

TOTAL IL-12 LEVELS ARE INCREASED IN PAEDIATRIC ATOPIC DERMATITIS: CORRELATIONS WITH AGE AND DISEASE SEVERITY

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Alterations in the immune response play an important role in the pathogenesis of atopic dermatitis (AD). We evaluated the role of Th1/Th2 cytokine imbalance in paediatric AD and its progression towards other allergic manifestations. The levels of total interleukin (IL)-12 (p70+p40), IL-12 p70 and soluble CD30 (sCD30) were measured in paediatric patients affected by AD and in age-matched, non-atopic subjects (controls). The serum levels of total IL-12 and sCD30 were higher in patients than in controls ($p=0.003$ and $p=0.053$, respectively). Total IL-12 and sCD30 were also particularly increased in patients with severe disease. Serum levels of total IL-12 were negatively correlated with patient age ($p=0.001$): they were significantly higher in patients younger than 30 months, as compared to age-matched controls and to older patients and controls. Total IL-12 and sCD30 production were not significantly correlated with disease outcomes (atopic march) or with a family history of atopy. Our data show that total IL-12 levels are strongly associated with Th2 activation and severe disease in children with AD. The increased production of total IL-12 at an early age might indicate a different immune modulation and suggests the possibility of new therapeutic approaches for allergic diseases, in particular AD, early in childhood.

Atopic dermatitis (AD) is a multifactorial disorder caused by hereditary and environmental factors and immunological abnormalities (1-2), characterized by a chronic inflammation of the skin. The prevalence of AD has increased in recent years, and 15–25% of children in Western countries are affected at some point during childhood (3-4). AD is often associated with other clinical abnormalities including allergic rhinitis, conjunctivitis and asthma.

Two forms of AD have been identified: an extrinsic form associated with increased IgE levels, which affects 70–80% of patients, also called atopic

eczema, and an intrinsic form that is not IgE-mediated (5-6). An associated eosinophilia is also present in AD. Disequilibrium between Th1/Th2 cells is important in the pathogenesis of atopic disorders, and a defect in the Th1/Th2 balance that favours Th2 cell development has been proposed. This hypothesis is supported by the presence of Th2-like cells in peripheral blood and local infiltrates from patients with various allergic diseases (7).

The high level of Th2 cell activation in AD patients is thought to be responsible for the clinical symptoms. Soluble CD30 (sCD30) is an 88-kDa

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protein released by Th2 cells through the proteolytic cleavage of the CD30 membrane protein; sCD30 production in serum has been associated with Th2 activation (8-9) and its role in atopic disorders has been described (7). CD30 expression on CD4⁺ T helper cells correlates with the Th2-cell phenotype and with the production of Th2-type cytokines. Elevated levels of sCD30 have been found in Th2-related diseases and in patients with AD (10).

The modulation of Th1/Th2 cells is also influenced by antigen-presenting cell (APC)-derived factors, such as interleukin (IL)-12. IL-12 is a heterodimeric cytokine produced mainly by dendritic cells, macrophages and monocytes. Composed of two subunits (a 40 kDa and a 35 kDa) forming the p70 heterodimer, IL-12 plays an important role in the regulation of Th1/Th2 cell activation. IL-12 p40 subunit can also exist as a monomer, a homodimer or a heterodimer associated with subunits other than p35 (11). The activities and roles of these different subunits in human diseases have yet to be evaluated.

IL-12 activates Th1 cells and promotes cell-mediated immunity (12). Cytokines such as IL-12 and IFN- γ are thought to have an inhibitory effect on the activation of Th2 cells. However, the mechanism of Th2 cell activation in atopic disorders has not been clearly elucidated.

