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The effect of insulin-like growth factor 1 on IL-12 expression

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ABSTRACT

Recently insulin-like growth factor 1 (IGF-1) and IGF-1 receptor (IGF-1R) is considered to have a role in the regulation of immune function. It may represent an important switch governing the type and amplitude of the immune response. Through the phosphatidylinositol-3-kinase (PI-3K) pathways IGF-1R regulated the IGF-1 antiapoptotic activity. Numerous studies have implicated PI-3Ks as a regulator of toll-like receptors (TLRs) signaling in antigen-presenting cells. Various TLRs ligands lead to the production of the regulatory Interleukin (IL)-12 related cytokines that affects the T-helper differentiation.
We investigated *in vitro* the effect of IGF-1 on IL-12p40 expression from stimulated peripheral blood mononuclear cells (PBMC) isolated from healthy donors. After resting about overnight the cells were stimulated with 100 ng/mL recombinant human IGF-1 with or absent of LPS (1 μg/mL). The effect of IGF-1 on IL-12p40 was assessed by qRT-PCR and ELISA methods. We also investigated the association between the IGF-1 stimulation and a bi-allelic promoter polymorphism (*IL12Bpro*) located at -2703bp of the transcription initiation site.
Our results showed that IGF-1 modulated LPS induced expression of IL12p40 through a slight upregulation of *IL12B* mRNA simultaneously with a slight downregulation on protein levels, depending on the presence of the rare GC allele of *IL12Bpro*. This dualism of the IGF-1 modulation effect can switch the type of immune responses in norm and pathology.

Key words: Insulin-like growth factor 1, Interleukin 12p40, human peripheral blood mononuclear cells, phosphatidylinositol–3-kinase

Introduction

According to the recent studies growth factors have been implicated for governing the type and amplitude of the immune response in norm and pathology. Insulin-like growth factor 1 (IGF-1) plays a key role in normal cell development, growth, metabolism, and homeostasis. Through IGF-1R, the proliferative activity of IGF-1 is mainly regulated by the mitogen-activated protein kinase (MAPK) signaling pathway and its antiapoptotic activity by the phosphatidylinositol– 3-kinase (PI-3K) pathway (Adams et al., 2000; De Meyts & Whittaker, 2002). It is considered to be involved in fundamental physiological and pathophysiological processes such as determining life span and coping with oxidative stress (Holzenberger et al., 2003). Serum IGF-1 level has an age-related modulation with a lower concentration in the elderly (Landin-Wilhelmsen et al., 2004; Friedrich et al., 2008), mediates most of the endocrine actions of growth hormone (GH) and is also influenced with sex hormones (Hall et al., 1999; Ruiz-Torres et al., 2002; Münzer et al., 2006). IGF-1 enhances diverse aspects of bone marrow function, including lymphocyte maturation (Clark et al., 1993), granulopoiesis (Merchav et al., 1988), and erythropoiesis (Kurtz et al., 1982). IGF-1R expression on the surface of T lymphocytes can be down-regulated after cell activation (Schillaci et al., 1998). IGF-1 signaling has been substantial in attenuation the myelosuppressive effects of powerful chemotherapeutic agents such as azidothymidine

(Murphy et al. 1992a, 1992b, 1992c). Detection of IGF-1 and IGF-1R mRNAs and the proteins they encode in peripheral blood mononuclear cells (PBMC) suggests that this pathway might serve some regulatory function in the immune system. Administration of GH and IGF-1 or driving the production of IGF-1 and IGF-2 using transgenic approaches in animals promotes both B and T cell development (Smith, 2010). Thus, there is a reason to explore the potential for this endocrine pathway as a regulator of immunity. The targeting of IGF-1/IGF-1R signaling as a strategy for altering the natural course of chronic inflammation may become an attractive means of managing autoimmune disease.

