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The impact of *IL18* gene polymorphisms on mRNA levels and interleukin-18 release by peripheral blood mononuclear cells*

Wpływ polimorfizmów genu *IL18* na stężenie mRNA oraz wydzielanie interleukiny 18 przez jednojądrzaste komórki krwi obwodowej

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Interleukin-18 (IL-18) is a pleiotropic cytokine playing an important role as a modulator of immune responses, found to play a role in pathogenesis of numerous inflammatory-associated disorders. In the present study a potential association between 7 common single-nucleotide polymorphisms (SNPs) spanning the whole *IL18* gene, gene expression and the release of IL-18 from the stimulated peripheral blood mononuclear cells (PBMCs) was investigated.

Materials/Methods: PBMCs were isolated from peripheral blood of 29 healthy volunteers, genotyped for the presence of *IL18* SNPs: rs1946518: A>C, rs187238: G>C, rs360718: A>C, rs360722: C>T, rs360721: C>G, rs549908: T>G, and rs5744292: A>G. IL-18 concentration and *IL18* mRNA levels were investigated after incubation of cells for 48 h with different stimulants (PHA, LPS, and anti-CD3/CD28 antibodies).

Results: After treatment with LPS and antibodies IL-18 concentrations were significantly lower in rs1946518AA homozygotes than in C allele carriers. When differences in *IL18* mRNA levels between non-stimulated and stimulated cells were analyzed, significantly decreased gene expression was noted in rs1946518 AA homozygotes (as compared with C allele carriers) in samples treated with PHA and LPS. Similar trends were observed in the case of rs187238 SNP; however, the differences reached statistical significance only after PHA treatment.

Conclusions: Our study supports the role of rs1946518 (-607A>C) and rs187238 (-137G>C) SNPs as genetic determinants of the observed variability in *IL18* expression.

Key words: **genetic polymorphism • interleukin-18 • peripheral blood mononuclear cells • stimulants**

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Streszczenie

IL-18 jest plejotropową cytokiną odgrywającą istotną rolę w modulowaniu odpowiedzi immunologicznej i w patogenezie wielu schorzeń o podłożu zapalnym. W pracy opisano związek między 7 jednonukleotydowymi polimorfizmami w genie *IL18* a ekspresją *IL18* na poziomie mRNA i wydzielaniem tej cytokiny przez stymulowane jednojądrzaste komórki krwi obwodowej (PBMC). PBMC izolowano z krwi obwodowej 29 zdrowych ochotników, u których oznaczono polimorfizmy *IL18*: rs1946518: A>C, rs187238: G>C, rs360718: A>C, rs360722: C>T, rs360721: C>G, rs549908: T>G i rs5744292: A>G. Stężenia IL-18 oraz poziomy mRNA analizowano po 48-godzinnej inkubacji z różnymi stymulatorami (PHA, LPS, przeciwciałami anty-CD3/CD28). Po zastosowaniu LPS i przeciwciał anty-CD3/CD28 stężenia IL-18 były istotnie statystycznie mniejsze u homozygot AA rs1946518 w porównaniu z nosicielami allela C. Gdy analizowano poziomy mRNA *IL18* między komórkami niestymulowanymi oraz stymulowanymi PHA i LPS istotnie obniżoną ekspresję genu stwierdzono u nosicieli genotypu AA rs1946518 w porównaniu do nosicieli allela C. Podobne zmiany obserwowano w odniesieniu do polimorfizmu rs187238, jednakże istotność statystyczną stwierdzono tylko dla komórek stymulowanych PHA. Wyniki naszej pracy potwierdzają rolę polimorfizmów rs1946518 (-607A>C) i rs187238 (-137G>C) jako genetycznych determinant wpływających na ekspresję *IL18*.

