Genetic studies in rheumatoid arthritis

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Chapter 7

Transcription of the *IL10* gene reveals allele-specific regulation at the mRNA level

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Abstract

Background

Interleukin-10 (IL10) is a cytokine with key regulatory and anti-inflammatory function involved in the pathogenesis of various diseases. Although the large interindividual differences in the production of IL10 have been extensively associated with polymorphisms and haplotypes of the IL10 gene, surprisingly little evidence exists that this variation is actually dictated by IL10 haplotypes.

Methods & Findings

Using the technique of allele-specific transcript quantification, the ratio between two alleles (A and G) of the IL10 gene was characterized in 15 healthy heterozygous individuals. Two groups were identified whereby donors in group 1 exhibited a 1:1 ratio, whereas those in group 2 exhibited a ratio > 1 ($P < 0.0017$). We found that donors heterozygous for haplotype IL10.2 (one of the four ancient IL10 haplotypes) were only prevalent in the group that showed higher allelic expression ratios.

Conclusion

In this study we show that IL10 alleles are indeed differentially transcribed in cells from heterozygous individuals and that IL10 haplotypes dictate production of IL10. These findings show that interindividual differences in IL10 protein levels can be explained at the transcriptional level.

Introduction

Interleukin-10 (IL10) has a key role in regulation of the immune response. Produced by a variety of cell types, including monocytes and B-lymphocytes, it is a potent upregulator of B-cell activation and differentiation (1), but has anti-inflammatory capabilities that can down-regulate TNFa, IL1, IL8 and IFNg (2,3). It has also been shown to be an essential growth factor for Tr1 cells (4).

Studies have shown that striking differences exist between healthy individuals in their ability to produce IL10 both at the constitutive level and following LPS stimulation of wholeblood culture in vitro. The relevance of these interindividual differences in IL10 secretion is supported by several studies. In the case of fertility, a 2-fold more prevalence of an IL10 genotype associated with low production (-2849AA) was observed among 73 women who remained childless as compared with the prevalence among 323 women with normal fecundity (5). In a study of first-degree family members of patients with meningococcal disease, families characterized by high production of IL10 had a 20-fold higher chance of a fatal outcome in comparison with families with low production of IL10 (6). These data along with other studies point to a genetic origin of enhanced IL10 production that is clinically relevant.

Around 50-70% of the interindividual differences in IL10 production can be attributed to genetic factors (6, 7). Polymorphisms in the promoter region of the IL10 locus exhibit functional relevance in a pleiotropy of diseases of infectious, autoimmune or immunosuppressive nature. Eleven promoter single nucleotide polymorphisms (SNPs) and two CA-repeat microsatellites (IL10.G and IL10.R) have been described. This has led to the identification of four common IL10 promoter haplotypes (8). One interesting SNP is -2849A/G (relative to the transcription start site) in the IL10 promoter. Two previous studies from our research showed that carriers of the -2849AA genotype (non-G carriers) produce significantly less IL10 than carriers of the AG and GG genotypes (G carriers) (5,9). The -2849AA genotype together with the IL10.R3 microsatellite tag the IL10.1 haplotype associated with low production of IL10. Interestingly, haplotypes of the IL10 promoter have been implicated in disease outcome as well. In a recent study, Lin et al. demonstrated that an IL10 promoter haplotype is an independent predictor of outcome of graft versus host disease after stem-cell transplantation (10).

The genetic characterization of the IL10 production phenotype was based on results from whole-blood cultures stimulated with LPS. Different amounts of secreted IL10 in response to a constant amount of stimulus showed a high correlation at the mRNA level, while the mRNA half-life between high and low producing donors was the same (8), indicating that secreted protein levels are regulated by differential transcription. However, the intraindividual variation in the amount of IL10 produced by one given individual is about 20% (11), and the associations between IL10 haplotypes and low or high producer phenotype are variable. Although current techniques, using statistic associations and artificial reporter constructs, may be effective in assigning a certain haplotype or a SNP as the marker of low/high IL10 production, we aimed at determining the exact nature of the relationship between IL10 production and cis-acting variations within the IL10 locus.

In general, both copies of human autosomal genes are assumed to be co-dominantly expressed. However, exceptions do arise in the course of normal development or when

promoter utilization or RNA stability is altered (12). Allelic imbalances were previously studied in situations of genomic imprinting when both maternal and paternal alleles are present, but one allele is expressed while the other remains inactive (13). Recent surveys of human and mouse genes suggest that the occurrence of allelic expression differences are more common than previously expected (14,15). Although differential allelic expression may be a potentially important mechanism underlying human phenotypic diversity, no data is currently available showing a direct functional effect of allelic imbalances. Here, we report the first study of genetic variations in the IL10 gene aimed at analysing the intrinsic relationship between genotype and phenotype at the transcriptional level by quantifying the rate of RNA synthesis.