Interleukin (IL)-12 is a regulatory mediator of the early innate immune response to intracellular microbes. Its most important action is to support differentiation of naïve $CD4^+$ helper T cells to the IFN-γ-producing (T_H1) subset. IL-12 exists as a disulfide-linked heterodimer of 35-kD (p35) and 40-kD (p40) subunits. The principal sources of IL-12 are activated dendritic cells (DCs) and macrophages. Many cells appear to synthesize the p35 subunit, but only phagocytes and DCs produce the p40 component and therefore the biological active cytokine. During the innate immune reaction to microbes, IL-12 is produced in response to toll-like receptors (TLR) signaling induced by many microbial stimuli, including lipopolysaccharide (LPS), flagellin, double-stranded RNA, CpG-DNA, and others (Abbas et al., 2007). Fukao was first to report PI-3K as a regulator of TLR signaling in DCs obtained from p85-/- mice (p85 - regulatory subunit of PI-3K). The researchers concluded that T_H1 responses are increased, while T_H2 responses are decreased in the p85-/- cells (Fukao et al., 2002a, 2002b). Recent work by Martin et al. indicated that the inhibitors of PI-3K and Akt suppress IL-10 production but augment IL-12 production in monocytes or PBMCs stimulated with agonists of TLR2, TLR4, TLR5, or TLR9 (Martin et al., 2005). Although there is ample evidence indicating this regulation, it is not yet clear which subtypes of PI-3Ks are activated (Hazeki et al., 2006). Thus, PI-3K activity may be central to the development of cell-mediated immunity by affecting IL-12 synthesis, or PI-3K may act by exerting an effect on counterregulatory circuits.

In regards to the regulatory role of the IGF-1/IGF-1R signaling, the objective of this study was to evaluate *in vitro* the effect of IGF-1 on the LPS induced cytokine expression of *IL-12B* in human PBMC. To clarify mechanisms of the modulation effects of IGF-1/IGF-1R signaling on *IL-12B* expression we searched for an association between the IGF-1 stimuli and a bi-allelic promoter polymorphism *IL12Bpro* located at -2703bp of the transcription initiation site of *IL-12B*.

Materials and Methods

Reagents

Lipopolysaccharide (LPS) from Escherichia coli serotype 026:B6, Histopaque-1077, and all culture reagents were obtained from Sigma, St. Louis, MO. Human recombinant IGF-1 was obtained from Gibco® by Life Technologies™ Co., Frederick, MD. Polystyrene materials were manufactured by Corning Inc., Corning, NY.

Blood donors and PBMC isolation

Eleven healthy volunteers were recruited for this study with average age of 40,6 ±16.8 years. The informing consent was obtained from each participant and authorization was given by the Ethics Review Board of the Faculty of Medicine, Trakia University. The peripheral venous blood (10 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 saline solution. After washing, the cell pellet was resuspended in RPMI-1640 medium (Sigma. St. Louis, MO).

Cell cultures and stimulation

PBMC ($2x10^6$) were cultured in sterile polystyrene tubes containing 2 ml RPMI-1640 medium. The cultures were supplemented with: 100 μg/ml gentamicin and 0.3 mg/ml L-glutamine. After an overnight of resting, cell cultures were stimulated with 100 ng/ml human recombinant IGF-1 with presence or absence of 1 μg/ml LPS. Nonstimulated cell culture was used as a control for each volunteer. Cultures were incubated at 37°C for 6 or 24 hours.

RNA isolation

Total RNA was isolated from the cell culture after the 6th hour from the stimulation. The cell pellet was re-suspended in Lysis Solution supplied with a column-based illustra RNAspin mini RNA isolation kit (GE-Healthcare, UK). The following steps for total RNA isolation were performed according manufacturer's instructions. The total RNA was quantified by spectrophotometrical analys.

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Reverse transcription

Synthesis of cDNA was performed manually according to manufacturer's instructions with High-Capacity cDNA Archive kit (Applied Biosystems, USA) that uses random primers and MutliScribe TM MuLV reverse transcriptase enzyme. Incubation conditions for reverse transcription was 10 min at 25°C followed by 2 hours at 37°C and was performed on a GeneAmp PCR System 9700 (Applied Biosystems, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a 7500 Real - Time PCR System (Applied Biosystems, Foster City, CA, USA). The following validated PCR primers and TaqMan MGB probes (Gene Expression Assay mix 6FAM-labeled, Life Technologies™ Co.) were used: *IL-12B* (assay ID: Hs00233688_m1) and eukaryotic 18S ribosomal RNA (Hs99999903_m1) as endogenous control.

