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Research Article

The interleukin-10 – 1082 G/A polymorphism: allele frequency in different populations and functional significance

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Abstract. Genotypic variation in the human interleukin-10 (IL-10) promoter may account for marked inter-individual variation in IL-10 production and may influence susceptibility to autoimmune diseases. The G/A polymorphism at position –1082 has been linked to high/low IL-10 producer status. We directly tested the functional significance of this polymorphism using DNA-binding assays and reporter gene assays, examined allele frequencies in two geographically distinct populations and assessed intra- and inter-individual variation in IL-10 production in vitro according to genotype. Functional analyses showed that the –1082 region contains a putative ETS-like transcription factor-binding site, and nuclear factors from a monocyte cell line bind to this region. Transient transfection studies in an Epstein-Barr virus-transformed B cell line indicated that the –1082 A allele confers a two fold increase in transcriptional activity of the IL-10 promoter compared to the G allele. There was marked inter-individual variation in IL-10 production by peripheral blood mononuclear cells in vitro, with no consistent effect of genotype.

Key words. Interleukin-10; cytokine; polymorphism; transcription factor; allele frequency; transcription.

Interleukin 10 (IL-10) is a regulatory cytokine produced by T cells, B cells, monocytes, macrophages, keratinocytes, eosinophils, mast cells and placental trophoblasts [1–5]. It plays a key role in steering the immune response away from an inflammatory, Th1-type response, via the suppression of macrophage activation. IL-10 potently down-regulates the production of macrophage pro-inflammatory cytokines such as IL–1, IL–6, IL–8, granulocyte-macrophage-colony-stimulating-factor and notably tumour necrosis-factor-α, via a feedback inhibition loop [6]. However, in addition to these anti-inflammatory effects, IL-10 promotes B cell activation, regulates immunoglobulin class switching and maintains B cell viability by inhibiting apoptosis [1].

IL-10 therefore has roles in the regulation of both cellular and humoral immune responses and is a candidate mediator in autoimmune disease. Elevated levels of IL-10 have been reported in association with several autoimmune diseases, including systemic lupus erythematosus (SLE) [7–11]. IL–10 is encoded in humans by a gene located on chromosome 1 [12]. The promoter spans a region of 5 kb upstream of the transcription start point, and is known to contain several polymorphisms [13–16]. At approximately 1.1 and 4 kb upstream, there are two microsatellite $(AC)^n$ repeats [13, 14] termed IL-10G and IL-10R, respectively. Each exists in multiple allelic forms

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according to the number of repeat units in normal controls, and there may be population differences in allele distribution [17]. In addition, the IL-10 proximal promoter contains three single-nucleotide polymorphisms (SNPs) of interest, at positions –819 (C/T, which may affect an oestrogen receptor element) [18], –592 (C/A, lying within a region exerting negative regulatory function) and –1082 (G/A, lying within a putative ETS-like transcription factor-binding site) [16]. Since this work was carried out, other SNPs have been identified in various populations [19–22].

There is marked inter-individual variability in IL-10 production in normal individuals, one plausible explanation for which would be an association between genotype and transcriptional activity. Indeed, studies of IL-10 production in mono- and dizygotic twins and unrelated individuals have indicated that variability in IL-10 production has a hereditary component of around 74% [23]. Correlations between genotype and IL-10 production *in vitro* have been reported; in particular, the G allele of the G/A polymorphism at position –1082 has been associated with a 'high IL-10 producer' phenotype compared to the A allele [24]. The other SNPs at –819 (C/T) and –592 (C/A) occur in strong linkage disequilibrium with the polymorphism at –1082. Eskdale and co-workers [17, 18] report that both the IL-10G 1.1 kb $(AC)_{16}$ microsatellite and the upstream IL-10R 4.4 kb $(AC)_5$ microsatellite repeat, in conjunction with the three SNPs are present in transmissible allelic combinations. Some individuals did not fit into the putative categories: one displayed the G-T-A SNP haplotype only previously seen in the Chinese population [25]. Direct sequencing of the promoter regions divulged three novel polymorphisms. Polymorphisms at –3538 and –1354 appear to be in strong linkage disequilibrium with the –1082 G/A polymorphism, so that –3538T occurs with –1354G and –1082A. Another SNP C/T at –1095 (–1090) has also been observed, but so far only in the Korean population [26]. Recent work by Gibson et al. [21] has identified seven novel SNPs at –3715 (A/T), –3575 (T/A), –2849 (G/A) –2776 (A/G), –2763 (C/A), –2100 (C/A) and –2050 (G/A) of the distal IL-10 promoter, which may constitute extended IL-10 haplotypes together with the proximal SNPs, and may correlate with IL-10 production and occurrence of SLE in African Americans. The influence of these allelic variations on transcriptional activity remains to be determined.

