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Cytokine responses to allergens during the first two years of life in Estonian and Swedish children

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Abstract

Background: The prevalence of atopic disease among children in the formerly socialist countries in Europe, with a life style similar to that prevailing in Western Europe 30-40 years ago, is low, whereas there has been a pronounced increase in industrialised countries over the last decades. The environment during infancy influences the risk of developing allergy for many years, perhaps even for life.

Objective: To investigate the development of allergen specific cytokine responses during the first two years of life in two geographically adjacent countries with marked differences in living conditions and incidence of atopic diseases, *i e* Estonia and Sweden.

Methods: The development of immune responses to the food (β-lactoglobulin (BLG) and ovalbumin) and inhalant (cat and birch) allergens were studied from birth up to the age of two years in 30 Estonian and 76 Swedish infants. Clinical investigation and skin prick tests were performed and blood samples obtained at birth and at 3, 6, 12 and 24 months.

Results: The levels of IL-5, IL-10 and IL-13 secreted by PBMC stimulated with BLG, ovalbumin and cat allergen in the Estonian and Swedish infants declined during the first 3 months of life. All cytokines then progressively increased in the Swedish infants, indicating the replacement of non specifically responding immature cord blood T-cells with specific T-memory cells, which are primed postnatally. The resurgence of allergen-specific responses in the Estonian infants was less marked. These differences were particularly notable for birch-specific T-cell responses, which correlated with development of atopic disease in the Swedish children.

Conclusions: The development of specific T-cell memory to food and inhalant allergens during the first two years of life differs between infants living in Sweden and Estonia, and mirrors the disparate patterns of expression of allergic disease which subsequently develops in the respective populations.

Key words; Immune responses, infants, allergens, cytokines, atopic disease, Sweden, Estonia

Introduction

The immune functions in children who later develop allergy and in those who remain non-allergic differ in several aspects [1]. The early immunoglobulin (Ig)E responses to allergens are eventually down regulated in infants who remain non-allergic, whereas they reach higher and more persistent levels in those who develop allergy [2]. Atopic diseases are generally characterised by excessive T-helper (Th)2-like immunity to allergens, with high production of interleukin (IL)-4 [3, 4], IL-5 [4, 5], IL-9 [4] and IL-13 [4, 6], accompanied by decreased [3] or similar [4-6] production of interferon (IFN)-γ. Although allergen specific Th1-like immune responses are present in healthy individuals, IFN-γ associated delayed type hypersensitivity is not observed. Therefore, factors counteracting Th1 like reactions are likely to be present and the anti-inflammatory cytokine IL-10 may be one such candidate [7]. Interleukin-10 also suppresses IgE production [8] and counter-regulates Th2-like immunity [9].

Several factors, including environmental factors, influence the development of the specific cytokine profile, and the immune response pattern is thought to be set during early childhood [10, 11]. The cytokine profile is more easily affected by external influences in naïve than in established responses [12], emphasizing the importance of events occurring during early childhood in determining responder phenotype in later life.

The prevalence of atopic disease among children in the formerly socialist countries in Europe, with a life style similar to that prevailing in Western Europe 30-40 years ago, is still low [13, 14], whereas there has been a pronounced increase in industrialized

countries over the last decades [15]. After the German reunification in 1990, the prevalence of hay fever and atopic sensitisation has increased in children in the former East Germany [16]. The unification has been associated with tremendous changes in everyday life towards Western conditions in the former East Germany. Although the causes of the increase of atopic diseases in Western Europe are unknown, several factors associated with a Western lifestyle, *e g* smaller family size [17], Western diet [18] and an overall diminished exposure to environmental microorganisms [19, 20] have been proposed as potential explanations. We have reported differences in the composition of the gut flora in Estonian and Swedish children [21], and also that microflora associated characteristics were disturbed in the gut from children with atopic disease [11]. Furthermore, we found differences in the gut flora as early as at five days of age between infants who did or did not develop atopic disease during the first two years of life [22].

Little is known regarding eventual regional differences in the setting of immune response patterns. Renz *et al* has reported dominating Th0-responses, defined as IFN-γ and IL-4/IL-5 secretion induced by phorbol myristate acetate (PMA) plus ionomycin in children growing up in Eastern Germany [23]. This was irrespective of their atopic status and age. The T-cell immune profile was age dependent in the children from Western Germany, with Th1- or Th2-responders dominating in the younger children (6-7 years) and Th0-responders dominating among the older children (9-11 years). Among the West German children, atopic disease was associated with a Th2-like cytokine profile. Strikingly, approximately one third of the children growing up in Western Germany was defined as non-responders, *i e* no detectable PMA plus ionomycin

induced IFN-γ, IL-4 or IL-5 responses, irrespective of age, whereas less then 1% of the children growing up in Eastern Germany were non-responders. However, comparative studies on allergen-specific T-cell responses in children living in industrialized and less developed countries with a high and low prevalence of atopic disease, respectively, have not been performed. In particular, the prospective development of T-cell responses have not been studied in these environments during the first years of life, when events critical for the establishment of persistent atopic disease are likely to occur.