Results

To distinguish between alleles of the IL10 gene, a panel of 58 unrelated, healthy individuals was genotyped for a SNP at nucleotide position +4259 (position relative to the transcription start site) present on exon 5/3'UTR region (Supplementary Material, Fig. S1). Given that this SNP is located within a part of the gene that is transcribed, it is possible to discriminate between allelic transcripts in a +4259 heterozygous individual. Thus, 15 heterozygous unrelated individuals were selected for further analysis.

To validate the allele-specific transcript quantification (12) procedure the intensities of the fragments in DNA mixtures with a known distribution of +4259A and +4259G alleles were measured along with genomic DNA controls. The ratio of the intensity of the bands equals the ratio in the DNA mixtures (Supplementary Material, Fig. S2a) with a high correlation factor (R = 0.999). Genomic DNA yielded an equimolar ratio with a median of 1.00 (25th percentile 0.97-75th percentile 1.035) as expected (Supplementary Material, Fig. S2b). Taken together these results show that any deviations from DNA equimolar ratio can be quantified as each allele acts as an internal control for confounding factors that alter the overall expression of that gene, including differences in tissue preparation, mRNA quality and environmental influences.

Quantification of the respective IL10 +4259A and IL10 +4259G allelic RNA transcripts for 15 individuals revealed that the median ratio of IL10 + 4259 A:G ranged from 0.93 to 1.13 (Fig. 1). In 33% of the individuals (2–6) both IL10 alleles contribute equally to the mRNA pool, whereas 67% showed a preferential expression of allele G or A, respectively (individuals 1 and 7–15). Individual 1 showed preferential expression of allele G significantly different from genomic DNA ($P < 0.0001$). In individuals whose A allele was preferentially expressed (7–15), the ratio of the transcripts as compared with genomic DNA varied from 1.03 to 1.13 ($P < 0.003$). A:G ratios of samples derived from blood collected at a second time-point for individuals 3, 11 and 15 yielded a ratio of 1.01, 1.15 and 1.145, respectively as expected (data not shown), further supporting evidence for allele-specific expression.

We therefore characterized two predominant groups, group 1 which does not show a significant deviation from genomic DNA ratio and group 2 which includes individuals showing preferential expression of allele A by 3–12%. Moreover, upon examination of nuclear families of individuals 13 and 14 (family 1 and family 2, respectively) exhibiting allelic variation in expression (Fig. 2), we observed that higher allele-specific expression in the IL10 gene is a heritable trait.

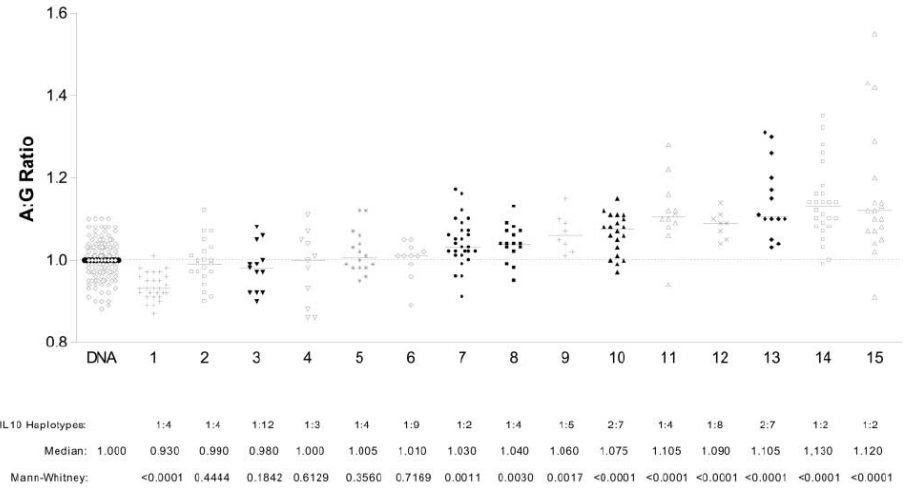

Figure 1. Determination of the relative contribution of the IL10 +4259A and IL10 +4259G alleles in LPS-stimulated whole-blood cultures of 15 healthy individuals. Each dot represents a sample of DNA or cDNA. Data are representative of a minimum of three independent experiments. All individuals (1–15) were subjected to independent whole-blood cultures stimulated with LPS, followed by separate RNA purification and cDNA preparation. P-values refer to significance test for differences (Mann–Whitney U-test) between every individual RNA transcript ratio to genomic DNA ratio (significance $P < 0.05$). Individuals 2–6 yielded a median ratio not significantly different from genomic DNA (Mann–Whitney $P > 0.2$), whereas individuals 1 and 7–15 yielded a ratio of $\neq 1$, significantly different from genomic DNA ($P < 0.0017$). Individually inferred haplotypes for each individual are indicated.

