A three base pair gene variation within the distal 5'-flanking region of the *interleukin-10 (IL-10)* gene is related to the *in vitro* IL-10 production capacity of lipopolysaccharide-stimulated peripheral blood mononuclear cells

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ABSTRACT. Interleukin-10 (IL-10) is an important multifunctional immunmodulator. There is evidence that IL-10 secretion is associated with certain genetic elements of the proximal *IL-10* gene 5'-flanking region. The allelic and genotypic comparison of IL-10 expression by lipopolysaccharide (LPS)- stimulated leukocytes (PBMC) with a recently discovered distal "indel" DNA-sequence variation at – 7400 bp revealed significant inter-individual differences in the IL-10 *in vitro* production capacity. Homozygotes lacking the three base pairs "GGA" (– 7400del) at this gene locus are characterised by high expression of IL-10 with a median of 1690pg/ml ($P \leq 0.009$). The allelic comparison supports this finding ($P \leq 0.002$). Further analysis of the haplotype – 7400/– 1087 showed that homozygotes for – 7400del/– 1087G may be classified as very strong IL-10 responders with a median IL-10 secretion of 2378 pg/ml ($P \leq 0.025$). When leukocytes were stimulated *in vitro* by dibutyryl-cAMP or infected with Epstein-Barr virus no significant inter-individual differences between the – 7400indel alleles or genotypes and the IL-10 *in vitro* production capacity were observed. Our findings further the understanding of the complexity of *IL-10* gene regulation in relation to defined regulatory gene variations.

Keywords: EBV, LPS, cAMP, interleukin-10, – 7400indel

INTRODUCTION

IL-10, an important cytokine in man, exerts a wide range of immuno-modulatory capacities [1]. It is involved in the regulation of inflammatory responses [2], human autoimmune diseases [3, 4] and other immune-associated processes [5-9].

Increasing attention has been drawn to the role of host variations in cytokine profiles in inflammatory and immune responses. Recent reports have shown that promoter polymorphisms of the *IL-10* gene may affect the cytokine expression profile of *IL-10* as well as the outcome or cure of disease Several studies have reported associations between *IL-10* promoter polymorphisms and risk of a diverse range of diseases including (for example) asthma, systemic lupus erythematosus, and rheumatoid arthritis, infectious diseases as well as transplantation complications [5, 9-23]. Polymorphisms in the promoter of the *IL-10* gene and their genetic variability are associated with inter-individual changes in *IL-10* production after stimulation with lipopolysaccharide (LPS) [13, 24-26]. Recent characterisation of the 5'-flanking region of the *IL-10* gene revealed a high number of single nucleotide polymorphisms (SNPs) as well as several short tandem repeats including the CA-dinucleotide repeats IL10.G & R. an allelic variation (inverted repeat IL10.IR) and an insertion/deletion of the 5'-flanking region [18, 16, 26-32]. So far the SNPs at – 597, – 824, – 1087 and the microsatellites IL10.G & R have been associated with changes in IL-10 secretion. Investigations of more distal promoter elements have also revealed correlations with IL-10 secretion [26].

In order to investigate the influence of far distal elements of the IL-10 promoter in relation to the IL-10 production capacity, the recently discovered – 7400indel DNA sequence variation, where a "GGA" is affected was analysed in detail here. We performed *in vitro* stimulation assays using LPS- or dibutyryl-cAMP (dbcAMP) -stimulated peripheral blood mononuclear cells (PBMCs) and EBV-

immortalised B-lymphocytes (LCL) from healthy blood donors (Caucasian).

DONORS AND METHODS

DNA Blood Samples, Isolation of DNA

All Caucasian samples were taken with no regard to sex or age and donors were free of any chronic diseases. The DNA of donors from Stuttgart, Germany was isolated from buffy coats obtained from the blood donation centre of Stuttgart.

DNA was purified from 200 µL whole blood using the Qiagen blood kit according to the manufacturer (Qiagen, Hilden, Germany) as described previously [33, 34].

PCR, electrophoresis and fragment analysis

The frequency of the DNA-sequence variation at – 7400bp was analysed using one fluorescent NED labelled primer "IL-10-7400 INDEL-NED" (Applied Biosystems, Perkin-Elmer, Foster City, CA) 5'-GAA GGA ACA TCT GAG CTG AGA GCT-3' together with a standard primer "IL-10-7400 INDEL" (Eurogentec, Seraine, Belgium) 5'-TTG AAC TCC TAG CTC AAG TAA TCC T-3' as described previously [33, 34].