The aim of the present study was to investigate the development of allergen specific cytokine responses during the first two years of life in two geographically adjacent countries with marked differences in living conditions and incidence of atopic diseases, *i e* Estonia and Sweden.

Material and Methods

Study group

The study comprises 30 Estonian children born during February 1997 – June 1998 at the Tartu University Women's' Clinic and 76 Swedish children born during March 1996 – October 1999 at the Linköping University Hospital. The children participated with their families in a larger prospective study regarding the influence of the environment on development of allergic diseases in children, described in detail elsewhere [24]. Inclusion in this study was based on availability of mononuclear cells, isolated from peripheral blood from the children.

At 3, 6, 12 and 24 months of age, the parents answered questionnaires regarding allergic symptoms and environmental factors, *e g* infections and diet. A pediatrician examined the children at 3, 6, 12 and 24 months in Estonia and at 24 months in Sweden. An experienced allergy research nurse examined the Swedish children at 3 or 6 and at 12 months. Atopic dermatitis was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as three or more episodes of bronchial obstruction during the last 12 months period, at least once verified by a physician. Allergic rhinitis/conjunctivitis was defined as rhinitis and/or conjunctivitis appearing at least twice within 1h after exposure to a particular allergen and not related to infection. Urticaria was defined as allergic if it appeared at least twice within one hour after exposure to a particular allergen. The cumulative incidence of allergic disease up to two years of age was 10 % (n=3) and 34 % (n=25) among the Estonian and Swedish infants, respectively. One Estonian child was diagnosed with

atopic dermatitis, one with atopic dermatitis and asthma and one child with asthma only. Among the 25 Swedish children with allergic disease, 24 had symptoms of atopic dermatitis and one had urticaria.

Skin prick tests (SPT) were performed at all follow-ups in duplicate on the volar aspects of the forearms with fresh skimmed cow's milk (lipid concentration 0.5 %) and thawed egg white. At 6, 12 and 24 months, the children were also tested with cat and dog allergen extracts and in Estonia also with mite allergen extract (*Dermatophagoides pteronyssinus*), at 12 months birch allergen extract was added to the panel and at 2 years timothy and in Estonia also cockroach (*Blatella germanica*) allergen. All extracts were standardised allergen extracts from ALK (Soluprick®, ALK, Hørsholm, Denmark), execpt the cockroach extract, which was from Bayer, Spokane, WA, USA. Histamine hydrochloride, 10 mg/ml, was used as positive and glycerol as negative control. The test was regarded as positive if the mean diameter of one of the wheals was at least 3 mm. Seventeen % (n=5) of the Estonian and 37 % (n=28) of the Swedish infants had at least one positive SPT up to two years of age (table 1). Two of the three Estonian infants with allergic disease up to two years of age had at least one positive SPT and the corresponding figure for the Swedish allergic infants was 19/25. These infants are referred to as infants with atopic disease.

Isolation of peripheral blood mononuclear cells

Venous blood samples were collected at birth (cord blood) and at all follow-ups in tubes with preservative-free heparin (Beckton Dickinson, Stockholm, Sweden). Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll Paque density gradient

(Pharmacia Biotech, Sollentuna, Sweden), and washed three times in RPMI-1640 (Life Technologies AB, Täby, Sweden) supplemented with 2 % fetal calf serum (Life Technologies AB). The cells from both Estonian and Swedish children were cryopreserved by standard methodology in 10 % DMSO (Sigma-Aldrich, Stockholm, Sweden), 50 % fetal calf serum and 40 % RPMI-1640. After cryopreservation, the cells collected from the Estonian infants were transported in liquid N_2 to Linköping and stored there until analysis.

Cell culture

After thawing, the cells were resuspended at $1x10^6$ viable cells/mL AIM-V serum free medium (Life Technologies) supplemented with 20 μM mercaptoethanol (Sigma-Aldrich). Duplicates of one mL aliquots ($1x10^6$ cells) were cultured at 37°C with 5 % CO_2 with medium alone, 10 000 SQU/mL Aquagen birch or cat extract (ALK), 100 μg/ml of β-lactoglobulin or ovalbumin purified grade IV (Sigma-Aldrich) or 2 μg/mL phytohaemagglutinin (PHA) (Sigma-Aldrich). **The endotoxin content in the birch extract was less than 0.5 EU/mL and 2.2 EU/mL in the cat extract as analysed by the chromogenic Limulus Amebocyte Lysate assay (Bio-Whittaker, Walkersville, ND, USA).** The priority of the allergens was set as follows; birch, BLG, cat and last ovalbumin. After 24 h and 6 days, the cultures were centrifuged at 400 x g for 5 minutes, and the supernatants were aspirated and stored in aliquots at -20°C. Allergen induced IL-10 and PHA induced IL-4, -5, -9, -10, -13 and IFN-γ secretion was analysed in the 24 h cultures, and allergen induced IL-5, -9, -13 and IFN-γ was analysed in the 6-day cultures. To enable measurement of allergen induced IL-4 secretion, separate 6-day

cultures were performed with a monoclonal antibody to the human IL-4 receptor; 2 μg/mL (clone 25463.111, R&D Systems, Abingdon, UK) [25].