An aliquot of 5 µl of the RT reaction was amplified in duplicate at a final volume of 20 µl using a TaqMan Universal PCR Master Mix (Thermo Scientific Fermentas™, Latvia) and Gene Expression Assay mix, containing specific forward and reverse primers and labelled probes for target genes and endogenous control. 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 1.3.1.

Relative quantitative evaluation of cytokine mRNAs was performed by the comparative ΔΔCt method. The mean ΔCt obtained in nonstimulated PBMC for each cytokine mRNA was used as calibrator. The results are presents as an n-fold difference relative to calibrator ($RQ=2^{-\Delta\Delta C_t}$).

Cytokine determination

Cytokine determination of IL-12p40 was obtained of the cell cultures. The quantity determination of IL-12p40 was performed by enzyme linked-immuno-sorbent assay (ELISA) kit in culture supernatants after the 6th and 24th hour from the stimulation, according to the manufacturer's protocol (Invitrogen™, Camarillo, CA). Color reaction developed was measured as OD units at 450 nm on an ELISA reader (Multiskan® EX, Thermo ELECTRON CORPORATION, Finland). The concentration was determined by using standard curve constructed with kit's standards and was expressed in pg/ml. The minimum detectable dose of Invitrogen™ Human IL-12p40/p70 ELISA kit is ≤2pg/mL.

DNA extraction and genotyping of IL12B polymorphisms

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 IL12B*pro* polymorphism was performed by amplification refractory mutation system (ARMS) - PCR. The sequences of used primers were: CTCTAA allele, marked as IL12B*pro*-1: 5'-TGT CTC CGA GAG AGG **CTC TAA** -3'; GC allele, marked as IL12B*pro*-2: 5'- TGT CTC CGA GAG AGG **GC**T GT-3' and IL-12B*pro* generic primer 5'-TGG AGG AAG TGG TTC TCG TAC-3'. The cycling parameters for IL12Bpro polymorphism were as follows: initial denaturation step of 15min at 95°C; 30 cycles of 30 sec at 95°C; 30 sec at 65°C and 30 sec at 72°C and final extension step of 7 min at 72°C.

PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems). PCR reagents were supplied by Fermentas, Latvia. PCR products were visualized by electrophoresis on an 2% agarose gel, stained with ethidium bromide (0.5 mg/ml). In each PCR run, heterozygous control and negative technical control (NTC) template was used to ensure accuracy. For quality control, 10% of random selected samples containing both cases and controls were analyzed a second time without finding any discrepancies.

Statistical analysis

Relative quantitative evaluation of mRNAs was performed by the comparative ΔΔCt method. As calibrators for the target gene IL-12p40 in monocytes was used the mean ΔCt obtained in the control not stimulated cell cultures after normalization to endogenous control 18S rRNA.

Results are presented as n-fold mean difference relative to calibrator ($RQ=2^{-\Delta\Delta C_t}$) and range (min-max value) calculated by 7500 system SDS software. Non-parametric tests were used because of the non-normal distributions of data for mRNA expression levels and protein levels. The data expressed as means ±SEs was compared by nonparametric Man-Whitney and Sign test on software platform STATISTICA7 (StatSoft™). Differences were considered significant when the p value was less than 0.05.

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Results

The effect of IGF-1 on LPS induced expression of IL-12B mRNA in human PBMC cultures

Results presented in Figure 1, demonstrated that human PBMC stimulated with recombinant IGF-1 with the absence of LPS did not enhanced significantly the *IL-12B* mRNA expression (RQ=0.74 ±0.08, mean ±SE). In PBMC cultures stimulated with LPS the expression of *IL-12B* mRNA was 67 times higher according to the nonstimulated (N) control cultures (RQ=67 ±23, mean ±SE, Man-Whitney test, $p<0.001$). In the presence of IGF-1 the LPS induced expression of *IL-12B* mRNA were no significantly different, compared to the cultures stimulated with LPS alone (RQ=67 ±20 vs. RQ=67 ±23).