AD is often the first step in the development of other allergic disorders, such as asthma, food allergy or allergic rhinoconjunctivitis, in the so-called atopic march (4). To manage AD appropriately, it is important to identify and characterize the immune mechanisms specific to the different phases of AD early in life. The aim of this study is to investigate the role of Th1/Th2 dysregulation in the pathogenesis of AD in children. We measured the levels of total IL-12 (p70 heterodimer + p40 subunit), IL-12 p70 and sCD30, and their correlation with other markers of immune activation, such as IgE levels, sensitization to allergens, eosinophil counts, and with clinical and anamnestic variables such as family history of atopy, disease severity and progression, and age. We wished to determine whether these variables could be useful as markers of disease or predictors of atopic progression.

MATERIALS AND METHODS

Patients and controls

Thirty-seven paediatric patients affected by AD (19 males, 18 females, ages 1–184 months) were studied.

None of the patients had been treated with antihistaminic drugs, oral steroids or disodium chromoglycate within one month of the study. Diagnosis was performed using the clinical criteria for AD proposed by Hanifin and Rajka (13) and the Scoring Atopic Dermatitis index (SCORAD) was used to define mild, moderate and severe cases (14).

Total IgE serum levels (Paper RadioImmunoSorbent Test, PRIST) and peripheral blood eosinophil counts were measured in all patients. Increased IgE levels were defined with reference to age: 2-5 years, >60 U/ml; 6-9 years, >75U/ml; 10-13 years, >155 U/ml; >13 years, >100U/ml.

Allergen skin sensitivity to the most common aeroallergens (dermatophagoides farinae, dermatophagoides pteronyssinus, cat fur, olive tree, poplar, wheat pollen, artemisia, alternaria, hazel) and food allergens (egg and milk proteins) was assessed using a skin prick test. Measurements of the skin reaction were performed on a semi quantitative scale (positive score from 1 to 4). Patients were dichotomized as mono- or unsensitized, or polysensitized to allergens (both food and aeroallergens), and as food-, aero-, or food plus aeroallergen sensitized.

Family history of allergy in one or more first-degree relatives and the presence of other allergic diseases in addition to AD (e.g., allergic rhinitis, rhinoconjunctivitis and asthma) were registered. Both allergic sensitization and the presence of other allergic diseases were successively re-evaluated at three years follow-up.

The control group consisted of 16 age-matched, non-atopic subjects (10 males, 6 females, age 8–127 months) with negative family or personal history for atopic diseases, who were not taking any medication at the time of the study. All families involved gave their informed consent prior to participation.

Serum collection and isolation of peripheral blood mononuclear cells

A venous blood sample was obtained from each subject. Serum was prepared within one hour of withdrawal and stored in aliquots at -70°C until assayed. In 20 patients and 8 controls, peripheral blood mononuclear cells (PBMC) were isolated to assess total IL-12 production in culture supernatants. IL-12 p70+p40 heterodimers are mainly produced by APCs (dendritic cells, monocytes, macrophages); both p35 and p40 production is stimulated by the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS), which was used to assess IL-12 production in cultures. PBMC were isolated from heparin-treated blood by centrifugation on a Ficoll-Hypaque density gradient, then washed and re-suspended in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated foetal calf serum, 50 UI/ml penicillin, 50

$\mu\text{g/ml}$ streptomycin and 0.2 mM L-glutamine (the latter four reagents were obtained from Sigma, St Louis, MO, USA). Cultures were prepared in triplicate in 96-well microtitre plates at a density of 1×10^6 cell/ml and stimulated with LPS (2.5 $\mu\text{g/ml}$, Sigma). After incubation for 20 hours at 37°C in a 5% CO₂ humidified atmosphere, supernatants were collected and frozen at -70°C until use.

Immunoassays to measure total IL-12, IL-12 p70 and sCD30 in serum and culture supernatants

