)] that were not polymorphic in any of the 52 Caucasian normal donors phenotyped for IL-10 production. No other sequence differences between our African-American and our Caucasian cohorts were identified.

*Determination of distal SNP haplotypes*

The proximal SNPs in the IL-10 promoter (−1082G/A, −819C/T, −591C/A) form three haplotypes in Caucasians (GCC, ACC, and ATA), and a fourth haplotype (GTA) has been reported in Southern Chinese (35). To determine the distal IL-10 promoter SNP haplotypes for the three sites with common polymorphisms, we genotyped the first cohort of 52 Caucasian donors. Four haplotypes were observed in donors homozygous at all three sites or heterozygous at only one site. To identify other possible haplotypes that may exist, we cloned the 4-kb promoter from 19 donors heterozygous at more than one of the common distal SNP sites. PCR-amplified promoter products were cloned into pGEM-T vector and 10 randomly chosen clones from each donor sequenced. Of the eight possible distal promoter SNP haplotypes, we identified six haplotypes in our first group of 52 Caucasian normal donors (Fig. 2). In this cohort, three haplotypes comprised of the common distal SNPs (TGC, AAA, and AGA), were present with the highest frequency (0.51, 0.26, and 0.13, respectively) while the other haplotypes, TAA, AAC, and AGC, were present with a low frequency (0.02, 0.05, and 0.04, respectively). In our second, independent Caucasian cohort and in our African-American populations (see below), we were able to unambiguously deduce the other two possible distal SNP haplotypes (TGA and TAC; Fig. 2). Therefore, all eight haplotypes for the three distal sites with common alleles (−3575T/A, −2849G/A, −2763C/A) exist in normal populations.

*IL-10 protein production is transcriptionally regulated in LPS stimulated leukocytes*

IL-10 mRNA and protein production in response to an LPS stimulus were determined in high and low IL-10-producing donors as described in *Materials and Methods*. Relative to GAPDH, the

[4] GenBank sequence X78437 lacks six nucleotides (GCTCAA) at position −1468 and two nucleotides (GA) at position −1684. These nucleotides were present in 28 donor sequences from two ethnic groups and in GenBank sequence U16720. Other non-SNP single nucleotide differences, deletions and insertions were present in the GenBank sequences relative to our consensus IL-10 sequence from both ethnic groups.

IL-10 mRNA level was consistently higher in high IL-10-producing individuals compared with low IL-10-producing individuals (Fig. 3, *a* and *c*). mRNA half-life determinations were comparable (Fig. 3, *b* and *d*), indicating that differences in IL-10 mRNA levels in this system are independent of mRNA stability.

### *5′ SNP haplotypes associate with IL-10 production*

We examined the genotype distribution in our normal phenotyped Caucasian donors to determine whether any haplotypes were associated with IL-10 production. Table II shows that among the three most common haplotypes, both AAA and AGA were each more frequently found in the low compared with the high IL-10 producers (AAA in 35% of low vs 17% of high producers; AGA: 19% of low vs 6% of high producers. Thus, the A-[G/A]-A haplotype was much more likely in IL-10 low producers ($p < 0.003$). More importantly, donors with two A-[G/A]-A haplotypes were 10-fold more frequent among the 26 low compared with the 26 high producers (42% vs 4%; $p < 0.007$; Fig. 4). Therefore, we extended this analysis by further dividing our low-producing donors into two nonoverlapping groups based on quantitative IL-10. Among donors in the lower half, which had a median IL-10 production of 543 pg/ml, an A-[G/A]-A haplotype was more frequent than among those in the upper half, which had a median production of 903 pg/ml (73% vs 35%, $p = 0.012$). The occurrence of two A-[G/A]-A haplotypes was also more common in the lower than in the upper half; despite the small sample size of 13 in each group, this distribution was statistically significant ($p = 0.044$). Not surprisingly, the TGC haplotype was significantly associated with high IL-10 production ($p < 0.05$).

### *Determination of extended SNP haplotypes*

Previous work has suggested that there may be association of proximal SNPs and SNP haplotypes with IL-10 production (26, 31, 33), and we considered the possibility that an extended SNP haplotype might show a stronger relationship to IL-10 production than the distal SNP haplotype alone. Therefore, we constructed extended haplotypes based on analysis of genotypes in homozygous donors or donors with a single heterozygous site and on analysis of direct sequence information of cloned promoter products. We identified nine extended haplotypes that include both the proximal and the distal IL-10 promoter SNPs (Fig. 2). Within these extended haplotypes, the TGC distal haplotype is linked to all three proximal haplotypes. However, among the 104 DNA strands analyzed, both the AAA and AGA distal haplotypes were found only with the AGCC proximal haplotype. Conversely, the GATA proximal haplotype was found exclusively with the TGC distal haplotype.