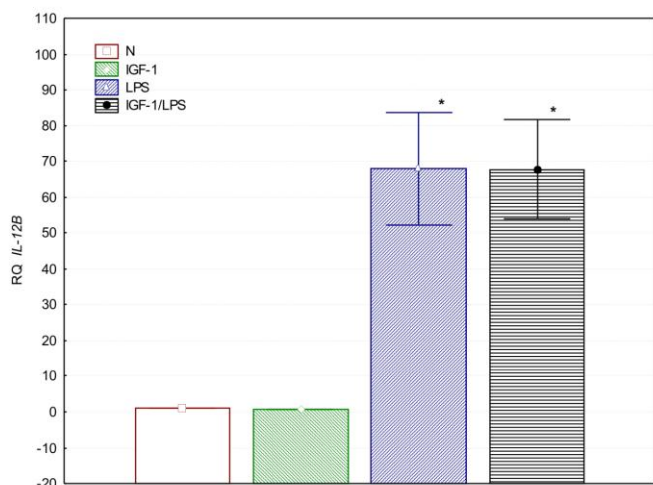

Figure 1. *Expression of IL-12B mRNA in human PBMC cultures on the 6th hour from the stimulation with 100ng/ml hrIGF-1 and 1µg/ml LPS. The results are presents as an n-fold difference relative to calibrator* $RQ=2^{-\Delta\Delta Ct}$ *(mean ± SE). N – nonstimulated control PBMC cultures; IGF-1 – PBMC cultures stimulated with IGF-1 alone; LPS – PBMC cultures stimulated with LPS alone; IGF-1/LPS –PBMC cultures stimulated with both IGF-1 and LPS; *Significantly higher than in nonstimulated cultures, p<0.05.*

It should be noted that tested individuals did not respond in the same direction. In details, 50% off the tested healthy volunteers responded with 10% to 58% of upregulation on *IL-12B* mRNA expression in cultures stimulated with both IGF-1 and LPS (IGF-1/LPS PBMC cultures), compared to the cultures stimulated with LPS alone (LPS PBMC cultures). The rest 50% off the participants responded with 9% to 30% of downregulation on *IL-12B* mRNA expression in the IGF-1/LPS PBMC cultures, compared to the LPS PBMC cultures (Table 1). We found statistically significant differences between the two subgroups according to the responses – upregulation 32±9% vs. downregulation 17 ±3%, $p=0.009$ (Man-Whitney test, mean ±SE).

The effect of IGF-1 on LPS induced expression of IL-12p40p cytokine in supernatants from human PBMC cultures

Results presented on Figure 2, show protein levels of the regulatory cytokine IL-12p40 in supernatants collected on the 6th and 24th hour after stimulation. On the 6th hour, PBMC stimulated with recombinant IGF-1 in the absence of LPS didn't decreased significantly the production of the IL-12p40 protein versus nonstimulated (N) control (5.7 ±1.7 pg/ml vs. 8.7±2.9 pg/ml). PBMC stimulated with LPS show slight upregulation in comparison of control cultures (36.2 ±8 pg/ml vs. 8.7 ±2.9pg/ml).