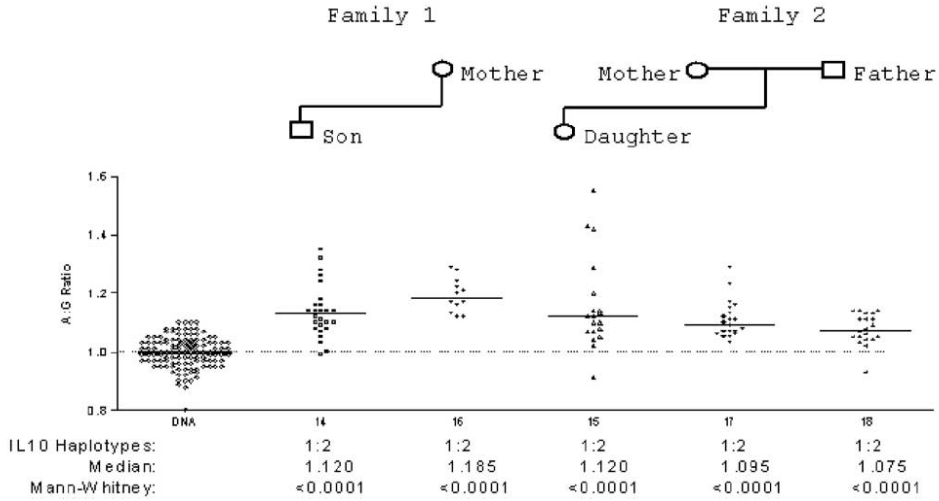

Figure 2. Inheritance pattern of altered IL10 allelic expression. The relative contribution of the IL10 +4259A and IL10 +4259G alleles in whole-blood cultures stimulated with LPS was investigated in two pedigrees (families 1 and 2), as shown, in which the same trait is prevalent implying an underlying inheritance pattern. Each dot represents a sample of DNA or cDNA. Data are representative of a minimum of three independent ASTQ experiments. P-values refer to significance test for differences (Mann–Whitney U-test) between every individual transcription ratio to genomic DNA ratio (significance $P < 0.05$).

Given the extensive associations between IL10 production and IL10 SNP genotypes, we hypothesized that the regulatory determinant could be one of the already characterized IL10 SNPs found in the promoter region (16). Therefore, we analysed if one of the SNPs' genotypes was always homozygous in the 1:1 donors and always heterozygous in the 1: ≠ 1 donors. Table 1 shows that this is not the case. Although none of the SNPs show an individual effect, a combination of alleles may identify which haplotype dictates differential expression. Haplotype frequencies for the population comprising of 58 individuals were subsequently inferred using the estimation maximization (EM) algorithm in SNPHAP (Fig. 3). Two haplotypes were assigned to each heterozygous individual ($P \geq 0.9$ were taken as true haplotypes). The level of linkage disequilibrium (LD) between all SNPs was very high, D' values were close to 1 (data not shown), thus only 13 out of the 29 possible haplotypes were prevalent in the population. IL10.1 to IL10.4 are the four most common haplotypes previously identified (17) and comprise 75% of total chromosomes.

Table 1. IL10 genotypes and haplotypes of 15 unrelated individuals included in the ASTQ analysis and three family members (16–18)

