Short communication

THE INFLUENCE OF JNK AND P38 MAPK INHIBITION ON IL-12P40 AND IL-23 PRODUCTION DEPENDING ON IL12B PROMOTER POLYMORPHISM

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Abstract: The interleukin-12p40 gene (IL12B) encodes the p40 polypeptide chain, which, together with p19, composes IL-23. A bi-allelic promoter polymorphism (IL12Bpro) located at -2703 bp of the transcription initiation site has been reported to show associations with IL-12p40 production. To elucidate the dependence of IL-12p40 and IL-23 production on IL12Bpro polymorphism in relation to MAPK signal transduction pathways, we examined the effect of JNK and p38 inhibition on the secretion of these cytokines by stimulated peripheral blood mononuclear cells (PBMC) from healthy donors with 1.1 and 1.2/2.2 IL12Bpro genotypes. Stimulation with LPS and C3bgp resulted in approximately equal IL-12p40 production from PBMC with the 1.1 and 1.2/2.2 genotypes. The inhibition of JNK and p38 before stimulation significantly upregulated IL-12p40 production by PBMC with the 1.1 genotype, but did not influence IL-12p40 production from PBMC with the 1.2/2.2 genotype. Cultures of PBMC with the 1.1 genotype produced significantly more IL-12p40 than PBMC with the 1.2/2.2 genotype after stimulation with PHA. Inhibition of p38 kinase upregulated p40 production only in cultures with the 1.1 genotype. Decreased IL-23 production was observed in C3bgp-stimulated cultures after the inhibition of p38 regardless of the genotype of the tested cells. We concluded

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Abbreviations used: C3bgp – C3 binding glycoprotein; IFN-γ – interferon gamma; IL – interleukin; IL12B – the gene for p40 chain of IL-12; LPS – lipopolysaccharide; MAPK – mitogen-activated protein kinase; SAC – Staphylococcus aureus cells; PBMC – peripheral blood mononuclear cells; PHA – phytohemagglutinin
that IL-12p40 and IL-23 expression, which is mediated by the p38 and JNK intracellular signaling pathways, is influenced by IL12Bpro polymorphism.

**Key words:** IL-12p40, IL-23, IL12B, Promoter polymorphism

**INTRODUCTION**

Interleukin-12p40 (IL-12p40) is produced by activated antigen-presenting cells in response to a variety of proinflammatory stimuli, such as LPS, SAC, peptidoglycan and CpG oligonucleotides. The p40 polypeptide chain forms the bioactive heterodimeric cytokine IL-12p70 after binding with IL-12p35 and IL-23 when it binds with IL-23p19 [1, 2]. IL-12p70 is a key immunoregulatory cytokine that governs the Th1 immune response polarization [3], whereas IL-23 selectively activates memory T cells to secrete IFN-γ and IL-17 [2, 4, 5]. IL-12p40 is secreted at a 50-fold excess relative to IL-12p70 by cells in a murine shock model [6], and at a 10- to 20-fold excess by stimulated human peripheral blood mononuclear cells [7]. The IL-12p40 chain may also form the homodimer IL-12p80, which serves as an IL-12p70 and IL-23 antagonist by competing for binding at the receptor complexes of both cytokines [1]. The ability of rmIL-12p80 to antagonize IL-12p70 activity in vitro is 20-fold greater than that of IL-12p40 [8]. Also, the levels of natural IL-12p80 are 20-40% of the total IL-12p40 levels in the serum in murine models of systemic infection [8]. Proper balance between IL-12p40-related cytokines is essential in immunoregulation, and it controls the appearance of pathological Th1-mediated autoimmune and inflammatory diseases [1].

IL-12p40 synthesis is strictly regulated, and restricted to the cell types producing IL-12p70 and IL-23 [2, 7]. The human gene for IL-12p40 (IL12B) is located at chromosome 5q31-33, and has the GenBank accession number AY008847. Recently, a complete genomic sequence analysis of the IL12B gene identified several polymorphisms, including a complex promoter polymorphism yielded by a 4-bp insertion combined with an AA/GC substitution at -2703 bp, upstream of the transcription initiation site [9, 10]. This CTCTAA/GC polymorphism is commonly referred to as the IL12B promoter (IL12Bpro) polymorphism with rs17860508. Associations between the IL12Bpro polymorphism and the severity of cerebral malaria and childhood asthma, and susceptibility to type 1 diabetes have been described [9-12].