The quality and amount of PCR products was estimated on a 3% Metaphor Agarose (FMC) gel in 0.5 × TBE using ethidium bromide to visualise DNA and a 100 bp length standard (Gibco BRL, Breda, The Netherlands). The samples were then mixed in the appropriate dilution with a loading buffer containing bromphenol blue, formamide and a CXR-labelled length standard (Promega, Woods, Madison, USA). The length standard visualised as red signals has a band spacing of 20 bp in the range between 60 to 200 bp and a spacing of 25 bp between 200 to 400 bp. 1.5 µL of the diluted amplimer/loading buffer mixture was analysed on a 4.5% denaturing acrylamide gel as recommended by the manufacturer of the sequencer (ABI 377: Applied Biosystems, Perkin-Elmer, Foster City, CA). For detection of the PCR product from NED-labelled primer (yellow colour) the filter set D was used. Data were collected and processed using GENESCAN software according to the manufacturer. The electropherogrammes generated from the processed gel images were in addition manually assessed for accurate size determination and the estimation of quality of the allelic peaks. Fragment Length Polymorphism-PCR (FLPPCR) was verified by conventional PCR and sequenced as described elsewhere [29]. In further analysis only samples were included where the intensity of the PCR-products gave relative fluorescence units between 500 and 4000 for the main peak.

The fragment length data were analysed using Microsoft Excel and afterwards by Arlequin software (http://www.bioinf.mdc-berlin.de/projects/hap/) [35, 36]. The statistical significance of Hardy-Weinberg differences and genotype frequencies were determined using exact test procedures by Arlequin software; allelic description used for IL-10 – 7400indel was: no deletion – 7400in (allele 01); three nucleotide deletion "GGA" – 7400del (allele 02) (– 7400del also called allele 02 for computer based analysis (two-digit code)) [33].

Cell culture and stimulation of PBMCs

PBMCs (Peripheral blood mononuclear cells) were isolated from buffy coats obtained from the blood bank in Stuttgart, Germany using Ficoll separation solution (Ficoll-Paque Plus, Pharmacia Biotech, Freiburg, Germany). Cells were centrifuged at 3 300 rpm for 10 min to separate white and red blood cells from plasma. The plasma was removed, white cells taken and overlaid with Ficoll solution. After centrifugation the cells were washed twice with PBS (Sigma, Deisenhofen, Germany).

The isolated PBMCs were counted and resuspended in RPMI 1640 cell culture medium (Sigma) at a concentration of 10^6 cells/mL completed with 1% of Penicillin, Streptomycin, Glutamin (Sigma) and 10% FCS (Sigma) and cultured in 24 well cell culture plates (Costar, USA) for 24 hours at 37°C and 5% CO_2. After 24 hours the cells were centrifuged at 2 200 U/min for 10 min at 4 °C. The supernatants were taken and frozen at – 80°C.

Cells suspended in 1 mL media at a concentration of 10^6 cells/mL were stimulated in 1mM cAMP (dibutyryl-cyclic-adenosine-monophosphate) respectively 1 µg/mL LPS (lipopolysaccharide) (both Sigma) in medium mentioned above.

Infection of PBMCs with Epstein-Barr-Virus (EBV)

The marmoset-cell line B95-8 was raised to a density of 10^6 cells/mL in the same media as stated above for PBMCs for 3 days. Cells were centrifuged at 6 000 × *g* and supernatant containing Epstein-Barr-virus (EBV) was used for *in vitro* infection of B lymphocytes. 1×10^7 PBMCs were incubated with 1 mL of EBV containing supernatant for 1 hour at 37 °C. PBMCs were collected and grown for 7 days at 37 °C and 5% CO_2 in complete RPMI 1640 cell culture medium at a concentration of 10^6 cells/mL. At day seven Cyclosporin-A at 1 µg/mL was added together with fresh complete RPMI 1640. PBMCs were grown for an additional 7 days. Afterwards lymphoblastoid cells were expanded for two more weeks and analysed three times for the amount of IL-10 produced within 24 h.

