

Enhanced production of IL4 but not of IFN γ and IL10 by peripheral blood mononuclear cells from atopic children in response to CD2 plus CD28 stimulation

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Peripheral blood mononuclear cells from 16 children with atopic disease (range of IgE levels: 33 - 2892 kU/l) and 12 age matched controls were stimulated either with mAbs specific for CD3, CD2, CD3 plus CD28, CD2 plus CD28, with Tetanus Toxoid, SEA, or PHA plus PMA and their cell proliferation was determined. In addition, their cytokine production (IL2, IL4, IL10, IFN γ) following selected stimuli was measured. We found that the cells from atopics proliferated significantly better in response to CD2 stimulation than control cells, with no difference in response to CD3 or SEA stimulation. Furthermore, cells from atopics produced significantly higher amounts of IL4 than cells from controls, a difference most pronounced following CD2 plus CD28 stimulation. No differential production was found for IL10 and IFN γ . We conclude that in atopic children with moderately elevated IgE a hyperreactivity of the CD2 pathway of stimulation and a clear elevation of IL4 but not of IL10 or IFN γ production can be demonstrated.

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Antigen-specific IgE is the major effector molecule in the pathogenesis of allergic diseases by linking recognition of the allergen to the triggering of effector cells such as mast cells and basophils. In recent years it became clear that the production of IgE is regulated through B cell surface molecules such as CD40 as well as by several cytokines including IL2, IL4, and IFN γ . IL4 induces IgE production by promoting IgE germline transcription whereas IFN γ inhibits IgE production directly, as well as by inhibiting the synthesis of IL4 (1–3). The relative production of IL4 and IFN γ by helper T cells is therefore of decisive importance for the development of an IgE dominated immune response. This relative production of cytokines depends on poorly defined regulatory mechanisms which modulate the cytokine transcription of a resting T cell or of a proliferating T cell clone of a Th0 phenotype capable of producing an unrestricted cytokine profile. Likewise, the factors which direct the development of Th0 T cells into Th1 and Th2 helper subtypes producing IL2 and IFN γ or IL4, IL5 and IL10, respectively, in a restricted fashion are not completely understood. It is

clear, however, that IL4 and IFN γ again act on this step of immune regulation (3–6).

Earlier studies have documented an elevation of IL4 and a reduction of IFN γ production in patients with severe atopic disease both in adults and in children, suggesting that this imbalance of cytokine production may be causally related to elevated IgE levels in such patients (7–9). It was not clear, however, from these studies, why cells from such patients upon stimulation *in vitro* produced elevated amounts of IL4.

T cell activation is influenced by signalling through a large number of different cell surface molecules, some of which can transmit stimulatory or inhibitory signals, depending on the nature of the respective ligand and on the stimulatory conditions employed (10). So far, these signals have been mostly evaluated in terms of their effect on cell growth. We would like to postulate that they may also be involved in fine regulation of the cytokine response of a T cell. Consequently, we have asked previously, whether the cytokine pattern of T cells will differ depending on which surface structure is

used for stimulation. In these studies we have found that the CD2 and the CD28 structures appear to be of central importance for the production of cytokines because highest levels of several cytokines including, in particular, IL4 were produced consistently in response to CD2 plus CD28 stimulation (11). For IL4 production, stimulation with this antibody combination was by far superior to stimulation through CD3 plus CD28 or using the lectin PHA plus the phorbol ester PMA. With regard to the reported elevated IL4 production by cells of atopic patients, we asked now, whether this overproduction could eventually be explained by an hyperreactivity of the CD2 pathway in these patients.

