

Interferon-gamma pretreatment of peripheral blood mononuclear cells partially restores defective cytokine production in children with atopic dermatitis

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Interferon-gamma (IFN- γ) is considered an important determinant of the balance between T-helper type 1 and 2 cytokines and has been used experimentally for the treatment of atopic dermatitis. However, contrasting results have been reported relative to the Th-1/Th-2 cytokine profile in atopic patients. In this study, we examined cytokine production by polyclonally activated peripheral blood mononuclear cells (PBMC) from children with atopic dermatitis, and assessed the influence of *in vitro* IFN- γ pretreatment on these cells. A fraction of PBMC isolated from children with severe atopic dermatitis, as well as from age-matched controls, was initially exposed to IFN- γ . After washing, both treated and untreated cells were then put into culture either alone or with the addition of phytohemagglutinin (PHA) or phorbol myristate acetate (PMA) plus ionomycin. IL-4, IL-5, IL-10 and IFN- γ production were measured in the supernatants using commercially available ELISAs. PBMC from atopic patients produced more IL-4 ($P = 0.04$) and IL-10 ($P = 0.03$) and less IFN- γ ($P = 0.01$) than controls, when stimulated with PHA. Interestingly, in PMA + ionomycin stimulated cultures, the atopic cytokine profile was different with more IL-5 ($P = 0.0068$) and less IFN- γ production ($P = 0.00046$) than the control group. When cells were pretreated with IFN- γ , there were no significant differences between patients and controls. PBMC from children with atopic dermatitis show alterations in cytokine production, compatible in general terms with the Th-1/Th-2 model. Exposure of PBMC to IFN- γ before activation results in a reduction of these differences, so that cytokine production becomes similar in the atopic and normal groups.

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In recent years it has become increasingly evident that elevated IgE production in atopic disease is closely associated with dysregulation of cytokine production by T-cell subsets (1). The balance between interferon-gamma (IFN- γ) and interleukin-4 (IL-4), the hallmark cytokines of T-helper type 1 (Th-1) and Th-2 cell subsets respectively, has been shown to be important in the regulation of IgE production and probably in the expression of atopic disease. Several conditions favoring the development of Th-1 or Th-2 responses are currently under scrutiny, with the ultimate aim of repressing the Th-2 phenotype as a novel therapeutic target for atopy. It has been demonstrated that both types of cells can be de-

rived from a common, bipotential precursor CD4⁺ T cell (2), while in cloning experiments, IFN- γ and IL-4 have reciprocal regulatory effects on the development of respective Th-1 and Th-2 clones (3). Several other cytokines such as interleukin-12 (IL-12) and interleukin-10 (IL-10) which induce or inhibit IFN- γ production respectively, have also been involved (4). IFN- γ production from cord blood mononuclear cells stimulated with betalactoglobulin and ovalbumin, was associated with the development of atopic eczema to cow's milk and egg at one year of age (5), indicating a possible involvement of this cytokine in the pathogenesis of the disease. As IFN- γ has been employed in the treatment of atopic dermatitis with in-

conclusive results (6, 7) the underlying mechanisms of its action have to be particularly addressed. This is further implied by the conflicting reports with regard to cytokine production in atopic individuals, ranging from normal to markedly affected for both IFN- γ and IL-4 (8–13). To evaluate Th-1/Th-2 cytokine balance in atopic dermatitis as well as the possible effects of exogenously administered IFN- γ , we examined cytokine production of polyclonally activated peripheral blood mononuclear cells (PBMC) from normal and highly atopic children with and without *in vitro* pretreatment with IFN- γ .

