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INTERLEUKIN-6 PROMOTER HAPLOTYPES AND INTERLEUKIN-6 CYTOKINE RESPONSES

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Abstract

Genetic variations contribute to differences in the inflammatory response and are markers for disease risk and outcomes. We studied three single-nucleotide polymorphisms (−597 G →A, −572 G →C, and −174 G →C) in the IL-6 promoter to determine associations with *ex vivo* LPS-stimulated IL-6 production by leukocytes. We also measured nuclear protein binding to synthetic oligonucleotides representing the −174 polymorphic region, located in proximity to important transcription factor motifs. We determined genotypes at three sites in the IL-6 promoter by pyrosequencing of genomic DNA obtained from 49 healthy control subjects. To determine molecular haplotypes, cloned DNA fragments from heterozygous subjects were sequenced. IL-6 release by whole blood leukocytes was measured after 24 h of *ex vivo* LPS stimulation. We compared IL-6 concentrations between haplotypes using Kruskall-Wallis and adjusted for covariates by analysis of covariance. Electromobility gel shift assay was carried out by the incubation of nuclear proteins from cultured human mononuclear cells with oligonucleotides representing the alternate −174 alleles. The amount of nuclear protein binding was quantified by densitometry, which was compared using analysis of variance. Genotype and sequence analysis of genomic and cloned DNA characterized three haplotypes. *Ex vivo* IL-6 production was greatest in individuals who were homozygous for the haplotype containing guanine at −597 and −174. IL-6 production was least for individuals homozygous for the haplotype containing adenine at −597 and cytosine at −174. Nuclear protein bound more avidly to guanine-containing oligonucleotides representing the −174 position than to oligonucleotides containing cytosine at that position. The IL-6 promoter haplotype influences *ex vivo* IL-6 response to endotoxin. This effect may be due to a functional effect of the −174 G →C polymorphism.

Keywords

Lipopolysaccharide; inflammation; genotype

INTRODUCTION

Interleukin-6 (IL-6) is an important mediator of the acute phase response, and levels correlate with outcome from infection in a variety of models (1). Differences between individuals, both

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in cytokine responses and clinical outcomes, may be due to genetic polymorphism in addition to gender, age, and other well-known influences (2,3). Polymorphism in the IL-6 gene has been investigated for associations with and outcomes from acute and chronic diseases and has primarily focused on a single-nucleotide polymorphism (SNP) at the −174 position. This guanine to cytosine (G →C) SNP has been associated with clinical conditions ranging from juvenile rheumatoid arthritis and severe sepsis to insulin sensitivity and carotid artery intimal thickening (4–7). However, the effect of promoter SNPs on gene transcription may be more complex, and likely involves interaction among multiple polymorphic sites (8,9). Few studies have examined variants in locations other than this position in the IL-6 promoter. Studies that have examined other polymorphisms have primarily involved *in vitro* experimental conditions such as transient transfection experiments and may not reflect *in vivo* conditions (8). Although the −174 G →C base transition has been reported to affect gene transcription and is related to resting plasma IL-6 concentrations, there is evidence that this polymorphism is part of a haplotype of linked SNPs, including those at the −597 and −572 positions (8). Therefore, it is not clear which (one or more) of these SNPs may be important, as haplotype effects have only been examined using *in vitro* reporter gene constructs, and have not been confirmed with other techniques. Therefore, we sought to expand upon the existing observations of potential haplotype effects and possible functional relationships between IL-6 promoter SNPs, SNP haplotypes, nuclear protein binding, and endotoxin-stimulated IL-6 release by human leukocytes. We studied IL-6 production in response to endotoxin stimulation by whole blood leukocytes obtained from healthy volunteers and we analyzed individual SNPs in genomic DNA and clarified SNP haplotypes by cloning. We also performed electromobility gel shift assays (EMSA) using synthetic oligonucleotides representing the region encompassing the −174 G →C SNP. This site is located in proximity to important nuclear protein binding sites in the IL-6 promoter. We hypothesized that there were differences in LPS-stimulated IL-6 production according to promoter haplotype and that differential nuclear protein binding in the region of nucleotide −174 exists.