Słowa kluczowe: **polimorfizm genetyczny • interleukina 18 • jednojądrzaste komórki krwi obwodowej • stymulatory**

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Introduction

Interleukin-18 (IL-18) is a pleiotropic cytokine playing an important role as a modulator of immune responses. IL-18 was first discovered as a potent IFN-inducing factor, produced by macrophages and dendritic cells. IL-18 was the first cytokine found to activate Th1 cells to produce abundant IFN-γ (interferon-gamma) without T cell receptor engagement [8,9,13]. Constitutively produced as an inactive precursor (Pro-IL-18) by several cell types, IL-18 is activated in response to inflammatory and infectious stimuli. Pro-IL-18 expression is widespread, including monocytes, macrophages, dendritic cells, keratinocytes, articular chondrocytes, synovial fibroblasts and osteoclasts [2]. In addition to its effects on Th1 cells, IL-18 is a strong stimulator of the activity of natural killer cells and CD8+ lymphocytes. Together with IL-2, IL-18 can also stimulate the production of IL-13 and other Th2 cytokines [1]. IL-18 was found to play a role in pathogenesis of numerous inflammatory-associated disorders, including infections, autoimmune diseases, cancer, as well as metabolic syndrome and atherosclerosis [2,3,15].

Although the *IL18* gene was resequenced in different populations, only a few missense and mRNA splicing interfering single-nucleotide polymorphisms (SNPs) have been found. However, some variation within the 5' untranslated region (UTR) and 3' UTR was observed, which may cause differences in translation rate and mRNA stability, as well as variation within the proximal promoter that may cause alterations in transcription rate [11]. Two single-nucleotide polymorphisms at position -607 (rs1946518: C>A) and -137 (rs187238: G>C) in the promoter region were initially found to be associated with *IL18* gene promoter transcriptional activity [5]. In the most thorough investigation into the link between *IL18* variation and IL-18 serum levels, two of five investigated SNPs were significantly associated with circulating IL-18 levels. *IL18* polymorphism was also found to increase the risk of some diseases of inflammatory background, including asthma, rheumatoid arthritis, atherosclerosis, Crohn's disease, multiple sclerosis and type I diabetes [11]. In the present study we tried to investigate a potential association between 7 common SNPs spanning the whole *IL18* gene, gene expression and the release of IL-18 from the stimulated peripheral blood mononuclear cells (PBMCs).

Materials and Methods

Twenty-nine healthy volunteers (17 females and 12 males) were enrolled in the study after submission of their written informed consent. All subjects were Caucasian from the Pomeranian region of Poland. The study was approved by the local ethics committee.

Genomic DNA was extracted from whole blood samples using GeneMATRIX Quick Blood DNA Purification Kit (EURx, Poland). The subjects were genotyped for the

presence of 7 common SNPs spanning the whole *IL18* gene (rs1946518: A>C and rs187238: G>C in the promoter region, rs360718: A>C in 5'-UTR, in exon 1, rs360722: C>T and rs360721: C>G in intron 1, rs549908: T>G synonymous SNP in exon 4, and rs5744292: A>G in exon 6 (3'-UTR)), using allele-specific amplification or PCR-RFLP assays, as described by us in detail elsewhere [10].

PBMCs were isolated from peripheral blood of study subjects using the Ficoll Paque procedure. After centrifugation (30 min., 400 × g, at room temp.) the mononuclear fraction was collected, washed twice in PBS, suspended in RPMI enriched with 10% fetal bovine serum and incubated on a tissue culture plate (for 1 hour at 37°C, 5% CO_2) to get rid of adherent cells (monocytes). Non-adherent cells were then collected, counted and seeded at a density of 2×10^6/2 mL in 24-well plates. Stimulation of cells was performed by adding phytohaemagglutinin (PHA) at the concentration of 20 µg/mL or lipopolysaccharide (LPS) at 40 ng/mL or 40 ng/mL anti-CD3+ 10 µg/mL anti-CD28 antibodies for 48 h incubation periods at 37°C, 5% CO_2. Secretion of IL-18 by PBMC was determined in supernatants by the MBL human IL-18 immunoassay (ELISA) (Medical & Biological Laboratories Co., LTD, Japan) according to the manufacturer's protocol.

Total RNA was extracted from PBMCs collected after 48 h of incubation, using the RNeasy kit (Qiagen, USA). Reverse transcription was performed with the RETROscript First Strand Synthesis Kit (Ambion, USA), using Moloney murine leukemia virus (M-MLV) reverse transcriptase and random decamers in a total volume of 20 µl. Subsequently, cDNA probes were used as a template for a real-time quantitative PCR (QPCR) analysis. QPCR was performed in duplicate in the 7500 Fast Real Time PCR System (Applied Biosystems, USA), using a commercially available validated TaqMan assay for the human *IL18* gene (assay ID: Hs99999040_m1, Applied Biosystems, USA) and an endogenous control – *ACTB*. Calculations were performed using the ΔΔCt relative quantification method. All Ct values were normalized to the values obtained for the endogenous control for each sample. Fold change between groups was calculated from the means of the logarithmic expression values.

