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Effect of a Small Selective Inhibitor of C-Jun N-Terminal Kinase on the Inducible mRNA Expression of Interleukin-6 and Interleukin-18

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Abstract

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BACKGROUND: The expression of many inducible genes, involved in cell growth and differentiation as cytokine genes are regulated by receptor-activated intracellular signalling pathways, including the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase pathway.

AIM: We examined the involvement of the JNK signalling pathway in the regulation of the inducible interleukin-6 (IL-6) and interleukin-18 (IL-18) gene expression at the transcriptional level.

METHODS: Peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated with lipopolysaccharide (LPS) and C3 binding glycoprotein (C3bgp) with or without SP600125 and cultured for 6 h. After mRNA isolation, a qRT-PCR was performed.

RESULTS: Regarding IL-6 and IL-18 mRNA expression, donors were divided into two groups of high and low responders. SP600125 inhibited significantly IL-6 mRNA transcription in the high responder group and did not influence the transcription level in the low responder group. Concerning IL-18 mRNA, we detect the significant effect of SP600125 on the inducible mRNA in high responder group upon C3bgp stimulation.

CONCLUSION: JNK transduction pathway is involved in the production of IL-6 mRNA, after LPS and C3bgp stimulation. We suggest that the inhibition of JNK may be beneficial only for higher responding patients during the treatment of inflammatory and autoimmune diseases.

Introduction

The expression of many inducible genes, involved in cell growth and differentiation as cytokine genes are regulated by a receptor-activated intracellular signalling pathways including c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathway. JNK is a serine-threonine protein kinase that by phosphorylation activates c-Jun, a part of the transcription factor AP-1 [1]. Many target genes regulating the cell cycle, apoptosis and cell survival with AP-1 binding sites are regulated by JNK transduction pathway [2]. In immune cells, JNK regulates the transcription of a lot of inducible genes, including inflammatory cytokine genes [3]. More precisely, JNK is involved in the regulation of TNF- α ,

IL-12p40, IL-10 and IL-23 as is shown by previous studies of our laboratory [4], [5], [6].

Interleukin-6 (IL-6) is a part of the inflammatory response initiated by recognition of antigens referred to as pathogen-associated molecular pattern (PAMP) molecules to the pattern recognition receptors (PRR) expressed by immune cells. IL-6 is a proinflammatory and immunoregulatory cytokine with hormone-like activity – it is involved in immune regulation, inflammation, and oncogenesis [7]. During acute – phase inflammatory response liver cells secrete CRP, serum amyloid A, complement proteins and fibrinogen in response to IL-6 stimulation [8]. IL-6 also plays a key role in the humoral immune response by stimulation of proliferation of activated B cells and antibody production [7]. IL-6 induces the development of Th17 cells from naive T cells together

with TGF- β . Dysregulation and overproduction of IL-6 are connected to the development of autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA), in which Th17 cells are suspected as the major cause of the diseases [9]. Although many somatic cells and cells of the immune system produce IL-6 the major source of this cytokine are the activated monocytes/macrophages. IL-6 expression and transducing IL-6 receptor system are well understood [10]. Human IL-6 is synthesised as a protein containing up to 212 amino acids, including a 28-amino-acid signal peptide. The core protein is 20 kDa and glycosylation accounts for the size of 21 – 26 kDa of natural IL-6 [8].

Interleukin-18 (IL-18) is also a pro-inflammatory cytokine, but it is included in Th1 polarisation. It's inducible gene expression after recognition of PAMPs by PRR lead to the synthesis of an inactive protein (pro – IL-18). Pro – IL-18 (24 kDa) is converted into biologically active IL-18 (17.2 kDa) by another activation pathway in inflammasome complexes through caspase 1-mediated cleavage [11], [12]. IL-18 is involved in the development of successful Th1 cell-mediated and antitumor immune response through its ability to induce IFN- γ secretion [13]. Unlike IL-6, the mechanisms regulating IL-18 gene expression and processing remains unclear.