MATERIALS AND METHODS

Genotyping polymorphisms at the −597, −572, and −174 positions in the IL-6 promoter

The Institutional Review Board at the University of Texas Southwestern Medical Center approved this study and all subjects gave written informed consent for the study procedures. Genomic DNA from healthy control volunteers was extracted from buffy coats using the QIAamp DNA Blood Midi kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and DNA was stored at −20°C until amplified. Fragments containing each of the SNPs were individually amplified from genomic DNA by PCR using Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN). All amplifications were carried out in a PTC 200 thermal cycler (MJRe-search, Watertown, MA); PCR primers and cycling conditions for each of the SNPs are shown in Table 1. We used a PSQ 96 (Pyrosequencing AB, Westborough, MA) to identify polymorphisms at each of the three sites using PCR products amplified from genomic DNA. Pyrosequencing relies upon the energy that is made available by pyrophosphate production upon allele-specific chain elongation. This energy is used to enzymatically cleave a targeted substrate and produce light, which is detected and scored in an automated fashion. To confirm the molecular haplotypes (SNPs located on the same chromosome), cloned DNA fragments from heterozygous subjects were genotyped. Briefly, a 515-base pair DNA fragment encompassing the three SNPs was amplified from genomic DNA samples. PCR was performed using 1 unit of Fast Start DNA Taq Polymerase (Roche), 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 10 pmol of each primer. PCR product (6.7 ng) was cloned into a pCRII vector using a TA Dual Promoter Cloning kit (Invitrogen, Carlsbad, CA). Plasmids were purified using an alkaline lysis mini prep protocol and were sequenced by the chain termination method

using a primer specific for the T7 promoter (10). Sequencing reaction products were resolved on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Assay for LPS-stimulated leukocyte IL-6 production

Whole blood from the healthy volunteers was collected into EDTA Vacutainer tubes. Assays were performed after diluting the blood samples 9:1 in RPMI-1640 media supplemented with glutamine. Aliquots of 2 mL of diluted blood per well were equally divided into LPS-stimulated (5 ng/mL *Escherichia coli* LCD25; List Biological Laboratories, Campbell, CA) and nonLPS groups in 24-well plates (Corning Incorporated, Corning, NY). Each experimental condition was conducted in triplicate. Plates were then incubated in 5% CO_2 for 24 h at 37°C. Supernatants were then harvested and stored at −80°C until they were assayed for IL-6. These experiments were followed by a second set in which blood under similar conditions was incubated with a range of LPS concentrations (0, 0.05, 0.5, 5.0, and 50 ng/mL).

EMSA

Peripheral blood mononuclear cells were isolated using a two-step procedure with single gradients at each step (11). Specifically, we followed an initial enrichment over a Ficoll-Paque gradient (density = 1.070 g/mL), with a second step using a slightly hyperosmolar Percoll gradient (density = 1.064 g/mL), as described below. From each donor, 15 mL of venous blood was collected (EDTA; Becton Dickinson, Franklin Lakes, NJ), diluted 1:1 with cold phosphate-buffered saline (PBS), added to 25 mL of Ficoll-Paque (Pharmacia Research, Piscataway, NJ), and centrifuged at 400*g* for 30 min at room temperature. Suspended mononuclear cells were washed with PBS and centrifuged for 10 min at 400*g*. The cell pellet was transferred into the Percoll gradient and centrifuged for 15 min at 400*g*. The mononuclear cells (98% viability by trypan blue exclusion) were suspended in 1 mL of RPMI-1640 media supplemented with glutamine and antibiotics and were cultured for 48 h in humidified 5% CO_2 at 37°C. The cells were then washed twice with serum-free RPMI, suspended in 10 mL of RPMI 1640, and stimulated with LPS (1μg/ml) for 30 min. After centrifugation at 1500*g* for 5 min, the cell pellet was transferred to a 1.5-mL Eppendorf tube and was suspended in 1 mL of cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 tablet of protease inhibitors [Roche], and 1 mM dithiothreitol [DTT]). After 15 min, 67 μL of Nonidet NP-40 (Sigma, St Louis, MO) was added and cells were vortexed for 30 s. The resulting homogenate was centrifuged for 30 s in a microfuge, the supernatants were removed, and the nuclear pellet was suspended in 100 μL of ice-cold buffer B (20 mM Hepes, pH 7.9, 0.4 M KCL, 1 mM EDTA, 1 mM EGTA, and 1× protease inhibitors). The tube was vigorously rocked at 4°C for 45 min on a shaking platform. The nuclear protein extracts were centrifuged (16,000*g*) for 5 min at 4°C and quantified (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA) (12).