The DNA sequence around position –1082 of the IL-10 promoter contains a GGAA/G motif, which corresponds to a consensus sequence common to the ETS transcription factor family [16]. As previous data suggested that the IL-10 –1082 polymorphism was independently associated with the observable differences in IL-10 production *in vitro* [23], we chose to study the functional significance of this polymorphism using electrophoretic mobility shift assays and transient transfection assays. Additionally, we have analysed the allele frequencies of this point mutation in two disparate populations (a UK Caucasoid control population and a Hong Kong Chinese population of SLE patients and controls) and assessed its influence on IL-10 protein production.

Materials and methods

Patients and control populations

Group 1: DNA samples were drawn from the predominantly Caucasoid population of the south-western region of the UK, comprising 98 healthy Caucasoid individuals (73 female, 25 male, age range 16–72 years). Group 2: DNA samples were obtained from the southern-Chinese population; (i) 116 Hong Kong Chinese SLE patients (108 female, 8 male, age range 18–68 years) and (ii) 97 Hong Kong Chinese controls (94 female, 3 male, age range 19–71 years).

Diagnoses of SLE were made according to established criteria [27]. Controls in group 2 were age- and sex-matched healthy volunteers with no obvious autoimmune disorders. Genomic DNA was isolated from peripheral blood of all subjects using standard methods: either phenol/chloroform extraction of DNA [28] from peripheral blood mononuclear cells (PBMCs) prepared by density centrifugation, or DNA extraction from whole blood using a non-enzymatic method previously described [29] and in some cases using a kit according to the manufacturer's instructions (Boehringer Mannheim, Germany). DNA was resuspended in TE buffer (pH 8.0) and its concentration determined using GeneQuant (Amersham Pharmacia).

Polymerase chain reaction heteroduplex analysis

One hundred nanograms DNA was then used in a 50 µl polymerase chain reaction (PCR) and subjected to heteroduplex analysis as previously described [30]. To confirm evidence of polymorphism, DNA from normal control individuals was amplified as described and PCR products sequenced directly on an ABI sequencer.

PBMC culture

PBMCs from 23 normal control donors of AA, GG, AG IL-10 –1082 genotype were obtained by density centrifugation, and used in stimulation assays to measure inter and intra-individual variation in IL-10 production. PBMCs were seeded at 10^7 cells/well and cultured for 48 h in the presence or absence of 5 µg/ml concanavalin A (con A) (Sigma) in 5 ml RPMI 1640 + 10% foetal calf serum (FCS) (Life Technologies, Paisley, UK). After culture, lymphocytes were pelleted by centrifugation and supernatants collected for quantification of IL-10 by ELISA. This procedure was repeated a total of four times for each individual, at consecutive 7-day intervals.

ELISA for IL-10

The antibody pair (Capture-JES3-9D7 and Biotinylated detection -JES3-1298) was obtained from Pharmingen (San Diego, Calif.). IL-10 ELISAs were carried out according to the Pharmingen standard protocol using samples diluted in blocking buffer/Tween at various dilutions and with IL-10 reference standards at fixed concentrations of 100 and 500 pg/ml on each plate as an inter-assay control. The limit of detection was 16 pg/ml.

Characterisation of transcription factor binding

The human histiocytic lymphoma cell line U937 (85011440; ECACC, Salisbury, UK) was cultured in RPMI 1640 medium containing L-glutamine (Sigma-Aldrich, Poole, UK) supplemented with 10% FCS and 100 units/ml penicillin-streptomycin solution (Sigma). Cultures were split every 3–4 days and maintained at a density of 10^6 cells/ml in polystyrene cell culture flasks (Corning, Acton, Mass., USA). Cells were stimulated with the mitogen phorbol myristate acetate (PMA, P8139; Sigma) at a concentration of 100 nM/10^6 cells in 1 ml (61.6 µg/µl) for 24 h prior to preparation of nuclear extracts.

Nuclear extracts were isolated from 10^8 cells, stimulated for 24 h with PMA. Cells were pelleted and resuspended in 400 µl ice-cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT + 100 mM PMSF) prior to use and incubated on ice for 10 min. Cell membranes were destroyed by vortexing with 15 µl Nonidet-P40. Nuclei were pelleted and lysed in 20–100 µl cold buffer B (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 400 mM NaCl, 0.2 mM EDTA, 25% glycerol and 0.5 mM DTT + 100 mM PMSF) prior to use for 20 min. Cellular debris was removed by centrifugation at 4 °C. Nuclear extracts were frozen at –80 °C.

