

PHA-induced IL-12R β_2 mRNA expression in atopic and non-atopic children

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Summary

Background IL-12 is a strong inducer of Th1 responses. Stimulation via the CD2 receptor increases IFN- γ production and enhances the responsiveness of activated T-cells to IL-12, possibly due to an up-regulation of the signal transducing β_2 chain of the IL-12 receptor (IL-12R β_2). Atopic children have a reduced Th1-like immunity and a reduced CD2 expression. Our hypothesis is that atopic individuals have a reduced function of the CD2 pathway, causing reduced responsiveness to IL-12 and decreased IFN- γ production.

Objective The aim was to study the mRNA expression of the IL-12R β_2 chain, after stimulation via the CD2 pathway in peripheral blood mononuclear cells (PBMC), of atopic and non-atopic children, and to investigate correlations to the production of Th1 and Th2 cytokines.

Materials and methods The study included 23 skin prick test positive, and 9 non-sensitized, 12-year-old children. PBMC were stimulated for 24 h with phytohemagglutinin (PHA) (2 μ g/mL), which stimulates T cells through the CD2 pathway. Expression of IL-12R β_2 mRNA was analysed by quantitative real time PCR and the cytokine production was detected with ELISA.

Results Atopic and non-atopic children had similar baseline expression of IL-12R β_2 mRNA, whereas PHA-induced IL-12R β_2 mRNA expression was lower in atopic than in non-atopic children. The PHA-induced IL-12R β_2 mRNA expression correlated well with the PHA-induced IFN- γ production and with the IFN- γ /IL-4 ratio.

Conclusion PBMC from atopic children expressed less IL-12R β_2 mRNA than non-atopic children after stimulation via the CD2 pathway (PHA). This may indicate a reduced capacity to respond to Th1-inducing stimuli in atopic children.

Keywords: CD2, PHA, IL-12R β_2 , Quantitative Real Time PCR, ELISA, cytokine, atopic children

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Introduction

T helper cells can be divided into two subpopulations, T helper 1 (Th1) and T helper 2 (Th2), based on which cytokines they produce [1]. Human Th1 cells produce interferon (IFN)- γ and tumour necrosis factor (TNF)- β , thereby activating macrophages and causing delayed type

hypersensitivity reactions [2]. Human Th2 cells produce interleukin (IL)-4, IL-5 and IL-9, causing eosinophilia and promoting immunoglobulin class switching to IgE [2]. Several factors influence the development of T helper cells along the Th1 or Th2 pathway, but the cytokines present in the environment are considered to be the single most important factor [3]. The cytokines that promote one response are able to down-regulate the other, e.g. IL-12 promotes Th1 and inhibits Th2 responses [4], and IL-4 promotes Th2 and inhibits Th1 responses [5]. Allergy is a

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Th2 phenomenon [6], including activation of allergen-specific T cells and mast cells, infiltration of eosinophils and local IgE production [7].

IL-12 is a strong inducer of Th1 responses [4,8,9]. It is a heterodimeric cytokine produced by antigen-presenting cells. The effects of IL-12 include stimulation of cytolytic activity, proliferation and IFN- γ production by T and natural killer cells [9]. Lymphocyte responsiveness to IL-12 is dependent on the expression of the high affinity form of the IL-12 receptor [10,11]. The receptor is a heterodimer composed of β_1 and β_2 subunits, expressed on natural killer cells and activated T cells only [10,12]. The β_1 subunit is relatively constantly expressed, while β_2 , which is the signal-transducing component of the receptor, is highly regulated [10]. The two subunits together compose the high affinity form of the IL-12 receptor. It is induced when naive T cells differentiate along the Th1 but not the Th2 pathway, and therefore Th1 but not Th2 cells respond to IL-12 [11,13–15].

The response to IL-12 is also dependent on the CD2 receptor, i.e. stimulation via CD2 enhances the responsiveness of activated T cells to IL-12 [8,16]. This might be due to an up-regulation of the β_2 chain of the receptor, although this has not been shown directly. The monocyte-stimulated IFN- γ production by T cells is also CD2 dependent [17]. Furthermore, it has recently been shown that CD2 enhances the interaction between MHC and the T cell receptor [18,19], which favours Th1 responses [20]. Moreover, higher CD2 expression is found on Th1, as compared to Th2, cells [21].