We used the following single-stranded oligonucleotides (Invitrogen), which differed only at the nucleotide 8 base pairs from the 5′ end:

IL-6 −174 C; -181 5′GTCTTGC**C**ATGCTAAAGGACG3′-161

IL-6 −174 G; -181 5′GTCTTGC**G**ATGCTAAAGGACG3′-161

Complementary strands were annealed by mixing 2 μg of each oligonucleotide and 6 μL of 10× annealing buffer (500 mM Tris, 100 mM $MgCl_2$, and 50 mM DTT) in a 60-μL reaction, and then heated in a water bath for 6 min at 100°C and allowed to cool to room temperature. The reaction was conducted in a 20-μL volume containing 7 μg of nuclear protein extract, 1 μg of poly (dl-dC) (Sigma), 4 μL of 5× binding buffer [60 mM HEPES, 7.5 mM $MgCl_2$, 300 mM KCl, 1 mM EDTA, 2.5 mM DTT, 50% glycerol, and 4-(2-aminoethyl)-benzenesulphonyl

fluoride hydrochloride] and 25×10^4 cpm of ^{32}P-labeled oligonucleotide probe. The gel was run at 120 V for 90 min at room temperature. Competition was done with a 100-fold excess of unlabelled probe. Each gel was digitized and densitometry was measured using a ChemiImager 4400 (Alpha Innotech Corporation, San Leandro, CA).

Data presentation and statistical analyses

LPS-stimulated IL-6 production is presented as medians with the range in parenthesis. The IL-6 response to LPS challenge was compared across haplotypes and −597/−174 genotypes using nonparametric (Kruskal-Wallis *H* test) and parametric (analysis of covariance) methods. Analysis of covariance was used to adjust for blood monocyte count, and subject age and gender, as there is no nonparametric alternative for multivariate analysis. In the second set of experiments, the IL-6 response to increasing LPS exposure in subjects homozygous for haplotype 1 (G-G-G) was compared with the response observed in individuals that were homozygous for haplotype 3 (A-G-C) using repeated measures analysis of variance (ANOVA). Densitometry results were compared using ANOVA. *Post hoc* comparisons were done using the Bonferroni correction for multiple comparisons.

RESULTS

Frequency of IL-6 promoter haplotypes

Forty-nine healthy Caucasian subjects provided blood samples for stimulation experiments and DNA isolation. There were 24 females and 25 males and the median age was 31 years (range of 21–59 years). There were 29 subjects homozygous for guanine at both sites and seven subjects homozygous for adenine at −597 and cytosine at −174. Molecular haplotyping of the heterozygous subjects indicated that the −597 and −174 SNPs were in linkage disequilibrium. The −597A and −174C alleles occurred together and the −572 C-allele always occurred in the presence of guanine at nucleotides −597 and −174. Therefore, we observed only three of the eight theoretically possible haplotypes. The haplotype breakdown for the 98 chromosomes is shown in Table 2. Haplotype 2, defined by cytosine at the −572 position (G-C-G), was present in 14 (28%) of the subjects and 20 subjects (41%) carried haplotype 3 (A-G-C). There were three individuals who carried haplotype 2 and 3 (and are therefore represented in both haplotype 2 and 3 totals listed above).

Ex vivo LPS-stimulated IL-6 production by leukocytes is related to promoter haplotype

The median supernatant IL-6 concentration after LPS stimulation was 2737 pg/mL (range of 981-4766 pg/mL). In the absence of LPS, the supernatant IL-6 concentrations were less than 100 pg/mL in all cases and were not detectable in most cases. Supernatant IL-6 concentration after LPS stimulation was associated with haplotype. As shown in Figure 1A, there was no difference according to haplotype pairs (overall P value = 0.11 by Kruskal-Wallis) and the −572 variant was not associated with IL-6 release after controlling for the other SNPs ($P = 0.7$ by Kruskal-Wallis). We also analyzed the data according to the number of copies of haplotype 3 carried by the subjects. Blood from haplotype 3 homozygotes produced the least and blood from individuals homozygous for the wild-type variant produced the greatest amount of IL-6 after LPS stimulation (Fig. 1A; $P = 0.019$ by ANOVA, testing for a linear association between the number of copies of haplotype 3). We then controlled for the effects of age and gender and blood monocyte counts and observed that carriage of the haplotype number 3 remained associated with IL-6 production ($P = 0.03$ by analysis of covariance).