Cytokine measurement by ELISA

The concentration of IL-10 in the cell supernatants was measured by ELISA (Enzyme Linked Immunosorbant Assay) (IL-10 Eli-pair Trinova Biochem GmbH, Giessen) according to the manufacturer's instructions. The ELISA was performed on 96 well plates (Nunc). IL-10 concentrations were detected by a DigiScan ELISA-Reader (Asys Hightech) at 450 nm wavelength.

RESULTS

The allele – 7400del is related to high IL-10 production

Recently we described the – 7400indel gene variation and showed that the allele – 7400in is present with a frequency of 0.75 in Caucasians [29]. This allele was found in 58% of healthy donors in the homozygous state, whereas the allele – 7400del was less frequent at 8% in homozygotes.

To analyse the influence of certain alleles of this gene locus on the capacity of PBMCs to produce IL-10, leukocytes from healthy Caucasian blood donors were isolated by

Ficoll separation and stimulated *in vitro* with dbcAMP or LPS. Both reagents were used because it is already known that there are interindividual differences for LPS-dependent IL-10 expression and that IL-10 expression is regulated in a cyclic-AMP dependent manner [24-27, 37, 38]. The amount of IL-10 produced within 24 h was measured by ELISA and assessed as a function of the alleles of the – 7400indel gene locus *(Figure 1)*.

In addition, as known from our own studies, EBV-positive Burkitt lymphoma cells and EBV immortalised B lymphocytes are characterised by constitutive IL-10 expression [27, 28]. Therefore lymphoblastoid cell lines (LCL) were established by infection of B lymphocytes *in vitro* with the EBV strain B95/8. The amount of IL-10 produced by these LCLs was analysed 4, 6 and 8 weeks after infection and compared to the – 7400indel polymorphism.

LPS stimulated PBMCs of carriers of the allele with the deletion of the three nucleotides "GGA" at – 7400del (allele 02; – 7400del) produced a median of 1 099 pg/mL IL-10 compared with 682 pg/mL for carriers of the allele – 7400in (allele 01; allele – 7400in; carrying additional "GGA") *(Figure 1B)*. For dbcAMP the median amounts of IL-10 were 113 pg/mL and 140 pg/mL respectively *(Figure 1A)*, whereas the IL-10 expression of LCLs with these 2 alleles was similar: 390 pg/mL and 454 pg/mL respectively *(Figure 1C)*. The interindividual differences in IL-10 expression between the alleles of the – 7400indel gene locus were significant only when cells were stimulated with LPS, with an observed p-value of 0.002.

The genotype – 7400del/del is related to high IL-10 production

For the detection of a possible relationship between the genotypes of the – 7400indel gene locus and the capacity of PBMCs to produce IL-10, the amount of IL-10 produced after stimulation with LPS, dbcAMP or infection with EBV was compared as a function of the corresponding genotypes.

A statistically significant inter-individual difference in IL-10 secretion was found when comparing homozygous carriers of the allele 01 to carriers heterozygous (0102) or homozygous for the allele 02 *(Figure 2B)*. PBMCs of homozygotes carrying allele 02, stimulated with LPS, were characterised by a high production capacity of IL-10 (median = 1 690 pg/mL). The corresponding heterozygotes had a median IL-10 amount of 945 pg/mL, while homozygotes for allele 01 were found to produce lower IL-10 amounts at 563 pg/mL ($P < 0.005$) *(Figure 2B)*.