Since the distribution of IL-18 concentration and relative mRNA expression values were different from normal (Shapiro-Wilk test), they were analyzed in relation to the *IL18* genotype using the Mann-Whitney U-test. Differences in IL-18 concentration and *IL18* mRNA levels between non-stimulated and stimulated PBMCs were analyzed by means of the Wilcoxon test. All calculations were performed using Statistica 8.0 software (StatSoft, Warsaw, Poland).

Results

Concentration of interleukin-18 collected from supernatant after 48 h of incubation was similar in non-stimulated (mean ±SD: 25.0±15.6) and stimulated PBMCs (25.5±18.7 for PHA, and 26.9±14.5 for LPS). Only in the case of anti-CD3/CD28 stimulation did the values differ significantly from the non-stimulated control (30.0±18.10, p=0.026, Wilcoxon test). In contrast, *IL18* relative mRNA levels measured after 48 h were significantly decreased in cells treated with all stimulants (12.6% of relative expression detected in control probes for PHA, p=0.00001; 52.2% for LPS, p=0.007; 36.2% for anti-CD3/CD28, p= 0.011).

As rs360718 was fully linked to rs187238, and rs549908 to rs5744292 (100% concordance of obtained genotypes), those two SNPs were excluded from the subsequent analysis. *IL18* genotype had no influence on IL-18 release from non-stimulated PBMCs. However, significant differences were noted in IL-18 concentration between cells from patients stratified by rs1946518 genotype, treated with LPS and antibodies, i.e. mean IL-18 concentrations were significantly lower in AA homozygotes than in C allele carriers (Table 1). In the case of PHA treatment, rs360722 SNP was associated with IL-18 levels (lower in CC homozygotes *vs.* T allele carriers). No other associations were observed. In the case of *IL18* relative mRNA expression, no genotype-dependent differences were noted in non-stimulated or stimulated PBMCs (Table 2). However, when differences in *IL18* relative expression between non-stimulated and stimulated cells were analyzed for each sample instead of raw relative quantity values, significantly decreased gene expression was noted in rs1946518 AA homozygotes (as compared with C allele carriers) in samples treated with PHA and LPS (Table 3). Similar trends were observed in the case of rs187238 SNP; however, the differences reached statistical significance only after PHA treatment.

Discussion

In the present study we examined the potential effect of common SNPs spanning the *IL18* gene on gene expression and the release of IL-18 by *in vitro* stimulated peripheral blood mononuclear cells. The cells were treated with three different stimulators: phytohaemagglutinin, lipopolysaccharide and anti-CD3/CD28 antibodies.

To date, two SNPs, rs1946518: C>A and rs187238: G>C (at positions -607 and -137), in the promoter region of *IL18* have been most thoroughly studied. In the initial study by Giedraitis et al. [5], lower promoter activity was observed for rs1946518 A and rs187238 C alleles and they were consequently suggested to be 'low activity' alleles in contrast to rs1946518: A>C and rs187238 G – 'high activity' alleles. Giedraitis et al. [5] studied the transcriptional activity of *IL18* in HeLa 229 cells, transfected with the promoter region of the human *IL18* gene, containing SNPs rs1946518 and rs187238. The authors did not find significant differences in promoter activity between alleles without stimulation, but after stimulation with PMA/ionomycin they observed increased transcription activity associated with rs1946518 C and rs187238 G alleles. In that study the expression of *IL18* in PBMCs of multiple sclerosis patients was also studied, revealing slightly (non-significantly) increased mRNA levels in subjects carrying rs1946518 C and rs187238 G alleles. These observations are in concordance with our study, in which higher IL-18 release and *IL18* mRNA expression were associated with rs1946518 C and rs187238 G alleles, reaching statistical significance in the case of rs1946518 SNP in IL-18 release after stimulation with LPS and anti-CD3/CD28 antibodies (Tables 1, 2).

