

Low frequency of CD4⁺, but not CD8⁺, T cells expressing interferon- γ is related to cow's milk allergy in infancy

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Low interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) production in peripheral blood mononuclear cells (PBMC) from patients with atopic dermatitis and food allergy have been reported previously. However, it remains unclear whether the weak cytokine production is caused by the imbalance of specific T-cell subsets or by dysregulation of T-cell function. In the present study we investigated the intracellular expression of these cytokines at a single-cell level to clarify the background of the disruption. Twelve of 27 breast-fed infants (0.1–8.8 months of age) had challenge-proven cow's milk allergy (CMA), and 15 infants were studied as a healthy control group. PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The frequencies of the cells expressing intracellular IL-4, IFN- γ , and TNF- α were assessed using flow cytometry. In addition, at this time-point leucocyte subsets from the milk of mothers of these infants were evaluated using light microscopy. A lower number of CD8⁺ T cells and the defective capability of CD4⁺ T cells to express IFN- γ in infant's peripheral blood co-existed with a lower number of macrophages in their mother's milk.

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Cow's milk allergy (CMA) is defined as an immunologically mediated adverse reaction against cow's milk antigens. The symptoms of CMA may appear even during exclusive breastfeeding (1). Interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) are cytokines needed for adequate communication in the cellular network of the neonate. Both IFN- γ and TNF- α have an important role in activating antigen-presenting cells and T cells and in the achievement of oral tolerance against food antigens.

In previous studies, atopic and cow's milk allergic infants have been shown to secrete reduced amounts of IFN- γ and TNF- α (2–4). These defective cytokine productions have been shown to occur equally in different clinical manifestations of CMA (3,4). It has also been shown that in atopic children both secretion and expression of IFN- γ mRNA is reduced (5). However, these studies could not determine

whether the results observed were caused by imbalances in T-cell subpopulations with different abilities to produce cytokines, or possibly as yet uncharacterized intrinsic defects in IFN- γ production. Subsequently, the defect has been explained to lie in the decreased number of IFN- γ -expressing CD4⁺ and CD8⁺ T cells (6). Recently, evidence has been presented to show that correction of an impaired T helper 1 (Th1) response and IFN- γ production in atopic patients may be successful at the precursor T-cell level (7).

Immune components of maternal milk gain access to the infant's immune system and may affect it (8). In human milk the predominant cellular component is the breast milk macrophage; other cells include neutrophils (40–60%), lymphocytes (5–27%), a few eosinophils and basophils, and occasional monocytes (9,10). Human milk also contains cytokines such as

interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, TNF- α , transforming growth factor- β (TGF- β), IL-4, and IFN- γ (11). Although there is no direct evidence regarding the influence of breast milk cytokines on the recipient, breast milk is believed to impart specific immune advantages to the neonate through enhancement or induction of the still-developing neonatal immune system (8). We have previously shown that in breast milk of mothers with a cow's milk allergic infant, both cellular and cytokine composition are significantly different from those of mothers with healthy infants, and this might influence the health status of the breast-fed infant (12–14). Therefore, we wanted to test the hypothesis that the immunologic composition of maternal milk might influence the maturation of the immune system of the breast-fed infant.

The purpose of this study was to evaluate the intracellular expression of cytokines at a single-cell level in infants with CMA in comparison to healthy controls. We also evaluated the relationship between cytokine-expressing T cells in the breast-fed infant and the cellular composition of human milk.

Subjects and methods

Infants and their mothers

The study population consisted of 27 breast-fed infants (age-range 0.1–8.8 months) and their mothers. Twelve of the 27 infants had challenge-proven CMA manifested with urticaria or eczematous skin eruptions ($n=4$), gastrointestinal symptoms ($n=2$), or both skin and gastrointestinal symptoms ($n=6$). These symptoms had occurred during exclusive breast feeding in 11 infants under 1 month of age and in one infant at 2 months of age. Fifteen healthy, age-matched children were studied as a control group; they had no symptoms suggestive of CMA or atopic eczema. All the infants were born at term and had no other chronic diseases or infections at the time of the study. Among the mothers of infants with CMA or with healthy infants, 11 of 12 (92%) and one of 15 (7%), respectively, were atopic (hay fever, atopic dermatitis or asthma). The mothers were taking no medication at the time of the study.

The mothers gave written informed consent to participate in the study, which was approved by the ethics committees of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki.