Atopic children have lower numbers of lymphocytes forming rosettes with sheep erythrocytes and they also have a reduced responsiveness to phytohemagglutinin (PHA) [22]. This may be a primary defect, since the same phenomenon has been seen at birth in children with atopic heredity [23]. PHA activates lymphocytes via CD2 and the T cell antigen receptor [24], and CD2 is also the receptor for sheep erythrocytes [22]. We recently showed, using monoclonal antibodies and flow cytometry, that atopic children have a lower proportion of CD2⁺ lymphocytes [25]. A reduced function of the CD2 pathway could have important consequences for the development of allergy, since a decreased responsiveness to IL-12 would result in difficulties to induce Th1-type immunity [9,26]. T cell maturation may proceed slower in atopic than in non-atopic children [22]. It is possible that a slow maturation of CD2 expression leads to the failure to down-regulate the Th2 response that dominates in allergic diseases.

Our hypothesis is that atopic children have a reduced IL-12 responsiveness associated with a defective CD2 function, giving a deficient up-regulation of the IL-12 receptor β_2 chain (IL-12R β_2). This would lead to a disturbed Th1/Th2 balance. The aim was to study the

mRNA expression of IL-12R β_2 , after stimulation of peripheral blood mononuclear cells (PBMC) via the CD2 receptor pathway (PHA), in atopic and non-atopic children. The aim was also to investigate whether there is a connection between IL-12R β_2 expression and the production of different Th1 and Th2 cytokines.

Materials and methods

Subjects

The study includes 32, 12-year-old children identified from the International Study of Asthma and Allergies in Childhood (ISAAC), phase II, Linköping, Sweden. Skin prick tests (SPT) for birch, timothy, cat, dog and two house dust mites were performed. Nine children were SPT negative and 23 were positive for one or several of the allergens tested. The children were also examined for allergic symptoms, namely allergic rhino-conjunctivitis (ARC), asthma and atopic eczema. In the SPT negative group, one child had allergic symptoms and in the positive group all children, except one, had or used to have symptoms. Of the symptomatic children, 21 had ARC, 6 suffered from asthma and 12 had atopic eczema (several children had more than one symptom).

Skin prick tests were performed in duplicate on the volar aspects of the forearms with standardized birch, timothy, cat, dog and two house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/mL) was used as a positive control and albumin diluent as a negative control. The test was regarded as positive when the mean diameter was ≥ 3 mm.

The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping.

Cell preparation

Venous blood was drawn into heparin-treated tubes (Vacuette, Greiner Labor technik, Kremsmünster, Austria). PBMC were isolated as described in detail elsewhere [25].

Aliquots of 1 mL, containing 1×10^6 cells, were cultured in AIM-V serum free medium (Life Technologies AB, Täby, Sweden) with 20 μ M mercaptoethanol (Sigma-Aldrich, Stockholm, Sweden), with or without 2 μ g/mL PHA (Sigma-Aldrich). The cells were cultured at 37 °C with 5% CO₂ (Forma CO₂-incubator model 3862, Forma Scientific Inc., Marietta, OH, USA) for 24 h.

The supernatants were collected after centrifugation and stored at –70 °C. For later RNA extraction, the pellets were lysed with 300 μ L RLT Lysis buffer (from RNeasy™

96 RNA extraction kit, Qiagen, Hilden, Germany) and then stored at -70°C .

Enzyme-Linked Immunosorbent Assay

The production of IFN- γ , IL-4, IL-5, IL-10 and IL-13 was detected with Enzyme-Linked Immunosorbent Assay (ELISA). Costar 3690 plates (Corning Inc., Corning, NY, USA) were coated overnight at room temperature (20°C). The following mouse anti-human coating antibodies were used; IL-4, IL-13 and IFN- γ (clone no. 3010.211, 32116.11 and 25718.111, respectively, R & D Systems, Abingdon, UK), IL-5 (clone no TRFK 5, PharMingen, San Diego, CA, USA) and IL-10 (coating antibody, PeliPairTM reagent set, CLB, Amsterdam, the Netherlands). The concentrations used were $2\text{ }\mu\text{g/mL}$ for IFN- γ , $0.25\text{ }\mu\text{g/mL}$ for IL-5, and $4\text{ }\mu\text{g/mL}$ for IL-4, IL-10 and IL-13. Excess free antibodies were washed off (Wellwash Ascent, Labsystems, Stockholm, Sweden) with $4 \times 250\text{ }\mu\text{L}$ PBS-Tween (0.05% Tween 20 in PBS, pH 7.4), and the wells were incubated for 1 h at room temperature on a plate shaker (Thermostar, Labvision, Stockholm, Sweden) with $100\text{ }\mu\text{L}$ /well of low-fat milk to block non-specific protein binding sites.