Material and methods

Patients

Twenty-eight pediatric patients aged from one to 14 years attending an outpatient allergy clinic for symptoms of potentially allergic diseases were enrolled in this study after informed consent from the patients and/or parents had been obtained. The patients were evaluated by their clinical history and physical examination for the presence of atopy including allergic rhinitis, bronchial asthma, atopic dermatitis, and food allergy. Their family history was documented, and venous blood was collected for the determination of total IgE by ELISA (ES 600, Boehringer Mannheim, Vienna, Austria), antigen-specific IgE by a chemiluminescence immunoassay (ALK, Horsholm, Denmark; ref. 12), and the determination of *in vitro* stimulatory responses reported in this study. In addition, skin prick tests were performed in order to document antigen specific reactivity *in vivo*. For the skin prick test (SPT), freeze-dried purified, biologically standardized and immunochemically characterized allergen preparations (ALK) were used by the technique described by Pepys (13). Tests were controlled with NaCl (0.9%; neg. control) and histamine dihydrochloride (10 mg/ml; pos. control). A wheal reaction of the same size or bigger than that of the histamine control was regarded as positive. In school children, a standard screening panel of inhalant and/or nutritive allergens was tested by SPT including pollen, mites, moulders, animals and food allergens (total number of allergens: n=50). In younger children the allergens for the SPT were selected according to clinical presentation. Specific IgE antibody levels were determined for SPT-reactive allergens only. Depending on the presence of a positive family history of allergic diseases, an elevation of total IgE corrected for age, positive skin prick tests, elevated antigen specific IgE, and clinical signs of atopic disorders patients were classified either to the atopic group (n=16) or otherwise to the control group (n=12). Pa-

tients under immunosuppressive or specific hyposensitisation therapy were excluded. Mean IgE of atopics was 487 kU/l (range 33 to 2892 kU/l, with 6 patients having more than 200 kU/l), mean IgE for the controls was 43 kU/l. Their age distribution did not differ significantly (7.3 ± 3.0 for the atopics, 8.4 ± 4.8 for the controls, mean $\pm$ S. D.).

Cell stimulation and cytokine determination

Peripheral blood mononuclear cells (triplicate cultures of 1×10^5 cells/well) were stimulated in U-bottomed microtiter (Nunc, Roskilde, Denmark) plates either with the CD3 mAb OKT3 (Ortho, Raritan NJ; 1 μ g/ml), with the CD2 mAb combination OKT11 (ATCC; 0.1 μ g/ml) plus VIT13 (this institute, 2.5 μ g/ml), the CD28 mAb 5E8 (kindly provided by L. Aarden, Amsterdam; 100 ng/ml), staphylococcus superantigen A (SEA; Sigma, St. Louis, MO; 10 ng/ml), phytohemagglutinin A (PHA; Wellcome, Beckenham, England; 0.01 mitogenic units/ml), phorbol-myristate-acetate (PMA; Sigma; 60 ng/ml), or Tetanus toxoid (TT; Connaught Laboratories Limited, Ontario, Canada; 10 LF/ml). Cell culture medium was RPMI 1640 supplemented with L-glutamine, antibiotics, and 5% pooled human serum. The culture time was 96 h for cell proliferation assays and cultures were labeled for the last 18 h with 3H-thymidine. For cytokine induction the cultures were set up using the identical experimental conditions, except that they were terminated at 48 h after which the cytokines were measured using classical sandwich ELISAs as described recently (11). For measurement of IL10 in culture supernatants antibodies JES3-9D7 for coating and JES3-12G8 for detection were obtained from Pharmingen (San Diego, CA) and used with the identical protocol as for the other ELISAs.