Mitogen-activated protein kinases (MAPKs) are an important group of serine/threonine signaling kinases. They link transmembrane signaling with gene expression events in the nucleus. There is published evidence for the involvement of the c-Jun N-terminal kinase (JNK) and p38 MAPK in IL-12p40 and IL-23 production [13-15]. However, in the available literature, we have not found any information about a relationship between IL12Bpro polymorphism, MAPK activation, and IL-12p40 and IL-23 production.

In this study, we examined the effect of inhibiting the JNK and p38 MAPK signal transduction pathways on IL-12p40 and IL-23 production with reference
to the *IL12Bpro* polymorphism. We used the plant-derived C3 binding glycoprotein (C3bgp) and lipopolysaccharide (LPS) as stimuli preferentially activating monocytes, and phytohemagglutinin (PHA) as a stimulus activating predominantly T cells in PBMC. C3bgp is a novel immunomodulator isolated from the seeds of the parasitic plant *Cuscuta europaea* [16-18]. The newest studies revealed that C3bgp activates monocyctic cells via interaction with complement receptor type 3 (CR3, CD11b/CD18), and that its immunomodulatory action is mediated by the activation of the JNK and p38 MAPK transduction pathways [19, 20]. LPS, a major component of the outer membrane of Gram-negative bacteria, is a very potent activator of monocytes. After interaction with LPS-binding protein, LPS binds to CD14, a high affinity LPS receptor. CD14 lacks the capacity for transmembrane signaling; it activates signal transduction cascades via crosstalk with TLR4. LPS stimulation activates several intracellular signaling pathways including those of JNK and p38 MAPKs [21].

**MATERIALS AND METHODS**

**PBMC isolation and stimulation**

With the consent of the local ethics board, heparinized venous blood samples were taken from 36 healthy donors who had no autoimmune or inflammatory diseases according to laboratory and clinical data. Peripheral blood mononuclear cells (PBMC) were isolated via Histopaque-1077 (Sigma, St. Louis, Mo) density gradient centrifugation. 2 x 10⁶ PBMC were cultured in sterile polystyrene tubes containing 2 ml RPMI 1640 (Sigma, St. Louis, Mo). The cultures were supplemented with 10% FBS (Sigma, St. Louis, Mo), 100 U/ml penicillin, 100 µg/ml gentamicin and 0.3 mg/ml L-glutamine. The PBMC were stimulated with: 30 µg/ml C3 binding glycoprotein (C3bgp), isolated from a total water extract of the seeds of the parasitic plant *Cuscuta europaea*, as previously described [15]; 1 µg/ml lipopolysaccharide from *Escherichia coli* serotype 026:B6 (LPS, Sigma, St. Louis, Mo); and 20 µg/ml phytohemagglutinin (PHA, Sigma, St. Louis, Mo). The cultures were incubated at 37°C for 24 h.

**Inhibiting the JNK and p38 MAPK transduction pathways**

To inhibit c-Jun N-terminal kinase, we used the selective anthrapyrazolone inhibitor SP600125 (Sigma, St. Louis, Mo). It competitively inhibits JNK 1, 2 and 3 with > 20-fold selectivity versus a wide range of kinases according to Bennett *et al.* [22]. The inhibition of p38 was achieved with pyrinidyl imidazole inhibitor SB202190 (Sigma, St. Louis, Mo). It selectively inhibits the p38 alpha and beta isoforms, and completely blocks LPS-stimulated IL-6 mRNA expression at a concentration of 10 µM, as demonstrated by Manthey *et al.* [23]. As per their protocol, 20 µM SP600125 or 10 µM SB202190 were added to PBMC 1 h before stimulation. The SP600125 and SB202190 were dissolved in 100% dimethylsulphoxide (DMSO, Sigma, St. Louis, Mo), and the final concentration of DMSO in the cultures was 0.1%.
Cytokine determination
The levels of IL-12p40 and IL-23 were quantified using ELISA kits in culture supernatants, according to the manufacturer’s protocol (BioSource, Austria). The developed color reaction was measured as OD units at 450 nm. The concentration of cytokines was determined using a standard curve constructed with the kit’s standards, and expressed in pg/ml. The minimum detectable concentration for IL-12p40 with the ELISA kit was less than 2 pg/ml, and for IL-23 was less than 15 pg/ml.