Individual	Sex	Age (y)	RNA A:G (median)	*P*-value	IL10 polymorphisms									IL10 haplotypes	
					A + 4259G	T + 1582C	C − 592A	C − 819T	G − 1082A	A − 1330G	A − 2763C	A − 2849G	A − 3575T		
1	M	45	0.930	<0.0001	AG	CT	CA	CT	AG	AG	AC	AG	TA	4(L)	1(L)
2	M	27	0.990	0.4444	AG	CT	CA	CT	AG	AG	AC	AG	TA	4(L)	1(L)
3	M	28	0.980	0.1842	AG	CC	CC	CC	GG	AA	AA	AA	AA	12(L)	1(L)
4	F	32	1.000	0.6129	AG	CC	CC	CC	GG	AA	AA	AG	AA	3(L)	1(L)
5	F	24	1.005	0.356	AG	CT	CA	CT	AG	AG	AC	AG	TA	4(L)	1(L)
6	M	40	1.010	0.7169	AG	CC	CA	CT	AG	AG	AC	AG	TA	9(L)	1(L)
7	F	26	1.030	0.00011	AG	CC	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)
8	M	30	1.040	0.003	AG	CT	CA	CT	AG	AG	AC	AG	TA	4(H)	1(L)
9	M	26	1.060	0.0017	AG	CC	CC	CC	GG	AA	AC	AG	TA	5(H)	1(L)
10	F	31	1.075	<0.0001	AG	CC	CC	CC	AG	AG	CC	AG	TA	2(H)	7(L)
11	M	28	1.105	<0.0001	AG	CT	CA	CT	AG	AG	AC	AG	TA	4(H)	1(L)
12	F	35	1.090	<0.0001	AG	CC	CC	CC	GG	AA	AC	AG	AA	8(H)	1(L)
13	F	27	1.105	<0.0001	AG	CC	CC	CC	AG	AG	CC	AG	TA	2(H)	7(L)
14	M	43	1.130	<0.0001	AG	CC	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)
15	F	32	1.120	<0.0001	AG	CC	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)
16	F	75	1.185	<0.0001	AG	ND	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)
17	F	67	1.095	<0.0001	AG	CC	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)
18	M	71	1.075	<0.0001	AG	CC	CC	CC	AG	AG	AC	AG	TA	2(H)	1(L)

ND, not determined. Each individual's A:G RNA ratio (1–18) is expressed as median. To determine if significant differences exist between RNA ratio and genomic DNA ratio, Mann–Whitney *U*-test was carried out (significance level $P < 0.05$). Nine IL10 SNPs including IL10 +4259 (exon 5/3'UTR), IL10 +1582 (intron 3); proximal promoter SNPs IL10 -592, IL10 -819, IL10 -1082, IL10 -1330 and distal promoter SNPs IL10 -2763, IL10 -2849 and IL10 -3575 were genotyped, and haplotypes for each individual are indicated. L is assigned to the lower IL10 transcribing allele and H to the higher transcribing allele.

Analysis of individual haplotypes in Table 1 shows that the IL10.2 haplotype is only present in the donors that exhibit a >1 ratio ($P < 0.0017$). IL10.2 is therefore proposed to be the 'high' haplotype, responsible for higher IL10 transcription and thus higher IL10 protein levels.

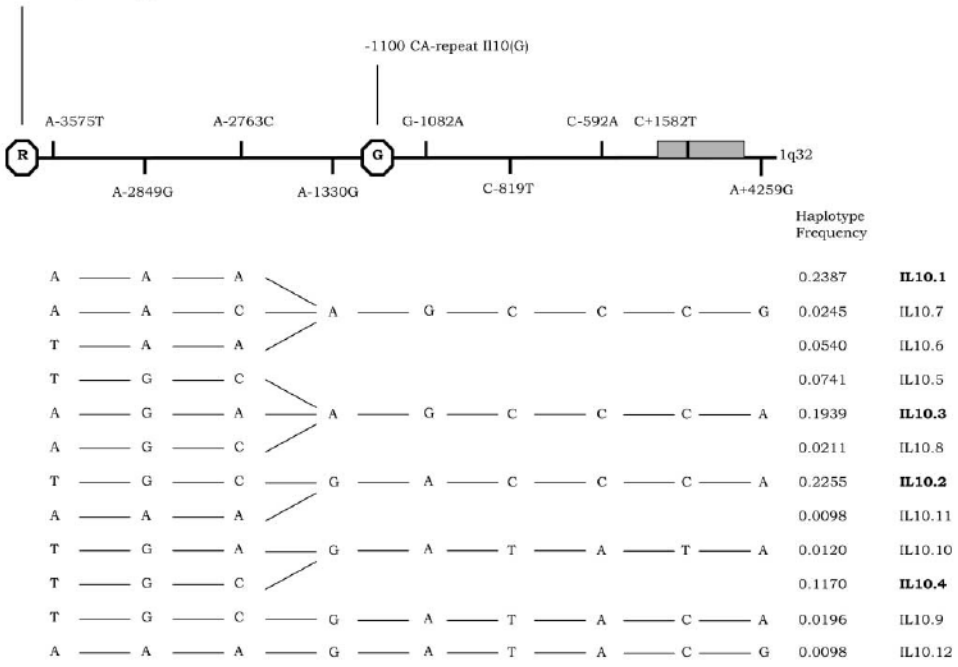

Figure 3. Haplotype frequencies of nine IL10 polymorphisms including seven promoter SNPs in 58 normal Caucasian individuals. SNPs were denoted respective to the transcription start site. In total, 13 haplotypes were inferred using EM algorithm in SNPHAP. Haplotypes IL10.1, 2, 3 and 4 are previously identified ancient haplotypes in the Caucasian population (17) and account for 75% of variation within our population. The proximal region of the IL10 promoter shows less variation than the distal part.