In this regard, our study was designed to investigate the involvement of the JNK transduction pathway in the regulation of the expression of IL-6 and IL-18 mRNA and identify the anthracycline inhibitor as a novel potential regulator of LPS-induced cytokine production in human blood mononuclear cells.

Methods

PBMC isolation

With the approval of the local ethics board, blood samples were taken from 18 healthy donors 30-40-year olds 9 male and 9 females. Informed consent was obtained from each participant. The peripheral venous blood (10 ml) was collected in sterile tubes with ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 (Sigma-Aldrich-Merck, Darmstadt, Germany) density gradient centrifugation. The interface containing PBMC was harvested and washed twice with cold RPMI-1640 medium.

Cell cultures and stimulation

PBMC (1×10^6 cells/ml) cultures were carried out in RPMI-1640 (Sigma-Aldrich-Merck, Darmstadt, Germany) supplemented with: 10% FBS, 100 U/ml penicillin, 100 μ g/ml gentamycin and 0.3 mg/ml L-

glutamine. The cells were stimulated with: 30 μ g/ml C3 binding glycoprotein (C3bgp) isolated as described previously [14] or 1 μ g/ml Lipopolysaccharide (LPS) from *Escherichia coli* serotype 026: B6 (Sigma-Aldrich-Merck, Darmstadt, Germany). The concentrations of the stimuli used were determined, according to Stanilova et al., [15] and Takahashi et al., [16]. As shown by our previous study of inducible cytokine gene expression transcripts, the maximum mRNA quantities were reached after 6 h of stimulation and were independent of stimuli used [5]. Therefore, PBMC cultures were incubated at 37°C for 6 h. After incubation, the cultures were centrifuged at 1800 rpm for 10 min. The cell pellet was separated, and the RNA was isolated.

Inhibition of JNK MAPK pathway

One hour before the stimuli addition, some of PBMC cultures were pre-treated with an inhibitor of JNK kinase. For inhibition of c-jun N-terminal kinase, we used the selective anthracycline inhibitor SP600125 (Sigma-Aldrich-Merck, Darmstadt, Germany). It competitively inhibits JNK 1, 2 and 3 with > 20-fold selectivity vs the wide range of kinases according to Bennett et al., [1]. SP600125 is dissolved in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich-Merck, Darmstadt, Germany), and the final concentration of JNK inhibitor in cell cultures is 20 μ M. Non-stimulated cell cultures were used as controls. Therefore, our experiment included the following PBMC cultures: non-stimulated, stimulated with LPS, stimulated with LPS and pre-treated with SP600125, stimulated with C3bgp, stimulated with C3bgp and pre-treated with SP600125.

RNA extraction and reverse transcription

Total RNA from cell culture pellet was isolated using innuPREP blood RNA isolation kit AJ Roboscreen (Leipzig; Germany) with the additional step of treatment with DNase I to remove traces of genomic DNA. The total RNA was quantified by spectrophotometrical analysis. Synthesis of cDNA was performed manually according to the manufacturer's instructions with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) that uses random primers and MultiScribe™ MuLV reverse transcriptase enzyme. Incubation conditions for reverse transcription were 10 min at 25°C followed by 2 h at 37°C and was performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a 7500 Real-Time PCR System (Applied Biosystems). The qRT-PCR primers and probes were purchased from

Primerdesign, UK and Termoscientific as pre-designed inventoried assay reagents. The following validated PCR primers, and TaqMan MGB probes (6FAM-labeled) were used: IL-6 (assay ID: Hs00985639_m1) and for IL-18 mRNA (AX-7948-00-0200). As endogenous controls were used eukaryotic 18S ribosomal RNA (Hs999999_s1) and β 2 microglobulin (Hs00187842_m1). An aliquot of 5 μ l of the RT reaction was amplified in duplicate in a final volume of 20 μ l using a TaqMan Universal PCR Master Mix and Gene Expression Assay mix, containing specific forward and reverse primers and labelled probes for target genes and endogenous controls (Applied Biosystems). The thermocycling conditions were: initial 10 min incubation at 95°C followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. PCR data were collected with Sequence Detection System (SDS) software, version 2.3.

