Reduced production of IL-12 and IL-12-dependent IFN-gamma release in patients with allergic asthma

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Reduced Production of IL-12 and IL-12-Dependent IFN-γ Release in Patients with Allergic Asthma

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In atopic patients, allergen-specific T cells have acquired the Th2 phenotype, which is considered to be responsible for the class switch to IgE Ab formation. Because IL-12 is a key cytokine for the induction of Th1 responses, a reduced capacity to produce this cytokine could lead to aberrant Th2 development. Therefore, we examined the production of IL-12 in whole blood cultures from patients with allergic asthma (n = 15) in comparison with nonatopic control subjects (n = 15) to different stimuli. After stimulation with Staphylococcus aureus Cowan I strain (SAC) we observed a 2.6-fold reduction of IL-12 p70 production in the patient group (p < 0.005). This was not due to a general failure of monocytes from these patients to produce cytokines, because the production of IL-12 was normal. SAC also induced the production of IFN-γ, which was blocked by neutralization of IL-12. In line with the reduced levels of IL-12 secretion, the patient group showed a 3-fold reduction of IL-12-dependent IFN-γ production (p < 0.005). The amounts of IL-12 and IFN-γ were positively correlated in both the patient (R = 0.51 at 0.05% SAC and R = 0.64 at 0.01% SAC) and the control groups (R = 0.64 at 0.05% SAC and R = 0.70 at 0.01% SAC). The IFN-γ:IL-12 ratio was not different between patients and control subjects, indicating a normal response to IL-12. Diminished production of IL-12 and IFN-γ could not be explained by an increased production of IL-10, because in SAC-stimulated cultures IL-10 was hardly induced in both groups. Furthermore, after stimulation with Escherichia coli, the production of IL-10 was similar in patients and control subjects.

Adequate immune responses to various pathogens require a controlled regulation of Th cell development. Th1 cells produce IL-2, IFN-γ, and TNF-β; activate macrophages; and cause delayed-type hypersensitivity reactions, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13; cause eosinophilia; and induce a Ig class switch to IgG1 (mouse) or IgG4 (human) and IgE (1–3). In allergic diseases such as atopic dermatitis and allergic asthma, allergen-specific T cells have acquired the Th2 phenotype (4–9). These Th2 cells are considered to be responsible for the enhanced production of IgE Abs, a characteristic of these patients. Factors that control Th responses include genetic background, the type and dose of the Ag, and soluble factors in the microenvironment. In addition, APC play a critical role in determining whether a Th1 or Th2 response will develop. Apart from membrane-expressed molecules (such as MHC class II, CD80, and CD86), soluble factors secreted by the APC are extremely important. IL-12 p70, a heterodimeric cytokine consisting of two subunits, p40 and p35, has been shown to be instrumental for the development of Th1 responses, supposedly through its ability to induce the Th1 cytokine IFN-γ by T cells and NK cells (10–12), although IFN-γ-independent effects have also been reported (13, 14). Other accessory cell-derived factors, such as IL-10 and PGE2, negatively influence Th1 cytokine production (15, 16). Perhaps most importantly, IL-10 and PGE2 contribute to Th2 responses by their capacity to strongly reduce the production of IL-12 (17, 18). The major producers of IL-12 are probably monocytes or macrophages and dendritic cells, whereas only low amounts of IL-12 are produced by B cells and granulocytes (19–22). The absence of IL-12 facilitates the development of Th2 responses (12, 23). Therefore, the capacity of monocytes and dendritic cells to produce IL-12 is a crucial factor in the regulation of Th development. In HIV-infected individuals, a reduced production of IL-12 corresponds with the reported shift from Th1 to Th2 or Th0 cytokine production (24–26). We examined the hypothesis that in patients with allergic asthma, a reduced production of IL-12 also contributes to the observed bias of T cells from bronchoalveolar lavage fluid and bronchial mucosa, as well as PBMC, toward Th2 cytokine secretion (4, 9, 27).

Materials and Methods

Ab and reagents

Anti-IL-12 mAb C11.79 and C8.6 (28) were provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). mAb 20C2, specific for IL-12 p70, was a kind gift from Dr. M. K. Gately (Hoffmann-La Roche, Nutley, NJ). Anti-IL-10 mAb B-710 and B-N10 were a kind gift from Dr. J. Wijdenes (Daclone, Besancon, France). Recombinant human (h) IL-10 was a kind gift from Dr. R. de Waal Malefijt (DNAX, Palo Alto, CA). IFN-γ was obtained from Boehringer Ingelheim (Alkmaar, The Netherlands). Neutralizing anti-IFN-γ mAb MD1 was a kind gift from Dr. P. H. van der Meide (TNO, Rijswijk, The Netherlands). Staphylococcus aureus Cowan I strain (SAC) (Pansorbin) was obtained from Calbiochem (La Jolla, CA), and

Abbreviations used in this paper: SAC, Staphylococcus aureus Cowan I strain; h, human.