The plates were washed as before and $50\text{ }\mu\text{L}$ of samples or standards; recombinant human IFN- γ , IL-4 and IL-13 (R & D Systems), IL-5 (PharMingen) and IL-10 (CLB) were added to duplicate wells. A control using AIM-V serum free medium with $20\text{ }\mu\text{M}$ mercaptoethanol was also included. The plates were incubated and washed as before.

Biotinylated detection antibodies, goat anti-human IFN- γ , IL-4 and IL-13 (R & D Systems), rat anti-human IL-5 (clone no JES1-5A10, PharMingen), and IL-10 (CLB), were then added ($50\text{ }\mu\text{L}$ /well). All were diluted in high performance ELISA-dilution buffer (CLB), to the final concentrations; $0.20\text{ }\mu\text{g/mL}$ for IFN- γ , $1\text{ }\mu\text{g/mL}$ for IL-5 and $0.50\text{ }\mu\text{g/mL}$ for IL-4 and IL-13. The antibodies to IL-10 were diluted 1 : 100. This was followed by another incubation and wash.

Streptavidin-poly horseradish peroxidase ($50\text{ }\mu\text{L}$ /well, $0.1\text{ }\mu\text{M}$, CLB) was added, and the plates were incubated as before, but for 30 min, and then washed. Thereafter 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) was added ($50\text{ }\mu\text{L}$ /well), and incubated as before but for 30 min in the dark. $1.8\text{ M H}_2\text{SO}_4$ ($50\text{ }\mu\text{L}$ /well) was finally added to stop the reaction. The amount of substrate converted to product was thereafter detected as optical densities (OD) at 450 nm in an Anthos ht II ELISA reader (Anthos ht II, Labdesign, Täby, Sweden). Values were expressed as pg/mL deduced from the ODs of the reference serum curve after subtracting the blanks. The sensitivity limits for quantitative determinations were 2 pg/mL for IL-10, 3 pg/mL for IL-4 and IL-5, 12 pg/mL for IFN- γ and 16 pg/mL for IL-13.

RNA extraction

Total ribonucleic acid (RNA) was isolated according to the RNeasyTM 96 Protocol (Qiagen), for isolation of total RNA from animal cells. In brief, cells were lysed with RLT lysis buffer, mixed with ethanol, and applied to a RNeasy 96TM well plate. Contaminants were washed away by wash buffers, the membrane was dried and the RNA was eluted in $2 \times 30\text{ }\mu\text{L}$ RNase free water.

Reverse transcription (RT) PCR of mRNA

RNA was converted to complementary DNA (cDNA) using an oligo dT-primer, random decamer primer and M-MLV reverse transcriptase for 1 h at 42°C followed by 10 min at 75°C , according to the Reverse-it protocol of the supplier (Abgene Advanced Biotechnologies Ltd, Epsom, UK). The samples were run in a GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Quantification of IL-12R β_2 mRNA with quantitative real-time PCR

To quantify the amount of mRNA present in the samples, the ABI PrismTM 7700 Sequence Detector (Perkin Elmer Applied Biosystems) was used. Oligonucleotide primers designed for human IL-12R β_2 and rRNA sequence were bought from MedProbe (Oslo, Norway) (Table 1). The rRNA sequence was as recommended by Perkin Elmer (forward primer: CGGCTACCACATCCAAGGAA, reverse primer: GCTGGAATTACCGCGGCT, probe: TGCTGGC ACCAGACTTGCCCTC). The TaqMan probes had the fluorophore FAM (6-carboxy-fluorescein) at the 5'-end and the quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3'-end and were obtained from MedProbe. Both primers and probes were HPLC-purified.