Cytokine analysis

The levels of IL-4, -5, -9, IFN-γ [4], IL-10 and IL-13 [11] were analysed as described in detail elsewhere. Values were expressed as pg/ml deduced from a standard curve. The sensitivity limits for quantitative determinations were 2 pg/mL for IL-13 and IL-4, 4.7 pg/mL for IL-10, 6.3 pg/mL for IL-5, 8 pg/mL for IL-9 and 25 pg/mL for IFN-γ. Samples below the sensitivity limit were given half the cut off value for statistical analyses.

Statistics

As the cytokine levels were not normally distributed even after logarithmic transformation, non-parametric tests, corrected for ties, were used. Comparisons between paired groups were performed with Wilcoxon signed rank test and between unpaired groups with Mann-Whitney *U*-test. A probability level of <5 % was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA).

Ethics

The Regional Committee for Human Research at the University Hospital of Linköping and the Ethics Review Committee on Human Research of the University of Tartu approved the study.

Results

Interferon-γ, IL-5, IL-10 and IL-13 secretion from cord blood mononuclear cells (CBMC) was frequently detected in response to the food allergens BLG (fig 1) and ovalbumin (data not shown), and to the perennial inhalant allergen cat dander (fig 2). The levels were similar in Estonian and Swedish newborns. Interleukin-5, IL-10 and IL-13 responses decreased or tended to do so during the first three months of life both in Estonian and Swedish infants (fig 1-2). Cytokine responses from CBMC to the seasonal allergen birch were uncommon, except for IL-13, and the levels were low during the first six months in both Estonian and Swedish infants (fig 3). There was no association between month of birth and birch induced cytokine responses.

After three months of age, IFN-γ, IL-5, IL-10 and IL-13 secretion from PBMC in response to all allergens increased in the Swedish infants (fig 1-3). The responses remained low in the Estonian infants, however (fig 1-3). From 6 months, cat allergen (fig 2), and from 12 months of age, birch allergen (fig 3) induced IFN-γ, IL-5, -10 and -13 secretion was, or tended to be, higher in Swedish than in Estonian infants. Similar trends were observed for BLG induced IFN-γ, IL-5, -10 and -13 secretion (fig 1). The differences remained when comparing Swedish children with and without atopic disease with the Estonian children (data not shown). The number of Estonian children whose cells were stimulated with ovalbumin was too low ($n \leq 4$ at all ages) to enable statistical comparisons between the groups.

Allergen induced IL-4 and IL-9 secretion was only sporadically detected and was generally low both in Estonian and Swedish infants (data not shown). Allergen induced IL-9 secretion increased or tended to do so during the first 24 months in the Swedish children, however (for birch see fig 4). No differences were observed between the two groups regarding allergen induced IL-4 secretion, whereas allergen induced IL-9 secretion tended to be higher among the Swedish children after the age of 12 months (data not shown).

Phytohaemagglutinin stimulation was used as a positive control and viability check of the cells. PHA induced cytokine secretion were generally similar in Estonian and Swedish infants (table 2). The PHA induced IFN-γ secretion from CBMC was higher in Estonian (median=488, range:12-4191 (pg/ml)) than in Swedish (median=110 range:12-4166 (pg/ml)) children (p=0.036), however, whereas PHA induced secretion of IFN-γ, IL-4, IL-5, -9, -10 and -13 often was, or tended to be, higher in the Swedish infants at 24 months of age.

Birch induced cytokine responses were generally higher in Swedish infants with, as compared to without, atopic disease (fig 4). The differences reached statistical significance for IFN-γ at 6 and 12 months, for IL-4 at 24 months, IL-5 and IL-13 at 12 months and IL-9 at 6 and 12 months. Similar trends were observed for birch induced IL-5 at 24 months, IL-10 at 6 and 12 months and IL-13 at 6 and 24 months. The cytokine responses induced by the other allergens, *i e* BLG, cat and ovalbumin, were similar among Swedish children with and without atopic disease at all ages. However, high IL-4 and IL-5 responses to BLG were observed in milk SPT positive children at 24 months

of age, with a similar trend for IL-13 (table 2). At 12 months of age, milk sensitized children showed low BLG induced IFN-γ production (table 3). Since ovalbumin stimulated cultures were available from fewer children, findings for this allergen did not reach statistical significance. The low number of Estonian children with allergic disease and/or positive SPT makes statistical comparisons within this group unreliable. No differences between allergic and non-allergic or SPT positive and negative children were observed among the Estonian children, however (data not shown).