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2 Abbreviations used in this paper: SAC, Staphylococcus aureus Cowan I strain; h, human.
Patients and control subjects

Fifteen patients with allergic asthma, 4 females and 11 males (mean age 35 yr, range 21–54 yr), were recruited from the Outpatient Department of Pulmonology. Asthma was defined according to the criteria of the American Thoracic Society (30). Patients with asthma had a history of paradoxisms of dyspnea and coughing but were studied during a stable phase of the disease. The median forced expiratory volume in 1 s was 87% of predicted value (range 57–114%). The median provocative concentration causing a 20% fall in the median forced expiratory volume in 1 s (pc20) was 2.4 mg histamine/ml (range 0.01–16 mg/ml, n = 11). Histamine threshold was not determined in patients with spirometry-induced bronchoconstriction or in patients with predicted percentage of median forced expiratory volume in 1 s less than 70% on the test day (n = 4). Patient selection was based on positive results of a skin-prick test and a radioallergosorbent test for at least one inhalant allergen and a total IgE of more than 500 IU/ml established during the previous 3 yr. The geometric mean of serum total IgE was 1068 IU/ml. Excluded were patients treated with systemic corticosteroids, antihistamines, and theophylline and patients who had received hyposensitization therapy. Most patients received low-dose inhaled corticosteroids (<800 µg) and inhaled β2-agonists. No medication was taken 12 h before blood sampling. The study was approved by the local medical ethics committee, and written consent was given by all patients times and in agreement with the Declaration of Helsinki.

Whole-blood cultures

Whole blood was obtained by venipuncture in sterile blood-collecting tubes containing sodium heparin (VT-100SH tubes, Venoject, Terumo Europe N.V., Leuven, Belgium). To prevent spontaneous production of cytokines by endotoxin or endotoxin-like substances present in culture media, Iscove’s modified Dulbecco’s medium was ultraltrifed by means of a hollow-fiber dialyzer (31) (Hemoflow F5, Fresenius A.G., Bad Homburg, Germany). Whole blood was diluted 1:10 in ultrafiltrated Iscove’s modified Dulbecco’s medium supplemented with 0.1% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 15 IU/ml sodium-heparin (Leo Pharmaceutical Products B.V., Woesp, The Netherlands). Diluted whole blood was cultured in triplicate in 200-µl flat-bottom culture plates (Nunc, Roskilde, Denmark) and stimulated with SAC (0.05% and 0.01% w/v) or heat-killed E. coli (10³/ml). Supernatants were harvested after 24 h of culture, and cytokine levels were determined. It is important to note that the elapsed time between the moment of blood withdrawal and start of the culture is negatively correlated with the production of IL-12. Therefore, extreme care was taken to obtain blood from patients and control subjects simultaneously. In addition, blood was put into culture as quickly as possible, routinely within 1 h after withdrawal.

Assays for cytokines

IL-10 p70 ELISA. Flat-bottom microtiter plates (Maxisorb, Nunc) were coated overnight with mAb 20C2 (0.5 µg/ml in 0.1 M carbonate buffer, pH 9.6, 100 µl/well). All subsequent incubations were in 100-µl vol at room temperature. The plates were washed twice with PBS supplemented with 0.02% (v/v) Tween-20 and incubated for 30 min with PBS containing 2% (v/v) cow’s milk as a blocking step. After washing, biotinylated, purified mAb C8.6 was added (final concentration 0.25 µg/ml) together with IL-12-containing samples diluted in high performance ELISA buffer (CLB, Amsterdam, The Netherlands) for 1.5 h. Thereafter, the plates were washed five times and incubated with polyclonal sheep anti-IL-6 (CLB) diluted 1/10,000 (according to the manufacturer’s instructions) in PBS containing 2% (v/v) cow’s milk for 0.5 h, washed, and developed with a solution of 100 µg/ml 3,5,5’-tetramethylbenzidine (Merck, Darmstadt, Germany) with 0.003% (v/v) H₂O₂ in 0.1 M sodium acetate, pH 5.5 (100 µl/well). The reaction was stopped by adding an equal volume of 2 M H₂SO₄ to the wells. Plates were read at 450 nm in a Titertek Multiskan reader. Background absorbance at 540 nm was subtracted. rhIL-10 p70, provided by Dr. S. F. Wolf (Genetics Institute, Inc., Cambridge, MA) was used as a standard; the detection limit was 0.5 pg/ml (twice the background). This assay does not detect p40 (provided by Dr. S. F. Wolf) up to 20 ng/ml.