Statistical analysis

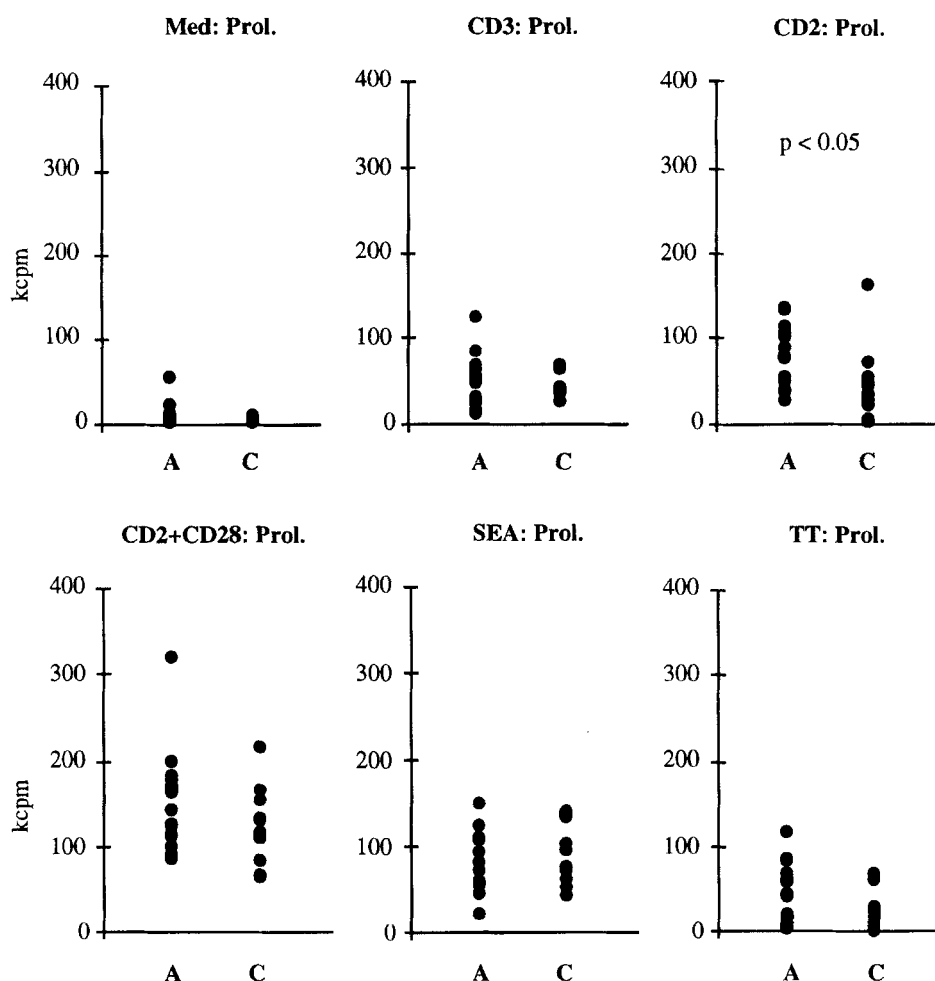
Data were analyzed using the Kruskal-Wallis test for comparison of the atopic group with the control group for a given functional response.

Result

Proliferative response of mononuclear cells from atopic patients

Mononuclear cells were cultured in medium alone or stimulated under various conditions as shown in Fig. 1 and the cell proliferation was determined on day four. Cells from atopic patients responded with a significantly higher proliferative response upon CD2 stimulation than control cells (72.0 ± 37.4 kcpm (m $\pm$ S. D., n=16) vs. 44.4 ± 42.2 kcpm (n=12), ($p < 0.05$). In addition, cells from the patient group tended to proliferate stronger in medium

Fig. 1. Proliferative response of MNCs from atopics (A) or controls (C) as measured by ^3H -thymidine incorporation and expressed in thousands of counts-per-minute (kcpm) following cell culture in medium alone (Med.: Prol.) or in response to various stimuli as indicated (f. i. "CD3: Prol." indicates: proliferation in response to CD3 stimulation).



alone and in response to CD2 plus CD28 or tetanus toxoid stimulation, however, these differences were not statistically significant. The response to CD3 (45.6 ± 29.2 kcpm vs. 43.3 ± 13.3 kcpm) and to superantigen SEA stimulation (80.2 ± 34.4 kcpm vs. 90.8 ± 33.4 kcpm) were also similar, (Fig. 1).

