

Reduced IL-2-induced IL-12 responsiveness in atopic children

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Atopy may be associated with a reduced T-cell function particularly regarding maturation of T helper 1 (Th1) responses. We hypothesized that atopic children may have a reduced capacity to up-regulate the β_2 subunit of the interleukin-12 (IL-12) receptor (IL-12R β_2 , the signal-transducing component). The study included 38 children followed from birth to the age of 7 years. Twenty one had a cumulative history of atopic disease, whereas 17 had none. Sixteen out of 21 children also had atopic symptoms within the past year (current), out of whom 10 children had atopic airway symptoms. The expression of IL-12R β_2 mRNA was analyzed by quantitative real-time PCR and the secretion of interferon- γ (IFN- γ), IL-5 and IL-10 was assessed by enzyme-linked immunosorbent assay (ELISA). Children with current atopic airway symptoms and high levels of total IgE up-regulated IL-12R β_2 mRNA expression less than non-atopic children with low IgE levels after IL-2 stimulation. This was accompanied by a low IL-2- and IL-12-induced IFN- γ production, possibly reflecting the reduced capacity of atopic children to up-regulate the IL-12 receptor. As IL-2 is needed to initiate and sustain immune responses and IL-12 promotes Th1 responses, this may contribute to the Th2-skewed pattern in atopic children.

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Immune responses to allergens are regulated by competing T helper 1 (Th1) and Th2 cell populations (reviewed in 1). Atopic disease is associated with Th2-skewed immune responses to allergens (reviewed in 1). Interleukin-12 (IL-12) is produced by antigen-presenting cells and promotes Th1 responses (2). The receptor (IL-12R) is composed of β_1 and β_2 subunits, primarily expressed on activated natural killer (NK) cells and activated T cells (3, 4). The β_2 subunit is the signal-transducing component of the receptor and is highly regulated, in contrast to the β_1 subunit, which is relatively constantly expressed (5). Together the subunits compose the high affinity form of the IL-12R, which is induced when naïve cells differentiate along the Th1 pathway (5). The IL-12R β_2 expression of human T cells is up-regulated by the Th1 cytokines interferon- γ (IFN- γ) and IFN- α , but down-regulated by the Th2 cytokines IL-4, IL-13 and the anti-inflammatory cytokine IL-10 (5).

Furthermore, Th2 cells do not express IL-12R β_2 on their surface (5).

It has previously been observed that IL-12-induced IFN- γ production is lower in atopic than in non-atopic individuals (6–11). The underlying mechanisms are not known, however and it may be hypothesized that an impaired expression of the IL-12R β_2 subunit in atopic individuals results in a reduced IFN- γ production. We recently observed that atopy was associated with a reduced phytohemagglutinin (PHA)-induced IL-12R β_2 mRNA expression in 12-year-old children (12).

We hypothesize that the reduced IL-12-induced IFN- γ production in atopic children is caused by a deficient up-regulation of the IL-12 receptor β_2 chain (IL-12R β_2). The aim of this study was to investigate IL-12R β_2 mRNA expression after stimulation of peripheral blood mononuclear cells (PBMC) with IL-2 or IL-12 alone, or in combination, in atopic and

non-atopic children. Moreover, the production of the Th1 cytokine IFN- γ , as well as the anti-inflammatory cytokine IL-10 and the Th2 cytokine IL-5 was studied. Thirty-eight children were prospectively followed from birth to the age of 7 years. The cumulative history of atopic disease was recorded, as well as atopic symptoms within the past year.

Materials and methods

Subjects

The investigation included 38, 7-year-old children who previously participated in a prospective study of T-cell function in atopic children in Linköping, Sweden (13). That study comprised 54 children selected from an original cohort of 172 children based on the availability of separated PBMC and a clear atopic ($n = 25$) or non-atopic ($n = 29$) cumulative history of atopic disease at the age of 18 months, as defined in (14). All 54 children were asked to take part in the 7-year follow-up and 38 agreed to participate.