Relative quantitative evaluation of cytokine mRNAs was performed by the comparative $\Delta\Delta C_t$ method. The mean ΔC_t obtained in nonstimulated controls for each cytokine mRNA was used as a calibrator, after normalisation to the average of endogenous controls 18S rRNA and β 2 microglobulin. The results are calculated as an n-fold difference relative to the calibrator ($RQ = 2^{-\Delta\Delta C_t}$).

Statistical analysis

The data were presented as a mean \pm standard error. One-way ANOVA test was used to compare the mean values between all stimuli used, followed by post hoc Fisher LSD test to analyse the effect of JNK inhibition on LPS or C3bpg induced gene expression. Differences were considered significant when the p values were < 0.05 .

Results

IL-6 mRNA expression

Results presented in Figure 1, showed that the transcription of IL-6 mRNA was upregulated after stimulation with both LPS and C3bpg. Moreover, the healthy donors were divided into two groups – individuals with high ($RQ > 200$) (6 healthy donors) and with low ($RQ \leq 200$) (12 donors) levels of the inducible IL-6 mRNA. In the high responder group, the level of mRNA expression in stimulated cultures RQ value varied between 219-2387 for LPS and 394-4728 for C3bpg stimulation. The gene expression was significantly affected by a used stimulus in high responder group ($F = 3.68$; $df = 3$; $p = 0.029$) in contrast to low responder group ($F = 1.16$; $df = 3$; $p = 0.35$). In the low responder group, the RQ value is 11-120 for LPS and 13-165 for C3bpg, respectively. We

found statistically significant inhibition of IL-6 gene transcription in cells pretreated with SP600125 compared to cultures without JNK inhibitor in high responder group – LPS vs. LPS + SP600125 $p = 0.014$; C3bpg vs. C3bpg + SP600125 $p = 0.014$ (Fisher LSD test). In the same experimental condition, we did not detect the inhibitory effect of SP600125 on the IL-6 gene transcription in the low responder group.

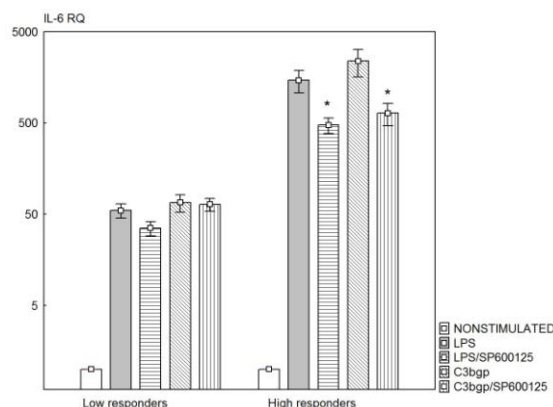


Figure 1: Levels of IL-6 mRNA transcription in PBMC of 18 healthy donors after stimulation with 1 μ g/ml LPS and 30 μ g/ml C3bpg, with or without 20 μ M JNK inhibitor SP600125. The results are presented on a logarithmic scale. The cells were harvested at 6 h after stimulation. After mRNA isolation, a qRT-PCR was performed, and the results were calculated as an n-fold difference relative to calibrator $RQ = 2^{-\Delta\Delta C_t}$. The results are presented as mean \pm standard error; * $p < 0.05$ for stimulus vs stimulus + SP600125

IL-18 mRNA expression

The levels of the inducible IL-18 mRNA were significantly lower (more than 100-fold) than those of IL-6 mRNA after stimulation with LPS and C3bpg. Regarding IL-18 mRNA expression individuals were also subdivided into two groups: high responders (8 healthy donors) (RQ between 1.201-1.979 for LPS and 1.324-3.249 for C3bpg) and low responders (10 donors) (RQ varied between 0.720-1.147 for LPS and 0.454-0.882 for C3bpg), Figure 2.

