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Altered signaling through IL-12 receptor in children with very high serum IgE levels

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ABSTRACT

An alteration of Th1/Th2 homeostasis may lead to diseases in humans. In this study, we investigated whether an impaired IL-12R signaling occurred in children with elevated serum IgE levels divided on the basis of the IgE levels (group A: >2000 kU/l; group B: <2000 kU/l). We evaluated the integrity of the IL-12R signaling through the analysis of phosphorylation/activation of STAT4, and mRNA expression and membrane assembly of the receptor chains. At a functional level, a proliferative defect of lymphocytes from group A patients was observed. In these patients, an abnormal IL-12R signaling was documented, and this finding was associated with abnormal expression of the IL-12Rβ2 chain. Our data indicate that in patients with very high IgE levels the generation of Th1 response is impaired, and that this abnormality associates with abnormal IL-12R signaling.

1. Introduction

The identification of distinct $CD4^+$ T helper cells (Th1 and Th2) exerting peculiar functions and differing on the basis of the production of a unique cytokine profile greatly contributed to our understanding of the intimate mechanism implicated in the different type of host immunity. Th1 cells produce interferon (IFN)-γ and interleukin (IL)-2 and, predominantly, promote cell-mediate immune responses, whereas Th2 cells that produce IL-4, IL-5 and IL-13 provide help for some B cell responses as IgG1 and IgE production [1,2]. Overall, an appropriate immune response mostly relies on a well orchestrated Th1/Th2 dichotomy, whose hallmark is based on the capability of the individual subset to work in an autocrine fashion leading to amplify its own cell development and to cross-regulate the other subset development and activity [3,4]. It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance. Moreover, the susceptibility to infections by certain pathogens is associated with low levels of IFN-γ [5]. Thus, alteration of Th1/Th2 homeostasis, also involving further regulatory T cells as Th17, may lead to diseases in humans [3,6]. A Th1 response is implicated under ordinary circumstances in resistance to several intracellular pathogens, but an excessive Th1 response is associated with different autoimmune diseases, as rheumatoid arthritis [7,8], type I diabetes [9] or multiple sclerosis [10]. On the contrary, a Th2 dominated response, usually involved in the response to extracellular pathogens as parasitic or helminths, is associated with allergic disorders and the progression of chronic infections as AIDS [11].

The dimeric cytokine IL-12, produced by B cells and macrophages, plays a pivotal role for the induction of a Th1 response [12]. Its cloned receptor consists of two subunits, IL-12Rβ1 and β2, both required for high affinity binding to IL-12 and full cytokine responsiveness [13]. The receptor is up-regulated during T-cell activation and IL-12Rβ2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rβ1 is constitutively expressed in resting cells [14]. The transducing element of the receptor is the IL-12Rβ2 chain that functionally interacts with members of the family of Signal Transducers and Activators of Transcription (STAT), and in particular STAT4 [15]. This transcription factor is promptly phosphorylated on tyrosine residues upon receptor triggering [15]. Th1 cells develop in the presence of IL-12 and STAT4 signaling and secrete mainly IFN-γ [16]. Moreover, experimental evidence using the knock-out technology supports the concept that IL-12R/STAT signaling pathway plays a role for the induction of a Th1 response [17,18].

The aims of our study were to investigate at a functional level whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels, and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus. In particular, the activation of STAT4 molecule that follows IL-12R triggering, and the analysis of gene transcription and membrane assembly of the receptor itself were investigated in allergic children divided on the basis of the amount of serum IgE.

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2. Materials and methods

2.1. Subjects

Twenty patients with elevated IgE levels and history of allergy were enrolled into the study. Sixteen patients were affected by asthma, 3 of them also by rhinitis, and 4 had a history of atopic dermatitis. The patients divided in two subgroups on the basis of IgE levels: group A consisted of 10 patients, 10 males, range of age 5–15 years, with very high serum IgE levels (>2000 kU/l, range 2152–5000 kU/l); group B consisted of 10 patients, 9 males, range of age 6–15 years, with high serum IgE levels (IgE value between the age specific mean ± 2 SD and 2000 kU/l, range 93–1152 kU/l) (Table 1). Twenty healthy controls, 16 males range of age 6–15 years (IgE range 85–100 kU/l), were also studied. Informed consent was obtained when required. All patients enrolled into the study did not receive any treatment, including steroid or non-steroid drugs, in the month before entering into the study. No difference was found between group A and B in either the number per year or the severity of allergic manifestations. In all patients, the clinical features persisted for more than 2 years.