Discussion

IL10 is a crucial player in the immune system. Genetic differences in IL10 production have been associated extensively with the pathology and outcome of diseases. The aim of this study was to determine whether the mechanism driving these associations is differences in allelic expression. We show here for the first time that alleles of the IL10 gene are indeed differentially transcribed in LPS-stimulated whole-blood cultures of heterozygous individuals, a finding which supports the functional existence of transcriptionally distinct IL10 haplotypes in humans and questions the functional relevance of currently known SNPs.

IL10.2 harbours a polymorphism that is associated with increased expression at the mRNA level. This implies that IL10.1 and IL10.7 harbour the SNP associated with lower transcription rate. By extrapolation, high or low phenotype has been assigned to each haplotype and indicated in Table 1. Our data can best be explained if the IL10.4 haplotype consists of two haplotypes, a haplotype associated with high and one associated with low allele-specific IL10 expression.

Our data are in accordance with previously published data on LPS-induced IL10 production. We observed that the -3575A/-2763A haplotype is associated with lower IL10 production (16). In our panel, this combination of -3575A/-2763A haplotype forms part of the larger haplotypes IL10.1, 3 and 12, which are responsible for lower allele-specific IL10 transcription. We can therefore correlate these haplotypes to low IL10 protein levels, providing further evidence that this finding is in line with all data previously obtained. The -3575T/-2849G/-2763C haplotype, which tags IL10.2, IL10.4 and IL10.5, is associated with high IL-10 production, while the -3575A/-2849A/-2763A haplotype, which tags IL10.1 and IL10.12, is associated with low IL-10 production. According to the data in Table 1, both IL10.1 and IL10.12 drive lower transcription of mRNA transcripts, and both IL10.2 and IL10.5 express higher allelic transcriptional levels, further supporting our analysis.

Our finding that the IL10.4 haplotype may either be a low or a highly transcribed haplotype and the occurrence of an individual with ratio <1 suggests the presence of a bidirectional allelic imbalance that could result from allelic heterogeneity of one or more cis-acting regulatory polymorphisms present in the coding, intronic and/or other non-coding regulatory sequences (14,18) or from DNA methylation, histone acetylation and other epigenetic factors (19,20). Alternatively, the marker SNP could be detecting a single causal polymorphism that is actually responsible for the differential expression of IL10 RNA transcripts, but that does not show unidirectional allelic imbalance when it is not in complete LD.

Prime candidates are SNPs that are present in the regulatory region of the IL10 gene. However, our current knowledge of regulatory elements in the human genome is far from comprehensive and, beyond their coding sequences, most genes are not well annotated. As the positions of all regulatory sequences for any given gene are generally unknown, comprehensive direct analyses of such elements are currently impossible. Moreover, the SNPs used in this study span only about 8 kb of the IL10 gene, a region that does not cover the average length of a high LD block in world populations of similar origin (21). It would be interesting to determine the average length of the LD block surrounding the IL10 gene and attempt to identify regulatory sequences within.

In summary, we propose that a common SNP in high but not complete LD with haplotype IL10.2 dictates differences in IL10 expression. This cis-acting variation(s) may alter the functional motifs in the promoter region or work indirectly by modifying the activity of the gene product. However, the magnitude of difference in expression level that is likely to be biologically relevant is unclear. It is plausible that different cell types present in peripheral blood dilute the effect seen, such that investigating specific cell types would yield larger differences in IL10 differential transcription. However, in our system of LPS-induced whole-blood cultures, the main producers of IL10 are monocytes and macrophages, and so far no data exist to support monoallelic expression of monocytes. A more likely alternative, in our view, is that if a particular allele is always transcribing at a faster rate, subtle differences can have an exponential effect resulting in large changes in gene expression patterns. We therefore conclude the mechanism behind higher IL10 gene expression levels is caused by subtle differences in allelic expression regulated by cis-acting polymorphism(s) on distinct IL10 haplotypes.

Materials and Methods

Reagents

All reagents were from Invitrogen, Life Technologies, Gaithersburg, MD, USA unless otherwise specified.