Cytokine production of mononuclear cells from atopic patients in response to mAb or PHA plus PMA stimulation

Previously we had observed that CD2 plus CD28 stimulation was a highly efficient way to induce IL4 production from freshly isolated mononuclear cells *in vitro* (11). In order to clarify whether the reported hyperproduction of IL4 by cells from atopic patients (7-9) could be attributed to an hyperreactivity of the CD2 pathway of stimulation, cells were stimulated either through CD3 plus CD28 or through CD2 plus CD28 with mAbs or with PHA plus PMA for 48 hours. Levels of IL2, IL4, IFN γ , and IL10 were determined subsequently in the supernatants. For the CD3 and CD2 mAb stimulation additionally a CD28 mAb was included during stimulation, since we had found previously that CD28 co-stimulation increased IL4 and IFN γ production 3 - 4 fold and IL2 synthesis

60 - 100 fold, making measurement of IL4 production *in vitro* possible from every donor tested (11).

As shown in Fig. 2, highest absolute amounts of IL2 were produced in response to PHA plus PMA stimulation and atopics produced about 2-fold more IL2 than controls (2.1 ± 1.2 ng/ml vs 1.0 ± 0.9 ng/ml, $p < 0.01$). No difference in IL2 production between both groups were detected following CD3 plus CD28 or CD2 plus CD28 stimulation.

IL4 production of cells from atopics was significantly higher than of control cells under all stimulatory conditions tested (Fig. 2). Mean values of IL4 were 39 ± 30 pg/ml for atopics and 11 ± 5 pg/ml for controls in the case of CD3 plus CD28 stimulation ($p < 0.01$), 162 ± 108 pg/ml and 49 ± 20 pg/ml following CD2 plus CD28 stimulation ($p < 0.001$), and 51 ± 30 pg/ml and 22 ± 16 pg/ml following PHA plus PMA stimulation ($p < 0.01$), respectively. As noted earlier, CD2 plus CD28 stimulation resulted in the highest absolute amounts of IL4 induced.

For IFN γ production, higher absolute levels of cytokine were induced via CD2 plus CD28 than via CD3 plus CD28 (Fig. 3), whereas PHA plus PMA stimulation was a very poor inducer of IFN γ (not shown), with no difference between atopics and