Specific high-sensitivity enzyme-linked immunoassays (EIA) were used to measure total IL-12 (p70+p40, Endogen, Cambridge, MA, USA), IL-12 p70 (Endogen) and sCD30 (Ki-1 antigen, Dako, Glostrup, Denmark) in serum and culture supernatants. The method is based on a sandwich-enzyme immunoassay using a monoclonal antibody specific for the molecule of interest, which has been pre-coated onto a microtitre plate. After incubation of samples and washing to remove the unbound proteins, an enzyme-linked polyclonal antibody, specific for a different epitope of the molecule of interest, was added to the wells. Following a wash to remove the unbound antibody-enzyme reagent, a substrate solution was added. After an incubation period, colour development was stopped and the intensity of the colour was measured. A standard curve was prepared and unknown values were determined using a specific software. The detection limits of the assays were: < 5 pg/ml for total human IL-12, < 3 pg/ml for human IL-12 p70, and 1 U/ml for human sCD30.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation. Differences among groups were assessed by the Student's t test, Mann-Whitney U-test, and the analysis of variance (ANOVA) followed by *post hoc* tests, as appropriate. Fisher's exact test was used to evaluate contingency tables. Spearman rank correlation coefficient was used for correlation analysis. $P < 0.05$ was considered significant. All tests were two tailed. SPSS software (SPSS Inc., Chicago, Ill., USA) was used for the analyses.

RESULTS

Clinical data

Patients and controls did not differ in age (Table I). Twenty-five patients (68%) had elevated total IgE serum levels; this percentage is in agreement to previous reports (5). Twenty-one patients (57%) had no family history of atopy and 16 (43%) had one or more first-degree relatives who were affected by atopic diseases.

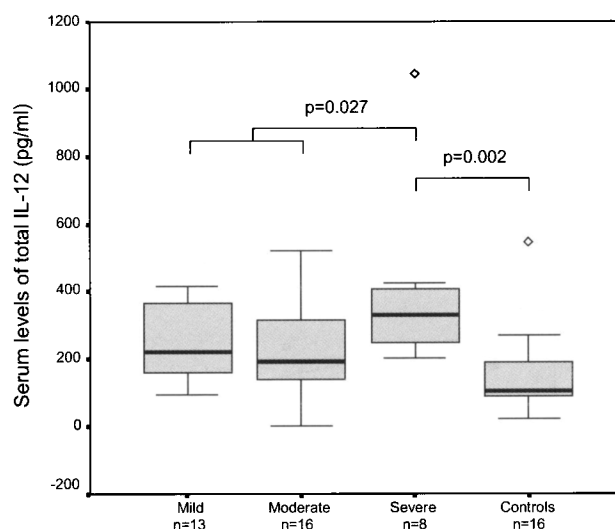


Fig. 1. Serum levels of total IL-12 in patients with varying severity of AD (mild, moderate and severe), and in the control group ($p=0.004$). In the box plot, IL-12 levels are displayed as medians (central line), interquartile ranges (boxes representing the 25th–75th percentiles); lines extend out to the largest and smallest non-outlier value, and extreme values are shown, marked by $\diamond$. p values between groups were calculated by Dunnett's *post hoc* test.

Twenty-eight (76%) of the 37 patients were sensitized to more than one allergen (food or aeroallergen), seven (19%) were unsensitized, and two (5%) were monosensitized (to food allergens). Total serum IgE levels and eosinophil counts were significantly higher in the patients who were polysensitized to allergens (IgE, 455.93 ± 748.54 UI/ml; eosinophil counts, $521.75 \pm 411.32/\mu\text{l}$) than in the monosensitized or unsensitized patients (IgE, 31.69 ± 6.74 UI/ml; eosinophil counts, $253.45 \pm 158.21/\mu\text{l}$; $p=0.007$ and $p=0.008$, respectively).

Twenty-seven percent (10/37) of the patients also had allergic asthma or rhinoconjunctivitis at the time of the study. All patients affected by allergic asthma or rhinoconjunctivitis were polysensitized (Table II). Patients affected by allergic asthma or rhinoconjunctivitis were significantly older than patients affected exclusively by AD ($p=0.027$).