Materials and methods

Study population

Twenty-five children, 17 boys and 8 girls, aged 3 months to 13 years (mean 5.9, median 4.35) were included in the study. Of these children, 15 (11 boys, 4 girls, mean age 5.4 years) were suffering from moderate to severe atopic dermatitis, as assessed by clinical examination, IgE > 700 mg/dl, and positive RAST and/or skin prick tests for more than one allergen. Children receiving systemic corticosteroid treatment at the time of the study were excluded. The control group consisted of ten children (mean age 6.9 years), with a negative personal and family history for atopy, normal IgE levels for their age and negative RAST. They were not suffering from any acute or chronic disease and were not receiving any drugs at the time of the study. These children were either outpatients referred to the hospital for a check-up or inpatients of the surgical clinics admitted for a scheduled, minor surgical procedure. In all cases, blood was taken prior to any therapeutic manipulation, from the venipuncture performed for scheduled tests and informed consent was obtained from the parents.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were obtained from whole blood by Ficoll density centrifugation. Cells were washed twice with Hanks balanced salt solution (HBSS) and resuspended in complete culture medium (CM) consisting of RPMI-1640 with Glutamax, 10% heat-inactivated fetal bovine serum, 50 μ g/ml gentamycin, and 10mM HEPES buffer (Gibco, Grand Island, NY), at a concentration of 10^7 cells/ml. Half of the PBMC suspension was subsequently incubated with recombinant human IFN- γ (specific activity 3×10^7 U/mg, kindly provided by Boehringer Ingelheim Hellas, Athens, GR) at a concentration of 10^3 U/ml for 1 hour at 37°C, after which cells were washed twice again in 50 ml of HBSS. Minimal or no residual

IFN- γ was measured in cell suspensions after washing. Both IFN- γ treated and untreated cells were put into culture at a density of 2×10^6 cells/ml, both alone and with the addition of either 5 μ g/ml phytohemagglutinin (PHA) or 10 ng/ml phorbol myristate acetate (PMA) plus 1 μ M ionomycin (Sigma Chemical Co, St. Louis, MO), in a humidified, 5% CO₂ incubator, at 37°C for 72 hours. Supernatants were collected 24 hours after the initiation of the culture, for the determination of IL-4, IL-5 and IL-10 and at 72 hours for the determination of IFN- γ , at the time of their optimal concentrations according to initial experiments and previous publications (14). These were stored at -70°C until assayed.

Cytokine assays

Levels of IFN- γ , IL-4, interleukin-5 (IL-5) and interleukin-10 (IL-10) in the supernatants of the cultures were measured using commercially available enzyme immunoassays (Cytoscreen, Biosource Int., Camarillo, CA) according to the manufacturer's instructions. The minimum detectable levels of the cytokines were 7 pg/ml, 12 pg/ml, 10 pg/ml and 14 pg/ml respectively.

Statistical analysis

Non-parametric tests were used for statistical evaluation of results. The Man-Whitney U-test was used for comparisons of atopic vs non-atopic subjects, while the Wilcoxon matched pairs test for comparisons between IFN- γ treated and non-treated cells. All quoted values represent mean $\pm$ standard deviation.

Results

Cytokine production

PHA-stimulated PBMC from atopic children produced more IL-4 (115.3 ± 76.1 vs. 29.8 ± 11.7 pg/ml, $P = 0.04$) and IL-10 (305.3 ± 125.4 vs. 36.8 ± 51.9 pg/ml, $P = 0.03$) and less IFN- γ (5543.6 ± 2547.5 vs. 9702.5 ± 2169.9 pg/ml, $P = 0.01$) than normal subjects. There was no statistically significant difference in the production of IL-5 (115.5 ± 104.8 vs. 59 ± 23.5 pg/ml) (Fig. 1).

When PMA plus ionomycin was used for stimulation, no differences were found in the production of either IL-4 (patients 109.0 ± 32.8 pg/ml, controls 150.0 ± 167.8 pg/ml) or IL-10 (patients 94.5 ± 57.1 pg/ml, controls 84.0 ± 76.7 pg/ml). On the contrary, patients produced more IL-5 and less IFN- γ than controls (403.8 ± 83.6 pg/ml vs 183.1 ± 79.7 , $P = 0.0068$ and 8728.0 ± 288.9 pg/ml vs 13333.3 ± 1527.5 pg/ml, $P = 0.00046$, respectively) (Fig. 2).