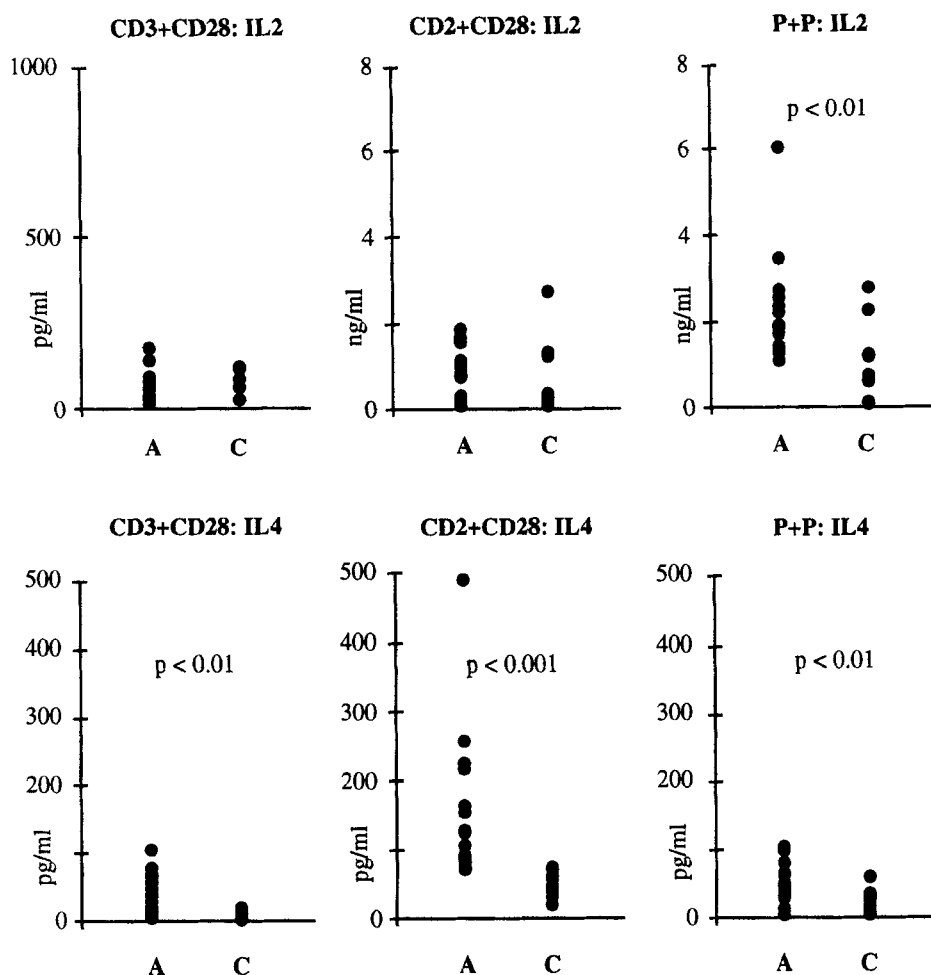


Fig. 2. IL2 and IL4 production of MNCs from atopics (A) or controls (C) in response to the stimuli as indicated (f. i. "CD3+CD28: IL2" indicates: IL2 production in response to CD3 plus CD28 stimulation; "P+P" indicates: PHA plus PMA stimulation). Culture time was 48 hours after which the cytokines were measured in the cell free supernatants by ELISA.

controls. Similarly, IL10 production did not differ significantly between cells from both study groups. Again, highest levels (493 ± 354 pg/ml with atopics, 377 ± 326 pg/ml with controls) were seen following CD2 plus CD28 stimulation. PHA plus PMA stimulation resulted in the production of only low levels of IL10 (not shown).

Finally, we calculated the ratios of IL4/IFN γ production, of IL4/IL2 production, and of IL4 production/cell proliferation, in each case following CD2 plus CD28 stimulation, and compared these ratios between atopics and controls (Fig. 4). All three ratios tended to be higher in the patient group, the difference, however, was only significant ($p < 0.001$) for the ratio IL4 production/cell proliferation (mean values for IL4/IFN γ production: 5.1 ± 5.1 vs. 6.7 ± 12.9 ; for IL4/IL2 production: 0.37 ± 0.35 vs. 0.22 ± 0.17 ; for IL4 production/cell proliferation: 1.06 ± 0.48 vs. 0.43 ± 0.20).

Correlation analysis between levels of IgE *in vivo* and IL4 induced *in vitro* (in response to CD2 plus CD28 stimulation) did not reveal a significant association (Fig. 5, top diagram). In accordance with this analysis, the mean IL4 production in response to CD2 plus CD28 stimulation of the six atopics with

IgE levels above 200 kU/l (mean 1152 kU/l) was not higher than that produced from cells of the remaining 10 subjects in the atopic patient group (mean IgE levels 88.9 kU/l).

As IL4 production was localized previously to preactivated "memory" cells (14, 15) and as the number of such cells would be expected to increase with age, we looked for a possible correlation between IL4 production and age, however, no such correlation could be found in our study group (Fig. 5, bottom diagram).

Discussion

Previously, several studies reported elevated IL4 and diminished IFN γ production *in vitro* by mononuclear cells from atopic patients in response to lectin or ionophore plus phorbol ester stimulation and this imbalance of cytokines has been linked to patients increased IgE production *in vivo* (7-9). It is not clear to date, why such patients respond with increased IL4 production to *in vitro* stimulation.

We and others have reported previously that freshly isolated cells from normal individuals stimulated with CD2 plus CD28 release more IL4 than