Thirty-seven percent (10/27) of the patients without other allergic manifestations developed allergic asthma or rhinoconjunctivitis in the subsequent three years. Patients who exhibited this atopic march tended to have higher IgE levels (90%

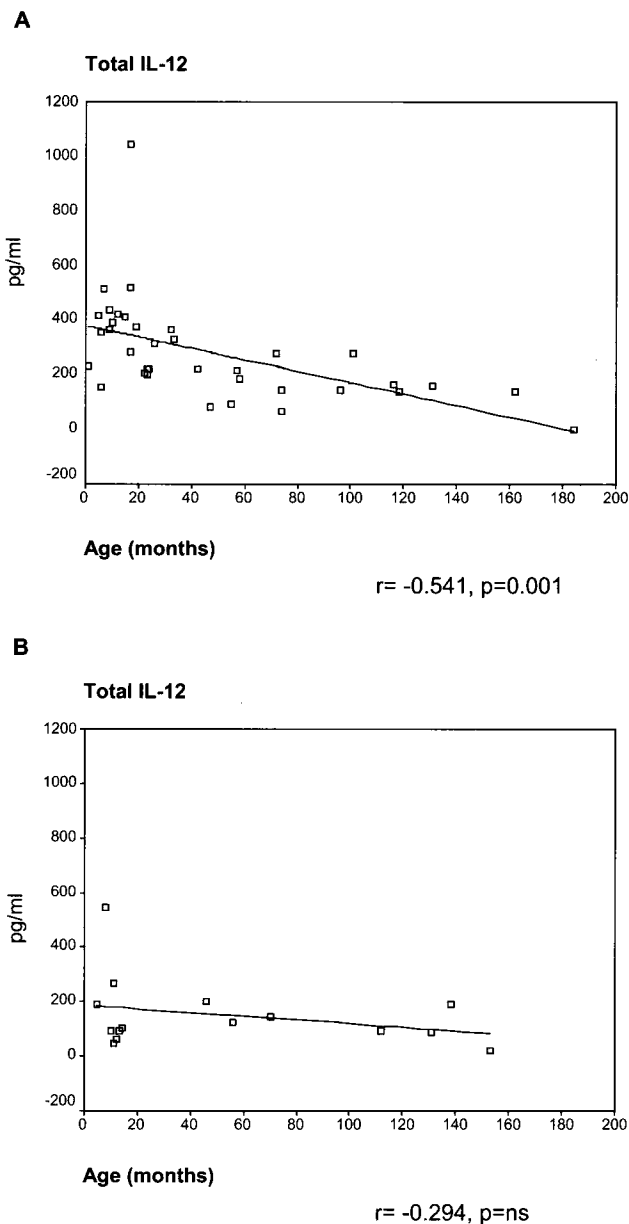


Fig. 2. Correlations between serum levels of total IL-12 and age in patients with AD (A) and in the control group (B).

versus 53% of patients with no allergic progression), polysensitization to allergens (100% versus 65% of patients with no allergic progression) and family history of allergy (50% versus 35%).

sCD30 levels and IL-12 production in serum and culture supernatants

Serum levels of sCD30 and total IL-12 were

higher in patients than in the controls ($p=0.053$ and $p=0.003$, Table I). PBMC from patients also produced higher quantities of total IL-12 than cells from non-atopic controls ($p=0.053$).

Fig. 1 shows the relationship between disease severity and serum total IL-12 levels: IL-12 was particularly increased in severe cases, as compared to mild/moderate disease and controls ($p=0.004$, Anova). sCD30 shows the same trend ($p=0.028$). Serum total IL-12 levels were significantly correlated with blood eosinophil counts in patients ($r=0.568$, $P=0.027$). IL-12 p70 was present at low levels in serum and was detectable in 24% of patients and 56% of controls. ($p=\text{ns}$, Table I).

Patients affected exclusively by AD (Table II) had significantly higher production of total IL-12 in both the serum ($p=0.040$) and in the culture supernatants ($p=0.016$) than patients with allergic asthma or rhinoconjunctivitis. IL-12 and sCD30 production were not significantly correlated with disease outcomes (i.e., allergic progression towards asthma or rhinoconjunctivitis during the three-year follow-up) or with a family history of atopy.

Serum total IL-12 level was negatively correlated with age in patients ($r=-0.541$, $p=0.001$) (Fig. 2A), but not in the controls (Fig. 2B).