The Hyper-IgE Syndrome (HIES) was excluded by the absence of typical clinical and immunological features according to the clinical score for HIES (Table 1) [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed. Other conditions accompanied by elevated serum IgE concentration, including AIDS, helminths and parasitic infections were also excluded by clinical and laboratoristic features.

The study has been approved by the Institutional Review Board.

2.2. Cell culture and proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation by standard procedure and cultured in triplicates (2×10^5/well). Cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories, Detroit, MI), phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co., St. Louis, MO). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody (Ortho Diagnostic, Raritan, NJ). To evaluate allogeneic response, patients responder cells (1×10^5) were stimulated with an equal amount of irradiated stimulator cells from controls in a standard one-way mixed lymphocyte reaction assay. Cell mixtures were cultured in 96-well round-bottom microtiter plates (Becton Dickinson, San Jose, CA) for 5 days and harvested 18 h after [^{3}H]thymidine pulsing.

Table 1
Clinical characteristics of patients divided in group A and group B included in the study.

Patients	Gender	Age	Clinical features	HIES score	Serum IgE levels (kU/l)
1	M	6	Asthma	0	<2000
2	M	6	Asthma	1	<2000
3	M	7	Asthma	1	<2000
4	M	9	Asthma, rhinitis	8	<2000
5	M	11	Asthma	4	<2000
6	M	8	Asthma	1	<2000
7	F	15	Asthma	4	<2000
8	M	6	Asthma, rhinitis	8	<2000
9	M	6	Atopic dermatitis	8	<2000
10	M	10	Asthma	0	<2000
11	M	7	Atopic dermatitis	10	<2000
12	M	5	Atopic dermatitis	10	>2000
13	M	5	Asthma	13	>2000
14	M	7	Asthma	13	>2000
15	M	8	Asthma, rhinitis	13	>2000
16	M	10	Asthma	13	>2000
17	M	15	Asthma	10	>2000
18	M	12	Asthma	13	>2000
19	M	5	Atopic dermatitis	10	>2000
20	M	7	Asthma	10	>2000

2.3. Generation of Th1 cell lines

Th1 cell lines were generated by stimulating PBMC with PHA (8 μg/ml) or, in a few experiments, with PHA + IFN-γ (1000 U/ml, ICN, Biomedical, OH) for 72 h in complete tissue culture medium. These cells usually widely express high affinity IL-12R.

2.4. Analysis of STAT4 activation

PHA-induced blasts were made quiescent by 12 h incubation in RPMI supplemented with 2.5% FCS at RT, and further stimulated with rIL-12 (Genetics Institute, Cambridge, MA) at a concentration of 10–100 U/ml for 10 min. After the appropriate stimuli, $3–5 \times 10^6$ cells were lysed in buffer containing 20 mM Tris, pH 8, 10% glycerol, 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mM sodium orthovanadatum (Na3Vo4), 5 μg/ml leupeptin and 5 μg/ml aprotinin. Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes and then blocked with 5% bovine serum albumin. Immunoblotting was performed by a 2–4 h incubation with anti-STAT4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using chemiluminescence (ECL system, Amersham, Buckinghamshire, England). The low migration supershifted form of STAT4 indicates the presence of the protein in its activated/phosphorylated form [21]. Densitometric analysis was performed to evaluate the overall amount of the protein and the amount of its supershifted form.

2.5. Membrane expression of β1 and β2 chains of IL-12R on T cells

After washing in PBS, cells were incubated for 20 min sequentially with murine anti-β1 or anti-β2 chain (25 μl) of IL-12R (kindly provided by Dr. Jerome Ritz, Dana Farber Cancer Institute, Boston, MA), IgG1 isotype control Ab, 10 μl FITC-conjugated goat anti-mouse IgG Ab (Becton Dickinson, San Jose, CA), and 5 μl anti-CD4 PE Ab (Becton Dickinson, San Jose, CA). After staining, the expression of IL-12Rβ1 and β2 on CD4$^+$ cells was determined with flow cytometer (Becton Dickinson) by gating on the CD4$^+$ population.