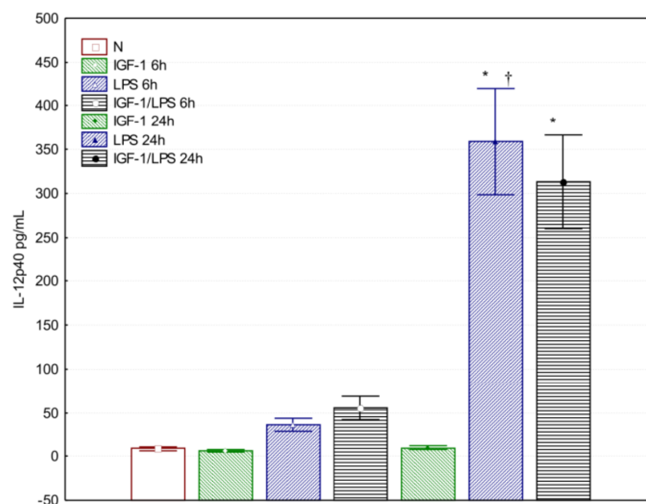

Figure 2. *IL-12p40 production by human PBMC cultures on 6th and 24th hour from the stimulation of the cultures stimulation with 100ng/ml hrIGF-1 and 1µg/ml LPS. The results are presents as pg/ml (mean ±SE). N – nonstimulated control PBMC cultures; IGF-1 – PBMC cultures stimulated with IGF-1 alone; LPS – PBMC cultures stimulated with LPS alone; IGF-1/LPS –PBMC cultures stimulated with both IGF-1 and LPS; †Significantly higher then in IGF-1/LPS PBMC cultures on 24th hour p<0.05; * Significantly higher then in PBMC on 6th hour p<0.0001.*

There was an upregulation from IGF-1 on the LPS induced expression of IL-12p40 protein on the 6th hour after stimulation (36.2±8 pg/ml vs. 55.4±20 pg/ml). The tested individuals did not respond in the same direction. Almost

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91% off the healthy volunteers responded with 10% to 56% of upregulation on protein level in the mixed IGF-1/LPS PBMC cultures in comparison with the LPS alone cultures. At the rest 9% there was very slight downregulation with 10% lower protein level in the IGF-1/LPS cultures versus the LPS cultures (Table 1).

Table 1. *Effect of IGF-1 on IL-12 expression in relation to the variable responses of tested individuals. N – nonstimulated control PBMC cultures; IGF-1 – PBMC cultures stimulated with IGF-1 alone; LPS – PBMC cultures stimulated with LPS alone; IGF-1/LPS –PBMC cultures stimulated with both IGF-1 and LPS; * compared with Sign test; ^ compared with Man-Whitney test.*

	N	IGF-1	LPS	IGF-1/LPS	n (%)	IGF-1/LPS vs. LPS	
***IL-12B* mRNA**							
RQ=$2^{-\Delta\Delta Ct}$ (mean ±SE)	1 ±0.00	0.7 ±0.1	67 ±23	67 ±20	100		
IGF-1 up	1 ±0.00	0.8 ±0.2	53.4 ±15	70.1 ±16	50	32 ±9%	*p*=0.009^
IGF-1 down	1 ±0.00	0.7 ±0.1	82.3 ±45	65.4 ±38	50	17 ±3%	
IL-12p40 protein							
6th hour (pg/ml) mean ±SE	8.7 ±2.9	5.7 ±1.7	36.2 ±8	55.4 ±20	100		*p*=0.013*
IGF-1 up	10 ±4.5	6.6 ±3	39 ±15.5	47.5 ±16	91	21 ±8%	
IGF-1 down	10.4	1.5	13.8	12.5	9	10%	
24th hour (pg/ml) mean ±SE	8.7 ±2.9	10 ±2.1	360 ±89	313 ±88	100		*p*=0.03*
IGF-1 up	10.6 ±5	10.1 ±5	318 ±147	387 ±162	28	28 ±11%	*p*=0.014^
IGF-1 down	8 ±4	10.1 ±4	375 ±112	285 ±92	72	34 ±7%	

On the 24th hour, PBMC stimulated with recombinant IGF-1 alone didn't enhance significantly the production of the IL-12p40 protein (10±2.1 pg/ml vs. 8.7 ±2.9 pg/ml). The dynamics of the LPS induced IL-12p40 protein production from the 6th hour to the 24th hour indicated significantly approximately ten times higher protein level of IL-12p40 in the LPS supernatants – LPS^{6th} 36.2±8 pg/ml vs. LPS^{24th} 360±89 pg/ml, p<0.0001 (Man-Whitney test). We found a slight downregulation from IGF-1 on the LPS induced expression of IL-12p40 protein. There was statistically significant differences for the IL-12p40 protein levels in the IGF-1/LPS versus the LPS cultures – 313±88 pg/ml vs. 360±77 pg/ml (Sign test, mean ±SE *p*=0.03). The tested individuals did not respond in the same direction, where at 28% off the healthy volunteers responded with upregulation from 10% to 44% of protein levels for the IGF-1/LPS cultures in comparison to the LPS cultures alone. The other 72% off the participants responded with downregulation from 12% to 75% of IL-12p40 protein levels in the IGF-1/LPS PBMC cultures versus the LPS alone (Table 1). There was statistically significant difference between the two subgroups depended on the response at the 24th hour – upregulation 28±11% vs. downregulation 34±7%, *p*=0.014 (Man-Whitney test, mean ±SE).

