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Epstein–Barr virus and cytomegalovirus are differentially associated with numbers of cytokine-producing cells and early atopy

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Clinical and
Experimental
Allergy

Summary

Background We have previously shown that Epstein–Barr virus (EBV) seropositivity, at 2 years of age, was inversely related to IgE-sensitization and that this effect was enhanced when EBV is combined with cytomegalovirus (CMV) seropositivity. We hypothesize that early exposure to EBV or CMV will affect the cytokine balance in the individual.

Objective The aim of this study was to relate the cytokine profile in peripheral blood mononuclear cells (PBMC) to the EBV and CMV serostatus and IgE-sensitization in children at 2 years of age.

Methods Seventy-five children were followed prospectively from birth until 2 years of age. Their EBV and CMV serostatus was correlated to the numbers of IFN- γ , IL-4, IL-10 and IL-12-producing PBMC following PHA stimulation *in vitro*. Skin prick tests and allergen-specific IgE antibodies were used to assess IgE-sensitization.

Results In the study cohort, there was an inverse association between EBV seropositivity and IgE-sensitization but not with CMV seropositivity. Following linear regression analysis, we did not detect any statistically significant associations between children with IgG antibodies against EBV at 2 years of age and the investigated cytokines. However, there was a non-significant tendency to a positive association between high numbers of all individual cytokine-producing cells and EBV seropositivity. Children who were CMV seropositive had significantly higher numbers of IFN- γ and lower numbers of IL-4-producing cells compared with CMV negative children. There was a significant, positive association between the number of IL-4-producing cells and IgE-sensitization.

Conclusion Taken together our results indicate that infections with EBV and CMV in different ways will interact with the immune system and may protect children from developing early atopy.

Keywords childhood, CMV, cytokines, EBV, IgE-sensitization

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Introduction

Epstein–Barr virus (EBV) and cytomegalovirus (CMV) are persistent viruses that are shed for a long time after the primary infections. The chronic nature of both viruses has been shown to exert an effect on the immune system [1]. The successful coexistence of the viruses with their hosts requires a variety of mechanisms of anti-viral immune evasion. Examples include genes encoding for IL-10-like proteins which may down-regulate the antigen proces-

sing/presentation capacity of dendritic cells/macrophages. Thereby the host T cell system may be switched off, similar to that observed for Treg cells [2, 3]. In addition, both viruses have been shown to polyclonally activate B cells to produce antibodies with multiple specificities. Thereby specific antigens are hindered from cross-linking the B cell receptor, as seen for helminthic infections [4].

In developing countries, the majority of young children have asymptomatic CMV and EBV infections early in life

[5, 6]. This is in contrast to industrialized countries where the primary EBV infection often is delayed, sometimes until puberty [7, 8] resulting in EBV-associated infectious mononucleosis (IM) in about half of those infected at this age. Patients with IM exhibit a Th0-type cytokine profile with high levels of IFN- γ and IL-12 as well as IL-4 [9, 10]. The CMV infection is mostly subclinical in immune-competent individuals and T cells from CMV-seropositive donors mainly produce Th1 cytokines, such as IFN- γ but also IL-4 [11, 12]. This suggests that both CMV and EBV can influence the development of functionally distinct immune responses especially when the nascent immune system encounters these viruses.

The herpes viruses, especially CMV and EBV, have been given attention for a possible role in allergy. EBV infection in the first years of life has previously been associated with a lower prevalence of IgE antibodies [8], while other studies have shown an association between CMV but not with EBV seropositivity at 4 years of age and elevated IgE to selected allergens [13, 14]. An antagonism between EBV and CMV was suggested [13, 14]. Recently, a multi-centre study of adults, with or without allergy, showed that allergic subjects more seldom were seropositive to CMV/EBV than non-allergic ones [15]. *In vitro* cellular studies have shown that activated CD8⁺ cells in CMV-seropositive adults with allergic asthma were reduced in numbers compared with findings in CMV-seropositive non-atopic adults [16]. Recently, Barton *et al.* presented a study showing an up-regulation of the innate immunity in mice with latent murine equivalents of human CMV and EBV [17]. In addition to the type of immune response elicited, the magnitude of the virus-specific response is of importance. It has been demonstrated that CMV-specific CD4⁺ and CD8⁺ T cells are a large proportion of the memory compartments of exposed subjects [18, 19].