2.6. Analysis of IL-12Rβ2 chain RNA expression

Total cellular RNA was prepared using Trizol reagent method (Sigma Chemical Co., St. Louis, MO); 1 μg of total RNA was reverse transcribed into cDNA using Expand™ Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim, Germany). The cDNA was PCR amplified (94 °C, 1 min; 55 °C, 1 min; 72 °C,1 min for 30 cycles) using specific primers for IL-12Rβ2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. Each PCR mixture consisted of 3 μl of cDNA, 1 μl of each primer (concentration from Kathy), 0.2 mM dNTP and 2.5 U of Taq DNA polymerase (Life Technologies Ltd., Paisley, Scotland). These reactions were carried out in a buffer

containing 25 mM $MgCl_2$, 200 mM Tris–HCl and 500 mM KCl. To monitor the amount of RNA, β-actin mRNA expression was used. PCR products were separated in a 1% agarose gel and viewed after ethidium bromide staining.

2.7. Statistical analysis

The significance of differences was evaluated by Wilcoxon rank sum test for unpaired data. All the data were obtained from at least three distinct experiments performed in a 6 months period.

3. Results

3.1. Proliferative responses

Fig. 1A illustrates the proliferative response to CD3 cross-linking (CD3 X-L) performed at optimal antibody concentration, that mimics *in vivo* antigen exposure in patients and controls. Group A patients showed a significantly lower response than controls (mean ± SD: 23,200 ± 6402 *versus* 49,690 ± 4398 cpm in controls, $p < 0.05$). In contrast, patients of group B had a higher proliferative response not significantly different from controls. Similarly, the proliferative response to PHA was lower in group A than in the other groups (mean ± SD: group A, 58,790 ± 11,690 cpm; group B, 106,500 ± 10,800 cpm; controls 93,070 ± 4455 cpm. A *versus* B and A *versus* controls: $p < 0.01$). No difference was found in the proliferative assays with the other stimuli. As depicted in Fig. 1B, the allogeneic response was comparable in the three groups.

3.2. Analysis of STAT4 tyrosine phosphorylation/activation

IL-12/IL-12 receptor signaling plays a crucial role in Th1 induction. To evaluate whether the low response to CD3 X-L associated with a normal allogeneic response was due to an impaired Th1 generation, we next investigated IL-12R signaling by analyzing supershift of the transcription factor STAT4, that promptly occurs after receptor triggering by its own cytokine and indicates protein tyrosine phosphorylation of the molecule [21]. Fig. 2A shows a representative experiment out of six performed indicating that in controls rIL-12 stimulation for 10 min of PHA-induced cell lines induces STAT4 supershift, due to the appearance of a slower migrating form representing the phosphorylated molecule. By contrast, in all patients of group A there was no supershift of STAT4, and the molecule appeared as a single form of 84 kDa. Fig. 2B illustrates the densitometric analysis representing the overall amount of STAT4 and the amount of its supershifted form. The protein was expressed in patients and controls in a comparable amount. IL-12 stimulation induced the supershift only in control cells and not in patient cells. Fig. 3A shows that IL-12 stimulation of cell

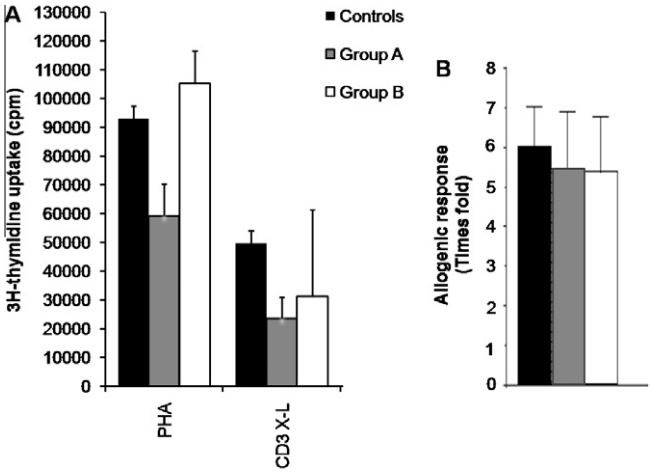

Fig. 1. Proliferative responses in patients and controls. Patients were divided on the basis of IgE levels (group A, $n = 10$, IgE >2000 kU/l; group B, $n = 10$, IgE value between the age specific mean ± 2 SD and 2000 kU/l; controls, $n = 20$). (A) Proliferative response to PHA (8 μg/ml) and CD3 cross-linking (CD3 X-L), performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody. Each column represents the mean value ± SD. (B) Proliferative response to allogeneic stimuli. Results are expressed as the mean value ± SD and indicate the times fold increase over the background.