Construction of radioactive probes

Lyophilised oligonucleotides (Bioline) 20 base pairs in length, corresponding to the upper and lower complementary strands of the DNA sequence of interest, were made up to a concentration of 2 µg/µl with nuclease-free water (Promega, Madison, Wis.). Bottom-strand oligonucleotides were then diluted 1:20 to a working concentration of 100 ng/µl, top strands in a 1:10 dilution to 200 ng/µl. Probes were labelled with γ^{32}P (ICN Biomedicals, Costa Mesa, Calif.) if necessary and then purified using a Stratagene radio-oligonucleotide purification column. The radioactivity of 1 µl purified radioactive eluate was measured in a scintillation counter and the amount of probe required to give 40,000 counts per sample was calculated.

Electrophoretic mobility shift assays

A 5-µl probe mixture containing 2 µl 50% glycerol (Sigma), 2 µl 10 × binding buffer (200 mM HEPES-KOH, 250 mM KCl, 10 mM DTT, 1% Nonidet P-40) and the required amount of radioactive probe (IL-10KG or IL-10KA) and water was made for each test sample. This was mixed with varying amounts of nuclear extract, polydIdC (non-specific competitor to reduce background; New England Biolabs) and water to a final volume of 20 µl.

Samples were electrophoresed at a constant 30 mA in 1 × TBE. Gels were then blotted onto paper, dried on a gel dryer at 80 °C and left to expose 24–48 h onto X-ray film. Film was processed in a developer and transcription factor/DNA probe complexes viewed on a light box.

Luciferase reporter gene assays

The construct PGL2-IL10 was a kind gift from Rene de Waal Malefyt at DNAX. This contained 5777 bp of the IL-10 promoter ligated into the PGL2 luciferase reporter vector (Promega), which could be digested at either end of the IL-10 promoter insertion with *Sma*1 and *Xho*1 (New England Biolabs) to yield linear fragments. These were then sequenced on an ABI 370 sequencer using oligonucleotides 300 bp apart on both DNA complementary strands along the length of the promoter (10 µM; Bioline). The genotype at position –1082 was confirmed as GG. PGL2-IL10 was digested with *Xho*1 and *Spe*1 to yield an IL-10 fragment of 1.86 kb (upstream from the start site of IL-10 transcription), containing the –1082 site of interest. This 1.86 kb fragment was then subcloned into PGL3-basic as follows. PGL3-basic (Promega) was digested with *Xho*1 and *Nhe*1 (New England Biolabs) and the truncated IL-10 fragment religated into it (PGL3-IL101.86G) using T4 DNA ligase. A ligation mixture (2 µl) was used to transform Top 10F competent *Escherichia coli* (Invitrogen) and screening for plasmids containing the correct IL-10 fragment was carried out using primers that flank the multiple cloning site of PGL3-basic (RV3 5′ ctagcaaaataggctgtccc and GL2 5′ ctttatgtttttg-gcgtcttcca) and also with primers that flank the IL-10 –1082 point mutation (1082F 5′ ccaagacaacactactaaggc, 1082R 5′ tgtaagcttctgtggctgga). Conditions for both PCRs were 94 °C for 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; followed by one cycle of 72 °C for 5 min.

The construct PGL3-IL101.86G was subjected to site-directed mutagenesis using a Stratagene Quickchange Site-Directed mutagenesis kit (Stratagene, La Jolla, Calif.) to mutate the base pair from G to A at position –1082 of the IL-10 promoter. Successful mutagenesis was confirmed by DNA sequencing. Both constructs were used to test the transcription potential of –1082G and –1082A alleles. All plasmids were prepared using Qiagen Maxi Prep kits.

Transfection

The Epstein-Barr virus (EBV) transformed human B cell line MAS B was a kind gift from Dr Richard Kay,

University of Dundee. Cultures were maintained in RPMI 1640 supplemented with L-glutamine, 10% FCS, 100 U/ml penicillin and streptomycin with 10 mM Hepes, 1 × MEM, 10 mM sodium pyruvate (Life Technologies) and split twice weekly.

MAS B lymphocytes were split at 4×10^5/ml 24 h prior to transfection in 175-cm^2 tissue culture flasks. Cells in log phase were washed and resuspended at $5-15 \times 10^6$ in a 0.4 cm electroporation cuvette (Life Technologies) in a final volume of 800 µl. Cells were transfected with 2 µg sea pansy renilla luciferase (pRL-tk) and 20 µg construct DNA in a Gibco Cell Porator (Life Technologies) at 300 V, 1600 µF (high resistance), then incubated in a six-well plate for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere. Transfections were performed using a negative (promoterless PGL3-basic vector) and SV40 positive control (PGLcontrol vector) to establish the baseline. Cell lysates were obtained and subjected to analysis in a Bio-Orbit luminometer (Turku, Finland) using the Promega Dual Luciferase system.