Diagnostic criteria

At the 7-year clinical follow-up, the children were seen by an experienced research nurse and the parents were asked questions about any on-going atopic symptoms. The research nurses scored the severity of any on-going atopic eczema according to the atopic dermatitis (SCORAD) index (15). A parent answered a questionnaire focusing on allergic symptoms after the age of 18 months including similar questions regarding symptoms within the past year (= current symptoms). The definitions used were: atopic dermatitis (AD) = a persisting or relapsing itching rash which was coming and going for at least 6 months; bronchial asthma (AB) = at least one episode of obstructive bronchitis after the age of 2 years verified by a physician together with an asthma diagnosis in the examined records; allergic rhinoconjunctivitis (ARC) = rhinitis and/or conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection; and gastro-intestinal allergy = vomiting and/or diarrhea on at least two separate occasions after intake of a certain offending food. Urticaria was regarded as allergic if appearing at least twice within 1 h after exposure to a particular antigen.

The records from primary care units, private pediatricians and the Paediatric Clinic were examined if the parents answered that their child had been seen by a physician, due to asthma,

wheezing, skin problems, urticaria or any suspect allergic reaction. The questionnaires and the records were evaluated by one of us (HZ) and the children were then classified as non-atopic or atopic. The results of the laboratory data, except for the skin prick tests (SPT), were not known at the time of the classification.

Skin prick tests

Single SPT at 7 years of age were performed using standardized extracts of cat, dog, horse, birch, timothy grass, mugwort, house dust mites (*Dermatophagoides farinae* and *pteronyssinus*) and mold (*Cladosporium herbarum* and *Alternaria*) (Solu-Prick SQTM, ALK, Hørsholm, Denmark). SPT were performed in duplicates at 18 months, using fresh hen's egg white and cow's milk and standardized extracts of cat, birch, house dust mite (*D. farinae*) and timothy grass (Solu-Prick SQTM). Histamine hydrochloride, 10 mg/ml, was used as a positive control and albumin diluents as a negative control (both at the 18-month and the 7-year follow-up). The wheal size was marked with a filter pen after 15 min and transferred onto a micropore tape for measuring. The SPT were regarded as positive when the mean wheal diameter was at least 3 mm. Antihistamines were not allowed within 3 days before the SPT.

Clinical diagnosis and allergic sensitization

The children were examined at approximately 7.5 years (range: 7.25–7.75). From birth, the cumulative history of atopic disease yielded 21 atopic children and 17 non-atopic children. Four children with a cumulative history of atopic disease within the first 18 months of life had not shown any atopic symptoms after that age. Thus, atopic symptoms appeared in 17/38 children (8 AB, 15 AD and 9 ARC) between the age of 18 months and 7 years.

Sixteen children reported current atopic signs and seven of them had more than one atopic disease. Ten children had current airway symptoms (6 AB, 8 ARC), 12 had current AD and one child also had urticaria after exposure to egg. Seven of 12 children with AD had on-going mild atopic eczema at the follow-up according to the SCORAD index. Three of the asthmatic children with current symptoms used inhalation steroids regularly and two during respiratory infections.

At 18 months, 11/38 were SPT positive to egg white and 1/38 also to cow's milk. At 7 years, SPT were performed in 37/38 children, of whom 25 were SPT negative and 12 were positive for

one or several of the allergens tested: cat, $n = 7$; timothy grass, $n = 4$; horse, $n = 3$; birch, $n = 4$; dog, $n = 2$; house dust mite (*D. pteronyssinus*), $n = 2$; mugwort, $n = 1$. One child with a positive SPT at 18 months had a declined SPT at 7 years, but had a high total serum IgE level. This child was SPT positive to several aero-allergens at the age of six. All but two SPT positive children were symptomatic within the past year. The two non-symptomatic children used to have symptoms at a younger age. In the SPT negative group, five children had current atopic symptoms (1 AB, 3 AD, 1 AD plus ARC).

IgE antibody analysis

Total serum IgE levels (kU/l) were analyzed with the fluoroenzyme immunoassay UniCAP[®], according to the manufacturer's recommendations (Pharmacia Diagnostics, Uppsala, Sweden).

Cell preparation

Venous blood was drawn into heparin-treated tubes (Vacuette, Greiner Labor technik, Kremsmünster, Austria). PBMC were isolated as described in detail elsewhere (13).