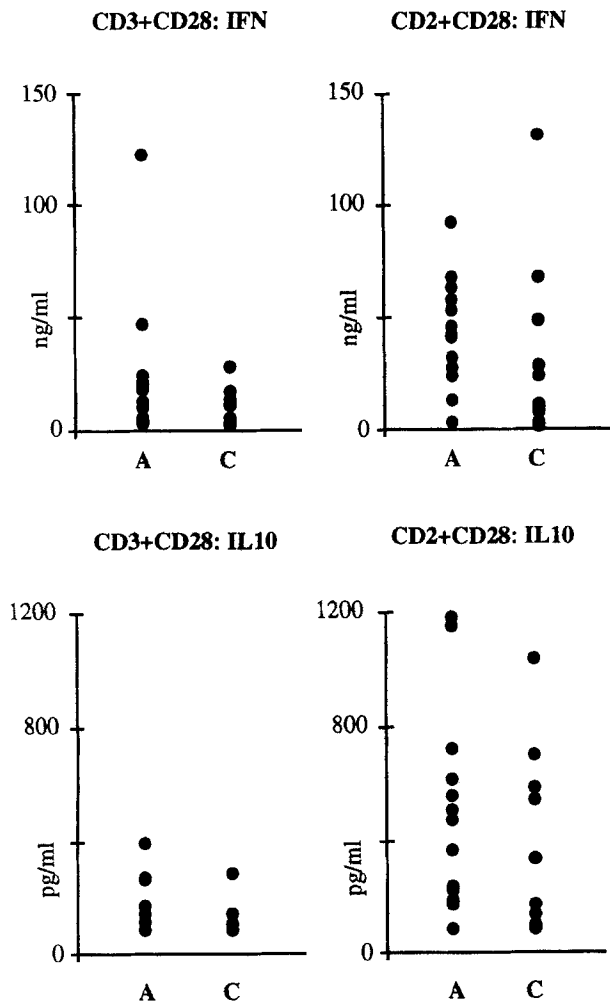


Fig. 3. IFN γ and IL10 production of MNCs from atopics (A) or controls (C) in response to the stimuli as indicated and explained in legend to Fig. 2.

when stimulated with either CD3 plus CD28 or PHA plus PMA stimulation (11, 16). As the above cited earlier studies investigating the cytokine profile of cells from atopic patients had used Ca⁺⁺ iono-

phore plus PMA, PHA plus PMA, or PHA for stimulation, we asked whether a better defined stimulation of the cells through CD3, CD2 and CD28 would allow to correlate IL4 hyperproduction with a potential hyperreactivity of anyone of these activation pathways, in particular that of CD2 plus CD28 stimulation. Furthermore, elevated IL4 levels were found previously only with cells from severely but not from mildly atopic patients (9), and in another study only patients with very high IgE levels had been included (8). Therefore, we were interested, whether similar findings could be demonstrated with cells from patients with clinically mild disease as well.

The most significant results of this study are: a) Cells from atopics proliferated stronger in response to CD2 stimulation than cells from controls (Fig. 1); b) Cells from atopics responded with higher IL4 production than cells from control individuals, a difference, which was most clearly seen in response to CD2 plus CD28 stimulation (Fig. 2); c) No differences were found for the production of IFN γ and IL10 regardless of which stimulation was employed (Fig. 3); d) Highest absolute IL2 levels were found in response to PHA plus PMA stimulation, whereas CD2 plus CD28 stimulation proved to be the most efficient method of induction for the cytokines IL4, IFN γ , and IL10 (Figs. 2 and 3, data not shown).

The observed elevation of IL4 production in response to CD2 plus CD28 stimulation appears to be due to two different mechanisms. First, we found evidence for hyperresponsiveness of the CD2 pathway of stimulation in atopics, because not only IL4 production but also cell proliferation was increased following this stimulation (Fig. 1). In addition, proliferation in medium alone and in response to tetanus toxoid tended to be higher in atopics, although this difference was not significant. Presumably, in both situations, interaction of CD2 with its ligand CD58 is also involved (17). Under presumably rela-