Statistical analysis

Allele frequencies were compared using χ^2 analysis. Statistical differences in IL-10 production according to –1082 genotype were assessed by Mann-Whitney analysis. Promoter activities were assessed using the Kruskal-Wallis test. p values <0.05 were considered significant.

Results

Identification of nuclear factors binding to the –1082 site

The G/A point mutation at –1082 lies within a putative transcription factor-binding site.

To understand the nature of the factors binding at this site, nuclear extracts from PMA-simulated cells were prepared. The human histiocytic lymphoma cell line U937 can be induced by overnight PMA stimulation to produce IL-10 (fig. 1). Electrophoretic mobility shift assays (EMSAs) were carried out using synthetic 20 bp oligonucleotides corresponding to the wild-type IL-10 allelic sequences and with a variety of oligos containing different mutated bases around the ETS-like core.

Figure 2 is an EMSA using nuclear extracts from the U937 monocytic cell line. Oligo sequences are shown in table 1. 1082G and –1082A alleles are indicated in bold. Competition assays were performed using radioactively labelled probe containing the IL-10 –1082G allele (lanes 1–6) and the IL-10 –1082A allele (lanes 7–12). Lanes 1 and 7 contain specific probe only; lanes 2 and 8 contain nuclear extract. The radiolabelled probe showed binding to at least four bands and similar DNA/protein complexes were observed for each allele. The binding was reduced competitively by the addition of excess cold unlabelled

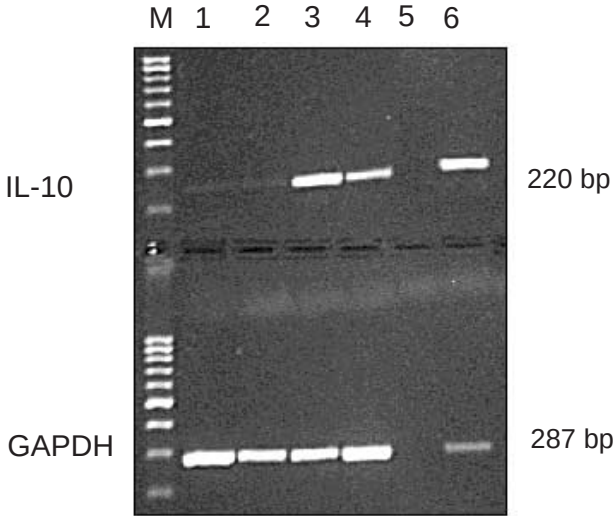

Figure 1. IL-10 mRNA production in the U937 cell line after stimulation with PMA Lanes: M, 100 bp DNA ladder; 1+2 unstimulated cells; 3+4 PMA stimulated; 5 negative water control; 6 positive control cDNA.

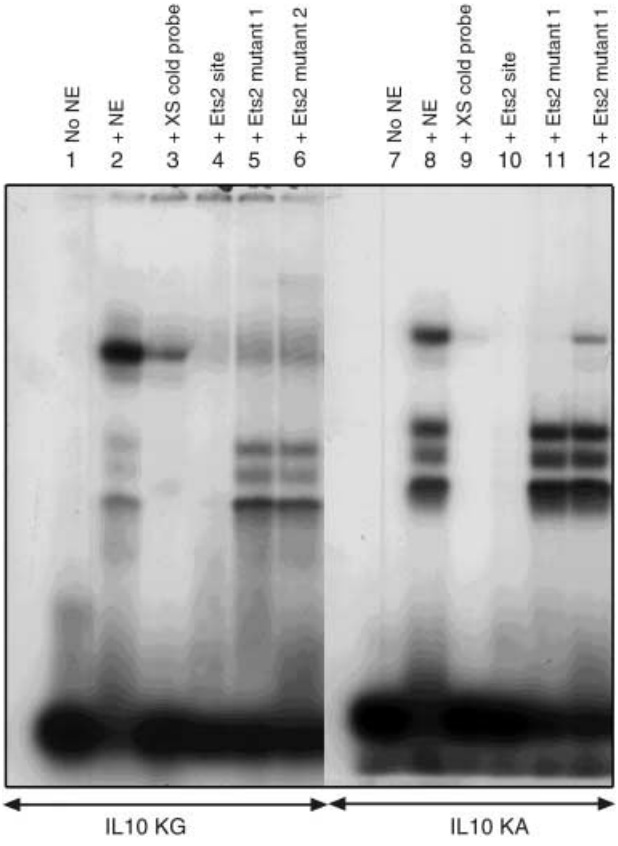

Figure 2. EMSA to determine the DNA sequence involved in binding to nuclear factors. The assays were performed three times. One representative experiment is shown. Competitions were performed using U937 nuclear extracts. Lanes 1–6 and 7–12 contain identical components except for the labelled probes used, i.e. IL-10KG (lanes 1–6) and IL-10KA (lanes 7–12). Lanes 1 and 7 contain no nuclear extract, i.e. labelled probe alone.