To further characterize the differences in IL-6 production according to genotype, we conducted additional experiments using blood obtained from 16 additional subjects homozygous for haplotype 1 (n = 9) or haplotype 3 (n = 7). Blood was exposed to a range of LPS concentrations (0, 0.05, 0.5, 5.0, and 50 ng/mL) for 24 h to determine whether the differences observed in the

previous experiments were dependent upon the LPS exposure level (Fig. 2; overall P value < 0.001 by repeated measures ANOVA).

Oligonucleotides containing guanine at the −174 position have greater affinity for nuclear protein that those containing cytosine

Nuclear protein from LPS-stimulated monocytes bound more avidly to the G-containing oligonucleotide than the C-containing oligonucleotides (Fig. 3, lanes 3 and 4; $P < 0.001$ by repeated measures ANOVA). Competition with cold G-containing probe (Fig. 3, lane 5) more effectively blocked hot G-probe binding than did cold C-containing probe (Fig. 3, lane 6; $P < 0.001$ by repeated measures ANOVA). Additionally, a nonspecific oligonucleotide failed to block DNA-protein complex formation (data not shown). To determine whether this region interacts with nuclear transcription factors that are considered important in IL6 expression, we conducted additional gel shift assays using antibodies to NF-κB, NF-IL6 (CEB/P), and c-JUN/AP-1 (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). There was no evidence of supershifting of the complex with any of these antibodies, suggesting that these transcription factors are not involved with IL-6 gene activation at this particular site.

DISCUSSION

During inflammation, IL-6 plays an important role in the initiation of the acute phase and other systemic responses (13). Locally, IL-6 is also considered to exert an effect by influencing inflammatory, immune, and coagulation processes (14). Because IL-6 is rapidly cleared from the circulation and is not stored in cells, its presence in the bloodstream requires gene transcription and new protein synthesis. The expression of IL-6 mRNA involves synergistic interaction of a number of transcription factors with the IL-6 promoter, including NF-κB and NF-IL6. Binding sites for these factors are present in close proximity to nucleotide −174 in the promoter (8). Polymorphisms, such as the −174 G →C SNP, which is located adjacent to the negative regulatory domain (nucleotides −173 to −151 relative to the transcription start site) and in close proximity to an NF-IL6 binding motif, seem to result in interindividual variation in IL-6 transcription and expression. Clinical studies have implicated the altered IL-6 response that is associated with the −174 G →C SNP in the risk and outcome from a variety of conditions (4,5,15,16). However, individual SNPs do not exist in isolation, but are genetically linked, forming distinct haplotypes. For example, 13 SNPs in the β2-adrenergic receptor were organized into only 12 (of a theoretically possible 8192) haplotypes (9). Therefore, phenotypic responses may depend upon the interactions of multiple SNPs closely linked on the same chromosome (molecular haplotype) (8,9,17). Our findings confirm the existence of SNP haplotypes in the IL-6 promoter and indicate that the −174 G →C SNP is an important contributor to reduced endotoxin-stimulated IL-6 release.

The potential for complex interaction among multiple SNPs in the IL-6 promoter was shown in a series of transfection experiments that studied the three SNPs examined here and an additional repeat sequence located in the −373 to −392 region (8). Terry et al. (8) showed that the importance of IL-6 promoter haplotypes might be related to cell type. There was little difference among promoter haplotypes in HeLa cells that were transfected with short (−211 to +31) or longer (−641 to +31) constructs. There were differences in luciferase activity observed when ECV304 cells were transfected with the longer constructs that included polymorphic sites representing the sites studied here with the highest activity seen in constructs containing the −174 G allele. Furthermore, the most marked differences that they observed were between the promoter constructs representing the −174G or −174C alleles, when all other polymorphic sites were kept constant. Using longer constructs (−550 to + 51) Fishman et al. (4) showed differences in transcription between −174G and −174C constructs, suggesting that the

influence of a given SNP on gene transcription is dependent upon the genetic context of the SNP.