The TaqMan Universal Master mix, including AmpliTaq Gold Polymerase, Amp Erase UNG (prevents reamplification of carryover-PCR products by removing any uracil incorporated into double stranded DNA, the enzyme is then inactivated at the hot start), dNTPs with dUTP, passive reference and optimized buffer components, and the 96-well reaction plates (MicroAmp Optical) were bought from Perkin Elmer. Preparations were done according to the instructions of the supplier. In short, reaction mix and samples were added in duplicate wells, supplied with optical caps and centrifuged briefly before entering the thermocycler. The thermal cycle conditions were, 50°C for 2 min followed by 95°C for 10 min (hot start) and then 40 cycles were run with 15 s at 95°C and 1 minute at 60°C , as recommended by Perkin Elmer for the SDS 7700 system.

Samples were run with primers and probe for the IL-12R β_2 subunit and for ribosomal RNA (rRNA) in two

Table 1. Description of primers, probe and amplicon

Target	Primer/probe	Sequence (5'-3')	Conc. (nM)	Position	T _m (°C)	Amplicon length (bp)
IL-12R β_2	FP	ACATTCTTGGACATAG T GAGGCC	50	379–401	58	80
	Probe	TCCTCCGTGGGACATTAGAATCA AATTTCAAA	125	405–436	69	
	RP	GTACATCTGCTCACGGAAGCC	50	438–458	58	

The sequences and positions for the primers and probe used for real time quantitative PCR. Final concentration and melting temperature (T_m) are shown for each primer and probe. FP = forward primer, RP = reverse primer. Letters in bold indicate an exon-exon junction, determined with the kind help of Dr René de Waal Malefyt. To avoid amplification of genomic sequences, it is desirable with primers hybridizing at exon-exon junctions of the mRNA, since these are separated by large introns in the genomic sequence.

different wells (Table 1). Water was included as no template control (NTC). The results from the rRNA reactions were used as internal controls, i.e. the amount of IL-12R β_2 was calculated relative to the amount of rRNA present in each sample. Standards, from which a standard curve was calculated, were included in each run. The slopes of the standard curves were -3.1 ± 0.3 (mean $\pm$ SD), indicating optimal reactions.

Statistics

Since the expression of IL-12R β_2 mRNA and the cytokine production were not normally distributed, non-parametric tests, corrected for ties, were used. Comparisons between unpaired groups were analysed with Mann–Whitney *U*-test, comparisons between paired groups with Wilcoxon signed rank test, and correlations were analysed with Spearman's rank order correlation coefficient test. A *P*-value < 0.05 was considered significant. Calculations were performed with a statistical package StatView 5.0 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Quantification of IL-12R β_2 mRNA expression

The amount of mRNA for IL-12R β_2 in PBMC stimulated with PHA was significantly higher ($P < 0.001$, Wilcoxon signed rank test) than in the control cells. No difference could be seen between the control cells from atopic (SPT positive) and non-atopic (SPT negative) children (Fig. 1). The amount present in the control cells was then withdrawn as background and the expression was compared between atopic and non-atopic children. The PHA-stimulated cells from atopic children (SPT positive) had a significantly ($P < 0.01$) lower expression of IL-12R β_2 than the cells from non-atopic (SPT negative) children (Fig. 2).

Production of IFN- γ , IL-4, IL-5, IL-10 and IL-13 and correlations with IL-12R β_2 mRNA expression

The production of IFN- γ was significantly ($P < 0.05$) lower in the SPT positive group, as compared to the SPT negative group (Fig. 3), whereas the IL-4, IL-5, IL-10 and IL-13 secretion were similar in both groups. The best correlations could be seen between expression of IL-12R β_2 mRNA and the amount of IFN- γ produced ($\rho = 0.68$, $P < 0.001$). The IFN- γ /IL-4 ratio also correlated well with IL-12R β_2 expression ($\rho = 0.57$, $P < 0.01$). All the other cytokines measured had a weaker correlation or no correlation at all.

Discussion

In this study, we have demonstrated that stimulation via the CD2 pathway (PHA) resulted in an up-regulation of IL-12R β_2 mRNA expression. This has previously not been shown directly, although blocking CD2-inhibited IL-12 induced proliferation and IFN- γ production [8]. Furthermore, stimulating PBMC with monoclonal antibodies to CD2, or to CD2 and CD28 in combination, resulted in an up-regulation of IL-12R β_2 mRNA expression (unpublished observations). Thus, our findings are in agreement with studies showing that IL-12 responsiveness, including IFN- γ secretion, is CD2-dependent [8,16,17]. Moreover, it has recently been shown that CD2 enhances the interaction between MHC and the T cell antigen receptor [18,19], which favours Th1 responses [20]. Furthermore, higher CD2 expression is found on Th1, as compared to Th2, cells [21].