Aliquots of 1 ml, containing 1×10^6 cells in RPMI-1640 (Invitrogen, Täby, Sweden) with 10% fetal Calf Serum (FCS) (Invitrogen), were cultured in medium alone or with 100 pg/ml IL-12 (R and D Systems, Abingdon, UK), 5 ng/ml IL-2 (R and D Systems) or with IL-2 in combination with high and low concentrations of IL-12 (100 pg/ml and 25 pg/ml, respectively). The incubation time was 72 h at 37°C with 5% CO₂ (Forma CO₂-incubator model 3862, Forma Scientific Inc., Marietta, OH, USA). Background responses, i.e. cultures with medium only, were subtracted from the IL-2- and IL-12-induced values.

The supernatants were collected after centrifugation and stored at -70°C. For later RNA extraction, the pellets were lysed with 300 µl RLT Lysis buffer (from RNeasy[™] 96 RNA extraction kit, Qiagen, Hilden, Germany) and then stored at -70°C.

Analysis of cytokine production

The production of IFN- γ , IL-5 and IL-10 was detected with enzyme-linked immunosorbent assay (ELISA), as described by Janefjord and Jenmalm (12). The amount of substrate converted to product was detected as optical densities (OD) at 450 nm in a VERSAmax tunable microplate reader (Göteborgs Termometerfabrik,

Göteborg, Sweden). Values were expressed as pg/ml deduced from the ODs of the reference serum curve after subtracting the blank values (RPMI-1640 with 10% FCS). The sensitivity limits for quantitative determinations were 3 pg/ml for IL-5, 5 pg/ml for IL-10 and 25 pg/ml for IFN- γ .

Analysis of IL-12R β_2 mRNA expression

RNA extraction, reverse transcription and analysis of IL-12R β_2 mRNA expression by quantitative real-time PCR were performed as described in Janefjord and Jenmalm (12), except that the reverse transcription was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA).

The amount of IL-12R β_2 was calculated relative to the amount of rRNA present in each sample.

Statistics

Because the expression of IL-12R β_2 mRNA and the cytokine production were not normally distributed, non-parametric tests were used. Comparisons between unpaired groups were analyzed using the Mann-Whitney *U*-test and between paired groups Wilcoxon's paired test was performed. Correlations were assessed with Spearman's rank order correlation coefficient test. Samples below the sensitivity limit of the ELISA were given half the cut-off value. A *p*-value < 0.05 was considered significant. Calculations were performed with a statistical package STATISTICA[™] 5.1 for Windows (StatSoft[®], Tulsa, OK, USA). If not otherwise described, median values and range are shown.

Ethics

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

Results

In the following comparisons, atopic symptoms within the past year are described as 'current' and a history of atopic symptoms within the first 7 years of life are defined as 'cumulative atopy'. A value above/below the median value of total IgE in serum is described as a high/low IgE at 7 years. The median value of total IgE (25–75 percentile) for all children was 72.2 (25.9–215), $n = 38$ for children with cumulative atopy 75.0 (39.6–418.0), $n = 21$, with current atopy 223.0

(50.4–529.0), $n = 16$ and in non-atopic children 71.1 (25.7–157.0), $n = 17$.

Blood samples were obtained from all children. Missing results of some parameters were due to low cDNA yield.

IL-12R β_2 mRNA expression in relation to cytokine production

The spontaneous expression of IL-12R β_2 mRNA strongly correlated to IL-12-induced IFN- γ secretion (Fig. 1a), but not with IL-12-induced IL-5 or IL-10 ($n = 35$, $\rho = 0.13$, $p = 0.46$ / $n = 35$, $\rho = -0.05$, $p = 0.79$, respectively). Furthermore, the IL-2-induced expression of IL-12R β_2