Table II. *Distribution of distal SNP haplotypes in high and low IL-10 producers*

| Haplotypes | | | Phenotype | |
|---|---|---|---|---|
| −3575 | −2849 | −2763 | Low | High |
| T | G/A | C | 21 | 32 |
| T | G/A | A | 0 | 2 |
| A | G/A | C | 3 | 6 |
| A | G/A | A[a] | 28 | 12 |

[a] A-[G/A]-A vs others; $p < 0.003$, $2 \times 2$ $\chi^2$ analysis.

As predicted from the distributions of the AAA and the AGA haplotypes and the absolute linkage of AGCC with these haplotypes in our population, the AAA-AGCC and AGA-AGCC haplotypes predominated in the low producer group (54% vs 23% in high producers, $p < 0.003$). A distribution analysis of the AGCC proximal haplotype alone also showed a higher frequency in the low producer group (low, 65%; high, 44%) but this distribution did not show as marked a difference between low and high IL-10 producers because of the TGC-AGCC, TAA-AGCC, and AAC-AGCC haplotypes, most of which were found in the high producer group. Indeed, if the proximal AGCC were predominantly responsible for the IL-10 production phenotype, then the distribution of distal haplotypes associated with AGCC should be the same in both high and low producers. Table III shows that, with AGCC held constant, the distal haplotypes do not occur with equal frequency in both high and low producers. The A-[G/A]-A distal haplotypes are much more frequently found in the low producers (82% vs 52%; $p = 0.032$).

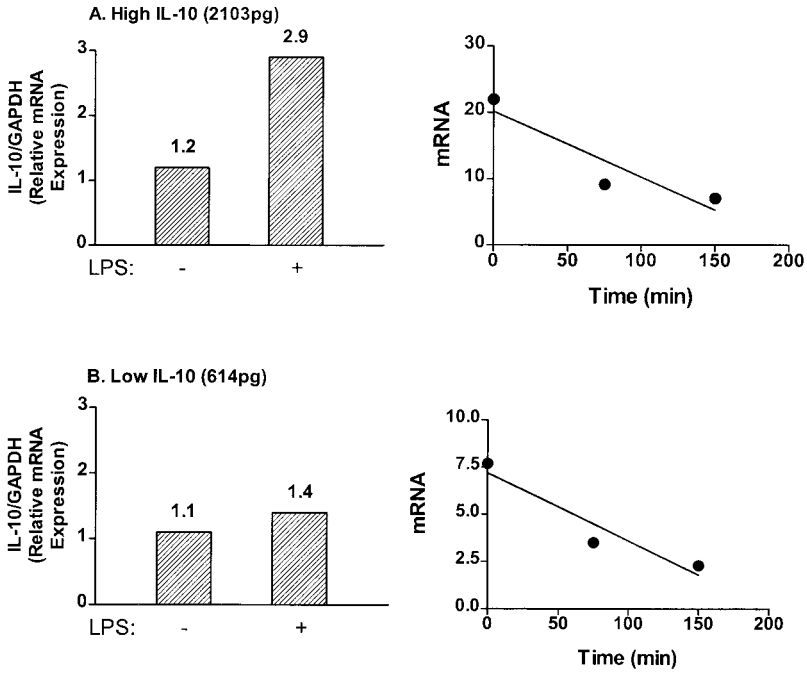


**FIGURE 3.** IL-10 mRNA levels and message stability. mRNA levels were determined by Northern blot analysis or by semiquantitative PCR in a high and low IL-10 producer, and mRNA half-life was determined after actinomycin D. mRNA level was independent of mRNA half-life as confirmed in a larger cohort (55).

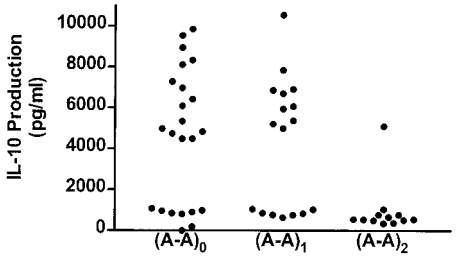


**FIGURE 4.** Production of IL-10 by donors, segregated by distal SNP haplotypes. Distal SNP haplotypes were established by cloning and direct sequencing and then correlated with LPS-induced IL-10 production. Homozygosity for haplotypes with an A allele at both −3575 and −2763 was strongly associated with low IL-10 production ($p = 0.007$).