Genomic DNA isolation and genotyping
Genomic DNA was extracted using a GFX genomic blood DNA purification kit (Amersham Biosciences, Buckinghamshire, UK), and stored at -80°C until use. Genotyping for the IL12Bpro polymorphism (rs17860508) was performed with amplification refractory mutation system (ARMS) PCR. 5’ primers specific for IL12Bpro alleles were used in combination with a generic 3’ primer. The sequence of the primer specific for the CTCTAA allele, marked as IL12Bpro-1 was 5’-TGT CTC CGA GAG AGG CTC TAA-3’; and that for the GC allele marked as IL12Bpro-2 was 5’-TGT CTC CGA GAG AGG GCT GT-3’. The generic 3’ primer used for the amplification reactions was 5’-TGG AGG AAG TGG TTC TCG TAC-3’ [24]. The cycling parameters were: an initial denaturation step of 15 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 65°C, and 30 sec at 72°C, with a final extension step of 7 min at 72°C. The PCR amplifications were performed in a total volume of 25 µl, containing 0.1-0.5 µg of genomic DNA, using Pure Tag Ready-to-Go PCR beads (Amersham, UK) in a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide. Positive (DNA from heterozygous individuals) and negative controls were used within each run of PCR amplification. A random selection of 10% of all the samples was repeated to ensure laboratory quality control.

Statistical analysis
The data was expressed as the means and the standard error of the mean (SEM). Student’s t-test was used to determine the statistical differences between the mean values. Differences were considered significant when the p-value was less than 0.05.

RESULTS
Genotype distribution
The genotyping of the donors recruited for the study showed that 14 (39%) are IL12Bpro-1 homozygotes, 18 (50%) are heterozygotes, and 4 (11%) are IL12Bpro-2 homozygotes. These genotype frequencies are similar to the previously published results for the distribution of IL12Bpro polymorphism in the Bulgarian population: 35% (1.1): 50% (1.2): 15% (2.2) [25].
IL-12p40 production depended on IL12Bpro polymorphism after JNK and p38 MAPK inhibition

The results of the analysis of IL-12p40 secretion in relation to the IL12Bpro genotypes are shown in Fig. 1. We found that PBMC cultures from individuals with the 1.1 and 1.2/2.2 IL12Bpro genotypes produced almost equal quantities of IL-12p40 after stimulation with C3bgp and LPS. The inhibition of JNK and p38 MAPKs clearly divided stimulated PBMC into two groups depending on their IL12Bpro genotype. The inhibition of JNK and p38 before stimulation with C3bgp and LPS resulted in IL-12p40 upregulated production from PBMC with 1.1 genotype (Fig. 1A and 1B). Under the same experimental conditions, the inhibition of both kinases did not affect the IL-12p40 production from PBMC with 1.2/2.2 genotypes. Significant differences in IL-12p40 production between PBMC stimulated with C3bgp and LPS and PBMC stimulated after JNK and p38 inhibition were detected only in IL12Bpro-1 homozygotes – C3bgp vs. C3bgp + SP600125, p = 0.042; C3bgp vs. C3bgp + SB202190, p = 0.007; LPS vs. LPS + SP600125, p = 0.044; LPS vs. LPS + SB202190, p = 0.013. Moreover, C3bgp- and LPS-stimulated PBMC with the 1.1 genotype produced significantly higher levels of IL-12p40 after JNK and p38 inhibition than did the
1.2/2.2 genotype: C3bgp + SP600125 (1.1 genotype) vs. C3bgp + SP600125 (1.2 genotype), p = 0.006; C3bgp + SB202190 (1.1 genotype) vs. C3bgp + SB202190 (1.2 genotype), p = 0.02; LPS + SP600125 (1.1 genotype) vs. LPS + SP600125 (1.2 genotype), p = 0.02; and LPS + SB202190 (1.1 genotype) vs. LPS + SB202190 (1.2 genotype), p = 0.04. By contrast, PHA-stimulated PBMC from 1.1 homozygotes produced significantly more IL-12p40 than PBMC with the 1.2/2.2 genotype, p = 0.049 (Fig. 1C). The inhibition of JNK MAPK did not significantly change the IL-12p40 production in either genotype. The inhibition of p38 kinase significantly increased the levels of IL-12p40 released from the 1.1 homozygotes (p = 0.046), and did not influence the cytokine production from PBMC with the 1.2/2.2 genotype.

