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Original Contribution

EFFECT OF MITOGEN - ACTIVATED PROTEIN KINASES JNK AND p38 INHIBITION ON THE INDUCIBLE PRODUCTION OF IL-12-RELATED CYTOKINES

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ABSTRACT

The expression of many inducible genes, involved in cell growth and differentiation as cytokine genes is regulated mainly by mitogen-activated protein kinases (MAPK)-signaling pathways. In this study we examined how JNK(SP600125) and p38(SB202190) MAPKs inhibitors influenced the inducible IL-12p40, IL-12p70 and IL-23 production. The quantity determination of these cytokines was performed by ELISA in culture supernatants. The inhibition of both JNK and p38 increased IL-12p40 production induced by all stimuli. The inhibition of p38MAPK downregulated IL-23 production and upregulated IL-12p40/p70 production in stimulated cells. We suggest the benefit of p38 control in the treatment of inflammatory and/or autoimmune diseases.

Key words: IL-12p40, IL-12p70, IL-23, JNK, p38

INTRODUCTION

During infection the type of the immune response developed is regulate by cytokines released from the activated immune cells. The pattern of the released cytokines is crucial and governs Thl or Th2 immune response polarization and successful defense against particular pathogen.

The expression of many inducible genes, involved in cell growth and differentiation as cytokine genes is regulated mainly by mitogen-activated protein kinases (MAPK) - signaling pathways. Each MAPK pathway is activated by a protein kinase signaling cascade. The MAPK pathways represent key routes through which extracellular stimuli are transmitted into nuclear responses. In immune cells, three MAPK-signaling cascades have been clearly characterized: extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38 [1]. Upon reaching the nucleus, the terminal MAPKs phosphorylate a number of key transcriptional factors including c-Jun, ATF-2, Elk-1, p53 and other [2]. MAPK pathways relay, amplify and integrate signals from a diverse range of stimuli and elicit an appropriate biological response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis in immune cells.

IL-12-related cytokines play a key role in the initiation and regulation of Thl adaptive and cell-mediated immune responses. Biologically active IL-12p70 is the principal immuno-regulatory cytokine that govern primary Thl immune response polarization through its ability to prime naive ThO cells for high IFN-γ production [3]. It is produced rapidly by the activated antigen - presenting cells in response to intracellular pathogens [4]. IL-12p70 is a 70-kDa heterodimer composed of two disulfide-linked glycosylated polypeptide chains of 40 (p40) and 35 (p35) kDa. Recently, Oppman et al. identified an IL-6/IL-12 - related subunit, pi9, which binds covalently with p40 to form other heterodimeric cytokine IL-23 (5). IL-23 has biological activities similar, as well as distinct from IL-12p70. Like IL-

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12p70, IL-23 induces T-cell proliferation, IFN-γ production and enhances protection against intracellular pathogens. This cytokine differs from IL-12 in target T-cell subset. While IL-12p70 acts on naive CD4+ T cells, IL-23 preferentially acts on memory CD4+ T cells and mediate secondary Thl immune response [5]. Cells that produce the biologically active IL-12p70 and IL-23 heterodimers secrete the isolated p40 chain at an excess of several fold to 1000-fold above heterodimer [6]. p40 chain form a homodimer IL-12p80 that serves as an IL-12p70 and IL-23 antagonist by competing for IL-12Rpi subunits of their receptors [3, 7].

Evidence for the involvement of MAPKs in IL-12 and IL-23 production is reported by many research teams [8, 9, 10]. There were contradictory results depending of the cell system and experimental condition used. For example, Ma et al. reported that activated JNK positively regulated LPS-stimulated IL-12p40 production in human monocytic cells and the inhibition of JNK resulted in decreased synthesis of this cytokine [8]. On the contrary Utsugi et al. reported that activated JNK negatively affects LPS-induced IL-12p40 production from human macrophages and inhibition of JNK by SP600125 dose dependently enhanced IL-12p40 production [9]. Apparently, the involvement of JNK and p38 MAPKs in inducible IL-12p40, IL-12p70 and IL-23 production remains in debates.