Discussion

Our findings in this prospective study show differences in the pattern of immune responses to allergens during the first two years of life in two geographically adjacent countries with marked differences in living conditions and incidence of atopic diseases, *i e* Estonia and Sweden. Notably, the levels of IL-5, IL-10 and IL-13 secreted by PBMC stimulated with BLG, ovalbumin and cat allergen in the Estonian and Swedish infants declined during the first 3 months of life. All cytokines then progressively increased in the Swedish children. The biphasic response pattern in the Swedish children is consistent with progressive replacement of non specifically responding immature fetal T-cells with specific T-memory cells, which are primed postnatally. This suggests that priming of long lived Th-memory cells may occur during this period, and this possibility is consistent with recent studies [10, 11]. These and other studies have reported the presence of allergen-responsive T-cells in cord blood, raising the possibility that T-cell priming may in some cases occur transplacentally [26]. However, more recent studies on the nature of T-cell responses to environmental allergens in newborns indicate that the bulk of responding cells represent functionally immature recent thymic emigrants (RTE), as opposed to conventional Th-memory cells [27]. The T-cell receptors utilised by RTE are structurally different to those on mature naive or memory T-cells [28], and facilitate promiscuous low affinity reactions to a wide range of peptides, triggering an initial burst of cytokine production followed by rapid apoptosis, in some cases accompanied by bystander activation of regulatory T-cells [27]. As these RTE progressively die out during early childhood, they are presumably replaced by functionally mature naïve T-cells with the capacity to generate genuine

memory responses. In support of this, cytokine responses to BLG, cat and birch declined between birth and 6 months and were then followed by resurgence up to two years in the Swedish children. This was most marked with the important seasonal inhalant allergen birch. Skin test reactivity to milk was associated with high BLG induced Th2-like responses to BLG at 24 months of age, and low IFN-γ responses at 12 months, in accordance with previous studies showing enhanced Th2-like responses to milk in sensitized children [29]. T-cell responses to birch correlated strongly with development of atopic disease in the Swedish children, with high IFN-γ responses at 6 and 12 months and high Th2-like responses from 12 months onwards in children with atopic disease.

We believe that the responses are allergen specific, although the allergen extracts are not endotoxin free, since we have earlier reported that immune responses to birch extract are related to birch allergy [11] and that cells from birch or cat sensitised children respond with Th2 like cytokine production after stimulation with birch or cat allergen extract, but not after stimulation with an unrelated allergen [4].

In contrast to the biphasic responses in the Swedish children, the resurgence of allergen-specific responses in the Estonian infants was much less marked, although the cells responded well to mitogenic PHA stimulation. Regardless of presence of atopic disease, the Swedish children showed a different development of cytokine responses to allergens than the Estonian children. Thus, the findings were similar both when comparing Estonian infants with Swedish allergic and non-allergic infants. In Estonia, there were

too few allergic children to allow any comparison between allergic and non-allergic individuals.

In a smaller long-term prospective study of development of immune responses to birch in Swedish children over the first seven birch pollen seasons, we have previously demonstrated that a transient early Th2-like response is down-regulated after the third pollen season, except in children who develop clinical allergy to the particular allergen [11]. It will be interesting to continue to follow the cohorts of the present study up to the age when clinical allergy to birch commonly develops.

The similar immune responses to allergens during the first 3-6 months of age in Estonian and Swedish infants indicate that differences in the environments of the two countries do not affect the very early immune responses. After that age, Swedish infants displayed stronger immune responses to all allergens than Estonian infants, and the responses included both Th1 and Th2 cytokines. We and others have previously hypothesized that the increase in prevalence and severity of atopic diseases during the last decades in industrialized countries is due to reduced maturation of Th1-like immunity to allergens, as a consequence of reduced microbial stimulation [20]. However, the simultaneous increases in the incidence of both allergic disorders, inflammatory bowel disease and autoimmune diseases in industrialised countries [30], and the low prevalence of autoimmune disease in less developed countries [31] may indicate that this is too simplified a theory and suggests a broader defect in immune regulation. For example, diseases associated with dysfunctional immune responses may be explained by an imbalance of effector T cells and regulatory T cells [32, 33]. Recent

studies suggest that regulatory T cells are important for the development of tolerance to allergens [34, 35]. Speculatively, the lower Th1 and Th2 cytokine production in the Estonian infants up to the age of two years could thus be explained by a more rapid induction of T-cell regulation. As regulatory T cells can be activated by microbial products to exert suppression [36] and their development may be particularly favored by the environment at the Peyer's patches in the gut [37], the less diverse gut flora of Swedish, as compared to Estonian, children [21, 22], may cause a less efficient induction of regulatory T cells. Unfortunately, we could not investigate this possibility as the study was designed at a time when the potential role of regulatory T cells was not fully appreciated.

In conclusion, the development of specific immune responses to food and inhalant allergens during the first two years of life differs between infants living in a country with a low and a high prevalence of allergy. Thus, both Th1 and Th2 cytokine production is lower in response to allergen stimulation in Estonian infants after 6 months of age, suggesting a more rapid induction of immune regulation.