IL-23 production depended on IL12Bpro polymorphism after stimulation

The results presented in Fig. 2A demonstrate that IL12Bpro-1 homozygotes produced significantly higher levels of IL-23 after C3bgp stimulation compared to the 1.2/2.2 genotype, p = 0.01. LPS and PHA stimulation resulted in a lower level of IL-23 production than with C3bgp stimulation. Nevertheless, as shown in Fig. 2B and C, PBMC from individuals with the 1.1 genotype produced significantly higher levels of IL-23 after stimulation with LPS (1.1 genotype) vs. LPS + SP600125 (1.2 genotype), p = 0.04, and similarly with PHA (1.1 genotype) vs. PHA + SB202190 (1.2 genotype), p = 0.04.

Fig. 2. IL-23 production by stimulated PBMC from healthy donors after the inhibition of JNK and p38 MAPKs in relation to the IL12Bpro genotype. The cells were treated for 1 h with 20 µM SP600125 (a selective inhibitor of JNK) or 10 µM SB202190 (a selective inhibitor of p38) or not treated with inhibitors, and then stimulated with 30 µg/ml C3bgp (A), 1 µg/ml LPS (B) or 20 µg/ml PHA (C). Cytokine production was determined in the culture supernatants by ELISA at 24 h. The data is expressed as means and standard error of the mean (SEM). ^P < 0.05, significantly higher IL-23 production by PBMC with the 1.1 genotype than by PBMC with the 1.2/2.2 genotype. *P < 0.05, significantly less or significantly higher IL-23 production by PMBC stimulated after JNK and p38 inhibition than by PBMC of the same genotype stimulated without prior inhibition.
significantly more IL-23 after LPS (p = 0.02) and PHA (p = 0.04) stimulation than the PBMC from the 1.2/2.2 donors (which did not produce a detectable quantity of IL-23). The inhibition of p38 MAPK before C3bgp stimulation led to a significant reduction in the level of IL-23 production, and this downregulation effect was independent of IL12Bpro polymorphism. Both the 1.1 and 1.2/2.2 genotypes produced significantly less IL-23 after p38 inhibition compared to C3bgp-stimulated PBMC with the same genotype: in the 1.1 genotype C3bgp vs. C3bgp + SB202190, p = 0.0002; while in the 1.2 genotype C3bgp vs. C3bgp + SB202190, p = 0.033. The inhibition of p38 kinase significantly decreased the level of IL-23 production in LPS- and PHA-stimulated cultures from individuals with the IL12Bpro 1.1 genotype. We found that JNK inhibition significantly increased IL-23 production in LPS-stimulated cultures from PBMC with the IL-12Bpro 1.1 genotype, p = 0.01. The inhibition of the same kinase increased IL-23 production in PHA-stimulated PBMC with the IL-12Bpro 1.1 genotype without a significant difference.

DISCUSSION

Many researchers have reported evidence for the involvement of MAPKs in IL-12p40 production, but there are contradictory results [13, 14, 25], and the exact role of MAPKs in inducible IL-12p40 expression is still a matter of debate. We hypothesize that the polymorphisms in the human IL12B gene could influence JNK and p38 MAPK control of IL-12p40 expression in human PBMC in response to mitogens and proinflammatory stimuli.