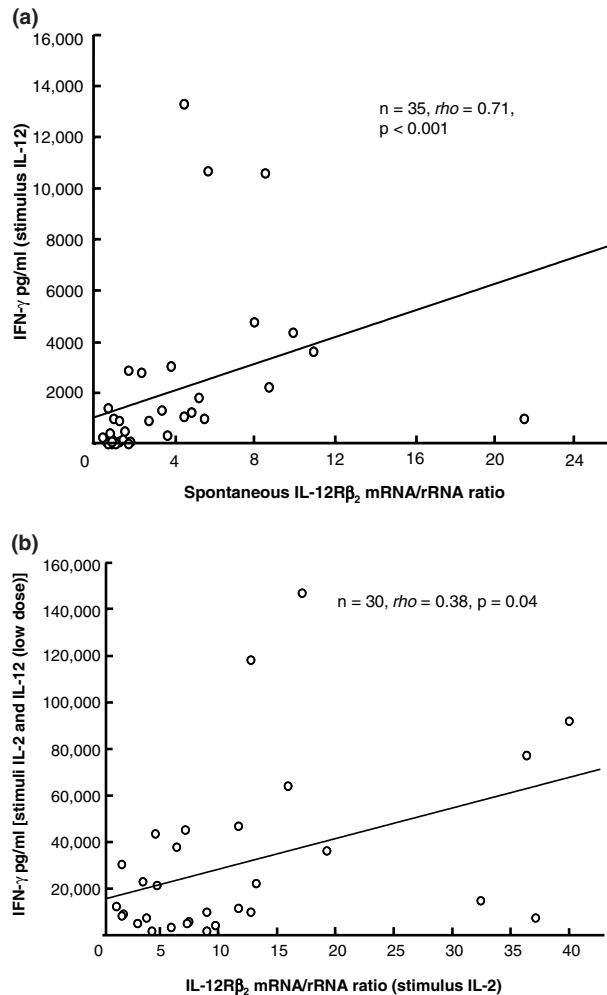


Fig. 1. (a) The correlation between the spontaneous expression of IL-12R β_2 mRNA and the IL-12-induced IFN- γ secretion at 7 years of age in all children (regardless of atopic symptoms). The correlation was analyzed with Spearman's rank correlation and the Rho value is given. (b) The correlation between the IL-2-induced expression of IL-12R β_2 mRNA and the IL-2/IL-12-induced (low dose) IFN- γ secretion at 7 years of age in all children (regardless of atopic symptoms). The correlation was analyzed with Spearman's rank correlation and the Rho value is given.

mRNA correlated with IL-2- and IL-12-induced (low dose) IFN- γ secretion (Fig. 1b). Addition of a high ($n = 29$) or low ($n = 27$) dose of IL-12 further enhanced IL-2-induced IL-12R β_2 mRNA expression, ratio: 7.4 (0.9–37.3) compared with 16.9 (1.7–89.4)/14.0 (4.1–52.5), respectively. The IL-2-induced IFN- γ and IL-10 production were also enhanced after addition of IL-12 (high/low dose, $n = 38$, $p < 0.0001$, data not shown). In contrast, addition of the high, but not the low, dose of IL-12 inhibited the IL-2-induced IL-5 secretion ($n = 38$, $p < 0.001$).

IL-12R β_2 mRNA expression and cytokine production in relation to atopic symptoms

Atopic and non-atopic children had similar spontaneous expression of IL-12R β_2 mRNA (Table 1). Children with current atopic airway symptoms and high levels of IgE had a significantly lower IL-2-induced IL-12R β_2 mRNA expression than non-atopic children with low IgE levels (Fig. 2). The expression of IL-12R β_2 induced by IL-2 together with the high dose of IL-12 correlated negatively to total IgE ($n = 32$, $\rho = -0.48$, $p = 0.01$), with a similar trend when IL-2 was used alone ($n = 30$, $\rho = -0.32$, $p = 0.09$). IFN- γ production after stimulation of PBMC with IL-2 and the low dose of IL-12 was lower in sensitized children with current airway symptoms [5622 pg/ml (1306–43633), $n = 7$ vs. 21242 (2982–118154), $n = 17$, $p = 0.01$] than in non-atopic children. Moreover, it was lower in children with a cumulative history of atopy and high IgE levels than in non-atopic children with low IgE levels (Fig. 3), as well as in children with current airway symptoms and/or skin symptoms and high IgE levels ($p = 0.04$ / $p = 0.02$, respectively, data not shown).

Total IgE correlated negatively to IL-2- and IL-12-induced (low dose) IFN- γ production ($n = 38$, $\rho = -0.40$, $p = 0.01$).

The IL-10 production after IL-2 stimulation alone was higher in children with a cumulative history of atopy than in children without an atopic history (Table 1). Neither the IL-2 nor the IL-2- and IL-12-induced IL-10 production correlated with total IgE levels (data not shown).

Children with a cumulative and/or current atopic history had similar IL-2- and IL-12-induced IL-5 production regardless of skin prick test reactivity and IgE levels (Table 1).