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Abbreviations

BLG	β-lactoglobulin
CBMC	cord blood mononuclear cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
PBMC	peripheral blood mononuclear cells
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
RTE	recent thymic emigrants
SPT	skin prick test
Th	T-helper

Table 1. Cumulative incidence of skin prick test positivity up to two years of age		
	Estonian	Swedish
Any SPT	5/30 (17%)	28/75 (37%)
Egg white	3/28 (11%)	24/76 (32%)
Cow's milk	1/30 (3%)	7/75 (9%)
Cat allergen	2/30 (7%)	4/75 (5%)
Birch allergen	0/30 (0%)	7/75 (9%)

Table 2. Phytohaemagglutinin induced cytokine secretion (median (range)) in pg/mL in cultures with mononuclear cells from Swedish (Swe) and Estonian (Est) children obtained at birth (cord blood (CB)) and at 3, 6, 12 and 24 months of age. Statistically significant differences between the groups are indicated with *($p<0.05$), **($p<0.01$) and ***($p<0.001$).

	CB		**3 m**		**6 m**		**12 m**		**24m**	
	Swe	Est	Swe	Est	Swe	Est	Swe	Est	Swe	Est
IFN-γ	110* (12-4166)	488* (12-4191)	256 (12-2810)	247 (12-546)	214 (12-3286)	159 (12-7282)	465 (12-4553)	959 (201-4901	1042 (12-6057)	109 (12-6950)
IL-4	1 (1-10)	1 (1-10)	1 (1-10)	1 (1-7)	2 (1-16)	1 (1-11)	6 (1-88)	1 (1-34)	13 (1-118)	1 (1-37)
IL-5	3 (3-100)	3 (3-15)	3 (3-78)	3 (3-7)	15 (3-97)	3 (3-32)	41 (3-959)	13 (3-62)	59** (1-450)	3** (1-128)
IL-9	4 (4-4)	4 (4-16)	4 (4-19)	4 (4-36)	4 (4-87)	4 (4-18)	9 (4-158)	4 (4-71)	21* (4-840)	4* (4-18)
IL-10	39 (2-222)	29 (2-400)	37 (2-461)	23 (2-103)	21 (2-88)	11 (2-62)	48 (2-218)	19 (2-166)	56*** (2-560)	5*** (2-16)
IL-13	18 (1-114)	53 (1-246)	23 (3-84)	14 (1-61)	32 (3-393)	20 (1-88)	80 (3-500)	42 (6-178)	110 (6-665)	51 (3-126)

Table 3. Cytokine levels (median (range), pg/mL) after stimulation with BLG in relation to milk sensitisation, at 12 months for IFN-γ and 24 months for IL-4, IL-5 and IL-13. *p<0.05 (Mann-Whitney U-test).

	Milk SPT+	n	Milk SPT-	n
IFN-γ	12 (12-55)*	6	39 (12-1610)	60
IL-4	3 (1-6)*	6	1 (1-5)	34
IL-5	55 (7-271)*	8	10 (3-140)	54
IL-13	50 (1-1721)$^{p=0.09}$	8	19 (1-240)	54

Legends to figures

Figure 1.

β-lactoglobulin induced IFN-γ, IL-5, -10 and -13 secretion from mononuclear cells isolated from cord blood (CB) or peripheral blood obtained at 3, 6, 12 and 24 months of age from Swedish (open circles) and Estonian (filled circles) infants. The cytokine levels are expressed in pg/mL and the median values are indicated. Statistical comparisons between the Estonian and Swedish groups were performed with Mann-Whitney *U*-test. Statistically significant differences are marked in bold and trends in plain text. The age related changes was calculated with Wilcoxon signed rank test and are indicated below each figure. The data was compared as follows, CB with 3 or 6 months data, 3 or 6 months with 12 months data and 12 months with 24 months data.

Figure 2.

Cat allergen induced IFN-γ, IL-5, -10 and -13 secretion from mononuclear cells isolated from cord blood (CB) or peripheral blood obtained at 3, 6, 12 and 24 months of age from Swedish (open circles) and Estonian (filled circles)infants. The cytokine levels are expressed in pg/mL and the median values are indicated. Statistical comparisons between the Swedish and Estonian groups were performed with Mann-Whitney *U*-test. Statistically significant differences are marked in bold and trend in plain text. The age related changes was calculated with Wilcoxon signed rank test and are indicated below each figure. The data was compared as follows, CB with 3 or 6 months data, 3 or 6 months with 12 months data and 12 months with 24 months data.

Figure 3.

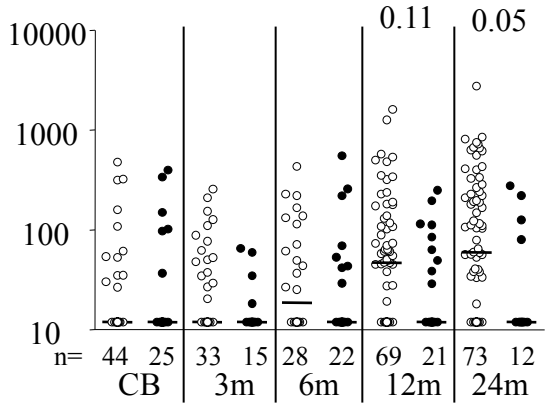
Birch allergen induced IFN-γ, IL-5, -10 and -13 secretion from mononuclear cells isolated from cord blood (CB) or peripheral blood obtained at 3, 6, 12 and 24 months of age from Swedish (open circles) and Estonian (filled circles) infants. The cytokine levels are expressed in pg/mL and the median values are indicated. Statistical comparisons between the Estonian and Swedish groups were performed with Mann-Whitney *U*-test. Statistically significant differences are marked in bold and trend in plain text. The age related changes was calculated with Wilcoxon signed rank test and are indicated below each figure. The data was compared as follows, CB with 3 or 6 months data, 3 or 6 months with 12 months data and 12 months with 24 months data.