In this study we examined the effect of JNK and p38 MAPK signal transduction pathways inhibition on the inducible IL-12p40, IL-12p70 and IL-23 production from human peripheral mononuclear cells. To this end we utilized the small cell permeable and highly selective pharmacological inhibitors of JNK and p38 MAPKs, SP600125 and SB202190.

MATERIAL AND METHODS

1. Reagents: Lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6, phytohemagglutinin (PHA), Histopaque-1077, specific inhibitors of JNK and p38 - SP600125 and SB202190, and all culture reagents were obtained from Sigma, St. Louis, Mo. Polystyrene materials were manufactured by Corning Inc., Corning, NY., and Nunc, Roskilde, Denmark. IL-12p40, IL-12p70 and IL-23 production was measured using commercially available kits purchased from BioSource, Austria. C3 binding glycoprotein (C3bgp) was isolated in our laboratory from total water extract of the seeds of the parasitic plant *Cuscuta europea* by affinity chromato-graphy as described previously [11].

2. Blood donors and PBMC isolation : 12 healthy volunteers were recruited for this study. The informing consent was obtained from each participant. The peripheral venous blood (20 ml) was collected in sterile tubes with ethylenediamine tetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 density gradient centrifugation. The interface containing PBMC was harvested and washed twice with cold RPMI-1640 medium. After washing the cell pellet was resuspended in the same medium. PBMC viability was tested with trypan blue exclusion; cells were counted and adjusted to $5xl0^6$ PBMC/ml in RPMI-1640.

3. **Cell cultures and stimulation:** 2×10^6 PBMC were cultured in sterile polystyrene tubes containing 2 ml RPMI 1640. The cultures were supplemented with: 10% FBS, 100 U/ml penicillin, 100 pg/ml gentamicin and 0.3 mg/ml L-glutamine. The cells were stimulated with: 30 pg/ml C3bgp, 1 μg/ml LPS and 20 pg/ml PHA. Cultures were incubated at 37°C for 18 h. After incubation the cultures were centrifuged of 1800 rpm for 10 min. Supernatants was separated and stored at -70°C.

4. Inhibition of JNK/SAPK and p38 MAPK transduction pathways: 20 μM SP600125 or IOμM SB202190 highly specific cell permeable inhibitors of c-Jun N-terminal kinase and p38 MAP kinase were added 1 hour before stimulation to PBMC cultures. SP600125 and SB202190 were dissolved in 100% dimethylsulphoxide (DMSO) and the final concentration of DMSO in cultures was 0.1%. To avoid the influence of DMSO on the cytokine synthesis, the nonstimulated PBMC cultures (controls) with 0.1% DMSO were seeded.

5. Cytokine determination: The quantity determination of IL-12p40, IL-12p70 and IL-23 was performed by ELISA in culture supernatants according to the manufacturer's protocol. Color reaction developed was measured as OD units at 450 nm. The concentration of each cytokine in pg/ml by kit's standard curve was expressed. The minimum detectable concentration of the IL-12p40 ELISA kit was less than 2 pg/ml, of IL-12p70 < 0.2 pg/ml and of IL-23 < 15 pg/ml.

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

RESULTS

1. Effect of JNK and p38 MAP kinases inhibition on C3bgp, LPS and PHA - induced IL-12p40 production from human PBMC: Results presented in Fig **1,** indicated that all stimuli used were capable to induce IL-12p40 production from human PBMC and significantly enhanced its quantity in the culture supernatants. Visibly, the inhibition of both JNK and p38 MAP kinases before stimulation with C3bgp, LPS and PHA significantly increased the level of IL-12p40 production compared to the stimulated PBMC nontreated with inhibitors.