IL-12 ELISA. This assay detects both p40 and p70 (18) and was performed in an identical manner to the p70-specific ELISA; however, the coating Ab was replaced by mAb C11.79 used at 2 µg/ml.

IL-10 ELISA. The assay was performed in an identical manner to the IL-12 ELISA except for the blocking step, which was omitted (18). For coating, mAb B-N10 was used at 0.5 µg/ml in PBS, and for detection, biotinylated mAb B-T10 was used at 0.125 µg/ml. rhIL-10 was used as a standard.

IFN-γ ELISA. IFN-γ levels were determined by a commercially available IFN-γ ELISA kit (CLB).

Statistical analyses

All data were compared by the Mann-Whitney U test (two tailed). p < 0.05 was considered significant. Correlations were tested using the Pearson correlation test of logarithmically transformed data.

Results

Production of IL-12, IL-10, and IFN-γ after stimulation with SAC or E. coli in whole blood cultures

To study the production of IL-12, we used whole-blood cultures because of the low production of IL-12 in isolated PBMC (18, 34). To analyze IL-12, IL-10, and IFN-γ production in patients with allergic asthma, both Gram-positive bacteria (SAC) and Gram-negative bacteria (E. coli) were used because of their capacities to induce differential cytokine secretion (Fig. 1). In E. coli-stimulated cultures, IL-12 p70 was produced in very low quantities but was strongly enhanced by the addition of IFN-γ or neutralizing IL-10 Abs (Fig.1A). In SAC-stimulated cultures, IL-12 was efficiently produced; this production was also enhanced by IFN-γ and slightly inhibited by neutralizing anti-IFN-γ but was not influenced by anti-IL-10 (Fig. 1D). IFN-γ was produced in higher amounts after stimulation with SAC than with E. coli, and its production was almost completely inhibited by neutralizing IL-12 Abs in both E. coli- and SAC-stimulated cultures (Fig. 1, B and E). As observed for the production of IL-12, neutralization of IL-10 had an enhancing effect on IFN-γ production in E. coli- but not SAC-stimulated cultures. This can be explained by the fact that IL-10 was induced only in E. coli-stimulated cultures (Fig. 1, C and F). The production of IL-10 could be inhibited by IFN-γ and enhanced by anti-IFN-γ. Neutralizing IL-12 Abs did not have an effect on IL-10 production. In short, IL-12 and IFN-γ were most efficiently produced in SAC-stimulated cultures, in which endogenous IL-10 does not play a role. Stimulation with E. coli results in a high production of IL-10, leading to strong inhibition of IL-12 and IFN-γ production.

Production of IL-12 p70 and IL-12-dependent IFN-γ is reduced in patients with allergic asthma

After stimulation of whole-blood cultures with 0.05% or 0.01% SAC, we observed a more than twofold reduction in secretion of bioactive IL-12 in the allergic asthma group (p < 0.005 and p = 0.01, respectively) compared with the control group (Fig. 2A, top). In the same cultures, the production of IL-6 was normal (Fig. 2B, bottom). SAC-induced IL-12 production causes the secretion of IFN-γ, because the production of IFN-γ can be blocked by neutralizing IL-12 Abs (Fig. 1, B and E). Therefore, we also examined IFN-γ levels in SAC-stimulated whole-blood cultures from patients and control subjects. The reduced production of IL-12 in patients was reflected in a threefold reduced production of IFN-γ (Fig. 2C, middle). To examine whether the production of IFN-γ is proportional to the production of IL-12, we calculated the ratio of IFN-γ to p70 for each individual. This ratio was not statistically different for patients and control subjects (not shown). In addition, both patients and control subjects showed a positive correlation
between the production of IL-12 and IFN-γ at the two different concentrations of SAC (Fig. 3). At 0.05% SAC, the correlation coefficient (r) was 0.51 (p = 0.05) for the patient group and 0.58 (p = 0.02) for the control group. At 0.01% SAC, r was 0.64 (p = 0.01) for the patient group and 0.70 (p = 0.004) for the control group. These results suggest that the response to IL-12, in terms of IFN-γ production, is not disturbed in patients with allergic asthma.