Cow's milk challenge protocol

The diagnosis of CMA was based on an adverse response to cow's milk challenge. Only those patients with suspected CMA were put on a cow's milk-elimination diet. After 2–4 weeks on a milk-elimination diet the patients were subjected to a diagnostic, open cow's milk challenge if they were free of symptoms and signs of CMA. The challenge was performed openly because placebo reactions have been shown to be rare in infants (15). During the elimination period, cow's milk had been strictly eliminated from the diet and the lactating mother was also on the cow's milk-elimination diet. If the infants needed extra formula in addition to breast milk, a tolerated formula (protein hydrolysate or amino acid-based formula) was given. The challenge was started with a drop of cow's milk on the skin or lips. Thereafter, cow's milk was given in increasing doses at 1-h intervals: on day 1, 2, 10, 50 and 100 ml; and on day 2, the normal milk intake appropriate to the infant's age was commenced. The challenge was stopped immediately if any adverse reaction occurred. The time of the reaction was defined as the time elapsed from the last given dose eliciting the specific symptoms. The cow's milk-allergic patients were followed up over the 1-week period of challenge, and all the infants in this study were followed-up over the next 2 years to detect any allergies that might develop in the healthy infants during that time. All the elimination diets as well as cow's milk challenges were performed based on clinical indications, not for research purposes.

Blood samples

For lymphocyte induction, blood was drawn into heparin always at the same time in the morning. Mononuclear cells consisting mainly of lymphocytes were isolated by Ficoll–Hypaque gradient centrifugation at 400 g (30 min at 20°C). The cells were resuspended in RPMI-1640 containing 5% fetal bovine serum (Sigma, Steinheim, Germany), antibiotics (penicillin 100 IU/ml and streptomycin 100 μ g/ml), and 2 mm l-glutamine (Sigma, Irvine, UK). After washing three times the cells were counted visually in Bürker chambers using a light microscope.

Intracellular staining of cytokines

Altogether, 1-ml of 1×10^6 peripheral blood mononuclear cells (PBMC) was incubated with 10 μ g/ml Brefeldin A, 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μ g/ml ionomycin (Sigma, St. Louis, MO, USA) at 37°C, in 5%

CO₂, for 4 h to activate the lymphocytes for cytokine production. Brefeldin A, as well as monensin, disrupts intracellular Golgi-mediated transport and allows cytokines to accumulate, yielding an enhanced cytokine signal that can be detected by flow cytometry (16). Spontaneous cytokine production in unstimulated controls was assessed in a similar manner, but without PMA and ionomycin. After activation the cells were stained with surface antigen-specific (CD8 or CD3) fluorescence-conjugated [fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PerCP monoclonal antibodies (mAbs) (Becton-Dickinson, San Jose, CA, USA) for 15 min. The cells were incubated with FACS[™] Lysing Solution (Becton-Dickinson) for 5 min at room temperature to lyse any remaining erythrocytes, fix the external epitopes and assist in permeabilization. Subsequently, the cells were centrifuged for 5 min at 500 g, and the Lysing Solution was changed to FACS[™] Permeabilizing Solution (Becton-Dickinson) in which the cells were incubated for 10 min at room temperature. After permeabilization the cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Sigma, Steinheim, Germany), following which the intracellular cytokines (IFN- γ , TNF- α and IL-4) were stained with fluorescence-conjugated mAbs (Becton-Dickinson) for 30 min. After washing with PBS+0.5% BSA, the cells were fixed in 1% paraformaldehyde (Sigma, Steinheim, Germany) and analyzed using a flow cytometer (FACScan; Becton-Dickinson).

Flow cytometric detection of intracellular cytokines

The data were collected and analyzed using a FACScan (Becton-Dickinson). Ten-thousand cells were acquired and the data was analyzed with CellQuest[™] (Becton-Dickinson) software. The lymphocytes were gated on forward-scatter (FSC)/side-scatter (SSC) dot-plots and the cytokine production was determined from that gate and displayed as single-color dot-plots. The results were expressed as the percentage of cytokine-expressing CD4⁺ and CD8⁺ T cells from the whole CD4⁺ or CD8⁺ T-cell populations.