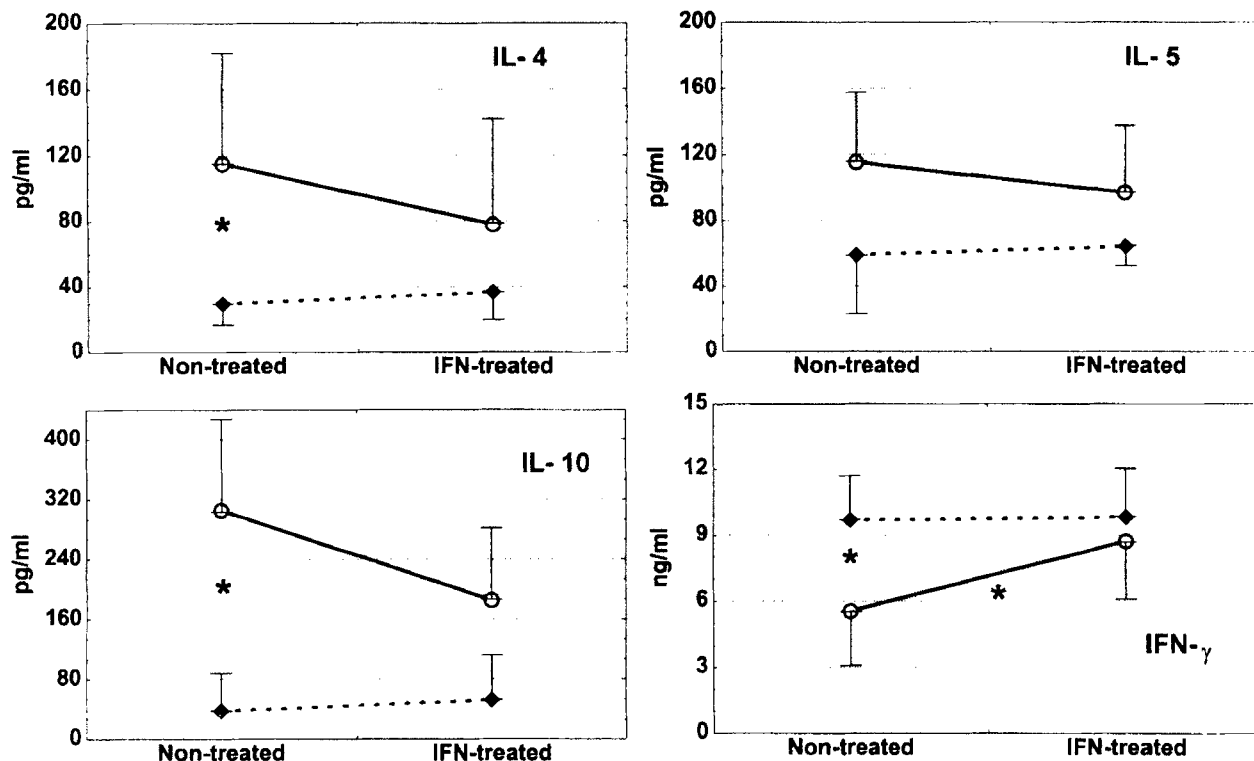


Fig. 1. Cytokine production (mean $\pm$ SD) by PHA-stimulated PBMC from atopic children (solid line) and healthy controls (dashed line). Statistically significant differences are marked with an asterisk. PBMC from atopic children produce more IL-4 and IL-10 and less IFN- γ than healthy controls. When cells are pretreated with IFN- γ , all differences between the groups are abolished. It is noteworthy that comparisons within the atopic group before and after the treatment reveal significant alteration only for IFN- γ .

Non-stimulated cells produced very low cytokine amounts, most of which were under the assay's detection range, with no differences between the groups (data not shown).

Effect of IFN- γ pretreatment on cytokine production

In contrast to the above, when we compared cytokine production in IFN- γ pre-treated cells of atopic vs non-atopic subjects, no statistically significant differences were found after either PHA or PMA + ionomycin activation (Figs. 1 and 2). Furthermore, the comparison of treated vs non-treated cells within each group revealed a marked increase of IFN- γ production in IFN- γ treated, PHA stimulated PBMC only in the atopic group (8714 ± 3161 pg/ml, $P = 0.005$), while no significant alterations were noted in other instances.