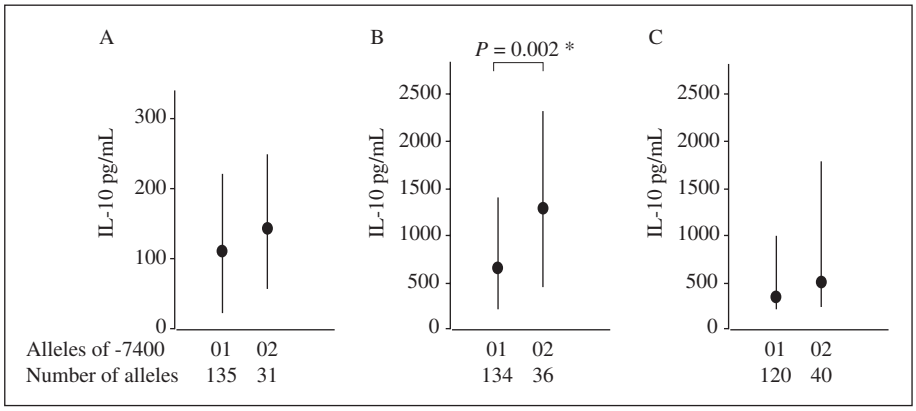

Figure 1

Expression of IL-10 in relation to the alleles of the – 7400indel gene variation of the 5'-flanking region of the *IL-10* gene.
PBMCs from healthy Caucasian blood donors were *in vitro* stimulated by dbcAMP (A), LPS (B), or immortalised by EBV (C) as described in Methods. Shown are the median and the semi-interquartile ranges of the IL-10 amount. Allele 01 represents the gene variation without deletion, while allele 02 represents the deletion at – 7400. *Mann-Whitney U-test.

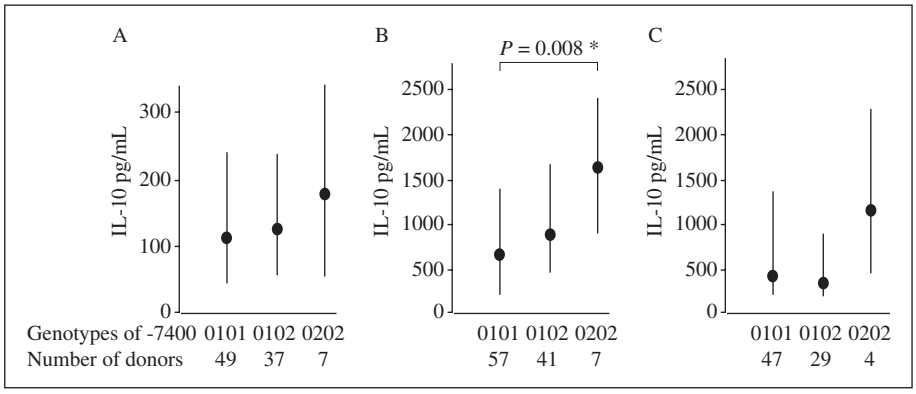

Figure 2

Expression of IL-10 in relation to the genotype of the – 7400indel gene variation of the 5'-flanking region of the *IL-10* gene.
PBMCs from healthy Caucasian blood donors were *in vitro* stimulated by dbcAMP (A), LPS (B), or immortalised by EBV (C) as described in Methods. Shown are the median and the semiinterquartile ranges of the IL-10 amount. 01 represents the gene variation without deletion, while 02 represents the deletion at – 7400. * Kruskal-Wallis test

Median IL-10 secretion of dbcAMP stimulated PBMCs was 112 pg/mL for homozygotes with the allele 01, 134 pg/mL for heterozygotes (0102) and 168 pg/mL for homozygotes with the allele 02 *(Figure 2A)*. These differences, however, were not significant, but may indicate, as seen with LPS, a dominant expression pattern for the allele – 7400del *(Figure 2B)*.

For LCLs the median IL-10 secretion of heterozygous carriers – 7400in/del was 400 pg/mL, a level similar to that of homozygous carriers – 7400in expressing 343 pg/mL *(Figure 2C)*. LCLs homozygous for the deletion – 7400del produced high amount of IL-10, with a median of 1191 pg/mL, although this was statistically not significant, probably due to the small sample number of LCLs carrying the genotype 0202 (n = 4).

– 7400del/– 1087G are related to high IL-10 production in vitro

In a number of studies it has been shown that proximal SNPs are related to the interindividual differences in the *in vitro* capacity to produce IL-10 [25, 24]. In addition there are indications that distal parts of the promoter are involved in this genetic network affecting IL-10 production [26]. Therefore we tested whether the proximal gene variation at – 1087 within the 5' flanking region of the *IL-10* gene together with the – 7400indel gene variation may be part of a haplotype reflecting potential co-operation of distal and proximal gene loci.

The haplotype – 7400in/– 1087A was found with the highest frequency (48%), whereas the haplotype – 7400del/– 1087A was very rare (1%). The genotypic characterisation of this haplotype revealed an absence of homozygous – 7400del/– 1087A; all variations were found to be in Hardy-Weinberg-Equilibrium *(Table 1)*.