Human breast milk samples

Collection of human milk and preparation of cell suspensions. The breast was washed with warm water and fresh human milk was collected by manual expression or with a breast pump, always at the same time (between 08.00 and 11.00 h). The volume of each milk sample was

measured and it was immediately centrifuged (400 g at room temperature for 15 min) to remove the lipids and the whey. The leucocytes were then collected and resuspended in RPMI-1640 containing 5% fetal bovine serum (Sigma, Steinheim, Germany), antibiotics (penicillin 100 IU/ml and streptomycin 100 μ g/ml), and 2 mm l-glutamine (Sigma, Irvine, UK). After four washes the cells were visually counted in Bürker chambers using a light microscope.

Cytospin preparations. Cytospin preparations of milk cells were obtained with a sample application of 1×10^5 cells [Cytospin3; Shandon, Life Sciences International (Europe) Ltd, Astmoor, Runcorn, Cheshire, UK]. The slides were treated with Vectapond[™] (Vector Laboratories, Inc, Burlingame, CA, USA) before sample application to improve the fixation of breast milk cells. The numbers of macrophages, monocytes, lymphocytes, neutrophils, and eosinophils were evaluated on slides stained with Wright–May–Grünwald–Giemsa (Wright–MGG, Merck Darmstadt, Germany, Oy Reagentia Ltd, Kuopio, Finland) application.

Statistical analysis

The Mann–Whitney *U*-test was employed to determine the statistical significance of the differences between continuous variables. Logarithmic transformations were used because of the skewed distribution. The chi-square test was applied to determine differences in proportions.

Results

Clinical characteristics of cow's milk-allergic infants and of controls

The geometric mean age of the infants with CMA was 2.9 [95% confidence intervals (CI): 1.6–5.1] months and of healthy infants 2.5 (95% CI: 1.0–6.1) months. The geometric mean ages were comparable between these two groups at the time of the study ($p = 0.52$). In clinical cow's milk challenge, one patient manifested urticaria immediately after introduction of a drop of cow's milk on his skin and lips. Therefore, the challenge was stopped without giving peroral doses of cow's milk to this patient. Six other infants developed either urticarial or eczematous skin eruptions, two had only gastrointestinal symptoms, and three infants reacted both cutaneously and gastrointestinally after peroral administration of cow's milk. The patients challenged perorally

reacted to a median total dose of 50 (range: 24–211) ml of cow's milk at a mean of 25 (range: 0.5–96) h after starting the challenge. The median of the last dose eliciting symptoms was 50 (range: 10–100) ml of cow's milk and the mean of the time from the last dose until the symptoms appeared was 1.6 (range: 0.5–3) h. An atopic constitution of the mother correlated with the development of CMA in the infant, $p = 0.0001$ (chi-square test).

Frequency of CD4⁺ and CD8⁺ T cells in healthy controls and infants with CMA

The frequency of CD4⁺ T cells in the overall lymphocyte population was comparable between the group with CMA and the healthy control group. The frequency of CD8⁺ T cells from infants with CMA, 20.5 (range: 13–28) %, was significantly lower ($p = 0.009$) than that from healthy infants, 25 (range: 19–32) %, as demonstrated previously (14).

To assess the effect of treatment with PMA and ionomycin on the staining of surface CD4 and CD8 molecules, we examined the frequencies of CD4⁺ T cells and CD8⁺ T cells in stimulated and unstimulated samples. The frequencies of CD4⁺ T cells and CD8⁺ T cells were comparable in stimulated and unstimulated samples. When comparing the results of the present study concerning surface molecule expression to results of our previous study (using comparable patient material), we found that the expression of CD4 and CD8 molecules did not decrease after fixation and permeabilization (14). The values for CD4 antigens were calculated from the values of CD3 and CD8 antigens.

Frequency of IFN- γ -expressing CD4⁺ and CD8⁺ T cells

There was a significant difference in the frequency of CD4⁺, but not CD8⁺, T cells expressing IFN- γ between infants with CMA and healthy controls. After 4 h of stimulation with PMA and ionomycin in the presence of Brefeldin A, the frequency of CD4⁺ T cells expressing IFN- γ in the group with CMA, geometric mean 4.4 (95% CI: 3.6–5.7) %, was significantly lower than that of the healthy control group, of 7.0 (95% CI: 5.5–10.3) % ($p = 0.006$) (Fig. 1a). In the CD8⁺ T cells the frequency of IFN- γ -expressing cells was comparable between the infants with CMA and healthy infants: geometric mean 14.4 (95% CI: 12.3–16.9) % and 18.0 (95% CI: 15.3–23.4) %, respectively ($p = 0.15$) (Fig. 1a). The cytokine expression from unstimulated samples was comparable to the background staining in all samples.