Figure 4.

Birch allergen induced cytokine secretion from Swedish infants without (open **squares**) and with (filled **squares**) atopic disease. Statistical comparisons between the groups were performed with Mann-Whitney *U*-test and statistically significant differences are marked in bold and trends in plain text.

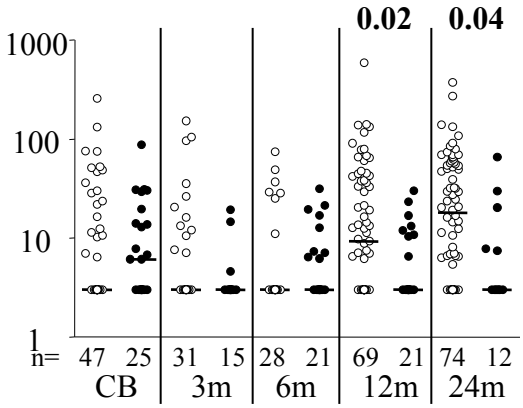
Fig 1

BLG IFN-γ



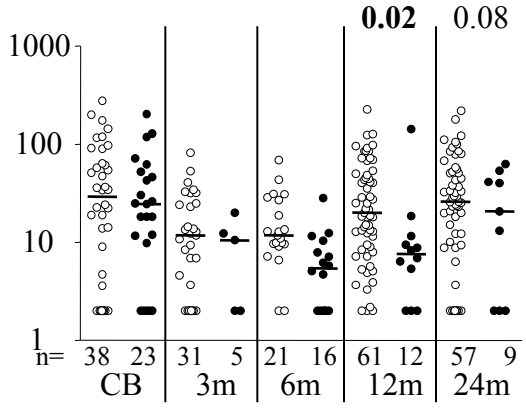
Swe
↑CB→3m p=0.01

BLG IL-5



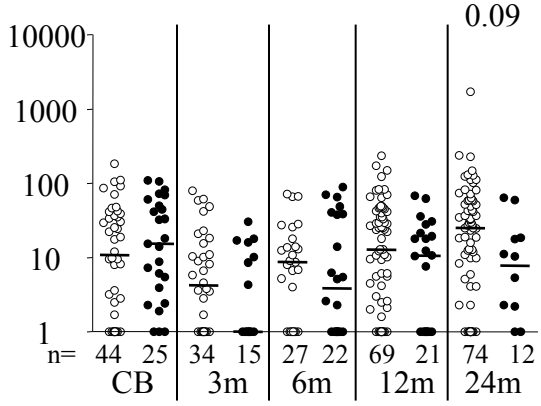
Est
↓CB→3m p=0.09
Swe
↓CB→3m p=0.02
↑3m→12m p=0.04
↑6m→12m p=0.1

BLG IL-10



Est
↓CB→3m p=0.1
Swe
↓CB→3m p=0.1
↓CB→6m p=0.1
↑3m→12m p=0.02

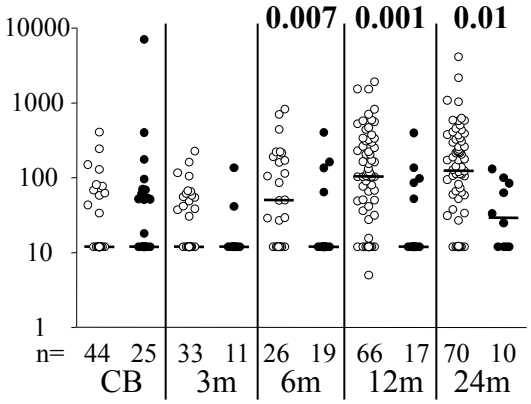
BLG IL-13



Est
↓CB→3m p=0.003
Swe
↓CB→3m p=0.04
↑3m→12m p=0.05

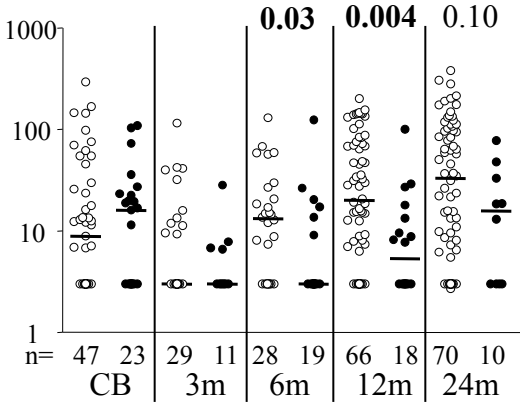
Fig 2

Cat IFN-γ



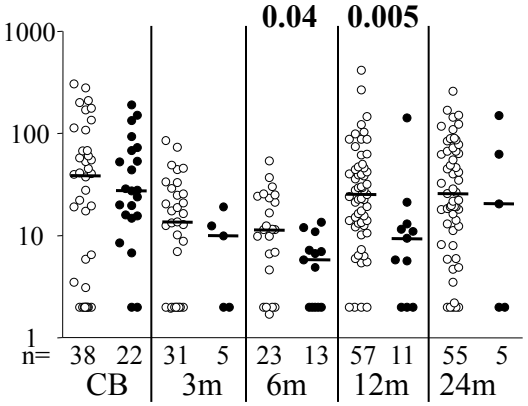
Est
↓CB→3m p=0.08
Swe
↑CB→6m p=0.049
↑3m→12m p=0.0004
↑6m→12m p=0.04