Discussion

There is no doubt that IL-4 and IFN- γ play an important role in the regulation of IgE synthesis. However, there are still many unanswered questions regarding both the mechanisms leading to cytokine dysregulation and its actual effects on atopy (15,

16). This is further complicated by the fact that several investigators have reported conflicting results on cytokine production by atopic patients, ranging from normal to severely affected (8–13).

These discrepancies can be attributed to differences in subject selection such as distinct hypersensitivity states, severity of disease, mono- or polysensitivity, seasonal variations and use of drugs. Protocol differences have also been the subject of debate since the selection of cell populations, such as PBMC, pure T cells or T-cell clones and selection of activator or other culture parameters could account for conflicting results (17).

In this study we have selected a homogeneous atopic population, i.e. polysensitive patients with moderate to severe atopic dermatitis and high IgE, who were not receiving any medication. There are certainly both advantages and drawbacks relative to our choice of unselected cell populations. It is increasingly argued that the Th-1/Th-2 paradigm reflects the extremes of the spectrum of helper T-cell activation, observed after the extensive selection applied to Th cells by cloning (18). Furthermore, CD8 T cells, NK cells and monocytes can inhibit, induce or consume Th-cell cytokines as well as produce their own so that the net cytokine amounts after acti-

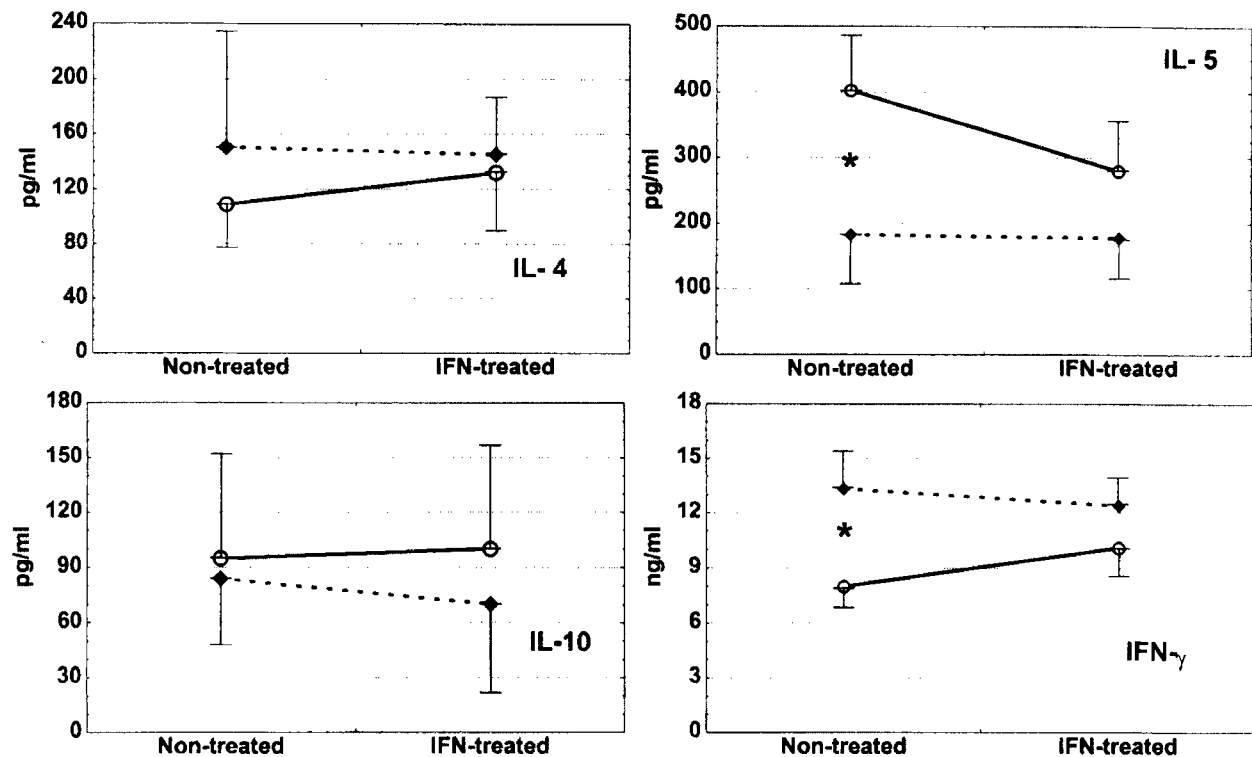


Fig. 2. Cytokine production (mean $\pm$ SD) by PMA + ionomycin-stimulated PBMC from atopic children (solid line) and healthy controls (dashed line). Statistically significant differences are marked with an asterisk. With such a stimulation, PBMC from atopic children produce more IL-5 and less IFN- γ than controls. These differences disappear when cells are pretreated with IFN- γ .