Genotype distribution of a bi-allelic promoter polymorphism IL12Bpro located at -2703bp of the transcription initiation site of IL-12B

The healthy volunteers from Bulgarian population recruited for the study were with genotype frequencies for the promoter polymorphism *IL-12Bpro* as follow: *IL-12Bpro-1.1*-18.2%: *IL-12Bpro-1.2*-54.5%: *IL-12Bpro-2.2*-27.3%.

IL-12B mRNA expression depended on IL-12Bpro polymorphism

Results on Figure 3 presented the *IL-12B* mRNA expression according to genotype distribution for *IL-12Bpro* polymorphism of the investigated healthy volunteers. We found that 40% there was an average 30% of upregulation from IGF-1 on the LPS induced *IL-12B* mRNA expression. For the rest 60% from the heterozygotes responded with an

average 8% downregulation on the *IL-12B* mRNA in the IGF-1/LPS PBMC cultures compared to the LPS PBMC cultures. 66% of all *IL-12Bpro-2* homozygotes responded with an average 34% IGF-1 upregulation and the rest 34% with an average 30% IGF-1 downregulation on the induced *IL-12B* mRNA expression. On the other hand fifty to fifty from the *IL-12Bpro-1* allele homozygotes responded with an average 20% of up- and downregulation from IGF-1 on the LPS induced cytokine expression. In contrast 45% of all *IL-12Bpro-2* allele carriers responded with an average 17% downregulation from IGF-1 on the induced cytokine expression and the other 55% of *IL-12Bpro-2* allele carriers did not respond to IGF-1 stimulation (Table 2). For the *IL-12Bpro-1* allele carriers 43% responded with an average 26% upregulation and 57% responded with an average 14% downregulation from the IGF-1 presence on the LPS induced *IL-12B* mRNA expression.

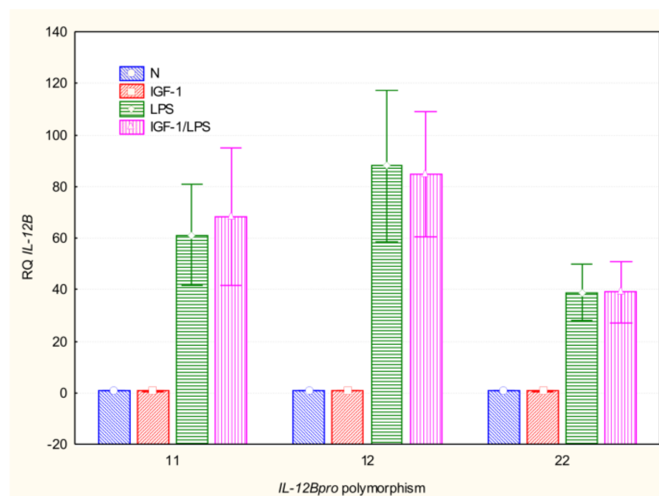

Figure 3. *Expression of IL-12B mRNA by stimulated human PBMC cultures in relation to the IL-12Bpro genotype, on 6th hour from the stimulation with 100ng/ml hrIGF-1 and 1µg/ml LPS. The results are presents as an n-fold difference relative to calibrator* $RQ=2^{-\Delta\Delta Ct}$ *(mean ± SE). N – nonstimulated control PBMC cultures; IGF-1 – PBMC cultures stimulated with IGF-1 alone; LPS – PBMC cultures stimulated with LPS alone; IGF-1/LPS –PBMC cultures stimulated with both IGF-1 and LPS.*