Subjects/DNA isolation

Blood samples were collected from 58 unrelated healthy Caucasian volunteers. DNA was isolated using a SOC-lysis and proteinase-K treatment of peripheral blood cells, followed by a phenol–chloroform extraction. From every heterozygous IL10 + 4259A/G (rs3024498) individual, two additional heparin-blood tubes were used to perform LPS-whole-blood stimulations, as described below.

Genotyping of IL10-polymorphisms (position relative to transcription start site)

The IL10 -3575A/T (rs1800890), -2849A/G, -2763A/C, -1082A/G (rs1800896) and -819A/C (rs1800871) polymorphisms were typed as previously described (22). An amount of 25 ng genomic DNA was used per polymerase chain reaction (PCR) for all SNPs. -592A/C (rs1800872) and -1330A/G (rs1800893) are in complete LD (R^2 = 1) with -819A/C and -1082A/G, respectively. All SNPs were annotated relative to the transcription start site according to the public reference sequence U16720.

The PCR for -592A/C genotyping was carried out using 0.6 U Taq polymerase with the buffer supplied by the manufacturer (Applied Biosystems), 2.5 mM MgCl2, 0.25 mM of each dNTP, 100 µg/ml bovine serum albumin and 0.25 µM of each primer in a 30 µl reaction. Sense primers were 5'-CTC AGTTAGCACTGGTGTAC-3' and antisense 5'-TGTTCCTA GGTCACAGTGAC-3'. DNA was denatured at 94°C for 5 min and cycling conditions were set at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 35 cycles and a final cycle of extension at 72°C for 7 min in a thermocycler (Applied Biosystems 9700). An aliquot of 10 µl of the PCR product was digested for 2 h with 1.5 U of Rsa I (MBI Fermentas) in a total volume of 25 µl at 37°C, yielding 480 bp for the C allele and 240 bp for the A allele.

The PCR conditions for the +4259A/G SNP were the same as for the -592A/C and were run for 40 cycles. Primers were sense 5'-ACTGAGCTTCTCTGTGAACG-3' and antisense 5'-AATAACAAGCTGGCCACAGC-3'. 12.5 µl PCR product was digested with 2.5 U per tube Fnu4HI (New England Biolabs) in a total volume of 50 µl yielding 416 bp for the A allele and 284 + 132 bp for the G allele.

The conditions for the +1582T/C (rs1554286) SNP PCR were set at 1 min 94°C, 1 min 60°C and 1 min 72°C for 40 cycles with sense primer 5'-CTACGGCGCTGTGTAAGTAGCAGATCAGA*T-3' (modified nucleotides are marked) and antisense primer 5'-AACCCACAAATGACTCACAAAT-3'. An aliquot of 10 µl PCR product was digested with 2.5 U of Bgl II in a total volume of 25 µl yielding 157 + 105 bp for the T allele and 130 + 105 + 27 bp for the C allele.

Quality control

Data for each SNP were reviewed independently to verify their quality. We considered each SNP to be validated and correctly genotyped for all individuals only if the genotype data met a series of strict criteria, including minimum signal intensity specifications and unambiguous genotype reading ability. No errors were detected in 10% of the samples that were randomly genotyped again.

LD mapping and haplotyping across the IL10 gene

GOLD (23) was used to determine pairwise LD between all SNPs. Haplotype frequencies were inferred using SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/). Crosschecking of the inferred haplotypes using available family data (data not shown) revealed that all haplotypes were accurate in areas of high LD (D' close to 1).

LPS-whole-blood cultures/RNA isolation

In total, 15 heterozygous unrelated individuals were selected for ASTQ on the basis of their heterozygosity with respect to the IL10 +4259 SNP. Approximately 20 ml of blood was collected at one time-point from each of 18 heterozygous IL10 + 4259A/G donors (15 unrelated and three family members, Table 1). Blood was collected from three individuals at a second time-point and an independent set of experiments was carried out.

For each donor, five wells containing 3 ml of blood was immediately diluted once in 3 ml of an aliquot consisting of endotoxin-free RPMI 1640 medium (Gibco) with 10% fetal bovine serum (Bodinco BV), 5% pen-strep (Bio-Whittaker) and 30 mg/ml LPS from Salmonella typhosa (Sigma). As a control, one additional well was subjected to the same conditions in the absence of LPS. These aliquots were incubated for 17 h at 37°C with 5% CO2.