vation could be considerably altered by their absence (14). It seems, therefore, that cloned or selected Th populations would be more suitable to focus on basic Th functions, while unselected populations are closer to *in vivo* conditions of the atopic disease.

Selection of activator is another important parameter. The use of antigen, while having obvious advantages (17), is impeded by the fact of very low levels of activation, especially in controls, so that results can be biased by irrelevant activation sources, such as the animal serum used in the culture, and the non-comparable number of cytokine-producing cells. Therefore, we used PHA and PMA plus ionomycin as polyclonal activators acting through different pathways. It has to be noted, however, that small differences could become undetectable by the high level of activation achieved by such potent stimuli, especially in the case of PMA + ionomycin. Nevertheless, in agreement with the general notion, our results indicate that in highly atopic children, cytokine production is altered towards the Th-2 phenotype. It is interesting that the range of affected cytokines was different depending on the polyclonal activator used. Thus, PHA discriminated patients and controls with regard to IL-4, IL-10 and IFN- γ production, while PMA + ionomycin did so for IL-5 and IFN- γ . This can be attributed to the different ac-

tivation pathways and/or the preferential activation of distinct cellular groups achieved by those molecules (19). PHA activates lymphocytes via antigen presenting cells and accessory molecules, while PMA + ionomycin bypasses cell surface physiological events, activating all cells in the culture, including monocytes which can be a significant cytokine source. Although differences in activation pathways by various polyclonal activators are widely known, this fact has not previously received attention, especially when comparing results from different research groups, and careful examination of the literature reveals this is, at least in some cases, related to contrasting conclusions (8–13, 20, 21).

On the clonal level, it has been shown that IFN- γ and IL-4 reciprocally regulate the development of Th-1 and Th-2 clones, respectively (3). In actual *in vivo* conditions with both cytokines coexisting, it is possible that their relative quantities would drive a response towards either end of the balance. With the above in mind we evaluated cytokine production in unfractionated PBMC preincubated with IFN- γ . To the best of our knowledge, such an approach has not been previously investigated. Our results show that this treatment was able to eliminate differences in IFN- γ production between patients and controls, as well as reduce, in the cases they existed, the differ-

ences in Th-2 cytokines (IL-4, IL-5 and IL-10), rendering them non-significant. Within the atopic group, this was achieved by a significant alteration after IFN- γ treatment of IFN- γ production, but with only minor alterations for the rest of the cytokines. This is not surprising, taking into account the relatively high variability of cytokine production, but indicates that such small differences may be missed by different study designs. Moreover, since the selected population did not include mild disease, it seems possible that other factors, either preceding or following T-helper cell activation contribute significantly in the expression of atopic dermatitis, several of which are currently under scrutiny.

The ability of IFN- γ to upregulate its own production in PBMC cultures of atopic children indicates that the Th-2 cytokine predominance in these patients is under certain conditions at least partially reversible. This is an important finding as it supports the proposed use of IFN- γ as a therapeutic modulator in atopic dermatitis. However, it is more likely that Th-1/Th-2 cytokine imbalance is only one aspect of atopic disease pathogenesis (15) and, while some encouraging results have been obtained in clinical trials (22), a better insight into the cytokine network interactions is necessary in order to rationalise and fine tune such attempts.

The Th-1/Th-2 model has proven quite useful for the understanding of a T-cell regulation of allergic responses. However, it seems that as in the case of many innovative concepts, it has been given a burden heavier than it can actually carry, resulting in increasingly documented challenges (16, 18). It is clear, that cytokine production in atopic disease needs to be evaluated by multiple approaches, especially under the prism of potential therapeutic interventions.

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