IL-12p40 protein production depended on IL-12Bpro polymorphism

As were described above for the IL-12p40 protein levels, on the 6th hour IGF-1 stimulus enhanced almost with 50% the LPS induced protein production of IL-12p40 (Table 1). According to the genotypes of the *IL-12Bpro* polymorphism, homozygotes for the *IL-12Bpro-1* allele responded with an average 60% upregulation while homozygotes for *IL-12Bpro-2* allele didn't show detectable response. 91% of heterozygotes correspond to an average 12 % upregulation and the other 9% with 10% downregulation. At last 91 % of *IL-12Bpro-1* and *IL-12Bpro-2* carriers respondedrespectively with an average 27% and 12% of upregulation and the rest 9% from the bth carriers with 10% downregulation on the LPS induced IL-12p40 cytokine production in the presence of IGF-1. The data on 6th hour for the protein levels in PBMC supernatants demonstrated much clear that *IL-12Bpro-1* allele was in an association with the IGF-1 upregulation of the LPS induced IL-12p40 production.

As for the IL-12p40 protein levels at the 24th hour, we found that 40% there was an average 20% IGF-1 upregulation on the LPS induced IL-12p40 protein expression. For the rest 60% from the heterozygotes responded with an average 20% downregulation on the IL-12p40 protein in the IGF-1/LPS PBMC cultures compared to the LPS PBMC cultures. *IL-12Bpro-2* homozygotes responded with an average 37% IGF-1 downregulation on the induced IL-12p40 protein production. 50 % from the *IL-12Bpro-1* allele homozygotes demonstrated an average 34% IGF-1 upregulation and the other 50% an average 62% IGF-1 downregulation on the induced cytokine production. 75% from the *IL-12Bpro-2* allele carriers responded with an average 23% IGF-1 downregulation while the rest 35% with an average 21% IGF-1 upregulation on LPS induced IL12p40cytokine production at the 24th hour. At last for the *IL-12Bpro-1* allele carriers, 62% responded with an average 23% IGF-1 downregulation and 38% with an average 21 % IGF-1 upregulation on the LPS induced cytokine production. According to these results it is obvious a tendency that *IL-12Bpro-2* allele was in strong association with downregulation while *IL-12Bpro-1* allele demonstrated both up and downregulation on the LPS induced IL-12p40 protein production at the 24th hour stimulation and IGF-1 signaling activation.

Discussion

A number of studies have demonstrated the importance of GH, IGF-1, and IGF-1R to many aspects of immune response and the inflammation, often linked to affect normal growth and patterns of tissue remodeling occurring in wound repair.

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Although these interactions could have been predicted from the well known harmful effects of chronic inflammatory disease on child growth and development, we now have gained critical insights into their basis mechanisms. Linking these biological functions is the complex interplay between cytokines and growth factors, including IGF-1 (O'Connor et al., 2008). In brief, proinflammatory cytokines seem to utilize several components of the IGF-1/IGF-1R pathway as well many of the cytokines share common signaling components, such as the Erk 1/2 MAP kinase.

Table 2. *Effect of IGF-1 on IL-12 expression by stimulated human PBMC cultures in relation to the IL-12Bpro polymorphism and the variable responses of tested individuals. For the promoter polymorphism IL12Bpro located at -2703bp of the transcription initiation site of IL-12B the CTCTAA allele was marked as IL12Bpro-1 and GC allele, marked as IL12Bpro-2.*

IL-12Bpro	allele				genotype					
	1	**n (%)**	**2**	**n (%)**	**1.1**	**n (%)**	**1.2**	**n (%)**	**2.2**	**n (%)**
***IL-12B mRNA* LPS vs. IGF-1/LPS**										
RQ=2$^{-\Delta\Delta Ct}$ mean	80 vs. 89		74 vs.73		61 vs. 68		88 vs. 84		38 vs. 38	
IGF-1 up	*26%*	43	-	55	*20%*	50	*30%*	40	*34%*	66
IGF-1 down	*14%*	57	*17%*	45	*20%*	50	*8%*	60	*30%*	34
IL-12p40 protein LPS vs. IGF-1/LPS										
6th hour pg/ml mean	33 vs. 38		47 vs. 51		9 vs. 15		42 vs. 48		39 vs. 40	
IGF-1 up	*27%*	91	*12%*	91	*60%*	100	*12%*	91	*10%*	100
IGF-1 down	*10%*	9	*10%*	9	-	-	*10%*	9	-	-
24th hour pg/ml mean	332 vs. 319		393 vs. 354		81 vs. 61		432 vs. 422		328 vs. 242	
IGF-1 up	*21%*	38	*21%*	35	*34%*	50	*20%*	40	-	-
IGF-1 down	*23%*	62	*23%*	75	*62%*	50	*20%*	60	*37%*	100