Discussion

Atopic children with current airway symptoms and/or skin symptoms and high IgE levels had

Table 1. Data of spontaneous, as well as cytokine-induced IL-12R β_2 mRNA expression and IFN- γ , IL-10 and IL-5 secretion

Stimuli	No.	Non-atopic	No.	Cumulative history of atopic diseases	No.	Current atopic diseases	No.	Current atopic airway symptoms
IL-12R β_2 mRNA/RNA ratio								
Spontaneous	14/17	1.8 (0.5–8.6)	21/21	2.8 (0.6–21.5)	16/16	1.7 (0.6–11.0)	10/10	1.7 (0.6–8.8)
IL-2	10/17	9.4 (0.9–30.2)	20/21	6.5 (1.2–37.3)	16/16	5.9 (1.3–37.3)	10/10	6.5 (1.3–15.8)
IL-12	11/17	4.2 (0.7–20.1)	20/21	4.5 (0–89.2)	16/16	7.5 (0–89.2)	10/10	7.5 (0–24.3)
IL-2/IL-12 high	13/17	15.9 (3.3–39.9)	19/21	18.1 (1.7–89.4)	15/16	12.2 (1.7–89.4)	9/10	11.9 (1.7–53.9)
IL-2/IL-12 low	10/17	18.0 (5.6–52.5)	18/21	14.0 (4.1–41.1)	14/16	13.7 (4.1–41.1)	8/10	6.4 (4.1–41.1)
p = 0.12								
IFN- γ secretion (pg/ml)								
Spontaneous	8/17	192 (65–912)	11/21	402 (71–2876)	7/16	402 (71–1867)	4/10	559 (84–1867)
IL-2	17/17	527 (101–68,068)	20/21	2996 (35–73,651)	16/16	2996 (35–73,651)	10/10	395 (82–46,816)
IL-12	15/17	1219 (74–10,628)	19/21	976 (43–13,259)	14/16	939 (43–13,259)	9/10	912 (43–13,259)
IL-2/IL-12 high	17/17	34165 (7704–218,737)	21/21	28500 (2317–228,049)	16/16	24576 (2317–228,049)	10/10	16230 (4912–228,049)
IL-2/IL-12 low	17/17	21243 (2982–118,154)	21/21	9003 (1306–146,751)	16/16	7736 (1306–146,751)	10/10	7736 (1306–146,751)
p = 0.02								
IL-10 secretion (pg/ml)								
Spontaneous	9/17	8.0 (6.4–92.3)	14/21	13.4 (4.9–110.3)	9/16	13.3 (5.5–110.3)	6/10	13.3 (5.5–110.3)
IL-2	9/17	9.1 (4.0–65.5)	18/21	11.6 (2.4–39.7)	14/16	10.7 (2.4–39.7)	8/10	12.6 (2.4–39.7)
p = 0.001								
IL-12	7/17	7.1 (3.0–22.8)	12/21	5.9 (2.4–28.6)	10/16	5.9 (2.4–28.6)	5/10	5.6 (4.7–13.4)
IL-2/IL-12 high	15/17	16.6 (8.9–105.4)	21/21	23.6 (7.2–71.1)	16/16	26.0 (7.2–71.1)	10/10	21.5 (7.2–53.1)
IL-2/IL-12 low	12/17	22.6 (5.0–45.3)	19/21	19.9 (4.6–46.7)	15/16	20.2 (4.6–46.7)	9/10	20.2 (5.5–46.7)
p = 0.06								
IL-5 secretion (pg/ml)								
Spontaneous	5/17	4.7 (3.3–9.3)	7/21	4.0 (3.1–16.3)	7/16	4.0 (3.1–16.3)	5/10	4.3 (3.9–16.3)
IL-2	17/17	38.5 (5.2–296.2)	20/21	31.8 (2.6–165.3)	15/16	31.2 (2.6–165.3)	10/10	34.2 (12.0–165.3)
IL-12	3/17	5.1 (4.3–5.8)	1/21	4.4	1/16	4.4	1/10	4.4
IL-2/IL-12 high	15/17	34.2 (3.9–181.5)	19/21	24.8 (2.3–71.3)	14/16	23.4 (2.3–71.3)	9/10	24.8 (2.3–47.7)
IL-2/IL-12 low	15/17	42.8 (3.4–116.0)	20/21	23.4 (3.0–89.6)	15/16	22.4 (3.0–89.6)	10/10	24.4 (3.0–76.8)