Cat IL-5



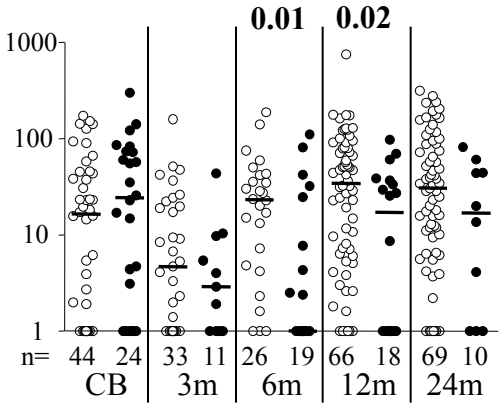
Est
↓CB→3m p=0.03
Swe
↓CB→3m p=0.008
↑3m→12m p=0.001
↑6m→12m p=0.1
↑12m→24m p=0.08

Cat IL-10



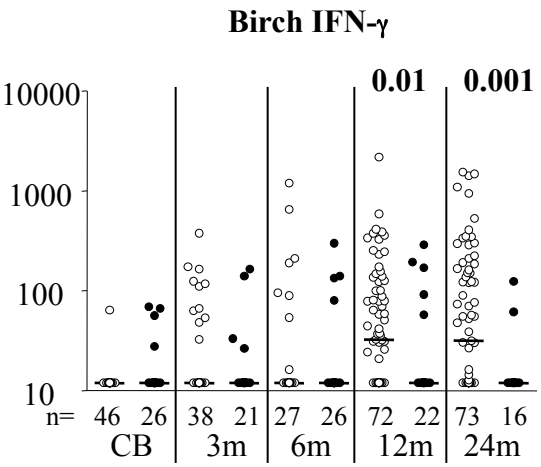
Est
↓CB→3m p=0.1
Swe
↓CB→6m p=0.1
↑3m→12m p=0.01
↑6m→12m p=0.06