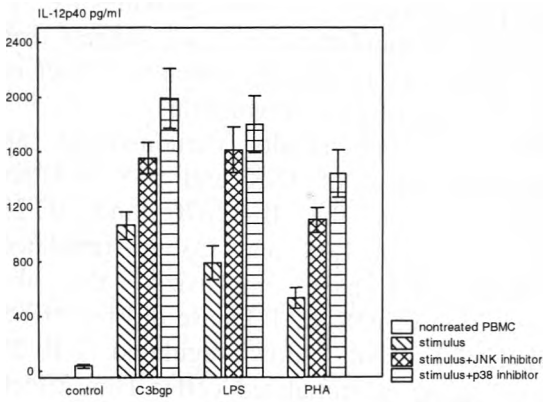

Fig.l: *Effect of the inhibition of JNK and p38 MAPKs on the inducible production of IL-12p40 by PBMC. The cells were pretreated for 1 h with 20 μM SP600125, 10 μM SB202190 and nontreated with inhibitors before stimulation with C3bgp (30 μg/ml), LPS (1 μg/ml) and PHA (20 pg/ml). Cytokine production was determined in culture supernatants by ELISA at 18 h. *P < 0.05 stimulus vs. stimulus + inhibitor.*

2. **Effect of JNK and p38 MAP kinases inhibition on C3bgp, LPS and PHA - induced IL-12p70 production from human PBMC:** All stimuli used enhanced the quantity of the IL-12p70 in culture supernatants, Fig. 2. The level of IL-12p70 secreted was relatively low, but significantly higher in comparison with spontaneous IL-12p70 production from nonstimulated PBMC. We found that the inhibition of JNK did not affect C3bp and PHA-stimulated IL-12p70 production but significantly reduced LPS-mediated IL-12p70 production. The p38

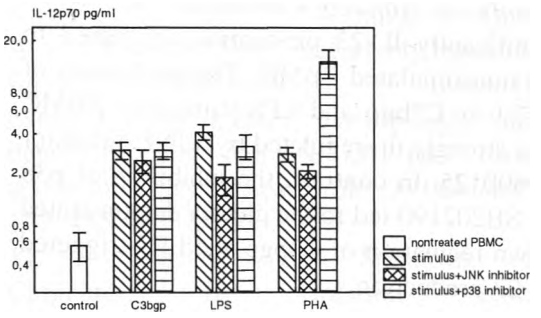

Fig. 2: *Effect of the inhibition of JNK and p38 MAPKs on the inducible production of 1L-I2p70 by PBMC. The cells were pretreated for 1 h with 20 μM SP600125, 10 μM SB202190 and nontreated with inhibitors before stimulation with C3bgp (30 pg/ml), LPS (1 μg/ml) and PHA (20 μg/ml). Cytokine production was determined in culture supernatants by ELISA at 18 h. * P < 0.05 stimulus* vs. *stimulus + inhibitor.*

MAP kinase inhibition had not any significant effect on the C3bgp- and LPS-stimulated IL-12p70 production. In contrast, the inhibition of p38 kinase before stimulation significantly increased PHA-stimulated IL-12p70 production.

3. Effect of JNK and p38 MAP kinases inhibition on C3bgp, LPS and PHA - induced IL-23 production from human PBMC: Our data showed that C3bgp was the strongest inducer of IL-23 production from human PBMC, Fig. 3. PHA failed to change