Reduced production of IL-12 by patients with allergic asthma is not due to enhanced secretion of IL-10

Because IL-10 is a known inhibitor of the production of IL-12, we examined whether the production of IL-10 was enhanced in the patient group. After stimulation with SAC, almost no IL-10 could be detected in either the patient or the control group (not shown). In addition, neutralizing IL-10 Abs had no effect on IL-12 production during SAC stimulation (Fig. 1D). Stimulation with E. coli results in significant IL-10 secretion, allowing direct analysis of IL-10 production. No difference in IL-10 production between patients and control subjects was observed (Fig. 4A). In the same E. coli-stimulated cultures, we also analyzed IL-12 p70 and IFN-γ production. Production of IL-12 p70 after E. coli stimulation is much lower than after SAC stimulation; in most individuals IL-12 p70 was not detectable. The production of IL-12 p70 was lower in the allergic asthma group (Fig. 4B) but did not reach statistical significance. However, IFN-γ production was again significantly reduced in the patient group at the two highest concentrations of E. coli (Fig. 4C; p < 0.05). These results indicate that the reduced production of IL-12 and IFN-γ by the patients cannot be attributed to an overproduction of IL-10.

Discussion

In the present report we show that production of bioactive IL-12 p70 is reduced in patients with allergic asthma after stimulation of whole-blood cultures with SAC or E. coli. Obviously, whole-blood cultures have the disadvantage that the complexity of a mixture of cells and plasma obscures analysis of the system. However, we previously showed that IL-12 production is extremely sensitive to manipulation of cells and plasma but to triggering of an inhibitory (and unknown) component upon purification of cells: low numbers of purified mononuclear cells strongly inhibit IL-12

![E.coli stimulation](image)

**FIGURE 1.** Whole blood (diluted 1:10) was stimulated with 0.01% SAC (A–C) or 10⁵ E. coli/ml (D–F) and the indicated additions (anti-IL-12 (a-IL-12) mAb C8.6 at 10 pg/ml, IFN-γ (IFNg) at 5 ng/ml, anti-IFN-γ (a-IFNg) mAb MD1 at 10 pg/ml, a control (ctr) mAb of the IgG1 subclass at 10 pg/ml, and anti-IL-10 (a-IL10) mAb BT-10 at 5 pg/ml). After 24 h of culture, supernatants were assayed for cytokine production. Results are expressed as the mean production of four normal donors ± SE. NT = not tested.
FIGURE 2. Decreased IL-12 p70 and IFN-γ but normal IL-6 production by patients with allergic asthma. Whole blood (diluted 1:10) was stimulated with the indicated concentrations of SAC. After 24 h of culture, supernatants were assayed for cytokine production. Individual and mean production of 15 patients with allergic asthma (○) and 15 nonatopic control subjects (□) are shown.

FIGURE 3. Positive correlation between IFN-γ and IL-12 p70 production in patients with allergic asthma (■) and control subjects (○). IFN-γ and p70 production after stimulation of whole blood with 0.01 or 0.05% SAC are shown (see Fig. 2, n = 15 for each group). The Pearson correlation coefficients (r) and p values of logarithmically transformed data are shown.

(but not IL-6) production when added back to autologous whole blood (data not shown). Others have used isolated mononuclear cells to obtain SAC-induced IL-12 production; in comparison with our whole-blood cultures, the production of IL-12 on a per cell basis is similar to that reported by D’Andrea et al. (28). Two other studies, however, reported an ~10-fold lower production of IL-12 on a per cell basis by mononuclear cells from healthy donors (35, 36). These results are consistent with our findings that IL-12 production is low in purified cells. Experiments with isolated cells have shown that purified monocytes but not CD14-depleted mononuclear cells or granulocytes can produce IL-12 and IL-6 following stimulation with LPS or SAC (18 and data not shown). Because the production of IL-6 can be regarded a measure for the number and competence of monocytes to produce cytokines, we calculated the IL-12 p70:IL-6 ratio for each individual. In the allergic asthma group, a lower IL-12 p70:IL-6 ratio was found (p < 0.02, not shown). In addition, the percentage of monocytes in isolated PBMC did not differ between patients and control subjects (19 and 21% monocytes, respectively), and the production of IL-6 and IL-10 was normal in the patient group. These results indicate that the reduced production of IL-12 was not due to a low amount of peripheral blood monocytes or a general failure of monocytes from patients with allergic asthma to produce cytokines. Although the patient and control groups were age and sex matched, we examined possible links of IL-12 production with these parameters. However, we did not find a correlation of the production of IL-12 with age or sex. Based on the observation that production of an important inhibitor of IL-12 production, IL-10, was not elevated and the fact that neutralizing IL-10 Abs do not influence IL-12 production in SAC-stimulated cultures, we conclude that IL-10 is not responsible for the observed diminished production of IL-12. Another possibility is that plasma components specifically influence IL-12 production, and differences between atopic patients and control subjects are due to differences in plasma constituents. This possibility was ruled out because addition of patient and control plasma to whole-blood cultures of control subjects did not lead to differential reduction of IL-12 production (not shown).