Similar results were obtained by Khripko et al. [6], who measured IL-18 concentrations in PBMCs after 48 h of LPS stimulation. IL-18 production by LPS-stimulated PBMC

Table 1. Interleukin-18 release from stimulated PBMCs in relation to *IL18* genotype

SNP	Genotype	Non-stimulated		PHA		LPS		Anti-CD3/CD28	
		Mean ±SD [pg/mL]	p	Mean ±SD [pg/mL]	p	Mean ±SD [pg/mL]	p	Mean ±SD [pg/mL]	p
rs1946518	AA	20.9±15.0	0.416	22.5±19.1	0.443	17.4±8.7	0.011	20.6±12.5	0.046
	AC+CC	26.9±15.9		26.9±18.9		31.3±14.6		34.0±17.6	
rs187238	GG	28.3±14.6	0.186	27.1±11.8	0.102	29.7±14.9	0.289	33.5±17.7	0.219
	GC +CC	21.5±16.4		23.9±24.5		24.1±14.0		26.3±18.5	
rs360722	CC	24.4±17.3	0.500	21.9±18.8	0.013	26.2±15.4	0.600	30.7±19.8	0.887
	CT+TT	26.9±9.0		36.9±14.2		29.3±12.0		27.5±11.2	
rs360721	CC	28.3±14.6	0.186	27.1±11.8	0.102	29.7±14.9	0.290	33.5±17.7	0.219
	CG+GG	21.5±16.4		23.9±24.5		24.1±14.0		26.3±18.5	
rs5744292	AA	23.5±12.6	0.776	25.5±15.6	0.585	24.9±10.8	0.394	23.3±11.8	0.066
	AG+GG	27.2±19.5		25.7±23.2		30.0±18.7		38.4±21.4	

All p values calculated by means of Mann-Whitney U-test.

Table 2. *IL18* mRNA expression in stimulated PBMCs in relation to *IL18* genotype

SNP	Genotype	Non-stimulated		PHA		LPS		Anti-CD3/CD28	
		Mean ±SD	p	Mean ±SD	p	Mean ±SD	p	Mean ±SD	p
rs1946518	AA	1.314±0.760	0.167	0.119±0.054	0.651	0.622±0.275	0.742	0.420±0.555	0.496
	AC+CC	1.197±1.640		0.169±0.193		0.654±0.343		0.457±0.418	
rs187238	GG	0.897±0.497	0.234	0.193±0.212	0.646	0.629±0.373	0.650	0.414±0.385	0.860
	GC +CC	1.594±1.938		0.104±0.048		0.656±0.268		0.484±0.526	
rs360722	CC	1.284±1.592	0.980	0.168±0.191	0.910	0.618±0.321	0.408	0.450±0.466	0.976
	CT+TT	1.074±0.623		0.122±0.073		0.733±0.311		0.437±0.419	
rs360721	CC	0.897±0.497	0.234	0.193±0.212	0.646	0.629±0.373	0.650	0.414±0.385	0.860
	CG+GG	1.594±1.938		0.104±0.048		0.656±0.268		0.484±0.526	
rs5744292	AA	1.074±0.666	0.678	0.582±0.256	0.163	0.426±0.474	0.272	0.102±0.057	0.402
	AG+GG	1.459±2.082		0.733±0.385		0.471±0.434		0.228±0.236	

Arbitrary units; all p values calculated by means of Mann-Whitney U-test.

was significantly greater in healthy donors carrying the rs1946518 CA genotype than in those with the CC genotype, and the 'low activity' rs187238 C allele frequency was greater in the group with a low level of IL-18 production [6].

The carriers of rs360722 T allele showed increased release of IL-18 after stimulation with PHA when compared to CC homozygotes. However, this observation is likely to be accidental, as that observation was not observed in the case of other stimulants or in mRNA level analysis. Moreover, no other SNPs (e.g. rs360718: A>C, rs360721: C>G, rs549908: T>G, and rs5744292: A>G) were found to be associated with IL-18 release or transcription rate.