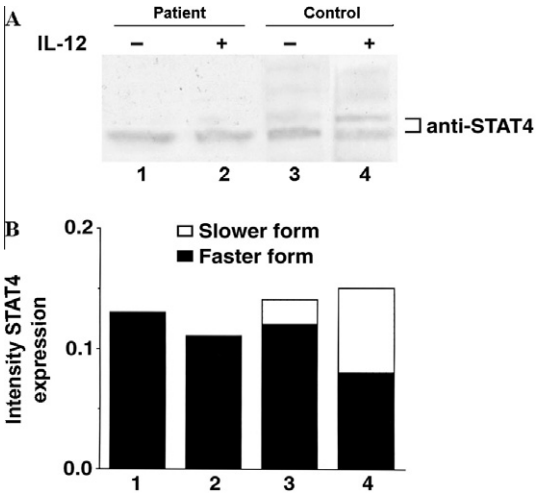

Fig. 2. Analysis by immunoblot of STAT4 protein in controls and patients with very high IgE levels (>2000 kU/l). (A) Representative experiment, out of 6, showing that rIL-12 stimulation induces in controls the appearance of a slow migrating phosphorylated form of the protein, whereas in patients only the 84 kDa protein is evident. PBMC from a patient (lanes 1 and 2) or control (lanes 3 and 4) were incubated with PHA for 72 h, and then further stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein, the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

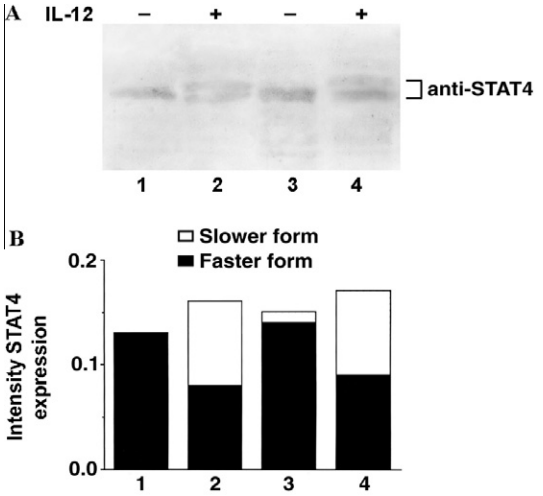

Fig. 3. Analysis by immunoblot of STAT4 protein in controls and patients with IgE values between the age specific mean ± 2 SD and 2000 kU/l. (A) Representative experiment, out of 3, showing that rIL-12 stimulation induces both in control and patient the appearance of a slower form of STAT4. PBMC were processed as indicated in Fig. 2 and Section 2. Lanes 1 and 2, patient; lanes 3 and 4, control. Cells were stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein; the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

lines obtained from patients of group B, induced the appearance of the slower supershifted form of STAT4 both in controls and patients to a similar extent.

3.3. IL-12R expression on T cells

The high affinity IL-12 receptor consists of β1 and β2 chains, the latter being up-regulated during cell activation and selectively expressed on Th1 cells. To determine whether the failure of STAT4 phosphorylation was due to decreased expression of IL-12 receptor, we analyzed the surface expression of IL-12R in T cell lines induced in the presence of PHA. The expression of both β1 and β2 chains was lower in group A than in the other groups. IL-12Rβ1 values, expressed as mean percentage of positively stained cells ± SD, were as follows: group A, 35.96 ± 7.3%; group B, 53.8 ± 6.6%; controls, 51.7 ± 6.1%. Similarly, a lower up-regulation of β2 chain in group A was observed as depicted in Fig. 4A. Mean percentage values ± SD of IL-12Rβ2 expression were 16.5 ± 3.0% in group A; 28.8 ± 3.7% in group B; 28.9 ± 1.6% in controls (A *versus* B and controls: $p < 0.05$). The mean fluorescence intensity was lower in the group A than in the other groups (Fig. 4B), differently from β1 whose intensity was comparable in the three groups (data not shown).