We have also shown that the expression of IL-12R β_2 was lower in atopic compared to non-atopic children, when PBMC were stimulated via the CD2 pathway by PHA. This agrees with the results of another study [12] which, by the use of *in situ* hybridization, showed decreased levels of IL-12R β_2 expression in ethmoid sinus tissue of patients with

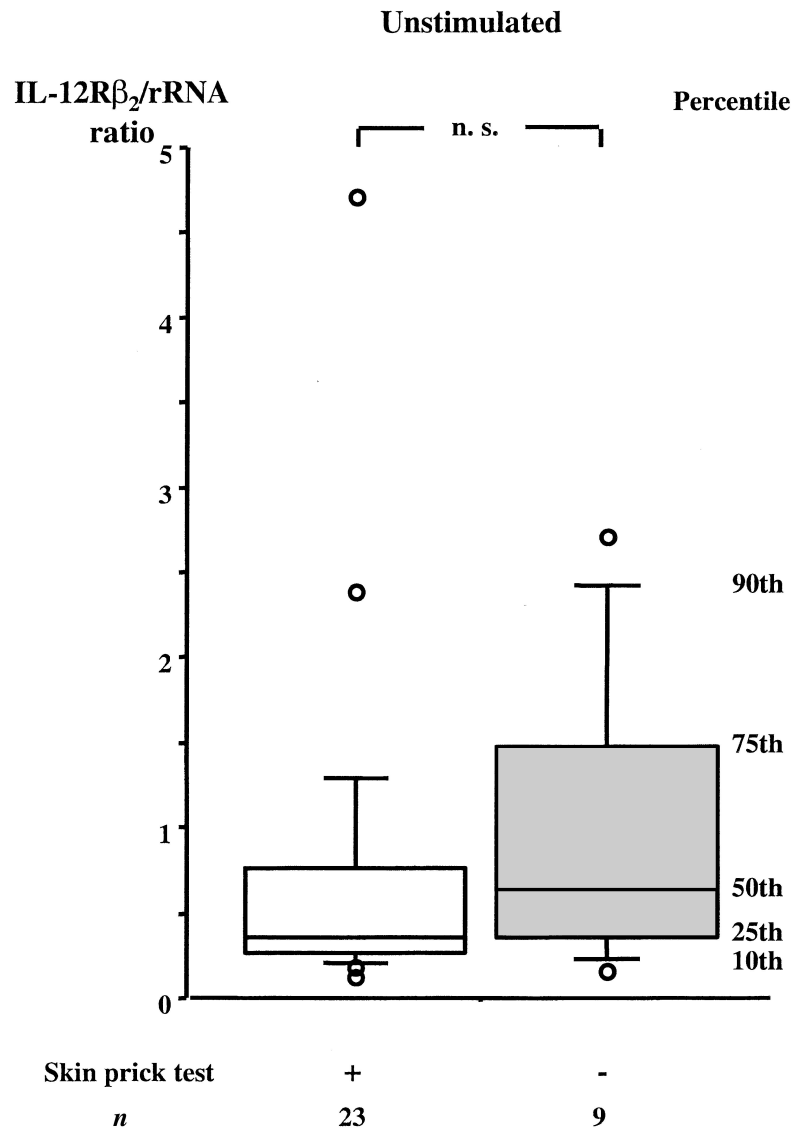


Fig. 1. Ratios of IL-12R β_2 mRNA/rRNA expression, in unstimulated peripheral blood mononuclear cells from 12-year-old children with positive ($n = 23$) and negative ($n = 9$) skin prick tests (SPT). The 10th, 25th, 50th, 75th and 90th percentiles are indicated, as well as outliers. The mRNA expression was quantified with real time PCR and the groups were compared using Mann–Whitney U -test.