In this study, for the first time, we investigated the effect of inhibiting the JNK and p38 MAPK signal transduction pathways on IL-12p40 and IL-23 production and how this relates to the IL12Bpro genotype. Initially, we demonstrated that PBMC from healthy donors with different IL12Bpro genotypes (1.1 vs. 1.2/2.2) produced almost equal quantities of IL-12p40 after stimulation with the monocytic activators LPS and C3bgp. The inhibition of JNK and p38 MAPKs resulted in upregulated IL-12p40 production in PBMC with the 1.1 genotype, but did not affect its production in the 1.2/2.2 genotype. Therefore, our results suggest that the inhibition of JNK and p38 MAPKs had a different effect on the IL-12p40 expression depending on the IL12Bpro polymorphism.

In general, there is a disagreement about the effect of IL12Bpro polymorphism on the inducible IL-12p40 production. The investigations of Müller-Berghaus et al. [24] showed that there were no significant differences in IL-12p40 production between IL12Bpro-1 homozygotes, heterozygotes and 2.2 homozygotes, realized by monocyte-derived dendritic cells after stimulation with CD40L. By contrast, Morahan et al. [9] revealed that heterozygosity in the IL12B promoter sequence reduced IL-12p40 mRNA synthesis in PBMC from asthmatic children. Tatebayashi et al. [10] demonstrated that homozygocity for haplotype TCTCTAA/-2403T was associated with lower IL-12p40 production by Derf1-stimulated PBMC, and that the transcription activity of the construct
with this haplotype was lower than that with the haplotype $2703^{GC}/2403^{C}$ in both healthy donors and asthmatic patients. An explanation for these discrepancies could be differences in the designs and settings of the studies, especially as the production of IL-12p40 was investigated in different cell types, the cells had been isolated from healthy donors or asthmatic patients, and different stimuli had been used. Variations in the experimental conditions could have affected the signal transduction pathways, in particular JNK and p38 MAPK activation. Despite these discrepancies, the authors indicated above clearly demonstrated that $IL12B$ promoter polymorphism is associated with gene transcription, although the precise molecular mechanism is unknown. Our results expanded the knowledge about the regulation mode driving the $IL12B$ expression, and accentuated the role of JNK and p38 MAPK in allele-specific IL-12p40 production.

We suppose that the observed discrepancies in the data presented by Utsugi et al., Ma et al. and Marriott et al. may have been influenced by the genotype of the tested cells [13, 14, 26]. We showed that: i) the inhibition of JNK upregulated IL-12p40 production only when the producer cells had the $IL12Bpro\ 1.1$ genotype (as demonstrated by Utsugi et al. [13]); ii) the inhibition of p38 kinase upregulated cytokine synthesis in PBMC with $1.1$ genotype (as demonstrated by Marriott et al. [26]); and iii) this inhibition did not influence IL-12p40 production when the stimulated cells had the $1.2$ genotype (as demonstrated by Ma et al. [14]).

As is well known, PHA mainly stimulates T cells from PBMC. Therefore, PHA-induced factors such as CD40L and/or IFN-γ from activated T cells could directly stimulate IL-12p40 expression from monocytes [27, 28]. Our results demonstrated that the activation signal mediated by T cell-derived factors resulted in different levels of IL-12p40 production depending on the $IL12Bpro$ genotype, in contrast to stimulation with monocyte activators such as LPS and C3bgp. This discrepancy could be due to the different activation of MAP kinases after PBMC stimulation with PHA or LPS and C3bgp. Moreover, the inhibition of p38 kinase led to a significant increase in IL-12p40 production levels from PBMC with the $1.1$ genotype. Several studies have shown that overexpression of IL-12p40/p80 has important roles in the development of the Th2-type immune response [29, 30]. The immune-enhancing Th1 agonistic functions of p40/p80 have also been described [31, 32]. Recently, the work of Kim et al. determined a dose-dependent influence of IL-12p40 on the specific CD8+ T-cell immune response: high doses of IL-12p40 caused a significant suppression of Th1 immune response development [33]. Our results suggest that p38 MAPKs are able to regulate IL-12p40 production in a different manner after T-cell activation depending on the $IL12Bpro$ polymorphism. We suppose that p38-mediated downregulation works as a protector for inappropriate IL-12p40 overexpression during Th1 immune response polarization in $IL12Bpro\-1$ homozygous individuals. The p38-mediated downregulation is lacking in the $1.2/2.2$
genotypes because of their significantly lower IL-12p40 production during T-cell activation (lower in comparison with the 1.1 genotype; Fig. 1C).