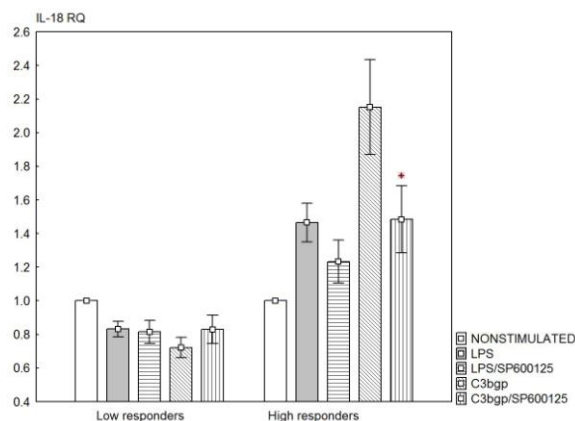


Figure 2: Levels of IL-18 mRNA transcription in PBMC of 18 healthy donors after stimulation with 1 μ g/ml LPS and 30 μ g/ml C3bpg, with or without 20 μ M JNK inhibitor SP600125. The cells were harvested at 6 h after stimulation. After mRNA isolation, a qRT-PCR was performed, and the results were calculated as an n-fold difference relative to calibrator $RQ = 2^{-\Delta\Delta C_t}$. The results are presented as mean \pm standard error; * $p < 0.05$ for stimulus vs stimulus + SP600125

The gene expression of IL-18 was also significantly affected by a used stimulus in high responder group ($F = 3.21$; $df = 3$; $p = 0.039$) in contrast to low responder group ($F = 0.89$; $df = 3$; $p = 0.46$). JNK inhibition significantly decreases C3bpg-induced IL-18 gene expression (C3bpg vs. C3bpg + SP600125 $p = 0.035$, Fisher LSD test) in contrast to LPS-induced gene expression (LPS vs. LPS + SP600125; $p = 0.43$) in high responder group. Also, upregulation of IL-18 gene expression was significantly higher after stimulation with C3bpg compared to LPS ($p = 0.04$).

Discussion

To investigate the involvement of JNK MAPK in the inducible IL-6 and IL-18 gene expression, we used a small selective inhibitor of JNK transduction pathway SP600125. The effect of JNK inhibition on the IL-6 and IL-18 mRNA expression we determined in an experimental model of LPS and C3bpg-stimulated human PBMC pretreated with inhibitor. Cell cultures were harvested on 6 h, and steady-state mRNA of IL-6 and IL-18 gene was evaluated by qRT-PCR.

We found that healthy donors were divided into two groups in terms of mRNA expression of both IL-6 and IL-18 genes—individuals with high mRNA expression (high responders) and those with low mRNA expression (low responders) of the target genes. In most cases, the same donors showed high or low IL-6 and IL-18 mRNA expression. The observed level of expression was distinct between two studied groups, showing principal differences of individual response in the regulation of IL-6 and IL-18 expression. Our experiments revealed that value for IL-6 after LPS and C3bpg stimulation varied between individuals more than a 10-fold range. This variability is much greater than would be expected due to technical reasons. It is obvious that mechanisms determining this variability of induced proinflammatory cytokine production should be searched elsewhere, probably in the individual genetic background and regulation of gene expression as well. Other authors also have a similar observation on the inter-individual variability in proinflammatory cytokine response, especially in response to LPS [17], [18]. Indeed, in their study Wurfel et al., discovered 80 genes that were differentially expressed in LPS-low and LPS-high healthy responders in the presence of LPS and 21 genes in the absence of LPS [19].