In order to answer the question about potential co-operation of distal and proximal gene loci genotypes – 7400indel/– 1087AG were related to the IL-10 *in vitro* production capacity after LPS stimulation. The genotype – 7 400/– 1 087 0101/AA was found to be related to a low median level of IL-10 of 442 pg/mL *(Figure 3)*. For the genotype – 7400/– 1087 0101/GG the median IL-10 level was 1 084 pg/mL. The genotype – 7400/– 1087 0202/GG was found to be related to a very high IL-10 level of 2378 pg/mL. Donors with other genetic backgrounds were characterised by a median IL-10 secretion of 812 pg/mL. These findings were statistically significant with $p \leq 0.025$ *(Figure 3)*. The – 1087AG gene variation in our study was related in a similar manner to IL-10 expression after LPS stimulation of PBMCs *in vitro* as published by other groups in that the – 1087G allele and – 1087G homozygotes were characterised by high IL-10 *in vitro* production capacity (data not shown) [24].

Table 1
Allele, Genotype and Haplotype frequencies of healthy Caucasian blood donors for the – 1087 and – 7400 gene loci of the 5'-flanking region of the *IL-10* gene. All frequencies are corresponding to the Hardy-Weinberg-equilibrium.

			Frequency
Alleles			
	– 1 087	A	51
	(*n* = 334)	G	49
	– 7 400	01	75.3
	(*n* = 372)	02	24.7
Haplotype			
		01A	48.8
	– 1 087/– 7 400	01G	34.7
	(*n* = 334)	02A	1.2
	02G		15.3
Genotypes			
		AA	26.3
	– 1 087	AG	47.5
	(*n* = 167)	GG	26.3
		0101	59.6
	– 7 400	0102	33.3
	(*n* = 186)	0202	7
	1 087/– 7 400	0101AA	25.2
	(*n* = 167)	0101AG	24.6
		0101GG	10.2
		0102AA	0.6
		0102AG	20.4
		0102GG	12.6
		0202AA	0
		0202AG	1.8
		0202GG	4.8

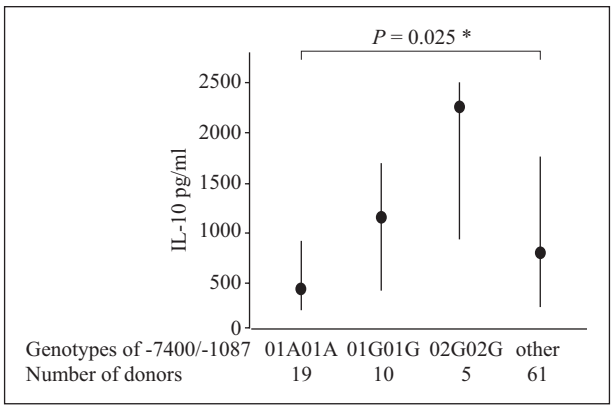

Figure 3
Expression of IL-10 in relation to the genotypes of the – 7400indel/– 1087 gene loci.
PBMCs from healthy Caucasian blood donors were *in vitro* stimulated by LPS as described in Methods. Shown are the median and the interquartile ranges. 01 represents the allele without deletion, while 02 represents the allele with the deletion; A represents the – 1087A allele, while G represents the – 1087G allele of the 5'-flanking region of the *IL-10* gene. * Kruskal-Wallis test.

DISCUSSION

It is known that proximal polymorphisms of the 5'-flanking region of the *IL-10* gene are related to IL-10 *in vitro* production capacity when cells are stimulated with Concanavalin-A or LPS. The haplotype – 1087/– 824/ – 597 GCC is related to high IL-10 and the haplotype – 1087/– 824/– 597 ATA is characterised by low IL-10 production *in vitro* [24-26]. More distal SNPs have been also been related to inter-individual differences in IL-10 production and found to sustain a hypothesis of a cooperation of elements in the IL-10 promoter [25, 26]. However, genetic variations of the 5'-flanking region of the *IL-10* gene are poorly characterised in particular with respect to far distal elements as well as different stimuli. Herein it is

shown that the recently described gene variation at – 7400 of the 5'-flanking region of the *IL-10* gene, called – 7400indel, is related to inter-individual differences in IL-10 production.