Cat IL-13

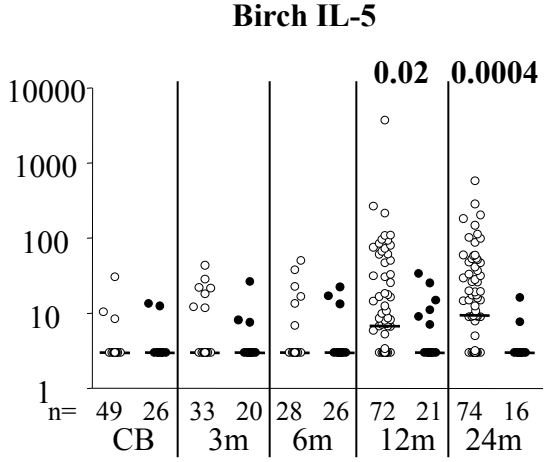


Est
↓CB→3m p=0.02
Swe
↓CB→3m p=0.002
↑3m→12m p=0.001
↑6m→12m p=0.05

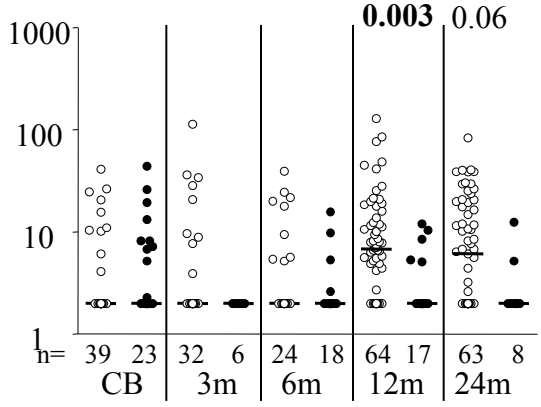
Fig 3



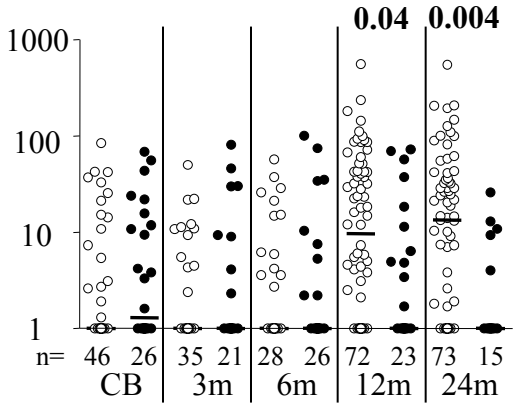
Swe
↑CB→3m p=0.08
↑12m→24m p=0.006



Swe
↑3m→12m p=0.02

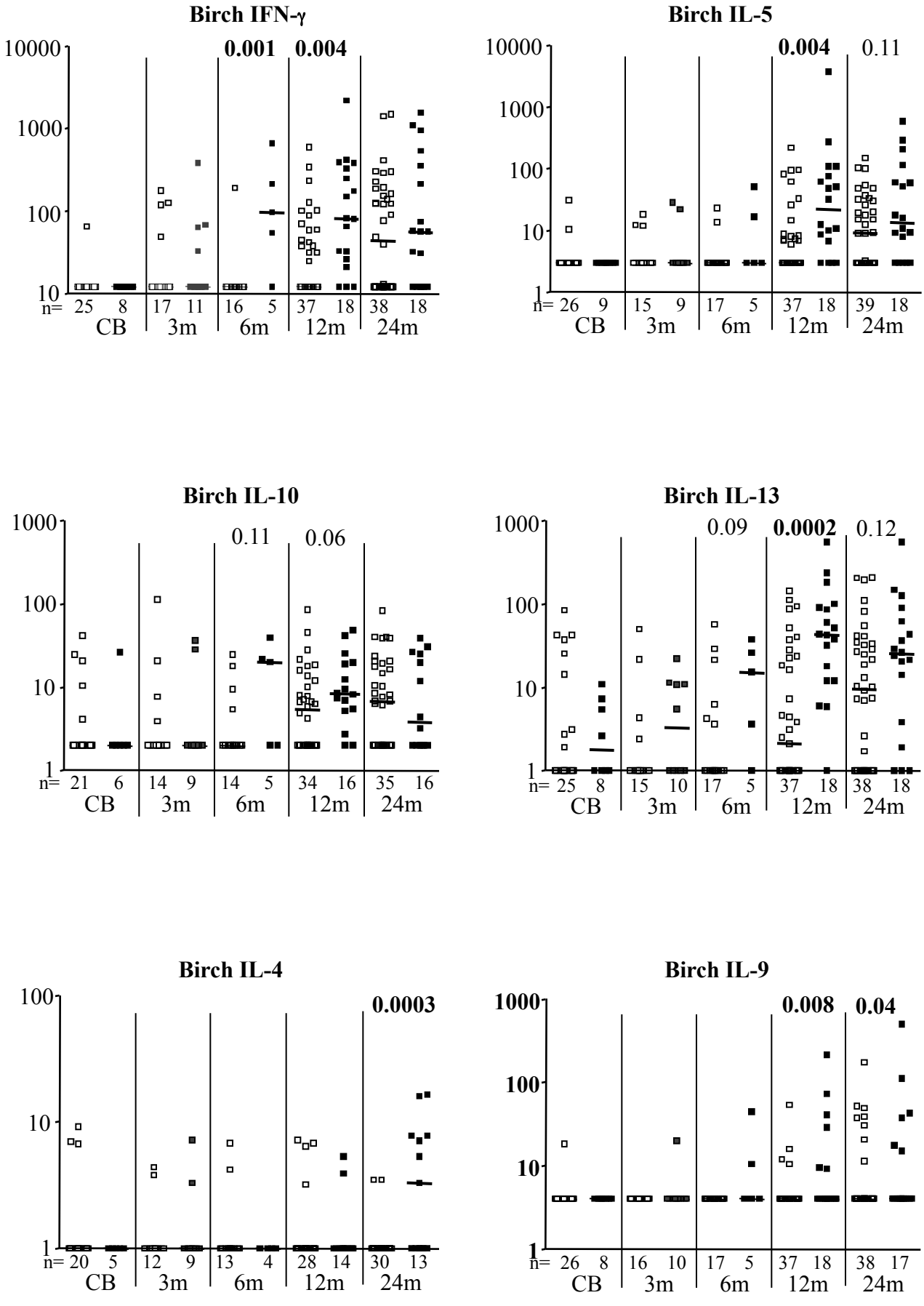


Swe
↑3m→12m p=0.02



Swe
↑3m→12m p=0.0001

Fig 4


Birch IFN-γ
10000
1000
100
10
0.001
0.004
n= 25 8 17 11 16 5 37 18 38 18
CB 3m 6m 12m 24m
Birch IL-5
10000
1000
100
10
1
0.004
0.11
n= 26 9 15 9 17 5 37 18 39 18
CB 3m 6m 12m 24m
Birch IL-10
1000
100
10
1
0.11
0.06
n= 21 6 14 9 14 5 34 16 35 16
CB 3m 6m 12m 24m
Birch IL-13
1000
100
10
1
0.09
0.0002
0.12
n= 25 8 15 10 17 5 37 18 38 18
CB 3m 6m 12m 24m
Birch IL-4
100
10
1
0.0003
n= 20 5 12 9 13 4 28 14 30 13
CB 3m 6m 12m 24m
Birch IL-9
1000
100
10
1
0.008
0.04
n= 26 8 16 10 17 5 37 18 38 17
CB 3m 6m 12m 24m