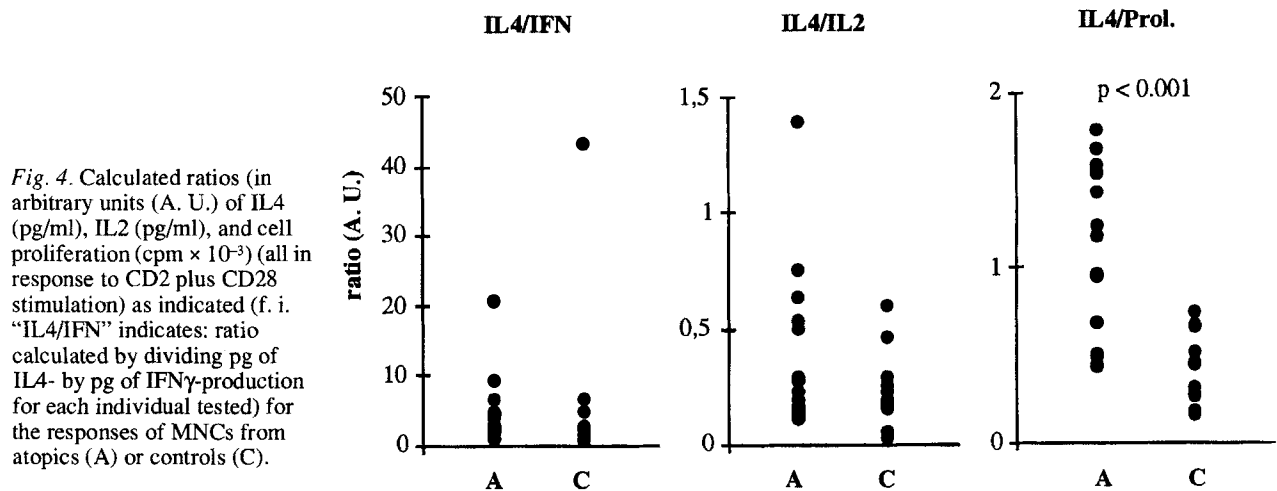


Fig. 4. Calculated ratios (in arbitrary units (A. U.) of IL4 (pg/ml), IL2 (pg/ml), and cell proliferation (cpm $\times 10^{-3}$) (all in response to CD2 plus CD28 stimulation) as indicated (f. i. "IL4/IFN" indicates: ratio calculated by dividing pg of IL4- by pg of IFN γ -production for each individual tested) for the responses of MNCs from atopics (A) or controls (C).

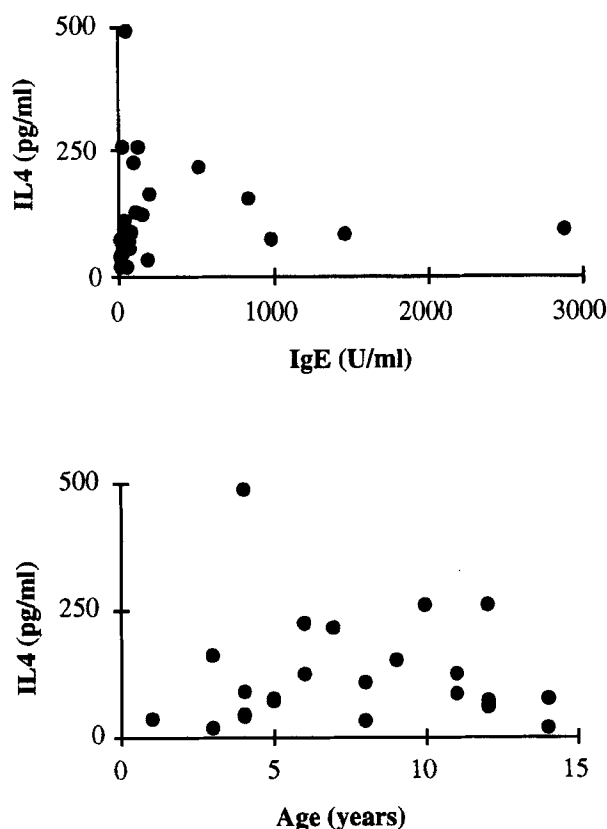


Fig. 5. IL4 production obtained *in vitro* (in response to CD2 plus CD28 stimulation) plotted against IgE *in vivo* (top diagram) and age of the subjects (bottom diagram) of all individuals included in this study (atopics and controls).

tively CD2-independent conditions, however, such as mAb stimulation through CD3 or stimulation with SEA, which both are strong stimuli signalling directly through the TCR complex, no difference in the proliferative response was found between atopics and controls (Fig. 1). As a second mechanism, however, there also seems to be a general upregulation of IL4 production in cells from atopic donors, regardless which pathway of stimulation is used. In support of the first proposed mechanism, however, it should be noted that the difference between atopics and controls was most outspoken following CD2 plus CD28 stimulation. This hyperproduction of IL4 was also evident when the ratio of IL4 production/cell proliferation in response to CD2 plus CD28 stimulation was calculated (Fig. 4). Increased IL4 production in response to polyclonal stimulation could be due to an elevated frequency of IL4 producing cells, due to increased production per cell, or both, and our data do not distinguish between these possibilities.