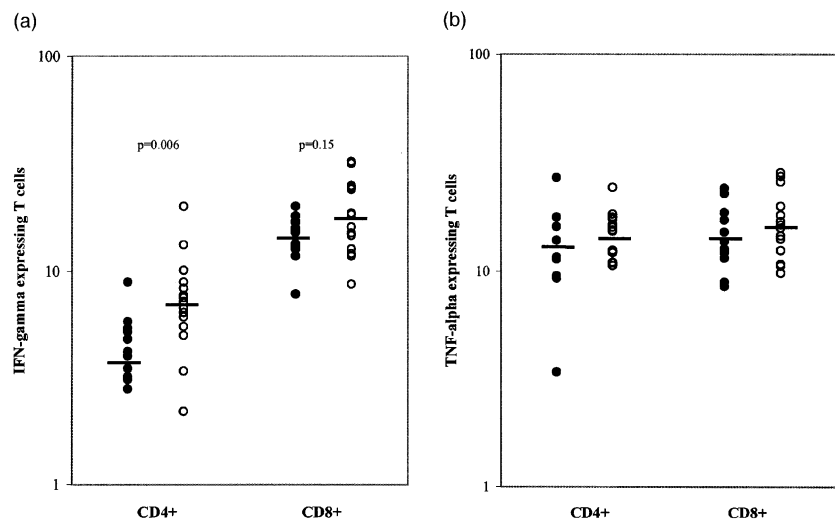
Frequency of TNF- α and IL-4-expressing CD4⁺ and CD8⁺ T cells

The frequency of CD4⁺ T cells expressing TNF- α was comparable between the two study groups ($p = 0.22$) (Fig. 1b). Also, the frequency of TNF- α -expressing CD8⁺ T cells was comparable between the infants with CMA and the healthy controls ($p = 0.43$) (Fig. 1b). The intensity of fluorescence for IL-4 was almost undetectable in both groups.

Cellular composition of maternal milk

The total number of breast milk macrophages was categorized as normal (>52%) or low (<52%). When low numbers (<52%) of macrophages were detected in the breast milk of the mother, the risk of the breast-fed infant devel-

Fig. 1. (a) Interferon- γ (IFN- γ)- and (b) tumor necrosis factor- α (TNF- α)-expressing CD4⁺ and CD8⁺ T cells (%) from infants with cow's milk allergy (CMA) (●) and healthy controls (○). Horizontal lines denote the geometric mean values. p -values were calculated using the Mann-Whitney U -test.



oping CMA was moderately high, according to a previous report (17). The low number of breast milk macrophages was associated with the development of CMA in breast-fed infants ($p = 0.003$; chi-square test).

Relationship between the cellular composition of maternal milk and intracellular expression of cytokines in the breast-fed infant

A low number of breast milk macrophages co-existed with a lower number of $CD8^+$ T cells, as well as with the lower frequency of $CD4^+$ T cells expressing IFN- γ , in infants with CMA. At the same time there was no difference in the expression of intracellular TNF- α in the infants with CMA and healthy control infants.

Discussion

In the present study we have shown that the total number of $CD8^+$ T cells was significantly lower in infants with CMA when compared to healthy infants. In addition, the total number of $CD4^+$ T cells was comparable between the two study groups, but the frequency of $CD4^+$ T cells expressing IFN- γ was significantly lower in infants with CMA. The frequencies of the cells expressing TNF- α were comparable between the two study groups. These results suggest that the capability of $CD4^+$, but not $CD8^+$, T cells to express IFN- γ is defective in CMA, and that a lower number of $CD8^+$ T cells are present in these patients. Moreover, the number of macrophages was evaluated in maternal breast milk, and a low number of macrophages in breast milk of mothers was found to be associated with the development of CMA in the breast-fed infant.

CMA, frequently the first manifestation of allergic pathology in early infancy, is usually considered to be a temporary, antigen-specific condition (1). It is an example of a condition with breakdown in the development of oral tolerance. The precise mechanisms leading to oral tolerance to dietary antigens in humans are not known. It may, however, normally be achieved by induction of neonatal T cells and macrophages, which become activated, develop into memory cells and start to produce cytokines, such as IFN- γ and TNF- α , which are needed for adequate communication in cellular networks in the neonate.