We observed that blood from individuals homozygous for haplotype 1 produced the greatest amount of IL-6. We also found that variation at −572 was not associated with endotoxin stimulated IL-6 release. All of the −174 C alleles were completely linked to the −597 A allele according to our genotyping of the cloned DNA fragments. This is in slight contrast to the findings of Terry et al. (4) who observed three of 182 subjects to carry either the −597G/−174C or −597A/−174G combinations—haplotypes that we did not observe. Nevertheless, our observations, combined with those of Terry et al. (4), indicate that these haplotypes are rare.

According to EMSA, the −174 C allele less avidly binds nuclear protein, indicating that this site may contribute to the observed differences in endotoxin-stimulated IL-6 production. Others have observed that naturally occurring SNPs in the 5′ regions of other cytokine genes, such as IL-1β and TNF-α have been associated with altered nuclear protein binding, findings that have been related to the incidence of conditions such as gastric cancer and severe malaria (18,19). Our observation of greater DNA-protein interaction with G-containing oligonucleotides than with C-containing oligonucleotides is consistent with the *in vitro* findings of Fishman et al. and Terry et al. (4,8) and suggests that increased DNA-protein interaction in this region is associated with increased IL-6 cytokine production. Therefore, although it is possible that the other SNPs examined here interact to alter IL-6 production in complex ways, the G →C SNP at −174 appears to contribute, in part, to the differences observed here. It is possible that our observations would differ had we used oligonucleotides of different lengths. The polymorphism does not appear to alter binding to three transcription factors that influence IL-6 transcription. NF-κB, c-JUN/AP-1, and NF-IL6 were not detected using antibodies to these proteins in supershift assays. This is perhaps not surprising, as this region does not contain known consensus sequences for these factors. However, the G-containing allele does have some homology to the GATA-1 consensus sequence (20). Although the GATA family of transcription factors is important in erythroid cell development, their role in leukocyte gene transcription is less well understood and seems limited to eosinophil and TH2 lymphocyte development (21).

A recent study examined LPS-induced IL-6 production relative to the −174 genotype and observed that blood from heterozygotes produced more IL-6 than either GG or CC homozygotes (22). Such an observation is difficult to explain and may reflect a type-1 statistical error. Alternatively, uncharacterized SNPs may have influenced these reported observations. It is also possible that the choice of experimental conditions, such as the endotoxin exposure level and duration, were not ideal to detect subtle genetic differences in the IL-6 response. Our observed differences in IL-6 production, only at the highest concentrations (5 and 50 ng/mL) in a range of LPS exposures, suggest that genetic differences may only influence responses at certain exposures, at least *in vitro*.

There is relatively little information regarding the association between these promoter polymorphisms and acute infectious/inflammatory conditions such as severe sepsis or septic shock, conditions in which IL-6 is considered and important mediator and outcome correlate (23). One recent series of 50 patients with sepsis implicates the −174 C allele SNP as a risk factor for poor outcome after sepsis (5). Thus, in the face of moderate to severe critical illness, a genetically determined decreased capacity to produce IL-6 may confer a survival disadvantage.

The region of the IL-6 promoter encompassed by these SNPs also contains an A_nT_n repeat region running from −373 to approximately −392 that may contribute to gene transcription rates *in vitro* (8). We observed four variant forms (A_8T_{12}, A_9T_9, $A_{10}T_{10}$, and $A_{10}T_{11}$) in the

sequenced individuals that did not appear to be associated with LPS-stimulated IL-6 responses. Were we to sequence all individuals, we may have observed an association between IL-6 release and this variant repeat. However, after careful examination of the *in vitro* experiments of Terry et al. (4), the −174 SNP seemed to have considerable influence on gene transcription, and the tandem repeat did not appear to be as important, findings similar to ours.

Based upon our observations, IL-6 promoter haplotypes, defined by the −597 and −174 SNPs, are associated with endotoxin-stimulated IL-6 production *ex vivo*. The −572 SNP appears not to influence IL-6 production in whole blood leukocytes. Furthermore, these observed differences in IL-6 production may be partly due to decreased DNA-protein interactions with promoters containing cytosine at the −174 position. It may be sufficient to interrogate the −174 variant alone in studies of clinical associations with IL-6 promoter polymorphisms.