Perhaps due to the efficient stimulatory conditions employed, we were able to demonstrate increased IL4 production also in this comparatively small group of patients with mild disease. It should be noted that only 6 of the 16 patients studied had IgE lev-

els above 200 kU (Fig. 5a), whereas the remaining 10 patients had normal or only mildly elevated levels. The mean IL4 production between these subgroups of patients was similar. These findings are at variance with those of an earlier study, where no imbalance of cytokine production was found in mildly diseased patients (9) and underline the value of employing optimal stimulatory conditions, when evaluating the cytokine profile of patient cells.

We were surprised to find no clear difference for IFN γ production in our system, which is at variance with earlier studies (7, 8). We would interpret this finding by the fact that our patients were not selected for extreme IgE levels but rather represented the average clientele of an outpatient allergic clinic. Demonstration of high IL4 despite normal levels of IFN γ production in these patients would, therefore, suggest that in patients with comparatively mild clinical disease and only moderately elevated IgE levels, the first change in the cytokine pattern is indeed restricted to IL4. Continuous presence of elevated IL4 levels *in vivo* would subsequently perhaps suppress the generation of IFN γ producing Th1 cells (18) and result in diminished IFN γ production later on. Such a mechanism would underscore the importance of IL4 for the development of cytokine dysregulation.

The levels of IL10 were approximately threefold higher after stimulation with CD2 plus CD28 than following CD3 plus CD28 stimulation, whereas PHA plus PMA did not result in measurable IL10 production in the majority of individuals tested. The IL10 levels were similar in patients and controls, whereas in rodents IL10 production is restricted to Th2 clones, human T cell clones of all subtypes apparently produce IL10 (19).

The CD2 pathway of T cell stimulation has received much interest since its initial discovery in 1984 (20). Since then, three different ligands for CD2 have been identified (CD58, CD59, CD48), of which CD58 and CD59 were found to bind to the outer portion of the CD2 molecule (21, 22). Using monoclonal antibody blocking studies, the importance of the CD2 pathway of stimulation has been established also *in vivo* (23, 24).

Although CD2 stimulation is dependent on the expression of a functional ζ chain of the CD3 complex, implying that the signalling pathways triggered by the TCR/CD3 complex and by CD2 may partially overlap, clear cut differences in secondary signals such as protein phosphorylation or Ca $^{++}$ mobilization patterns were reported between both pathways (25–27). Evidence for a functional hyporesponsiveness of the CD2 pathway was found with cells from patients with alcoholic liver disease (28) and Pirzer et al. reported recently that intestinal lymphocytes responded stronger to CD2 and weaker

to CD3 stimulation than peripheral blood control cells from the same individuals (29). Upon biochemical analysis CD2 was found associated with a number of other surface molecules, some of them implicated in signal transduction such as CD45, p56lck and p59fyn (30, 31). One could imagine that the extent to which CD2 molecules enter close associations with other molecules within the plasma membrane may differ during cell differentiation or in response to signals encountered under pathophysiological circumstances. In consequence, such a changed association could lead to an altered functional outcome of signalling through CD2 such as that observed in our study.

The existence of multiple ligands for CD2 argues for a role of CD2 in the selective modulation of the functions of T cells initially activated by antigen through the TCR. Hyperreactivity of the CD2 pathway under defined pathophysiologic conditions as demonstrated in this study emphasizes the importance of the various distinct T cell activation pathways and may lead to a better understanding of the pathomechanisms involved in the generation of an allergic immune response.

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