The capacity to produce cytokines, especially IFN- γ and TNF- α , is physiologically low in newborn infants (18). Optimal IFN- γ and TNF- α production seems to be important in the regulation of the infant's macrophage and T-cell function (19). Therefore, lack of them in the

infant or in the breast milk of the lactating mother (as another important source of cytokines for the infant) may dramatically disturb the development and maintenance of oral tolerance to dietary antigens (20). If the infant's T-cell function is depleted, or only weakly induced because of low cytokine production in infant or in mother's milk, exposure of a child to foreign proteins might lead to a local or systemic immunoinflammatory reaction (21,22).

An increased production of IL-4 and a decreased production of IFN- γ in atopic diseases has been demonstrated in many studies (2,23,24). It has also been reported that reduced IFN- γ production as a result of reduced IFN- γ mRNA expression is associated with an elevated serum immunoglobulin E (IgE) level in atopic patients (25). On the other hand, Tang and her colleagues (5) suggested that reduced secretion of IFN- γ was related not to abnormal regulation of transcription, but rather to a post-transcriptional defect in the mRNA expression of IFN- γ . These conflicting results may reflect differences in the subjects studied, but because all these studies used total PBMC, it was not clear whether the defective IFN- γ production is the result of a decrease in the number of T helper 1 (Th1)-like cells or in the amounts of cytokine produced from Th1-like cells. We approached this issue by determining IFN- γ expression at a single-cell level using cytometric analysis. Our results demonstrated that there were fewer $CD4^+$ T cells activated towards the Th1-like cytokine production, but it still remains to be shown if the Th1-like cells are also expressing less IFN- γ . A study by Nakazawa and his co-workers (6) demonstrated decreased frequencies of both $CD4^+$ and $CD8^+$ cells expressing IFN- γ in patients with atopic dermatitis. On the other hand, Jung and colleagues (26) reported a decreased proportion of IFN- γ -producing $CD4^+$ cells, but a normal proportion of IFN- γ -producing $CD8^+$ cells in atopic patients, which is also in agreement with the present results. These conflicting data may be a result of differences in patient selection and probably also because of small sample sizes. However, all these studies do agree that the T helper 1-like cell count is low in atopic diseases.

The risk of allergy development in newborns has previously been shown to correlate with decreased production of IFN- γ from cord blood mononuclear cells (27). A rapid suppression of T helper 2 (Th2) responses during the first year of life in non-atopic children, but consolidation of responses in atopic children associated with defective neonatal IFN- γ production, has been reported by Prescott et al. (28). This might be

caused by a deficiency of factors directing the Th1 responses. Impaired IFN- γ production by T cells from atopic individuals has been postulated not to be the result of an intrinsic, genetically fixed, defect to produce sufficient amounts of IFN- γ , but to be corrected in the presence of IL-12 at priming (7). Because the defective IFN- γ production seems to be a regulatory defect rather than an intrinsic genetic defect, a good candidate source of regulatory factors for the newborn is their mother's milk. As reported in this present study, the total number of breast milk macrophages was low in mothers with a cow's milk-allergic infant. This could probably lead to insufficient amounts of regulatory factors needed for the T-cell priming and maturation towards T helper 1 responses.

TNF- α has synergistic effects with IFN- γ and they are required for adequate communication in cellular networks and development of oral tolerance in the neonate (19). The results of the present study, of comparable expression of TNF- α in T cells between infants with CMA and healthy infants, indicate that there may be no defect in the number of TNF- α -expressing cells. On the other hand, our previous results, showing decreased production of TNF- α in the culture supernatants of PBMC from infants with CMA could indicate, together with this present finding, that either the amounts of TNF- α expressed by the cells are decreased in infants with CMA, or there might be some other defects in the secretion of the expressed cytokine (4). TNF- α is initially expressed as a membrane-anchored precursor which is proteolytically processed to yield the mature cytokine (29). The processing enzyme(s) which cleave TNF- α are unknown but there is some evidence that this processing is dependent on at least one matrix metalloproteinase-like enzyme (30). These proteolytic processes could be one place where the possible post-translational defects might be sought.

Taken together, a low frequency of CD4⁺, but not CD8⁺, cells expressing IFN- γ in the infants with CMA co-existed with a low number of macrophages in the milk of mothers of these infants. A direct relationship between these two phenomena remains to be shown.

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