allergic rhinitis. Moreover, the IL-12-induced IFN- γ production is low in PBMC from patients with atopic dermatitis [27] and allergic rhinitis [28]. In contrast to our results, another study [29] using RT-PCR, showed no difference between patients with allergic rhinitis and control subjects, considering the IL-12R β_2 mRNA expression in ethmoidal mucosae. On the other hand, the primers in that study amplified the IL-12R β_2 sequence where no known exon-exon junctions are situated, and therefore it is possible that genomic DNA also were amplified. The lower expression of IL-12R β_2 mRNA in atopic children supports our theory that they fail to up-regulate the β_2 receptor component. Since the responsiveness to IL-12 is dependent on this up-regulation [10,11], atopic children have a reduced ability to respond to IL-12. This in turn, could have important consequences in the development of

allergic diseases where increased IL-12 responsiveness should favour a shift from Th2 to Th1 response. Our results also support a decreased CD2 function in atopic children [25], which is likely to be a primary defect [23].

The correlation between IFN- γ production and IL-12R β_2 mRNA expression supports previous studies showing that IL-12 increases the IFN- γ production [9,11]. It has also been shown that treatment with IFN- γ augment the high affinity IL-12 receptor [30]. The good correlation seen between the IFN- γ /IL-4 ratio and IL-12R β_2 expression indicates that the up-regulation of the high affinity form of the receptor is preferably a Th1 phenomenon. This is also in line with our theory that up-regulation of the β_2 component of the IL-12 receptor is deficient in allergy.

In conclusion, we have demonstrated that atopic children express less IL-12R β_2 mRNA and this probably contributes