RNA isolation

The samples were harvested, red blood cells lysed, and centrifuged at 600g. Each cell pellet was washed once with 25 ml PBS, taken up in 1 ml RNA-Bee (Campro Scientific) and stored at -20°C for at least 18 h. These samples were thawed on ice and 100 µl chloroform was added. After the samples were vigorously shaken for 20 s, and incubated on ice for 60 min, they were centrifuged (13 200 rpm, 4°C) for 15 min. The upper aqueous phase was pipetted out into new tubes. An equal amount of isopropanol was added to each tube followed by incubation on ice for 15 mins. RNA was precipitated by centrifugation (13 200 rpm, 4°C) for 15 min. The supernatant was discarded and the pellets were washed with 500 µl 70% ethanol. Samples were vortexed and centrifuged (13 200 rpm, 4°C) for 5 min. Supernatant was discarded and pellets were dried in air for 5–10 min. RNA was solubilised in 40–50 µl DEPC-treated water by incubation in a 55°C water bath for 30 min.

DNAse-treatment/RT-PCR

RNA samples were treated with DNAse I, Amp. grade. Each DNAse-treated RNA sample was split into four equal volumes, each containing 2 µg RNA. Two fractions were reverse-transcribed into cDNA in a 40 µl volume at 37°C for 1 h using 400 U of M-MLV, 5x first-strand buffer, 0.1 M dithiotreitol, rRNAsin (Promega) 100 mM dNTP mix and either 1 µg of oligo $dT_{12\text{-}18}$, or 1 µg of random primers. The remaining two fractions were used as negative controls. The reaction was inactivated by incubation at 95°C for 10 min. In total, each blood collection from each individual yielded six RNA samples which were reverse transcribed into 12 cDNAs. All cDNA samples were tested several times in independent ASTQ experiments as outlined below. Samples that were not stimulated with LPS were excluded from the analysis as the intensities obtained were too low to be quantified.

Allele-specific transcript quantification

This method is essentially the same as previously described (12) with the exception of the use of a marker (IL10 +4259) which is present in exon 5/3'UTR region of the IL10 gene, thus allowing the post-transcriptional analysis of the expressed alleles.

cDNA samples were amplified by PCR with the IL10 +4259A/G PCR. An aliquot of 20 µl amplicon was mixed with 4 µCi [α-^{32}P]-dCTP (Amersham Biosciences) and 0.5 U Taq polymerase, followed by one PCR cycle with conditions set at 94°C for 5 min, 94°C for 1 min, 60°C 1 min, 72°C for 1 min and 72°C for 7 min. An aliquot of 15 µl labelled amplicon was digested with 2.5 U Fnu4HI in a total volume of 50 µl and incubated at 37°C for 2 h. All samples were ethanol-salt precipitated, dissolved and separated on a 5% polyacrylamide sequence gel. After electrophoresis, the gel was dried for 3 h in a preheated vacuum gel-dryer and incubated for 7 days in a Phosphor imager (Molecular Dynamics) cassette at room temperature. The exposed screen was scanned and computer analysed (ImageQuant).

Ratios of +4259A/+4259G transcripts were obtained by calculating the pixel values of digestion fragments of allele A (416 bp) versus allele G (284 bp). As the respective products are not of the same size, a correction factor was applied based on the respective GC content of each restriction product: GC content allele A = 168 bp; GC content allele G = 108 bp; Correction factor = 168/108 = 1.56 was applied to each cDNA ratio. As the values for these two bands reflect the synthetic products from a RT-PCR amplification of the same RNA preparation, controlling for variations in reaction conditions was unnecessary.

Genomic DNA controls

Each gel consisted of cDNA samples along with genomic DNA controls from six to eight independent individuals. As allele A and allele G are codominantly expressed at the genomic DNA level, A:G DNA ratio from heterozygous IL10 + 4259 individuals should be 1:1. The reliability of the cDNA data was determined by the average of the DNA ratios obtained. In each gel, RNA ratios were subjected to a correction factor equivalent to genomic DNA ratio to circumvent overestimation of differences. Gels with a genomic DNA ratio <0.95 and >1.05 were excluded from further analysis.

Statistics

Data were analysed using Graphpad prism and did not follow a normal distribution. The median value for RNA A:G ratio was calculated for each individual (Table 1) and a Mann–Whitney U-test was performed to detect significant differences between RNA ratios and genomic DNA ratio ($P < 0.05$).

References

(1) Rousset, F., Garcia, E., Defrance, T., Peronne, C., Vezzio, N., Hsu, D.H., Kastelein, R., Moore, K.W. and Banchereau, J. (1992) Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl Acad. Sci. USA, 89, 1890–1893.

(2) de Waal, M.R., Abrams, J., Bennett, B., Figdor, C.G. and de Vries, J.E. (1991) Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.J. Exp. Med., 174, 1209–1220.

(3) Katsikis, P.D., Chu, C.Q., Brennan, F.M., Maini, R.N. and Feldmann, M. (1994) Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J. Exp. Med., 179, 1517–1527.