Median and range for all samples analyzed are shown for mRNA and for positive samples for the cytokines. Comparisons between non-atopic children and children with a cumulative history of atopic disease, current atopic disease or current atopic airway symptoms were performed. Significant p-values ($p < 0.05$) as well as tendencies ($p < 0.15$) are shown. No.: number of samples analyzed for mRNA and number of positive samples (above cut-off level) for cytokines.

lower IFN- γ production after stimulation with IL-2 together with IL-12 than non-atopic children with low IgE levels. Moreover, it was lower in sensitized, i.e. SPT-positive children, with current atopic airway symptoms than in non-sensitized, non-atopic children.

Our findings are in agreement with other studies reporting reduced levels of IFN- γ after IL-12 stimulation of PBMC in atopic individuals (6–11). A recent study of atopic adults with high IgE levels showed a decreased tyrosine phosphorylation of STAT-4 (signal transducer and activator of transcription-4) (7). Furthermore, IL-12 was unable to induce IFN- γ production in Th2 clones of atopics and failed to induce phosphorylation of STAT-4 (16). IL-12 promotes IFN- γ production via STAT-4 (17). The mechanisms behind the reduced IFN- γ production and STAT-4 phosphorylation in response to IL-12 in atopic individuals are not known, but an impaired expression of the IL-12R β_2 subunit is an attractive hypothesis.

In support of this hypothesis, we observed that atopic children had significantly lower expression of IL-12R β_2 mRNA after IL-2 stimulation alone, compared with non-atopic children. As IL-2 is

needed to initiate and sustain immune responses (reviewed in 18), this may contribute to the Th2-skewed immunity in atopic children. The low IL-2-induced expression of IL-12R β_2 mRNA may cause the observed low IFN- γ production in atopic children after stimulation with IL-2 and IL-12 in combination. The IL-2-induced expression of IL-12R β_2 mRNA correlated with IL-2/IL-12-induced IFN- γ secretion, also supporting the hypothesis (Fig. 4). Our results are also in line with findings of a reduced IL-12R β_2 expression in tissue biopsies from ARC patients compared with healthy controls (19). Moreover, we have previously observed a reduced PHA-induced IL-12R β_2 mRNA expression in 12-year-old atopic children compared with non-atopics (12). Furthermore, mutations of the IL-12R β_2 gene in atopic children have been described (7).

Interestingly, we also found a negative correlation between the IL-2 and IL-12 induced, with a similar trend for IL-2-induced IL-12R β_2 mRNA expression and total IgE in all children, suggesting that the expression of IL-12R β_2 mRNA plays a role in the regulation of IgE. The inhibitory effects of IL-12 on Th2-associated immune responses are well known (2). Our

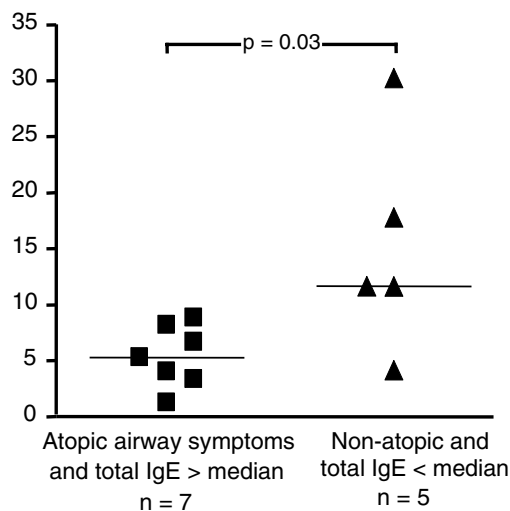


Fig. 2. The IL-2-induced expression of IL-12R β_2 mRNA at 7 years of age in children with current atopic airway symptoms and high total IgE (above the median value, ■) and non-atopic children with low total IgE (below the median value, ▲). The median values of the expression of IL-12R β_2 mRNA are shown.