Conversely, with the distal TGC held constant, there was no difference in the distribution of the TGC-GACC, TGC-GATA, and TGC-AGCC haplotypes between high and low producers. While not excluding a contribution of proximal promoter SNP haplotypes to IL-10 production, these results suggest that the predominant phenotype is determined by the distal SNP haplotype.

*SNP allele distributions in SLE patients*

To determine whether genetically determined capacity for IL-10 production might constitute a susceptibility factor for SLE, we examined the distal SNP haplotypes of 24 SLE patients and compared the distribution of these haplotypes to our high and low IL-10-producing normal donors. The distribution of SLE patient haplotypes was significantly different from low production normal donors ($p = 0.001$) and essentially the same as the high producers (Table IV).

To extend these observations, we determined whether analysis of SNP alleles at −3575, −2849, and −2763 could reveal significant associations with IL-10 production. We noticed that while both the G and A alleles at −2849 were equally represented in individuals producing high and low amounts of IL-10, there was a significant over-representation in the frequency of the A allele at both −3575 ($p = 0.02$) and at −2763 ($p = 0.009$) when analyzed individually in low producers compared with high producers (Table V).

Therefore, we examined the distribution of genotypes at −3575, −2849, and −2763 in 60 African-American SLE patients and 64 healthy ethnically matched controls. Table VI shows that while there was no significant difference in the genotype distribution at the first two sites, there was a significant difference between normal donors and SLE patients at −2763 ($p < 0.05$). As with our normal donors producing high levels of IL-10, the −2763A allele frequency was lower and there were significantly fewer −2763A homozygotes in SLE patients vs ethnically matched controls (5 in SLE, 16 in normal donors, $p = 0.026$).

Table III. *Distribution of AGCC-linked distal haplotypes in high and low IL-10 producers*

| Extended Haplotype | | Phenotype | |
|---|---|---|---|
| Distal | Proximal | Low | High |
| TGC | AGCC | 3 | 4 |
| TAA | AGCC | 0 | 2 |
| AAA | AGCC[a] | 18 | 9 |
| AGA | AGCC[a] | 10 | 3 |
| AAC | AGCC | 1 | 4 |
| AGC | AGCC | 2 | 1 |

[a] A-[G/A]-A—A-G-C-C vs others; $p = 0.032$, $2 \times 2$ $\chi^2$ analysis.

Table IV. *Distribution of IL-10 haplotypes in SLE patients*

| Haplotypes | | | | | | |
|---|---|---|---|---|---|---|
| −3575 | −2849 | −2763 | SLE | Low IL-10[a] | SLE | High IL-10 |
| T | G/A | C | 28 | 19 | 28 | 33 |
| T | G/A | A | 6 | 0 | 6 | 1 |
| A | G/A | C | 5 | 5 | 5 | 4 |
| A | G/A | A | 9 | 28 | 9 | 14 |

[a] A value of $p = 0.001$; $4 \times 2$ $\chi^2$ analysis.

*Allele distribution differs between Caucasians and African-Americans*

The lack of an effect of SNP −3575 in our SLE patients was initially puzzling. However, inspection of the normal African-American population at this site revealed a very high frequency of TT homozygosity and low frequency of AA homozygosity compared with Caucasian normal donors. Indeed, the −3575T allele frequency in our 64 African-American normal donors was significantly different not only from our Caucasian IL-10-low normal donors (0.84 vs 0.40) but also from our Caucasian IL-10-high producers (0.84 vs 0.65; $p < 0.00001$ and $p < 0.013$, respectively). To determine whether this might be related to the selection of these donors on the basis of IL-10 phenotype, we genotyped a second population of 128 randomly selected Caucasians. The genotype distributions at positions −3575 and −2763 between African-Americans and Caucasians were significantly different ($p < 0.00002$ and $p < 0.03$, respectively; Table VII). In particular, the −3575T allele frequency was significantly higher in the African-American normal donors (0.84 vs 0.69; $p < 0.0001$). This enrichment for −3575T and under-representation of −3575A, a genotype associated with high IL-10 production, in African-American normal donors compared with Caucasians suggests that heritable differences in IL-10 production capacity may represent one of the risk factors for the increased prevalence of lupus in African-Americans.