Table 1. Oligonucleotide probes used in EMSA.

Oligo	Sequence
IL10 KG-upper	5′ tttggga**g**gggggaagtaggg
IL10 KG-lower	5′ ccctacttcccc**c**tcccaaa
IL10 KA-upper	5′ tttggga**a**gggggaagtaggg
IL10 KA-lower	5′ ccctacttcccc**t**tcccaaa
WT ETS2-upper	5′cggccccgcgggcgcttccgggacgcag
WT ETS2-lower	5′ctgctgcccggaagcgcccgcggggccg
ETS2mutant-1-upper	5′ctgctgcccggttgcgcccgcggggccg
ETS2mutant-1-lower	5′ cggccccgcgggcgcaaccgggacgcag
ETS2mutant-2-upper	5′ ctgcgtcccttaagcgcccgcggggccg
ETS2mutant-2-lower	5′ cggccccgcgggcgcttaagggacgcac

probe (lanes 3 and 9), competition being more complete with the A than the G allele. To determine whether the sequence GGAA/G was specifically involved in binding of nuclear factors, competition assays were performed using excess competitor corresponding to the binding site of ETS2 (lanes 4 and 10), and with two ETS2 mutant oligos (lanes 5 + 6, 11 + 12) which contained disrupted GGAA cores.

The wild-type IL-10KG probe containing the consensus GGAG bound to nuclear factor(s), although binding was consistently less clean and specific than with the wild-type IL-10KA (GGAG) probe, suggesting that there may be a difference in binding between the two sequences. The competition assays using mutated ETS2 sequences indicated that for both IL-10 probes, the GGAG/GGAA consensus was the important site for protein interaction.

Genotyping

IL-10 promoter –1082 genotypes (G or A) were successfully assigned in all cases by comparison with standards of known IL-10 genotype.

Allele frequencies were compared using χ^2 analysis and are shown in table 2. Allele frequencies in the UK populations were similar to expected, but in the Hong Kong Chinese populations, there was marked skewing towards the A allele (freq. A = 0.96, freq. G = 0.04) [25]. There were no significant differences between SLE patients and controls.

Table 2. IL10 –1082 genotypes and allele frequencies in two populations. The distribution of –1082 alleles in the Hong Kong Chinese was significantly different to that of the Caucasoid population ($p<0.001$) as tested by χ^2 analysis.

	UK white (n = 98)	Chinese (n = 213)
Freq. A	0.52	0.96
Freq. G	0.48	0.04

Nature of the IL10 –1082 polymorphism in relation to transcriptional activity

Direct sequencing of the original PGL2-IL10 plasmid from which the 1.68 kb IL-10 fragment was excised and subcloned into PGL3-basic also yielded genotypes at the upstream IL-10G microsatellite, –819 and –592 loci, which were found to be 23 repeats (allele 10), –819C and –592C, respectively.

The guanine at position –1082 in the transfection construct PGL3-IL-10G was successfully mutated to adenine to produce the transfection construct PGL3-IL-10A.

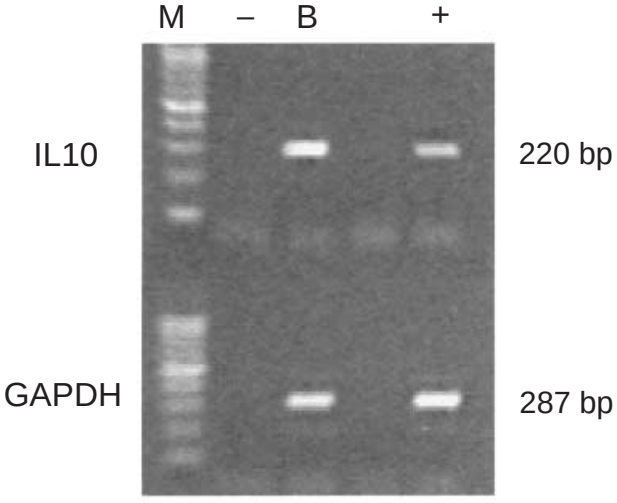

Figure 3. Basal IL-10 mRNA production in the MAS B cell line. Lanes: M, 100-bp DNA ladder; – negative water control; B MAS B cell line; + positive control cDNA.