AD presents commonly within the first two years of life, and later in childhood patients frequently develop other allergic symptoms and sensitization to aeroallergens. Therefore, the patients and controls were dichotomized as younger or over 30 months of age. This cut-off was considered because it divided patients and controls into two groups with no differences in mean age (age <30 months: patients, $n=20$, mean age 14.65 ± 7.7 ; controls, $n=9$, mean age 10.5 ± 2.9 months; age >30 months: patients, $n=17$, mean age 85.4 ± 44.7 ; controls, $n=7$, mean age 100.9 ± 43.0 months, Fig. 3) and homogeneous number of individuals, but with a significantly different sensitization status (sensitization to aeroallergens significantly increased in patients >30 months, $p=0.007$) and presence of other allergic symptoms (significantly increased presence of asthma/rhinoconjunctivitis in patients >30 months, $p=0.023$).

Total IL-12 and sCD30 levels were significantly different among age groups ($p<0.001$ and $p=0.001$, Anova) (Fig. 3); total IL-12 in serum was significantly

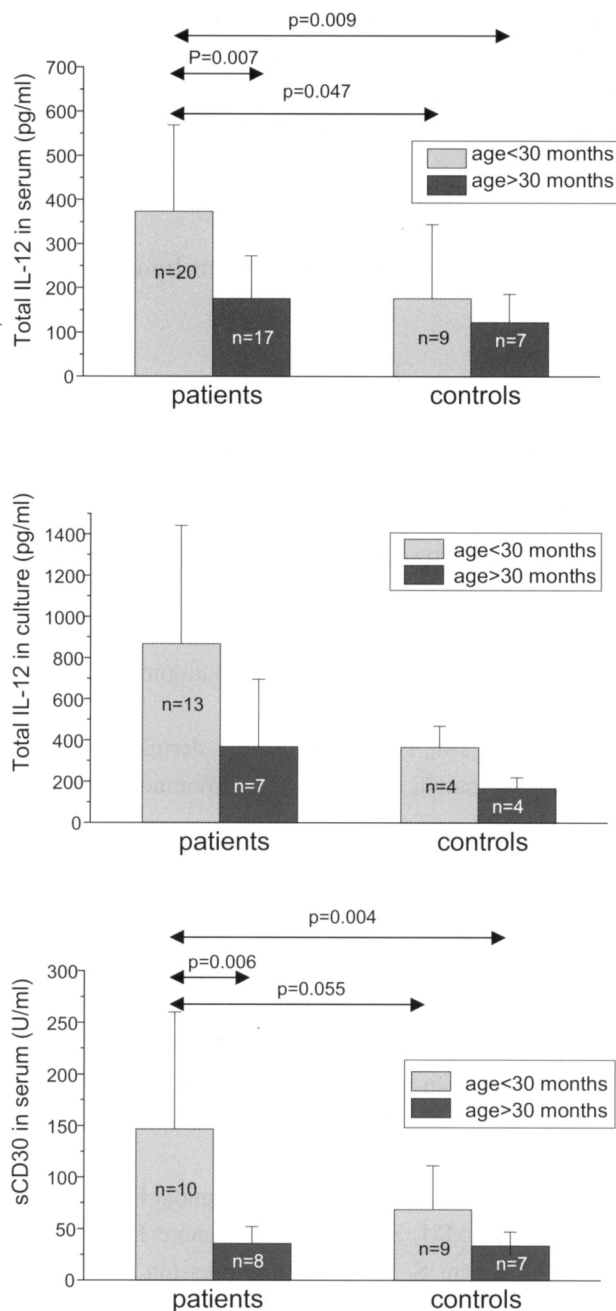


Fig. 3. Age, total IL-12 and sCD30 production: patients and controls were dichotomized as younger or over 30 months age, and differences among groups were tested by Scheffé post hoc test.

higher in younger patients as compared to over 30 months patients ($p=0.007$) and to controls ($p=0.047$ and $p=0.009$); serum sCD30 level were also higher in younger than in older patients ($p=0.006$) and than controls ($p=0.055$ and $p=0.004$). No difference

in total IL-12 and sCD30 production was found between patients over 30 months and age-matched controls (Fig. 3).