3.4. IL-12Rβ2 mRNA expression

We next analyzed the mRNA expression of IL-12Rβ2 chain in group A, where no STAT4 tyrosine phosphorylation was observed. The expression of the IL-12Rβ2 transcript in all experiment performed was different between patients and controls. In three experiments there was no induction at all of β2 transcript after of 18, 36 and 48 h PHA stimulation, as illustrated in a representative experiment in Fig. 5. Furthermore, no effect of IFN-γ was noted. These data were confirmed by five distinct experiments. In two cases there was mRNA expression, but in one case it was delayed appearing only after 48 h PHA stimulation, even though it

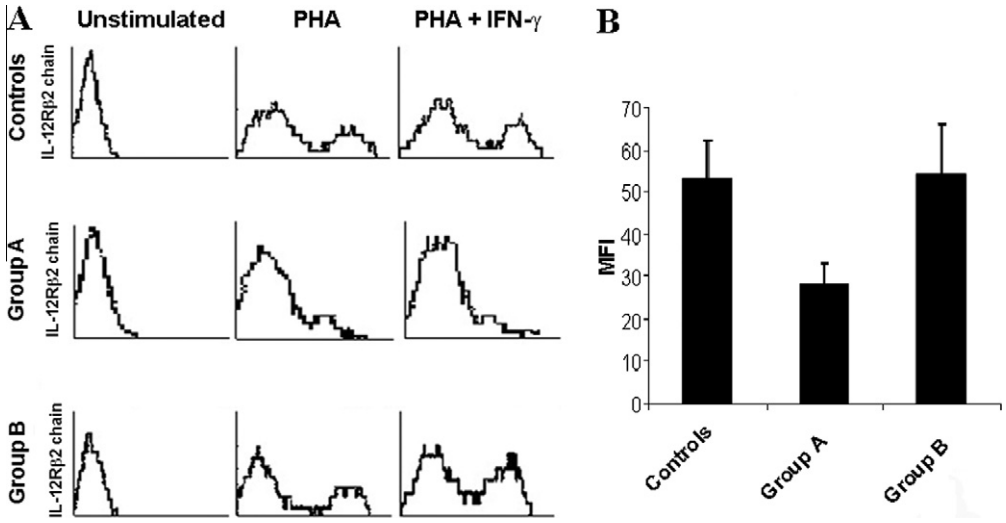

Fig. 4. Membrane expression of β2 chain of IL-12R on T cells. IL-12Rβ2 membrane expression on resting or T-cell blasts, induced by stimulation with PHA for 72 h in the absence or presence of IFN-γ, in controls and patients divided in two groups on the basis of IgE levels as indicated in Section 2. Dual colour fluorescence using FITC-conjugated anti-β2 and PE-conjugated anti-CD4 was performed. (A) Shows a representative experiment indicating the lower up-regulation of β2 chain in group A. The mean fluorescence intensity in the three groups is shown in (B). Each column represents the mean value ± SD. The intensity was lower in the patients of group A than in the other groups.

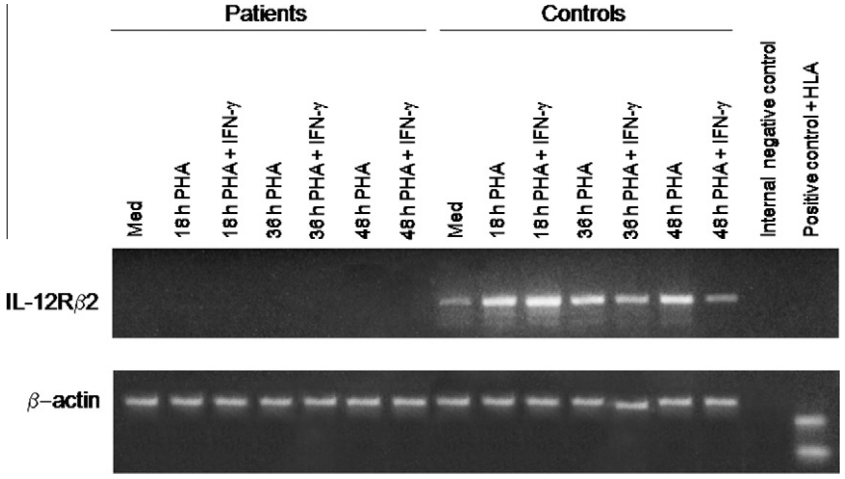

Fig. 5. mRNA expression of IL-12Rβ2 chain in controls and group A patients (IgE levels: >2000 kU/l). Representative experiment showing that in controls, β2 chain mRNA expression increased after 18 h of PHA stimulation. Lanes 1 and 8: freshly isolated PBMC. T-cell blasts were generated by 18, 36 and 48 h of PHA stimulation. IFN-γ upregulated β2 chain mRNA expression after short term PHA stimulation, but it was ineffective during longer stimulations. In group A there was no induction at all of β2 transcript. Furthermore, no effect of IFN-γ was noted.