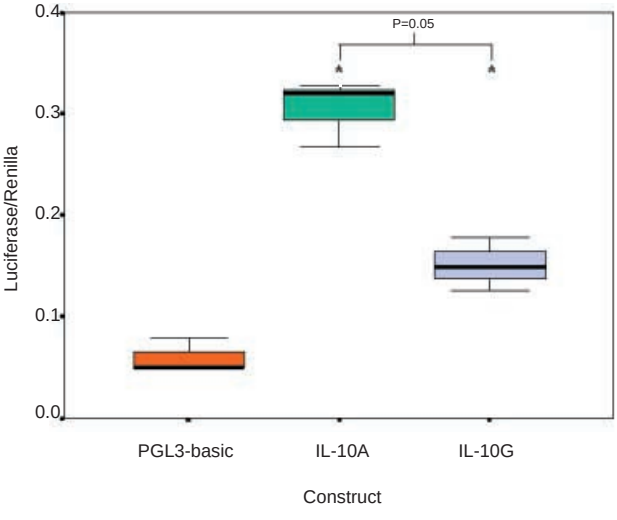

Figure 4. Activity of the two allelic forms of the IL-10 –1082 G/A polymorphism in MAS B cells. Black bars indicate the median. Three separate experiments were perfomed. One experiment in triplicate is represented for clarity.

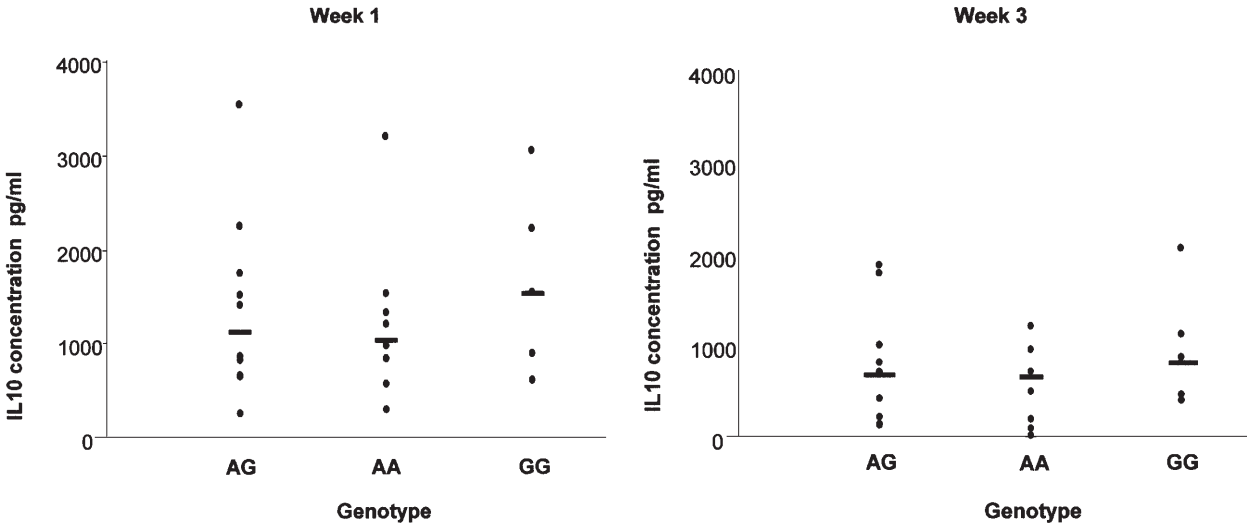

Figure 5. IL-10 production in 10^7 conA-stimulated PBMCs according to –1082 genotype. Black bars indicate the median. Weeks 1 and 3 are represented for clarity.

Transfections were initially performed in the U937 monocytic cell line but were unsuccessful, so the EBV-transformed B cell line (MAS B) was used. These cells have high constitutive IL-10 expression, as shown in figure 3. Transfections were performed nine times with each IL-10 construct in three separate triplicate experiments. IL-10 promoter activity was defined as the ratio of firefly luciferase/sea pansy luciferase. A representative experiment is shown in figure 4. The transfections demonstrated that the A allele was consistently associated with an approximately twofold increase in transcriptional activity compared to the G allele ($p = 0.05$, Kruskal-Wallis test).

Production of IL-10

IL-10 production by conA-stimulated PBMCs according to IL-10 –1082 genotype is shown in figure 5. This experiment was repeated at four different time points, each a week apart: weeks 1 and 3 are shown as representative experiments. PBMCs cells were extracted from each individual by density centrifugation and 10^7 cells were cultured on the same day at the same time for each individual every 7 days for 4 weeks with or without PMA. IL-10 in the supernatants after 48 h was detected using an in-house ELISA. Figure 5 demonstrates the IL-10 production according to genotype. There was marked inter- and intra-individual variation in IL-10 production over the 4-week period but there was no consistent correlation with IL-10 genotype when compared using the Mann-Whitney U test ($p > 0.05$).