References

1. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 H after the initiation of sepsis predicts mortality over 3 days. Shock 2002;17:463–467. [PubMed: 12069181]
2. Asai K, Hiki N, Mimura Y, Ogawa T, Unou K, Kaminishi M. Gender differences in cytokine secretion by human peripheral blood mononuclear cells: role of estrogen in modulating LPS-induced cytokine secretion in an *ex vivo* septic model. Shock 2001;16:340–343. [PubMed: 11699070]
3. Schroder J, Kahlke V, Book M, Stuber F. Gender differences in sepsis: genetically determined? Shock 2000;14:307–310. [PubMed: 11028548]
4. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 1998;102:1369–1376. [PubMed: 9769329]
5. Schluter B, Raufhake C, Erren M, Schotte H, Kipp F, Rust S, Van Aken H, Assmann G, Berendes E. Effect of the interleukin-6 promoter polymorphism (−174 G/C) on the incidence and outcome of sepsis. Crit Care Med 2002;30:32–37. [PubMed: 11902285]
6. Fernandez-Real JM, Broch M, Vendrell J, Gutierrez C, Casamitjana R, Pugeat M, Richart C, Ricart W. Interleukin-6 gene polymorphism and insulin sensitivity. Diabetes 2000;49:517–520. [PubMed: 10868978]
7. Rundek T, Elkind MS, Pittman J, Boden-Albala B, Martin S, Humphries SE, Juo SH, Sacco RL. Carotid intima-media thickness is associated with allelic variants of stromelysin-1, interleukin-6, and hepatic lipase genes: the Northern Manhattan Prospective Cohort Study. Stroke 2002;33:1420–1423. [PubMed: 11988625]
8. Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. J Biol Chem 2000;275:18138–18144. [PubMed: 10747905]
9. Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS, Nandabalan K, Arnold K, Ruano G, Liggett SB. Complex promoter and coding region β2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. Proc Natl Acad Sci USA 2000;97:10483–10488. [PubMed: 10984540]
10. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463–5467. [PubMed: 271968]
11. de Almeida MC, Silva AC, Barral A, Barral NM. A simple method for human peripheral blood monocyte isolation. Mem Inst Oswaldo Cruz 2000;95:221–223. [PubMed: 10733742]
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254. [PubMed: 942051]
13. Oldenburg HS, Rogy MA, Lazarus DD, Van Zee KJ, Keeler BP, Chizzonite RA, Lowry SF, Moldawer LL. Cachexia and the acute-phase protein response in inflammation are regulated by interleukin-6. Eur J Immunol 1993;23:1889–1894. [PubMed: 8344351]
14. Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunol Today 1997;18:428–432. [PubMed: 9293158]

15. Vickers MA, Green FR, Terry C, Mayosi BM, Julier C, Lathrop M, Ratcliffe PJ, Watkins HC, Keavney B. Genotype at a promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein. Cardiovasc Res 2002;53:1029–1034. [PubMed: 11922913]

16. Georges JL, Loukaci V, Poirier O, Evans A, Luc G, Arveiler D, Ruidavets JB, Cambien F, Tiret L. Interleukin-6 gene polymorphisms and susceptibility to myocardial infarction: the ECTIM study. Etude Cas-Temoin de l'Infarctus du Myocarde. J Mol Med 2001;79:300–305. [PubMed: 11485024]

17. Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G, Vovis GF. Haplotype variation and linkage disequilibrium in 313 human genes. Science 2001;293:489–493. [PubMed: 11452081]

18. El Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402. [PubMed: 10746728]

19. Knight JC, Udalova I, Hill AV, Greenwood BM, Peshu N, Marsh K, Kwiatkowski D. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet 1999;22:145–150. [PubMed: 10369255]

20. Merika M, Orkin SH. DNA-binding specificity of GATA family transcription factors. Mol Cell Biol 1993;13:3999–4010. [PubMed: 8321207]

21. Hirasawa R, Shimizu R, Takahashi S, Osawa M, Takayanagi S, Kato Y, Onodera M, Minegishi N, Yamamoto M, Fukao K, Taniguchi H, Nakauchi H, Iwama A. Essential and instructive roles of GATA factors in eosinophil development. J Exp Med 2002;195:1379–1386. [PubMed: 12045236]

22. Heesen M, Bloemeke B, Heussen N, Kunz D. Can the interleukin-6 response to endotoxin be predicted? Studies of the influence of a promoter polymorphism of the interleukin-6 gene, gender, the density of the endotoxin receptor CD14, and inflammatory cytokines. Crit Care Med 2002;30:664–669. [PubMed: 11990931]