Our results show that PBMC stimulated with IGF-1 alone uninfluenced the IL-12 expression both *IL-12B* mRNA and IL-12p40 protein. There was a moderate modulation effect of IGF-1 on the LPS induced *IL-12B* mRNA expression (Table 1). Thus might be due to the residual activation of signaling pathways, in spite of the overnight resting of the PBMC cultures. At the 6th hour of the LPS induced cytokine expression, we observed an upregulation of IL-12 protein production from IGF-1. That could be discussing as an early effect of sharing common signaling components between TLR and IGF-1R activation of MAPK and of nuclear factor-κB (NK-κB) pathway. Thus various components of the inflammatory process and the IGF-1 pathway share a complex relationship that manifests in several ways. As an example, IGF-1 can increase survival in rats treated with D-galactosamine and LPS, a strategy used to induce experimental acute hepatic failure (Hijikawa et al., 2008). IGF-1 additionally reduces the production of nitrous oxide by inhibiting the inductive effects of LPS and D-galactosamine on nitric-oxide synthase mRNA and protein levels in the

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liver. These effects seem to be independent of nuclear factor-κB (Hijikawa et al., 2008). In this line, at the 24th hour our results represented a significant downregulation from IGF-1 on the LPS induced expression of the regulatory cytokine IL-12 on protein levels. Thus the study was limited from the small number of the participants these data need confirmation.

Salminen and Kaarniranta assumed that the IGF-1 activation of PI-3K pathway was involved with inhibitory κB kinase α/β (IKKα/β) (Salminen & Kaarniranta, 2010). IKKα/β has been reported as a component of TLR signaling and thereby might be in competition with the IGF-1R signaling. Also Martin et al. indicated that the inhibitors of PI-3K and Akt suppress IL-10 production but augment IL-12 production in monocytes or PBMCs stimulated with agonists of TLR2, TLR4, TLR5, or TLR9 (Martin et al., 2005). Thus it's more likely the modulating effect of IGF-1 on LPS induced IL-12p40 expression to be due to posttranscriptional regulation repertoire or the survival of the PBMC cultures. There seems to be complex interactions between the components of TLR and IGF-1R signaling. Our work should be continued with valuation of PBMC survival under the influence of IGF-1 with or without the involvement of specific PI-3K pathway inhibitor for TRL and IGF-1R signaling.

The complex promoter polymorphism, yielded by a 4bp CTCT insertion combined with an AA/GC substitution at -2703bp upstream of the *IL-12B* transcription initiation site, has been reported to have association with the IL-12p40 production (Tatebayashi et al., 2005). Considering the limitations of this study we couldn't found clear association of *IL-12Bpro* polymorphism with the IGF-1 on the LPS induced *IL-12B* mRNA expression from human PBMC. In this study the presence of mutant allele *IL-12Bpro-1* was in moderate association mostly with an upregulation from IGF-1 stimuli on LPS induced cytokine expression of IL-12. *IL-12Bpro-2* allele carriers were in moderate association mostly with downregulation of IL-12 induced expression (Table 2).

In conclusion IGF-1 affected LPS induced cytokine expression of the regulatory IL-12 cytokine more likely on a posttranscriptional regulation influenced by *IL-12Bpro* polymorphism and of sharing common signaling components between TLR and IGF-1R signaling. Although we expect that the modulation effect of IGF-1 can affect the type and magnitude of immune responses under physiological or pathological condition.

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