## Discussion

We have identified seven novel SNPs in the distal region of the IL-10 promoter between −1.3 kb and −4.0 kb 5′ to the transcription start site. Of these, alleles at three sites (−3575 T/A, −2849 G/A, −2763 C/A) are commonly found in our populations, and we have found all eight possible combinatorial distal SNP haplotypes. Our data indicate that, taken together with the proximal SNP haplotypes, the distal SNP haplotypes form at least 11 extended haplotypes. Alleles at two of these polymorphic sites, −3575 and −2763, associate with IL-10 production in phenotyped normal donors and consideration of these alleles and extended SNP haplotypes resolves much of the controversy in the genetic associations of IL-10 production. Furthermore, alleles associated with high

Table V. *Association of −3575 and −2763 SNP alleles with IL-10 phenotypes*

| | −3575[a] | | −2849 | | −2763[b] | |
|---|---|---|---|---|---|---|
| | T | A | G | A | C | A |
| High IL-10 | 34 | 18 | 37 | 15 | 38 | 14 |
| Low IL-10 | 21 | 31 | 32 | 20 | 24 | 28 |

[a] A value of $p = 0.02$; $2 \times 2$ $\chi^2$ analysis.
[b] A value of $p = 0.009$; $2 \times 2$ $\chi^2$ analysis.

Table VI. *Distribution of IL-10 distal promoter SNP genotypes in African-American SLE patients*

| | −3575 | | | −2849 | | | −2763[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | TT | TA | AA | GG | GA | AA | CC | CA | AA |
| African-American SLE | 40 | 19 | 1 | 38 | 19 | 3 | 28 | 27 | 5 |
| African-American normal donors | 48 | 11 | 5 | 36 | 22 | 6 | 25 | 23 | 16 |

[a] A value of $p < 0.05$; $2 \times 3$ $\chi^2$ analysis.

IL-10 production are related to the SLE phenotype in African-Americans. This finding supports the identification of a region on human chromosome 1q just telomeric to 1q23 with linkage to the SLE phenotype in this ethnic group (39).

Several studies have found associations between the IL-10 promoter microsatellites and IL-10 production and between the proximal promoter SNPs and IL-10 production (27, 31, 33, 40). Given the variability found in these studies, differences in cell type and experimental conditions could explain these differences. Our data strongly suggest that an additional explanation is more likely, that is, that other promoter SNPs may influence promoter activity. We have found that several novel alleles and haplotypes, defined by −3575 and −2763 SNPs, associate strongly with IL-10 production. Specifically, the T-G-C distal haplotype is associated with high IL-10 production and the A-G/A-A distal haplotypes associate with low IL-10 production. The physical linkage of the T-G-C distal haplotype with all three proximal haplotypes in Caucasians might explain the variability between proximal IL-10 haplotypes and IL-10 production seen in other studies. However, because the association between the distal haplotypes and IL-10 production is not absolute (Fig. 4), additional factors or additional SNP sites, perhaps as part of even longer extended haplotypes, may also underlie some of the variation in IL-10 production levels.

Nonetheless, our studies suggest that the distal SNPs have important biological significance. Not only do these SNPs segregate significantly with IL-10 production in normal donors, but they also segregate with autoimmune disease. High IL-10 production is associated with the pathogenesis of autoimmune diseases in humans and in mouse models (1). IL-10 production is increased in RA and SLE patients in vivo and in monocytes and B-cells isolated from SLE patients and studied ex vivo (5, 8, 11, 12, 14–19). IL-10 can prevent spontaneous cell death of germinal center B-cells by inducing Bcl2 expression and results in increased production of autoantibodies by B-cells from SLE patients (4, 5). Our data indicates that there is a significant difference in the genotype distribution at −3575 and −2763 between normal donors and African-Americans with SLE, which implicates high IL-10 production in disease susceptibility.

Of course, one explanation for positive disease associations is population admixture and genetic heterogeneity, which reflects different prevalent genotypes in different ethnic groups. Indeed, our data show that Dutch Caucasian and African-American normal donors differ significantly in the SNP allele distributions. African-Americans show a much higher allele frequency of −3575T, which is associated with high IL-10 production, and it is interesting to speculate whether this difference may underlie, in part, the higher susceptibility of African-Americans to SLE. In such a case, it is also interesting to consider what control group is most appropriate when analyzing IL-10 SNPs in African-American SLE patients. Use of African-American controls would miss the potential importance of −3575T in IL-10 biology since the allele frequency is the same in normal donors (0.83) and in SLE (0.83). Use of the Dutch Caucasian controls would make the role of −3575T clear. In either case, compared with either control group, the significant under-representation of the −2763 AA homozygous genotype in African-American SLE patients indicates that genetically determined differences in IL-10 production contribute to SLE susceptibility as a prototypic autoimmune disease.