Discussion

Binding of nuclear factors to the IL-10 promoter

The large body of evidence from many genetic association studies and the handful of functional studies show that the regulation of IL-10 is complex and multi-factorial. This study has taken one previously identified biallelic polymorphism in the human IL-10 gene promoter and assessed its DNA-binding properties and influence on gene transcription *in vitro*, analysed its associations with IL-10 protein production in normals, and its allelic distribution in two distinct populations.

The IL-10 base transition at position –1082 lies within a putative ETS transcription factor-binding site [16], but the DNA sequence does not correspond directly to any of the published ETS consensus binding sites [31–33]. The IL-10KG probe containing the consensus GGAG bound to a nuclear factor, although binding was consistently less clean and specific than that of the IL-10KA probe, suggesting there is a difference in binding affinity between the two sequences. However, this would be difficult to test in our system, since an excess amount of protein would be required in the nuclear extract compared to the amount of probe DNA. Competition with excess competitor specific for the ETS2 binding site (fig. 2) competed out the labelled IL-10 probe containing the GGAA motif more readily than GGAG sequence, although the latter was reduced to some degree. The competition assays using mutated ETS2 sequences indicate that for both IL-10 probes, the GGAG/GGAA consensus is the important site for protein interaction.

Previous literature on the effect of the –1082 polymorphism on transcriptional activity is conflicting. Our findings are supported by those of Eskdale et al. [34], who

isolated the –1082 polymorphism against a constant haplotype background and found that the IL-10 –1082A allele was associated with the highest median IL-10 production in in vitro stimulation assays. Keijsers et al. [35, 36] also found a positive correlation between the –1082A allele and high IL-10 production in whole blood from rheumatoid arthritis patients stimulated in vitro with lipopolysaccharide. However, other groups have reported –1082G rather than –1082A in association with elevated IL-10 protein levels.

Crawley et al. [37] demonstrated that reporter gene assays using constructs containing particular SNP alleles have variable activities. They have recently published a study on the association of IL-10 –1082/–819/–592 haplotypes and juvenile rheumatoid arthritis. Those individuals homozygous for A (ATA/ATA) produced significantly less IL-10 than those without the ATA haplotype. However, the ATA haplotype was not associated with disease susceptibility. Luciferase reporter assays using a truncated version of the IL-10 promoter were performed. Constructs containing the haplotypes GCC, ACC, and ATA were transfected into U937 monocytes stimulated with dbcAmp (aAMP analogue). Significantly higher transcriptional activity was observed using the GCC IL-10 haplotype construct compared to that of the ACC and ATA constructs. Paterson [38] has also performed transient transfections, using truncated IL-10 promoter/PGL3basic constructs in different cell types, and demonstrated a significant difference in luciferase transcription between IL-10 haplotypes, which appears to be cell type dependent. The GCC haplotype exhibited consistently higher activity than ACC and ATA in B cells, but in dbcAMP-stimulated U937s, both the ACC and GCC haplotypes exhibited increased transcriptional activity compared to ATA.

The transcription factors involved in transcription of the IL-10 gene have been suggested to differ in different cell types. Work as yet unpublished by Kay and colleagues [personal communication; Second IL-10 workshop, Milan 1999] has identified several ETS transcription factors that bind this region of the IL-10 promoter, in different cell types. B cell lines have been reported to over-express transcription factors Pu.1 and ETS-1. In PBMCs, EMSAs show binding to Elk-1, Nef-1 and ETS-1, and are confirmed by antibody supershifts. ETS proteins can be regulated by methylation. For example, the human housekeeping genes Surf-1 and Surf-2 regulate binding of ETS transcription factors by differential methylation of cytosine residues within the dinucleotide sequence CpG [39]. In many cases, the methylation of CpG islands of most housekeeping genes results in gene silencing [40]. Subsequent additional EMSAs using methylated ETS2 oligos were performed and those containing methylated residues within the GGAA/G consensus region abolished binding (data not shown), further supporting evidence for an ETS transcription factor. Transcription of the IL-10 gene may be regulated by the methylation state of its promoter, but there is so far no direct evidence to support this.