Reduced production of IL-12 could be an inherited intrinsic monocyte defect. Atopy can be considered a multigenetic disorder influenced by environmental factors. Bronchial hyperresponsiveness and elevated serum IgE levels, two characteristics of the patients we studied, are associated with polymorphic markers located on chromosome 5q31–33 (37–39). This chromosomal region contains a cluster of genes encoding cytokines, including IL-3, IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating...
Reduced Production of IL-12 and IFN-γ in Allergic Asthma

Preliminary results suggest that overproduction of PGE₂ is not responsible for the reduced production of IL-12. Also, the Th2 cytokines IL-4, IL-13, and especially IL-10 negatively influence the secretion of IL-12 (17, 42). However, these cytokines also inhibit IL-6 and IL-10 production by monocytes (42-44). Because the production of IL-6 and IL-10 were unimpaired in the patient group, it is unlikely that the reduced production of IL-12 is due to a negative feedback by Th2 cytokines. In contrast, the Th1 cytokine IFN-γ stimulates IL-12 production (43, 45), creating a positive feedback on the production of IL-12. Therefore, a diminished response to IFN-γ by monocytes from patients with allergic asthma cannot be ruled out.

Lester et al. (46) reported that patients with atopic dermatitis produce normal levels of IL-12 in response to a bacterial superantigen. In the same patients, reduced production of IFN-γ by PBMC in response to PHA or to superantigen is observed, even in the presence of IL-12. In addition, IL-12-neutralizing Abs inhibit the production of superantigen-induced IFN-γ by PBMC from healthy control subjects but not patients, indicating that in these patients, reduced production of IFN-γ is caused by a diminished response to IL-12. Although we also found a reduced IL-12-dependent IFN-γ production in patients with allergic asthma, this was proportional to the diminished amounts of IL-12. The cellular source of IFN-γ could be both T and NK cells, since both cell types produce IFN-γ in response to IL-12 (47). Because the production of IFN-γ by isolated T cells (without the influence of monocytes) from patients with allergic asthma was not statistically different from that in the control subjects (not shown), we believe that the diminished production of IL-12 may be the primary defect in these patients, while the capacity to produce IFN-γ in response to IL-12 or polyclonal T cell stimulation is normal. In allergic asthmatic children, a reduced production of IFN-γ was reported after stimulation of PBMC with PHA (27). In that study it is very well possible that monocytes influenced the production of IFN-γ. The fact that IL-12 production is reduced in patients with allergic asthma may explain the observed skewing toward Th2 cell development not only after allergen exposure but also after exposure to bacterial Ags (48). Besides the influence of IL-12 on Th1 development, IL-12 also reduces the production of IgE independent of IFN-γ, as was shown for IL-4, plus hydrocortisone-dependent IgE production by B cells (49). In murine models of allergic airway hyperresponsiveness, the administration of IL-12 during immunization prevents subsequent bronchial Ag exposure-induced airway eosinophilia, IL-4 and IL-5 expression, Ag-specific IgE Ab formation, and airway hyperresponsiveness (50, 51). Interestingly, administration of IL-12 only during aerosol exposure also reduces the aerosol-induced effects on airways, independent of the presence of IgE (50). These data indicate that diminished production of IL-12 may enhance Th2 development, leading to elevated production of IgE, and, in an IgE-independent manner, to more severe airway hyperresponsiveness. Because allergens, e.g., proteins derived from house dust mite (Dermatophagoides pteronyssinus, DerpII) and grass pollen (Dactylis glomerata, crude extract) did not induce IL-12 (our unpublished results), the inclusion of bacterial components to induce IL-12 production during hyposensitization therapy may be worthwhile. In addition, during such treatment, IL-12 production can be up-regulated by PGE₂ synthesis inhibitors and histamine H₂ antagonists.

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References