As found by Marshall et al. [7] using semi-quantitative reverse transcription PCR, *IL18* gene expression may be elevated only in a short period (4–6 h) after stimulation of PBMCs with LPS or other antigens, and is then depressed below the constitutive level found in non-treated cells. In our study mRNA levels of *IL18* transcripts after 48 h of incubation were also significantly lower in stimulated cells when compared with non-stimulated controls. Although *IL18* expression was not significantly correlated with analyzed SNPs, a comparison between stimulated PBMCs and non-stimulated controls revealed that *IL18* transcription was significantly more depressed in rs1946518 AA homozygotes when compared with C allele carriers (Table 3). A similar association was observed in the case of rs187238 C 'low activity' allele, reaching statistical significance in the case of PHA stimulation.

In the largest study on *IL18* variation and *in-vivo* IL-18 levels, five tag SNPs (rs1946519: G>T, rs360717: C>T,

Table 3. Changes in *IL18* mRNA expression in stimulated PBMCs in relation to *IL18* genotype

SNP	Genotype	PHA		LPS		Anti-CD3/CD28	
		Mean ±SD	p	Mean ±SD	p	Mean ±SD	p
rs1946518	AA	–1.39±0.74	0.035	–0.69±0.74	0.017	–1.04±0.74	0.135
	AC+CC	–1.09±1.70		–0.47±1.68		–0.76±1.51	
rs187238	GG	–0.70±0.55	0.017	–0.12±0.24	0.115	–0.49±0.54	0.251
	GC +CC	–1.60±2.09		–0.94±1.91		–1.25±1.84	
rs360722	CC	–1.25±1.72	0.955	–0.63±1.61	0.659	–0.89±1.46	0.929
	CT+TT	–0.95±0.59		–0.23±0.34		–0.67±0.84	
rs360721	CC	–0.70±0.55	0.017	–0.12±0.24	0.115	–0.49±0.54	0.251
	CG+GG	–1.60±2.09		–0.93±1.91		–1.25±1.84	
rs5744292	AA	–1.03±0.67	0.474	–0.45±0.62	0.174	–0.71±0.69	0.781
	AG+GG	–1.36±2.21		–0.67±2.17		–0.99±1.86	

Arbitrary units; values present differences between relative quantity of IL18 mRNA in stimulated and non-stimulated PBMCs; p values calculated by means of Mann-Whitney U-test.

rs549908: A>C, rs5744292: A>G, rs4937100: T>C) were analyzed in 1288 patients with coronary artery disease (rs1946518 and rs187238 were not studied). Although two SNPs were significantly associated with circulating IL-18 levels, genetic polymorphism explained only 1.8% of the variability of IL-18 levels [12]. In the study of Zhou et al. [14], the polymorphisms rs1946519: G>T and rs1946518: C>A were significantly associated with serum levels of IL-18 in patients with sarcoidosis, but not in controls. In another study, Evans et al. [4] investigated the association between circulating IL-18 serum levels and rs187238: G>C SNP (described there as -137G>C) within the *IL18* gene in black South African women with cardiovascular disease, and revealed that IL-18 levels were not associated with *IL18* genotype. However, *IL18* gene polymorphism was also found to significantly increase the risk of some diseases of inflammatory background, including asthma, rheumatoid arthritis, atherosclerosis, Crohn's disease, multiple sclerosis and type I diabetes [11]. Our observation that IL-18 levels in PBMCs were genotype-dependent only after stimulation (in contrast to non-stimulated PBMCs) could be joined with the results of previous investigations, leading to a hypothesis that the impact of *IL18* polymorphism is only pronounced in particular conditions, after application of some stimulatory factors, *in vitro* as well as *in vivo*.

Our study supports the role of rs1946518 and rs187238 SNPs as the main genetic determinants of *IL18* expression variability and their greater significance compared to other SNPs within the *IL18* gene. However, genetic polymorphism is probably responsible only for a part of the observed differences in IL-18 production, and its diagnostic application seems to be limited. Each subject is the carrier of various polymorphisms and haplotypes in the *IL18* gene, often exerting opposite effects on IL-18 protein production. IL-18 production is the outcome of the influence of various polymorphisms and haplotypes. Therefore the functional significance of these polymorphisms *in vivo* may not be consistent with experimental studies due to different combinations of particular SNPs and haplotypes in various subjects.

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