(4) Kemper, C., Chan, A.C., Green, J.M., Brett, K.A., Murphy, K.M. and Atkinson, J.P. (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature, 421, 388–392.

(5) Westendorp, R.G., van Dunne, F.M., Kirkwood, T.B., Helmerhorst, F.M. and Huizinga, T.W. (2001) Optimizing human fertility and survival. Nat. Med., 7, 873.

(6) Westendorp, R.G., Langermans, J.A., Huizinga, T.W., Elouali, A.H., Verweij, C.L., Boomsma, D.I., Vandenbroucke, J.P. and Vandenbrouke, J.P. (1997) Genetic influence on cytokine production and fatal meningococcal disease. Lancet, 349, 170–173.

(7) Reuss, E., Fimmers, R., Kruger, A., Becker, C., Rittner, C. and Hohler, T. (2002) Differential regulation of interleukin-10 production by genetic and environmental factors—a twin study. Genes Immun., 3, 407–413.

(8) Eskdale, J., Gallagher, G., Verweij, C.L., Keijsers, V., Westendorp, R.G. and Huizinga, T.W. (1998) Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc. Natl Acad. Sci. USA, 95, 9465–9470.

(9) de Jong, B.A., Westendorp, R.G., Eskdale, J., Uitdehaag, B.M. and Huizinga, T.W. (2002) Frequency of functional interleukin-10 promoter polymorphism is different between relapse-onset and primary progressive multiple sclerosis. Hum. Immunol., 63, 281–285.

(10) Lin, M.T., Storer, B., Martin, P.J., Tseng, L.H., Gooley, T., Chen, P.J. and Hansen, J.A. (2003) Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. N. Engl. J. Med., 349, 2201–2210.

(11) van der Linden, M.W., Huizinga, T.W., Stoeken, D.J., Sturk, A. and Westendorp, R.G. (1998) Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. J. Immunol. Methods, 218, 63–71.

(12) Kaijzel, E.L., Bayley, J.P., van Krugten, M.V., Smith, L., van de, L.P., Bakker, A.M., Breedveld, F.C., Huizinga, T.W. and Verweij, C.L. (2001) Allele-specific quantification of tumor necrosis factor alpha (TNF) transcription and the role of promoter polymorphisms in rheumatoid arthritis patients and healthy individuals. Genes Immun., 2, 135–144.

(13) Reik, W. and Walter, J. (2001) Genomic imprinting: parental influence on the genome. Nat. Rev. Genet., 2, 21–32.

(14) Cowles, C.R., Hirschhorn, J.N., Altshuler, D. and Lander, E.S. (2002) Detection of regulatory variation in mouse genes. Nat. Genet., 32, 432–437.

(15) Bray, N.J., Buckland, P.R., Owen, M.J. and O'Donovan, M.C. (2003) Cis-acting variation in the expression of a high proportion of genes in human brain. Hum. Genet., 113, 149–153.

(16) Gibson, A.W., Edberg, J.C., Wu, J., Westendorp, R.G., Huizinga, T.W. and Kimberly, R.P. (2001) Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. J. Immunol., 166, 3915–3922.

(17) Eskdale, J., Keijsers, V., Huizinga, T. and Gallagher, G. (1999) Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. Genes Immun., 1, 151–155.

(18) Martone, R., Euskirchen, G., Bertone, P., Hartman, S., Royce, T.E., Luscombe, N.M., Rinn, J.L., Nelson, F.K., Miller, P., Gerstein, M. et al. (2003) Distribution of NF-kappaB-binding sites across human chromosome 22. Proc. Natl Acad. Sci. USA, 100, 12247–12252.

(19) Dillon, N. and Festenstein, R. (2002) Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. Trends Genet., 18, 252–258.

(20) Jaenisch, R. and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet., 33 (suppl.), 245–254.

(21) Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M. et al. (2002) The structure of haplotype blocks in the human genome. Science, 296, 2225–2229.

(22) Moraes, M.O., Santos, A.R., Schonkeren, J.J., Vanderborght, P.R., Ottenhoff, T.H., Moraes, M.E., Moraes, J.R., Sampaio, E.P., Sarno, E.N. and Huizinga, T.W. (2003) Interleukin-10 promoter haplotypes are differently distributed in the Brazilian versus the Dutch population. Immunogenetics, 54, 896–899.

(23) Abecasis, G.R. and Cookson, W.O. (2000) GOLD—graphical overview of linkage disequilibrium. Bioinformatics., 16, 182–183. 1762 Human Molecular Genetics, 2004, Vol. 13, No. 16