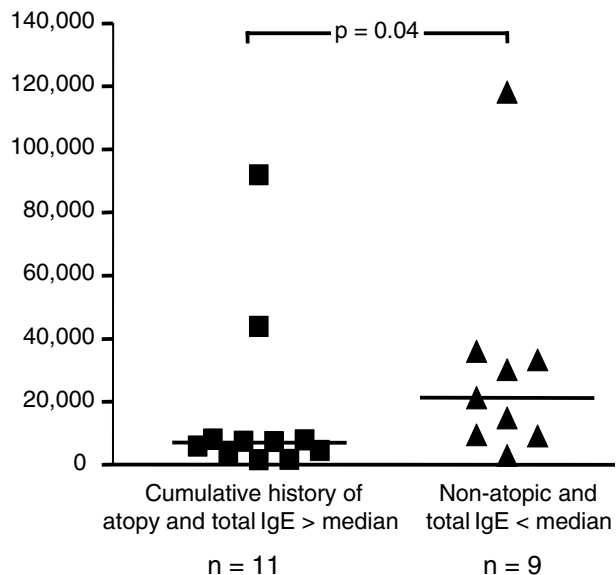


Fig. 3. The IL-2- and IL-12-induced (25 pg/ml) IFN- γ production at 7 years of age in children with high total IgE (above the median value) and a cumulative history of atopy (■) and non-atopic children with low total IgE (below the median value, ▲). The median values of the IFN- γ production are shown.

observation is supported by findings that IL-12 suppresses IgE synthesis in human helminth infections, in part, by regulating IFN- γ and IL-4 (20). In addition, we found that the IL-2- and IL-12-induced IFN- γ production was negatively correlated with total IgE. A negative

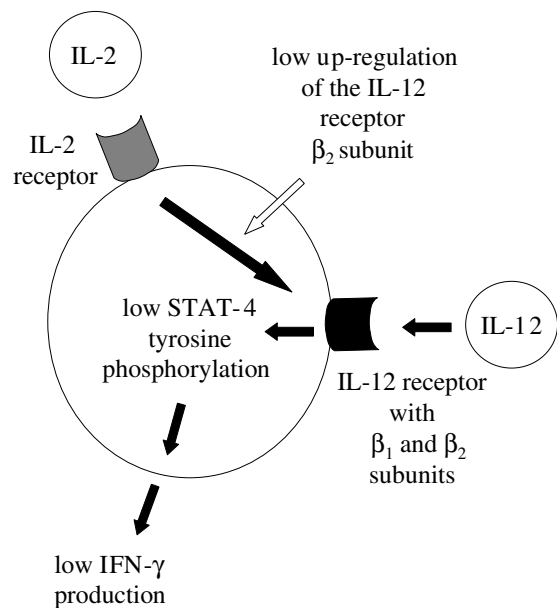


Fig. 4. T cell of atopic individual. Putative role of the mechanisms behind the low up-regulation of the IL-12 β_2 receptor and the low IFN- γ secretion in the atopic subject. Atopic individuals may have a reduced up-regulation of the β_2 subunit of the IL-12 receptor in response to IL-2. This would then lead to a decreased tyrosine phosphorylation of STAT-4 (signal transducer and activator of transcription-4) after exposure to IL-12, resulting in low IFN- γ secretion. As IL-2 is critical for the activation of T cells and IL-12 promotes Th1 responses the described sequence of events could thus contribute to a Th2-skewed immune pattern.

correlation between total IgE levels and IL-12-induced IFN- γ production was observed previously (6), although another study did not show this (8).

We found that IL-10 production after IL-2 stimulation alone was higher in children with a cumulative history of atopy from birth to 7 years of age than in non-atopic children. The involvement of IL-10, which has both anti-inflammatory properties (21) and stimulates humoral immunity (22) in allergic disease, is controversial. Increased spontaneous IL-10 mRNA expression in skin lesions has been reported in atopic patients (23). On the other hand, asthma is associated with low lipopolysaccharide-induced IL-10 production (24).

In conclusion, atopic airway symptoms and high IgE levels were associated with a reduced IL-2-induced IL-12R β_2 mRNA expression. Furthermore, IL-2 in combination with IL-12 induced lower IFN- γ production in atopic than in non-atopic children, possibly reflecting the formers reduced capacity to up-regulate the IL-12 receptor. As IL-2 is needed to initiate and sustain immune responses, this may contribute to the Th2-skewed immunity in atopic children.

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