Several important control elements have been defined interacting with trans-factors in the IL12B proximal promoter region. For example, an Ets protein DNA-binding sequence, an NF-κB site and a C/EBPβ-binding site have been identified [34-36]. There is a rapidly expanding body of literature regarding an AP-1 binding site [37], and AP-1 (c-Fos/c-Jun heterodimer) has been implicated as an inhibitor of IL-12 production via suppression of IL-12p40 mRNA expression. Mitsuhashi et al. demonstrated that coexpression of c-Fos (phosphorylated by p38 MAPK) and c-Jun (primary target of JNK MAPK) resulted in an additive inhibition of IL-12p40 transcription in RAW264.7 cells [38-40]. However, as described by Bhat et al. [41], p38 kinase activates ATF-2, which can bind to the AP-1 site in the proximal promoter region and trigger p40 gene expression. The newest study revealed that zinc finger Sp1 transcription factor binds with different affinity to CTCTAA and the GC allele of the IL-12p40 distal promoter region [42]. D’Addario et al. reported that p38 kinase phosphorylated Sp1 transcription factor and enhanced the expression of Sp1-dependent genes [43]. This data could at least partially explain the differences in the p40 response during p38 and JNK inhibition.

Bearing in mind that IL-12p40 is a subunit of IL-23, we also determined the effect of IL12Bpro polymorphism on IL-23 production. The current results confirm our previous finding of relatively low IL-23 production in stimulated PBMC [20]. As is well known, PHA-stimulated T cells produce IFN-γ. On the other hand, the most recent studies revealed that the IL-23p19 gene contains an interferon-stimulated response element (ISRE), and that IFN-γ inhibited IL-23p19 mRNA and IL-23 protein expression in murine bone marrow-derived macrophages [44]. We suppose that a similar mechanism mediated the poor IL-23 production in PHA cultures. Here, we specify that the inhibition of p38 MAPK had a downregulatory effect on IL-23 production in C3bgp-stimulated PBMC from the two tested groups – IL12Bpro-1 homozygotes and 2.2 heterozygotes/homozygotes. Consequently, the effect of p38 MAPK inhibition on the IL-23 production in these cultures is independent of IL12Bpro polymorphism. One explanation could be the involvement of specific p38 MAPK downregulation of p19 subunit expression. Similar results for the differentially regulated expression of p19 and p40 through MAPKs have been reported by Petro [15]. Alternatively, our previous findings showed that the upregulated IL-23p19 gene transcription and IL-12p40 production in stimulated human monocytes are not associated with high IL-23 protein production, indicating the presence of an additional intracellular regulating mechanism driving the successful IL-23p19/IL-12p40 heterodimerization [20]. In this regard, we suppose that p38 MAPK may be involved in regulating the assembly of the p19/p40 heterodimer.

In summary, this study elucidated the involvement of JNK and p38 MAPKs in IL-12p40 and IL-23 production and how it related to the IL12Bpro
polymorphism. The results revealed that JNK and p38 MAPK inhibition in PBMC stimulated with C3bgp and LPS significantly upregulated the IL-12p40 production in *IL12Bpro-1* homozygotes, and did not influence the IL-12p40 production in *1.2/2.2* genotypes. Also, p38 inhibition led to a significant increase in IL-12p40 production in homozygous *IL12Bpro-1* PBMC stimulated with PHA. p38 MAPK inhibition decreased IL-23 production independently of the *IL12Bpro* genotype in C3bgp-stimulated PBMC. We concluded that IL-12p40 and IL-23 expression, mediated by the p38 and JNK intracellular signaling pathways, is influenced by *IL12Bpro* polymorphism.

**Acknowledgments.** This study was supported by grant 4/2008 from the Fund for Scientific and Mobile Projects, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria

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