In this study for the first time, we showed that JNK inhibitor SP600125 selectively mediated its effect in PBMC from healthy individuals – SP600125 downregulated IL-6 gene expression in high responder group and did not affect the expression of this gene in the low responder group. Other

researchers did not divide its donors depending on the magnitude of IL-6 expression and reported that inhibition of JNK reduced IL-6 expression in a different type of human cells on the protein level [20], [21] IL-6 expression is tightly regulated transcriptionally by several transcription factors. NF- κ B and AP-1 are included between the major transcription factors responsible for IL-6 gene regulation [22]. Therefore, the downregulation of IL-6 gene expression by JNK inhibitor in high responder group is not an unexpected result. On the contrary, the lack of inhibitory effect of SP600125 on low responders indicates that in these individuals, the JNK transduction pathway may not be involved in inducible IL-6 gene expression. Additional experiments with inhibition of ERK and p38 is necessary to clarify whether these MAP kinases are involved in the regulation of IL-6 production in the low responder group.

At present, it is known that IL-18 mediated Th1 type of the immune response, because of its property to induce IFN- γ secretion from activated T cells and NK cells [13]. There is evidence that treatment with IL-18 has significant antitumor action due to enhanced cell-mediated immune response [11]. Also, IL-18 administration in mice inoculated with tumour cell line stimulated IFN- γ production and IL-12 independent Th1 cell-mediated antitumor immune response [23]. Our study indicated that SP600125 downregulated significantly IL-18 mRNA expression in PBMC after C3bpg, but not after LPS stimulation. There are few studies about JNK inhibition and its effect on IL-18 secretion. Similar results regarding the absence of an inhibitory effect of SP600125 after LPS stimulation was reported in primary human keratinocytes activated by β -defensins in the work of Niyonsaba et al., [24]. However, our results support the study of Miyauchi et al., reporting that JNK inhibition by SP600125 in human tubular epithelial cell from type 2 diabetic subjects significantly suppressed TGF- β -induced IL-18 expression [25] or Wang et al., showing that SP600125 inhibited IL-18 mRNA expression in murine peritoneal macrophages after heat shock response [26].

Currently, it is widely accepted that IL-6, together with TGF- β , induced the development of normal Th17 cells, whereas the additive presence of IL-23 and IL-1 lead to the development of pathological Th17 subset [27]. The pathological Th17 cells are recognised as essential cells responsible for the development of chronic inflammatory conditions and autoimmune diseases. The upregulated prolonged synthesis of IL-6, both genetically and epigenetically determined, has a significant role in the development of many diseases, including autoimmune and cancers. Dysregulated IL-6 production and subsequently, the presence of pathologic CD4 + Th17 cells were described in rheumatoid arthritis, multiple sclerosis, myeloma, systemic lupus erythematosus and others [9], [22]. Because of the pathologic role of IL-6 in the therapy of these diseases is used anti-IL-6R

monoclonal antibody tocilizumab that block IL-6 mediated signal transduction. There was an observation that tocilizumab alleviated diseases symptoms, but also several side effects are described, including hypercholesterolemia, acute pyelonephritis, inflammation of the upper respiratory system and parotid glands [28]. During immune response development of the Th1 type of the response suppressed the development of the Th17 cells by the secretion of IFN- γ [29]. Therefore, in the context of an autoimmune response downregulation of IL-6 expression by the SP600125 application may provide a desirable effect in the treatment of these diseases in high responding patients.

The results and conclusions in this article are based on studies conducted within 18 healthy donors and PBMC isolated from them.

In conclusion, the JNK MAPK transduction pathway is selectively involved in the production of IL-6 mRNA, after LPS and C3b_{gp} stimulation, and IL-18 mRNA after C3b_{gp} stimulation from PBMC isolated from higher responding individuals. We suggest that the inhibition of JNK may be beneficial only for higher responding patients during the treatment of inflammatory and autoimmune diseases mediated by the unbalanced Th1/Th17 type of immune response.

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