The *in vitro* stimulation of PBMCs with LPS or dbcAMP, and the immortalization of B lymphocytes with EBV reveals inter-individual differences in IL-10 secretion. These differences are probably caused by the length polymorphism due to the deletion of the three nucleotides "GGA" at – 7400 bp of the *IL-10* gene promoter. Only homozygotes for the deletion express high amounts of IL-10. Where the allele 01 is present, less than 50% of the maximal amount IL-10 is released. This may imply a repressor function of allele 01, that is abolished if the carrier is homozygous for the deletion *(Figure 2C)*. In addition, the differences in IL-10 production induced by different stimuli suggest that LPS activated pathways are related to different genetic elements within the 5'-flanking region of the *IL-10* gene. It has been suggested recently that genetic components might be responsible for more than 70% of the innate variation in IL-10 production, but a twin study published in 2002 indicated that this may be only 50% [21, 31].

The – 1087G/– 7400del haplotype in the homozygous state showed the strongest association ($P = 0.009$) with high IL-10 production *in vitro* upon LPS stimulation of PBMCs. Carriers of the homozygous genotype 0202/GG express twice as much IL-10 as donors homozygous for the genotype 0101/GG. This provides strong support for an the influence of the indel polymorphism on IL-10 secretion. The influence of the SNP at – 1087 is nevertheless evident in that PBMC from homozygous carriers of the haplotype – 1087A/– 7400in produce only about half as much IL-10 as homozygotes for the haplotype – 1087G/– 7400in. This indicates a degree of cooperation between proximal and far distal elements in the IL-10 5'-flanking region which – in this case – mutually affect each other.

Within the area of the – 7400insertion/deletion we detected no well known binding sites for transcription factors that could be involved in regulation of cytokines. However next to the deletion a potential c-ETS binding site was found (search with high matrix (90%) and core (100%) similarity) 10bp and 20 bp from this locus (GAAGGAC-GAGA *(GGA)* GGACAGAAG). In the area 100bp up – and downstream of the – 7400indel locus additional putative transcription factor binding sites are present such as for example GATA-binding factor 1, STAT-1 and ELK-1 showing up to 97% matrix similarity. The observation of a potential binding site for transcription factors of the ets-family is intriguing because ets-proteins have been shown to be differently affected by the – 1087 polymorphism in their binding to a corresponding IL-10 promoter fragment. It could also not be excluded that steric hindrance due to this insertion/deletion polymorphism, might lead to cooperating factors not working in the required way.

Our findings show that the deletion of the triplet "GGA" at the IL10.indel site at – 7400 bp is responsible for an augmentation in IL-10 secretion in LPS-stimulated PBMCs, but not for dbcAMP-stimulated PBMCs or EBV-immortalised B lymphocytes. The EBV-infected and immortalised B-cells of homozygous carriers of the deletion emphasise the complexity of the IL-10 expression regulation. Thus in this case we found a somewhat altered expression pattern compared to those seen after stimulation with LPS or dbcAMP. Carriers heterozygous for this gene variation produced the lowest amount of IL-10, which contrasts with the other observations *(Figure 2)*. This strengthens our hypothesis that different pathways of expression are induced depending on the stimulant used.

It has been shown that in cAMP-dependent regulation of IL-10 expression various elements like CRE (cAMP responsive elements) transcription factors CREB 1, ATF-1 (Activating Transcription Factor) and additional factors like C/EBPα and β (CCAAT/Enhancer Binding Protein) and their binding to three motifs of the promoter region are involved [37, 38]. However these promoter elements are probably too far from the indel locus or the – 1087 gene variation to influence inter-individual differences in IL-10 expression.

The rarity of homozygotes carrying the deletion at the IL-10.indel locus and their capacity to express extremely high IL-10 amounts might be an indication for evolutionary situation. Overexpression of IL-10 might lead to skewing towards Th 2 response that are not appropriate.

In summary the data presented here clearly show the involvement of far distal promoter elements of the 5'-flanking region of the *IL-10* gene with IL-10 production. Furthermore cooperation between far distal and proximal elements was demonstrated to influence IL-10 secretion after stimulation with LPS. This report is thus a step further towards elucidating the complex mosaic of IL-10 expression regulation via different stimulants that involves many polymorphic elements.

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