23. Bernard GR, Wheeler AP, Russell JA, Schein R, Summer WR, Steinberg KP, Fulkerson WJ, Wright PE, Christman BW, Dupont WD, Higgins SB, Swindell BB. The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. N Engl J Med 1997;336:912–918. [PubMed: 9070471]

Fig. 1. LPS-stimulated whole blood leukocyte production of IL-6

Whole blood from healthy individuals was exposed to endotoxin for 24 h as detailed in "Materials and Methods." The results of IL-6 release from blood obtained from 49 subjects and stimulated with 5 ng/mL LPS are shown according to haplotype (A) and according to the number of copies of haplotype 3 (B), which is defined by the presence of adenine at −597, guanine at −572, and cytosine at −174. IL-6 release was inversely related to the number of copies of haplotype 3.

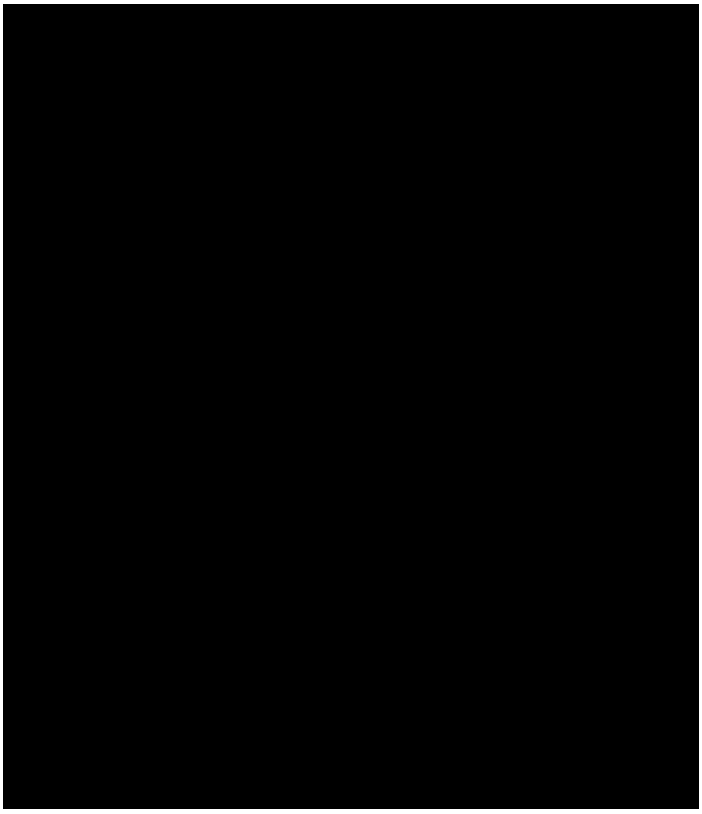

Fig. 2. Dose-response comparison of IL-6 responses to LPS stimulation

In a subsequent series of experiments, LPS-stimulated IL-6 production from subjects homozygous for haplotype 1 and subjects homozygous for haplotype 3 was compared over range of LPS exposures (0, 0.05, 0.5, 5.0, and 50 ng/mL). The IL-6 responses were similar at the lower LPS exposures but differed between genotype at the two highest exposures.