The perspective that different ethnic groups may differ in IL-10 genetics is supported by several recent reports. Although not accompanied by functional analysis, the SNP at −3575 was recently reported in normal Italian donors (41). The −3575T allele frequency was higher in the Italian population (0.75) than in our Dutch cohort (0.61) although not as high as in our African-American population (0.83). The rare SNPs at −2050 and −2776 were also noted, but interestingly other positions at nt −2769, −1349, −1255, −851, and −657 were polymorphic in Italian normal donors but not in either our Caucasian or African-American groups. These data indicate the presence of genetic heterogeneity within and between ethnic groups in the highly polymorphic IL-10 promoter and raise the possibility that, in different populations, different SNPs or SNP haplotypes may be important in regulating the expression of IL-10.

Promoter SNPs alter cytokine gene transcription in other systems. The −238 G/A and −308 G/A transitions in the TNF-$\alpha$ promoter affect transcription factor binding and expression of reporter genes, and associate with cytokine production and disease outcome in psoriasis (−238) and cerebral malaria (−308) (42–45). Similarly, the −81A/G and −590 C/T substitutions in the IL-4 promoter affect binding of nuclear transcription factors and result in higher levels of reporter gene expression (46, 47). The −590 SNP also associates with higher serum IgE levels in asthma (46).

Table VII. *Distribution of IL-10 distal promoter SNP alleles in African-Americans and Dutch-Caucasians*

| | −3575[a] | | | −2849 | | | −2763[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | TT | TA | AA | GG | GA | AA | CC | CA | AA |
| Dutch normal donors ($n = 128$) | 51 | 64 | 13 | 71 | 49 | 8 | 51 | 63 | 14 |
| African-American normal donors ($n = 64$) | 48 | 11 | 5 | 36 | 22 | 6 | 25 | 23 | 16 |

[a] A value of $p < 0.00002$; $2 \times 2$ $\chi^2$ analysis.
[b] A value of $p < 0.03$; $2 \times 2$ $\chi^2$ analysis.

Although posttranscriptional regulation by 3′-UTR elements has been shown in some systems (48), IL-10 message levels from high and low IL-10 producers show comparable half-lives but marked differences in quantity in our system. These observations support transcriptional regulation of IL-10 expression.

Many putative transcription factor binding sites exist throughout the 4-kb promoter region (21, 23, 49–52). For example, within the proximal promoter region, the −1082 SNP appears within a putative *ETS*-factor binding site, while the −592 SNP may be a STAT3 binding site (20, 33). In the distal promoter region the −3575 SNP occurs within a putative *Pit*-1 binding site. Given the association between female sex hormones and SLE, and the high female to male ratio (9:1) in the disease, it is noteworthy that expression of isoforms of *Pit*-1 is regulated in part by estrogen, and that estrogen regulates IL-10 expression in PBMC from SLE patients (53). The −2763 SNP lies within putative lymphocyte-specific factor and myeloid zinc finger binding sites.

Nonetheless both genetic and nongenetic factors must contribute to IL-10 production. Using studies of IL-10 production by monozygotic twins and first-degree relatives of meningococcal patients, Westendorp et al. (10) showed that genetic factors account for as much as 75% of interindividual differences in IL-10 production. This suggests that up to 25% of IL-10 production can be accounted for by environmental factors, and data showing that Icelandic SLE patients and their spouses had significantly higher numbers of IL-10-producing cells than unrelated controls support this conclusion (54).

In summary, we have identified seven novel SNPs in the distal region of the IL-10 promoter in individuals phenotyped for IL-10 protein production and have determined the existence of eight distal promoter SNP haplotypes. We have shown that extended promoter SNP haplotypes, which include distal SNPs as well as proximal promoter SNPs, segregate significantly with quantitative IL-10 production and that distal SNPs that associate with IL-10 production significantly associate with SLE in African-Americans. This study provides the basis for a replication study of distal IL-10 promoter genetic variants in larger African-American cohorts and other ethnic groups.

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