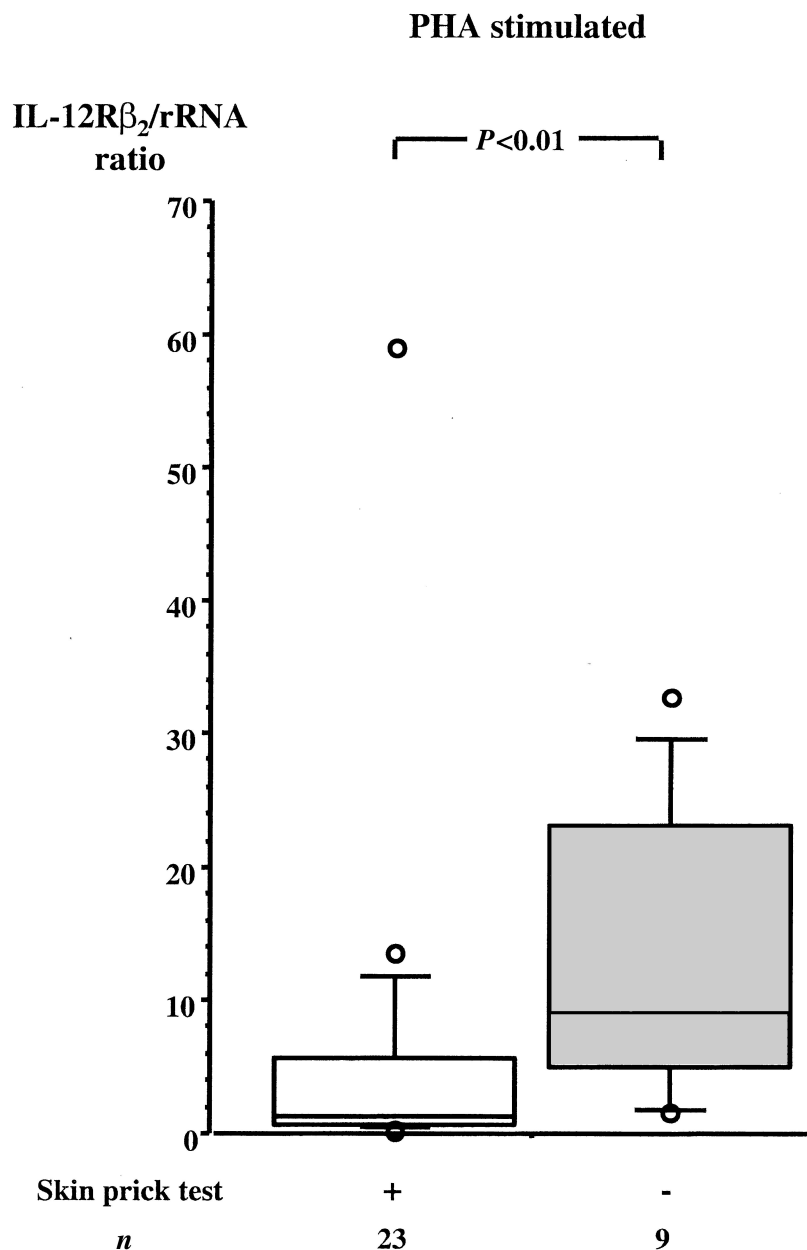


Fig. 2. Ratios of IL-12R β_2 mRNA/rRNA expression, in PHA-stimulated (2 μ g/mL) peripheral blood mononuclear cells, from 12-year-old children with positive ($n = 23$) and negative ($n = 9$) skin prick tests (SPT). The mRNA expression was quantified with real-time PCR and the groups were compared using Mann–Whitney *U*-test.

to their Th2-skewed immunity, since decreased expression of the IL-12 receptor may result in difficulties to induce Th1 immunity.

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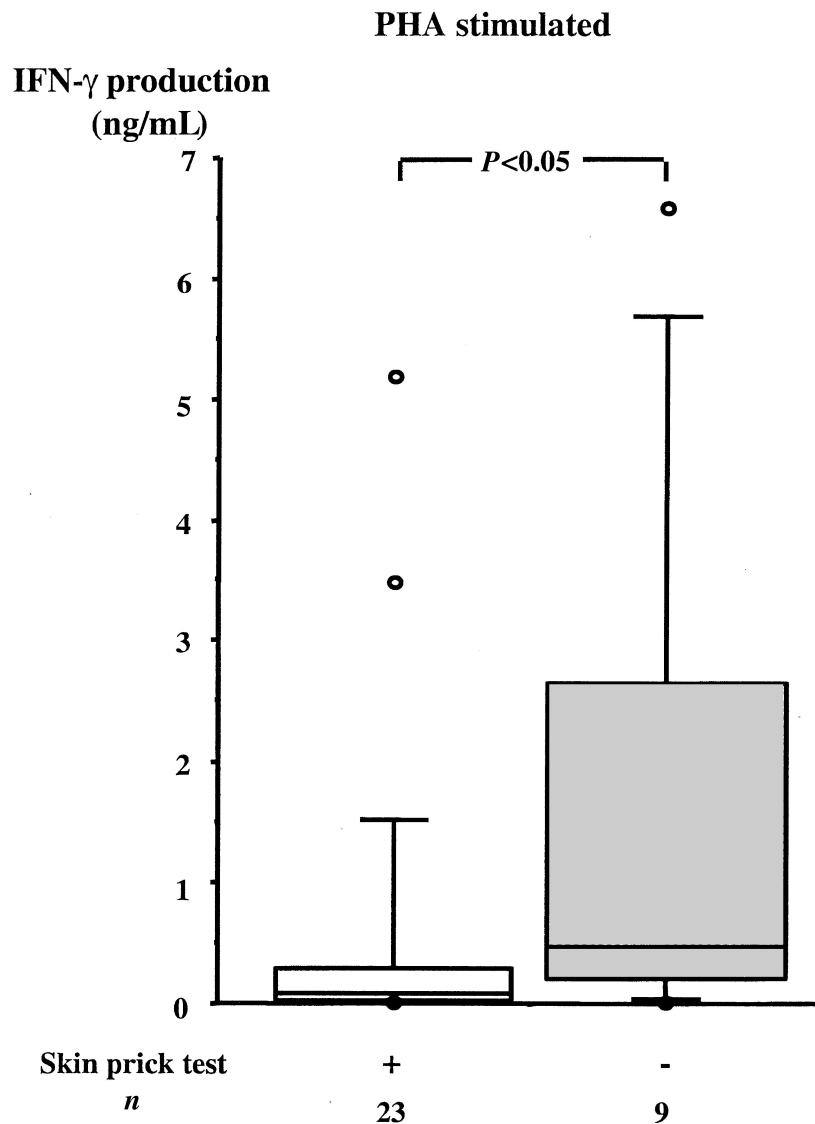


Fig. 3. IFN- γ production by PHA-stimulated (2 μ g/mL) peripheral blood mononuclear cells, from 12-year-old children with positive ($n = 23$) and negative ($n = 9$) skin prick tests (SPT). The cytokine production was detected with ELISA, with a sensitivity of 12 pg IFN- γ /mL, and the groups were compared using Mann–Whitney U -test.

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