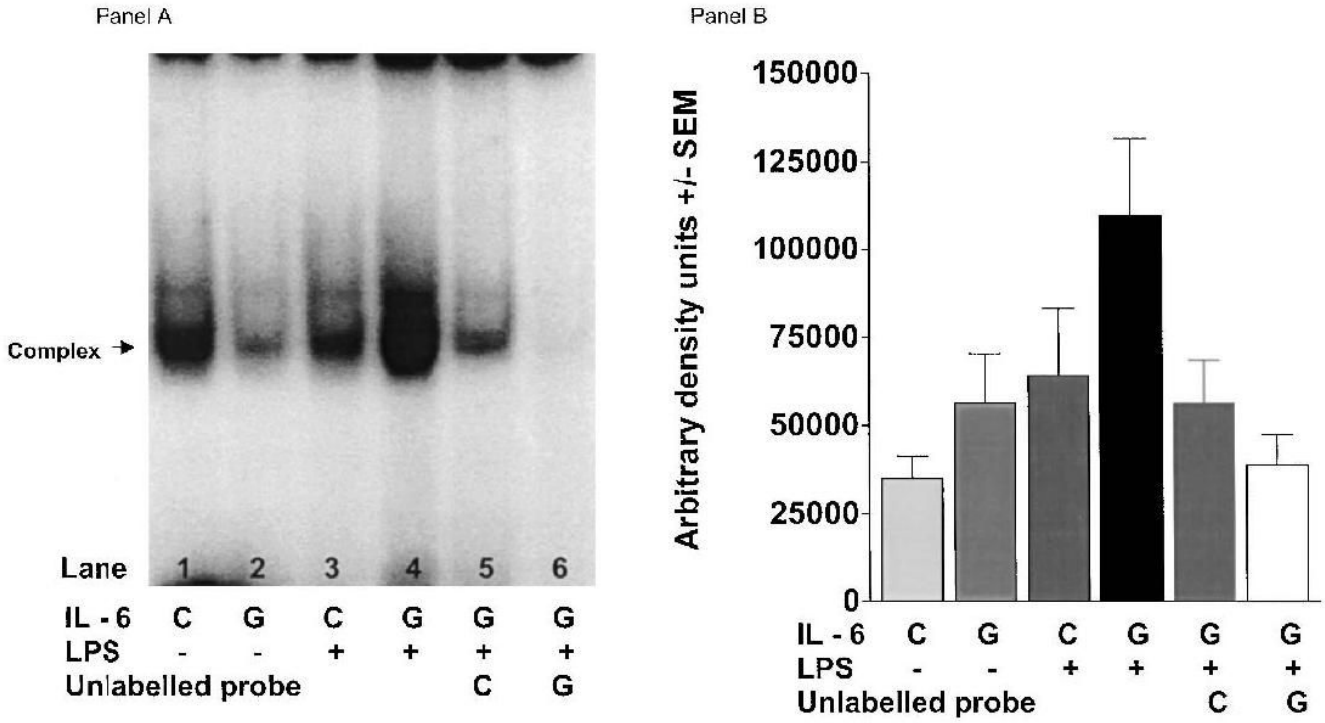

Fig. 3. EMSA for nuclear protein binding to oligonucleotides representing the IL-6−161 to −181 region.

Competition EMSA examining DNA-protein interactions with sequences representing the IL-6 −174-promoter region showed different binding patterns between the C- and G-bearing alleles. (A) The results of an experiment with nuclear protein isolated from monocytes from a single individual; (B) the overall results of experiments using monocyte nuclear protein from eight individuals.

Table 1

Primers and polymerase chain reaction conditions for Interleukin-6 promoter

Locus	PCR product length and concentrations	Primers	Cycling conditions
−174 G →C	111 nucleotides	Forward: 5′-CGCTAGCCTCAATGAC; reverse (biotinylated): 5′-CGGGTGGGGCTGATTGGAA; sequencing: 5′-CCCTAGTTGTGTCTTGC	95°C for 5 min, then 35 cycles of 95°C for 45 s and 55°C for 30 s, 74°C for 45 s followed by 74°C for 6 min
−572 G →C	158 nucleotides	Forward: 5′-GGAGACGCCTTGAAGTAAC: reverse (biotinylated): 5′-CTCTGACTCCATCGCAGCC: sequencing: 5′-GCAGTTCTACAACAGCC	95°C for 6 min, then 30 cycles of 95°C for 45 s and 55°C for 30 s, 74°C for 45 s followed by 74°C for 6 min
−597 G →A	158 nucleotides	Forward: 5′-GGAGACGCCTTGAAGTAAC; reverse (biotinylated): 5′-CTCTGACTCCATCGCAGCC, sequencing: 5′-GCAGTTCTACAACAGCC	95°C for 5 min, then 35 cycles of 95°C for 45 s and 55°C for 30 s, 74°C for 45 s followed by 74°C for 6 min
Promoter region for cloning	515 nucleotides	Forward: 5′-ACCTGGAGACGCCTTGAAG: reverse: 5′-GGGCTGATTGGAAACCTT	95°C for 5 min, then 35 cycles of 95°C for 55 s and 55°C for 30 s, 74°C for 45 s followed by 74°C for 6 min

Table 2

Haplotype frequencies

Haplotype 1 G-G-G	Haplotype 2 G-C-G	Haplotype 3 A-G-C
55 (56%)	16 (16%)	27 (28%)

Haplotype refers to the base combination at −597, −572, and −174, and the loci are indicated from left to right in descending order. The values are of a total of 98 chromosomes represented in the 49 subjects.