was also slightly appreciable after 36 h stimulation in the presence of IFN-γ. In the other case a faint signal was appreciable after 18 h of PHA stimulation, but it rapidly disappeared.

4. Discussion

In this study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction. In particular, defective supershift of the STAT4 molecule following rIL-12 stimulation of T-cell blasts was documented. Supershift of this molecule indicates its phosphorylation [21]. This finding was associated with a T-lymphocyte functional derangement characterized by low proliferative response to stimulations via TCR/CD3 complex, but with a preserved allogeneic response. The discrepancy between mitogenic and allogeneic stimuli in inducing cell proliferation has already been documented in mice in which the gene coding for 40 kDa subunit of IL-12 has been disrupted [17]. These mice are not able to generate most of the Th1 responses, including IFN-γ production and delayed type hypersensitivity response *in vivo*, but cytolytic response elicited by allogeneic stimuli was preserved, thus suggesting that the allogeneic response is dependent on a wider array of cytokines influences. Further evidence on the role of the IL-12/IL-12R signaling apparatus on the induction of Th1 responses comes from the functional studies on mice lacking STAT4 molecule, that represents a central signaling protein involved in IL-12R signaling [22]. Although there is evidence suggesting that the development of Th1 type responses may also take place in a STAT4 independent fashion [23,24], the STAT4 knock-out experimental model underlines the importance of the integrity of the IL-12/IL-12R signaling for the generation of a proper Th1 type response. Again, $STAT4^{-/-}$ mice exhibit a propensity to generate Th2 type cells [22]. It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut-off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by Hyper-IgE Syndrome (HIES). However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed.

The link between viral and bacterial infections and the pathogenesis of allergic asthma has represented for years an appealing area of clinical investigation, which is currently expanding in parallel with the worldwide increase of childhood asthma prevalence [25]. Longitudinal studies indicate that respiratory tract infections may predispose children to asthma [26]. Persistent wheezing seems to be related to increased IgE levels and eosinophils at the time of the first respiratory infection, thus suggesting that infections may trigger asthma attacks in already predisposed subjects [27,28]. However, in contrast to this, it has been shown that early infections may protect against the subsequent development of an atopic phenotype [29]. This hypothesis is also supported by the recently documented inhibitory effect on Th2 cell functions of Th1-released proinflammatory cytokines [30]. Public health measures, as hygiene programs to reduce foodstuffs contamination, active immunization programs, a better pharmacological control of infections, may certainly have contributed in limiting the immune system challenge by infectious agents in early childhood, even though hygiene hypothesis should be revisited in the light of recent data on the role of Toll like receptors and regulatory mechanisms [31]. However, in our study it should be noted that there were not striking differences between the three groups of subjects with regards to vaccination program, social habits and the number or severity of infections in the clinical history that preceded the appearance of allergic disorders.

However, it should be noted that abnormalities of IL-12 signaling pathway is not sufficient *per se* to cause an allergic disease in children, in that the prevalence of asthma, eczema and rhinoconjunctivitis is similar in patients with or without genetic alteration of IFN-γ or IL-12Rβ1 [32]. Our data could imply a link between infections and allergy in children, even though this matter is still under debate and no conclusive demonstration is available [31]. In the light of this consideration, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help ameliorate the overall management of these patients. In fact, there is evidence that patients with severe forms of allergic manifestations are more susceptible to respiratory infections, and vice versa infections may trigger acute episodes of asthma [25]. A defective induction of a Th1 response in patients with very high IgE levels may lead to a higher risk of infections, thus worsening the overall outcome.

Overall, our results indicate that children with very high serum IgE levels have functional and biochemical signs of an altered IL-12/IL-12 receptor signaling network.

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