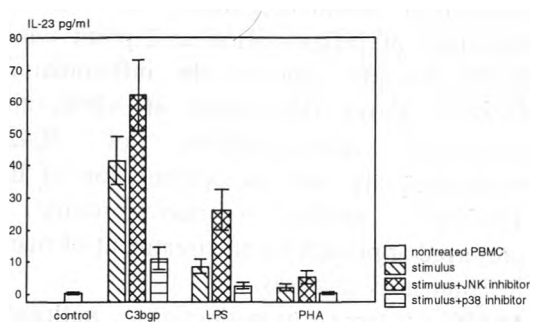

Fig. 3: *Effect of the inhibition of JNK and p38 MAPKs on the inducible production of IL-23 by PBMC. The cells were pretreated for 1 h with 20 μM SP600125, 10 μM SB202190 and nontreated with inhibitors before stimulation with C3bgp (30 μg/ml), LPS (1 μg/ml) and PHA (20 pg/ml). Cytokine production was determined in culture supernatants by ELISA at 18 h. * P < 0.05*

stimulus vs. *stimulus + inhibitor.*

significantly IL-23 production compared to the nonstimulated PBMC. The production of IL-23 by C3bgp and LPS-stimulated PBMC was strongly up-regulated by a JNK inhibitor SP600125. In contrast, the inhibition of p38 by SB202190 led to the clearly demonstrated down-regulation of C3bgp and LPS-triggered IL-23 production.

DISCUSSION

The present study elucidated the influence of JNK and p38 MAPKs inhibition on the inducible IL-12p40, IL-12p70 and IL-23 production. As well know the proper balance between IL-12p40, IL-12p70 and IL-23 plays a key immunoregulatory role and control the appearance of pathological Thl-mediated autoimmune and inflammatory diseases [3]. Previously, the development of inflammatory and autoimmune diseases was associated with high level of IL-12p70 production. Recently, Wiekowski et al. showed that transgenic expression of IL-23p 19 subunit resulted in the induction of multiorgan inflammation [12]. Further, Becher et al. demonstrated that immunized IL-12p35$^{-/-}$ mice were not resistant to experimental autoimmune encephalo-myelitis [13]. Cua et al., and Murphy et al., showed that IL-23 is required for central nervous system autoimmunity and is essential in joint autoimmune inflammation in collagen-induced arthritis [14, 15]. According to the authors IL-12p70 plays a subsequent immunoregulatory role in the late-stage of inflammation at a point when IL-23 strongly supports the inflammatory process. From this point of view, the successful downregulation of IL-23 simultaneously with an upregulation of IL-12p40/p70 production may provide a preferred approach for the treatment of many inflammatory diseases.

MAPK signal transductions pathways mediate the expression of numerous immuno-regulatory genes including IL-12 and IL-23 [8, 10]; therefore they are prime candidates for therapeutic intervention. In this study we used the pharmacological approach with specific inhibitors of JNK and p38 in order to clarify the effect of MAP kinases inhibition on the inducible IL-12p40, IL-12p70 and IL-23 production from stimulated PBMC. We have shown that the inhibition of both JNK and p38 MAPKs resulted in an upregulation of IL-12p40 production. As reported by Utsugi et al. JNK negatively regulates LPS-induced IL-12p40 production in human macrophages, because SP600125 dose-dependently enhanced IL-12p40 [9]. Similar results were reported by Ma et al. for p38 inhibition in human monocytic cells [8]. The excess of p40 produced *in vitro* contains 5-30% p80 homodimer [3]. It is well known that EL-12p80 may acts as an antagonist of IL-12p70 and IL-23 by competing for binding at the receptor complexes of both cytokines [3, 7]. Bearing in mind the above, we suggest that IL-12p80 has an anti-inflammatory effect in the immune response regulation.

Moreover, our results demonstrated an opposite effect of JNK and p38 MAPKs inhibition on the IL-12p70 and IL-23 production in LPS and C3bgp-stimulated PBMC. We have emphasized that the inhibition of p38 MAP kinase did not affect IL-12p70 but down-regulated IL-23 production in stimulated cells. This effect, combined with the strong up-regulation of IL-12p40 production, showed that the inhibition of p38 MAP kinase may have a favorable anti-inflammatory potential.

In conclusion, our study revealed that p38 MAPK inhibition downregulates IL-23 and upregulates IL-12p40/p70 inducible expression suggesting the benefit of p38 control in the treatment of inflammatory and/or autoimmune diseases.

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