Genotyping

In the Caucasoid population, the allele frequencies of the –1082 G/A polymorphism have been reported to be G = 0.51 and A = 0.49 [24], and the data presented in our report are similar (G = 0.48, A = 0.52) in the group of 98 normal control subjects. A recent study by Morse et al. [30] also reported similar allele frequencies in 500 normal controls (G = 0.48, A = 0.52). In the Hong Kong Chinese populations, the allele frequency was markedly different: the majority of individuals were homozygous AA at this locus. In another study published while our work was in progress, similar allele frequencies were found in another southern Chinese population [25]. We found no evidence of any association of the –1082 polymorphism with SLE in the Chinese population. Although caution is required with this interpretation because the number of SLE patients in our study was relatively small, our findings are consistent with other recently published data [18, 25, 41]. SLE is common and often severe in Hong Kong Chinese [42]. The rarity of the –1082 G allele in this population adds further evidence that this polymorphism is unlikely to be a strong genetic determinant of susceptibility to lupus. Lupus susceptibility has been linked to several genetic loci including the major histocompatibility complex HLA-DR2 [43] and to complement C4 null genes [44]. Genetic susceptibility to lupus is likely to be complex, and evidence that IL-10 polymorphisms contribute is weak and inconsistent. Eskdale et al. [45] and Ou et al. [46] suggest that certain microsatellite allelic combinations may be associated with renal involvement in lupus, whereas others report no significant association between microsatellite alleles and incidence of SLE [47]. Evidence from Mehrian et al. [48] suggests that bcl-2 and IL-10 alleles may together exert a synergistic effect in determining susceptibility to SLE. Particular IL-10G microsatellite alleles on their own may present a moderate risk but in combination with certain bcl-2-encoding alleles, the risk of developing disease may be 40 times greater. There appears to be no direct association of the –1082/-819/-592 haplotypes with disease, although some groups report increased frequencies of certain haplotypes with clinical subgroups of SLE [18, 21, 25, 47, 49].

Transient transfections

The transfection data suggest that there is increased transcription of the IL-10A construct compared to IL-10G in MAS B cells. The reporter gene assays performed in the MAS B cell line were designed to test primarily the effect of an A or G at –1082, independently of the other two

polymorphisms, which through direct sequencing were characterised as –819C and –592C. The IL-10G microsatellite region was characterised as having 23 repeats. The hypothesis was that the –1082G/A polymorphism independently influences IL-10 transcription, and our luciferase assay demonstrated a modest twofold difference in promoter activity of the ACC IL-10 construct (IL-10A) compared to that of the GCC (IL-10G) construct ($p = 0.05$). Other groups have performed transfection experiments with fragments of the IL-10 promoter and have conflicting data [38, 39], reinforcing the concept that the IL-10 gene must be differentially regulated in different cell types. Paterson [38] showed that the IL-10 haplotypes ACC = GCC > ATA when used in transient transfections of MAS B cells. We did not study constructs containing the ATA haplotype so we were unable to further compare activity with the ACC and GCC haplotypes in our system.

IL-10 production

Turner et al. [24] reported that the presence of the IL-10 –1082G allele was independently associated with higher IL-10 protein production in vitro. Other researchers have performed similar assays and reported conflicting associations between IL-10 production and genotype [35, 36, 50, 51]. Very recently, Helminen et al. [52] measured spontaneous plasma IL-10 levels in children by enzyme immunoassay. An association between IL-10 ATA haplotype and increased IL-10 levels was observed, which may confer protection against primary EBV infection. In our study, inter-individual variation in IL-10 production was high, but no consistent effect of genotype on IL-10 production was observed under these assay conditions. Intra-individual variation was also marked, so that previous studies in which assays have only been performed at one time point must be interpreted with caution. The protocol chosen to obtain lymphocytes for in vitro stimulation assays has been suggested to significantly affect the frequency and lineage of cells obtained. Conflicting previous results on IL-10 production by PBMC preparations may be explained by variations in the cell lineages present, which themselves are likely to have differing capacity to produce IL-10. Separation of individual cell lineages would be required to address this point; results using whole PBMC preparations must be clearly interpreted with caution.

The implications of innate differences in IL-10 production are of interest in studies of autoimmunity, tumour development and transplantation tolerance. The modest difference in transcriptional activity seen here between the IL-10A construct and the IL-10G construct may or may not be biologically significant.

In conclusion, we have shown that the IL-10 –1082 G/A polymorphism occurs within an ETS-like transcription factor-binding site. In our experiments, the IL-10A allele was linked to increased IL-10 gene transcription in MAS B cells, independently of the polymorphisms at –819 and –592. Additionally, this polymorphism occurs at markedly different frequencies in UK and Hong Kong populations. Further characterisation of the transcription factors binding this region of the IL-10 promoter is required, together with a more detailed investigation of the activity of IL-10 haplotype combinations in various cell types.

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