DISCUSSION

We studied specific immune characteristics in children with AD. Patients were characterized by high serum levels of IgE, increased number of eosinophils in the blood, and frequent sensitization to allergens. Serum levels of total IL-12 and sCD30 were higher in patients than in age-matched, non-atopic subjects. We found a clear association between severity of AD and serum levels of total IL-12 and sCD30. Serum levels of sCD30 and total IL-12 were independent of IgE levels.

Serum sCD30, used as a marker of Th2 activation (15), showed that these patients had Th2 activation. Cytokines such as IFN- γ and IL-12 generally have a negative influence on the development of Th2 cells. Reduced IFN- γ levels in atopic diseases (16) were confirmed in AD associated with asthma or rhinoconjunctivitis in our laboratory (data not shown). IL-12 is important in the early phases of the inflammatory response, especially in the activation of macrophages, and also induces antigen-activated T cell differentiation towards the Th1 phenotype by stimulating IFN- γ (12). A possible role of bioactive IL-12 (p70) in AD appears to contrast with the Th2 activation demonstrated in atopy. The increased levels of total IL-12 (p70+p40) that we observed can be attributed to an increase in the level of the p40 subunit, because IL-12 p70 did not differ significantly between groups. One possibility is that IL-12 p40, which can be produced at a higher level than p70 (17) and seems to have some biological activity, could play a role in Th2 activation. IL-12 p40 could regulate *in vivo* both physiologic (e.g. ageing) (18) and pathologic (e.g. pre-eclampsia, sepsis, autoimmunity or psoriasis) events (19-20) and could act as an antagonist of bioactive IL-12 in Th2-mediated diseases. It is possible that impaired IL-12 p70 production or activation of p40 production occurs in conditions of reduced IFN- γ production, such as AD.

Age is a known factor in the clinical evolution of AD. We found a significant negative correlation between age and total IL-12 production in young

patients. Previous studies have shown a correlation between age and physiologic IL-12 production in adults (18). In the neonatal period, variations in cytokine production and regulation might influence the development of immune mediated diseases, including atopy. A foetal Th1 activation is present in physiological conditions, while a Th2 activation early in life has been suggested in atopics (21) and an increased total IL-12 production from monocytes in children with allergic diseases was observed (22). Our data suggest that IL-12 dysregulation, probably related to monocyte activation by food allergens, could be important in the development of AD in the neonatal period, when the immature immune system of patients predisposed to AD might differently respond to allergic stimulation than non-atopic subjects. We found that serum total IL-12 and sCD30 levels were significantly higher in patients younger than 30 months of age; in later years, disease severity can be sustained by chronic stimulation and IL-12 could have a different role.

Sensitization could also play a role in the development of AD: sensitization to food allergens predominates in younger children, whereas older children display a different pattern of sensitization, which is sustained by both food and aeroallergens. It has been suggested that different mechanisms are responsible for the induction of tolerance to different food or aeroallergens (21, 23). IL-12 production in a heterogeneous group of patients with allergic diseases differs between food- and pollen-sensitized children (24). These studies and our data suggest that the early onset of AD might reflect different patterns of immune modulation, which might require a different therapeutic approach. This can also account for the evolution of the cutaneous symptoms of AD.

Total IL-12 levels did not differ between patients who also had other allergic diseases and controls, but were significantly higher in patients affected by AD exclusively. This result may be related with the significantly older age of the subgroup of patients who had other allergic manifestations, although we cannot exclude the possibility of a different effect of IL-12 dysregulation between AD and atopic asthma or rhinoconjunctivitis.

In conclusion, this study suggests that IL-12 is involved in the pathogenesis of AD. Levels of IL-12 and sCD30 change with age in children with AD;

this might explain differences in the time course of the disease, disease chronicity and the response to therapy, and may be useful for developing new diagnostic and therapeutic strategies.

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