We have previously shown that early exposure to EBV may protect against the development of early atopy [20] and that this effect is enhanced when EBV is combined with CMV exposure. We hypothesize that early exposure to EBV or EBV together with CMV might affect the development of functionally distinct T-helper cells. The aim of this study was to evaluate the associations between the number of IFN- γ , IL-4, IL-10 and IL-12-producing peripheral blood mononuclear cells (PBMC) after stimulation with phytohaemagglutinin (PHA), the serostatus against EBV and CMV and IgE-sensitization in children at 2 years of age.

Materials and methods

Subjects

A total of 75 2-year-old infants were selected for this study from a cohort of children participating in a larger, prospective study [20]. The cytokine profile of PBMC in

cord blood has been presented elsewhere [21]. One third of the children had two allergic parents, another third had an allergic mother and the last third had no parental history of allergy. Parental allergy was assessed with skin prick tests (SPT) before the delivery of the child. All children in the larger prospective study were skin prick tested and bled at 2 years of age. Circulating allergen-specific IgE and antibodies against CMV and EBV were analysed in the plasma samples obtained at this occasion. Children, from whom there were enough PBMCs recovered from blood samples to perform cellular studies, were included in this study. All infants were born full term (>35 weeks of gestation) at hospitals in Stockholm, were healthy and had birth weights within the normal range (mean 3.6, range 2.5–4.8 kg). The study was approved by the Human Ethics Committee at Huddinge University Hospital, Stockholm (Dnr 75/97, 113/97) and the parents provided informed consent.

Clinical evaluation

The children were followed from birth to 2 years of age and were clinically evaluated at ages 6, 12, 18 and 24 months by one paediatrician (C.N.).

Skin prick test

SPT (ALK, Copenhagen, Denmark) against egg white (Soluprick weight to volume ratio 1/100), cod (Soluprick 1/20), peanut (Soluprick 1/20), cow's milk (3% fat, standard milk), soy bean protein (Soja Semp[®] Semper AB, Stockholm, Sweden), cat, dog, *Dermatophagoides farinae*, birch and timothy (Soluprick 10 HEP) were performed at 24 months of age as previously described [20]. All parents were skin prick tested against the same inhalant allergens as the children and further against horse, rabbit and mugwort. The SPT was considered positive if the weal diameter was ≥ 3 mm after 15 min.

Blood sampling

Venous blood samples were collected when the children were 24 months old (mean 23.8, range 21.8–27.7 months). The blood samples were stored for a maximum of 24 h before PBMC were separated and isolated by Ficoll-Paque (Pharmacia-Upjohn, Uppsala, Sweden) gradient centrifugation. The cells were resuspended and frozen at $1\text{--}2 \times 10^7$ cells/mL in tissue-culture medium (TCM; i.e. RPMI 1640 Hepes containing 10% heat-inactivated fetal calf serum (FCS) and 25 $\mu\text{g/mL}$ of gentamicin and 2 mmol L-glutamine), supplemented with 10% dimethyl sulfoxide (DMSO) and gradually frozen at 1 °C/min to -70 °C in a freezing container (Nalgene Cryo 1 °C, Nalgene Company, USA) and were then stored in liquid nitrogen until

used in the ELISpot assay. Plasma was also separated and stored at -70°C pending analysis.

ELISpot

Frozen cells were thawed and diluted in TCM. The numbers of IFN- γ , IL-4, IL-10 and IL-12-producing cells were measured by the ELISpot after PHA stimulation. The reverse ELISpot assay was performed mainly as described previously [22]. Briefly, the anti-human IL-4, IL-12, IL-10 and IFN- γ monoclonal antibodies (MoAbs) were added onto nitrocellulose plates (Millipore Corp., Bedford, MA, USA) at a concentration $10\text{ }\mu\text{g/mL}$ and incubated overnight. Monoclonal antibodies used were M-450 for IL-4 (Endogen, MA, USA), 1-D1K for IFN- γ , IL-12-I for IL-12 and 9D7 for IL-10 (all from Mabtech AB, Nacka, Sweden). The cells were diluted to a concentration of 10^6 cells/mL in TCM alone or in TCM containing PHA ($1\text{ }\mu\text{g/mL}$, Murex Diagnostics Ltd, Dartford, UK) in round-bottomed tubes. After incubation for 4 h, the cells were transferred to the coated ELISpot plates and incubated for 42 h. Thereafter the cells were washed away and $100\text{ }\mu\text{L}$ of biotinylated MoAbs were added and incubated for 2 h at room temperature (RT). Finally, $100\text{ }\mu\text{L}$ colour-developing buffer (Bio-Rad, Richmond, CA, USA) was added and incubated at RT until dark spots emerged. After development, the plates were dried and spots were counted using a computerized ELISpot counter (Autoimmun Diagnostika GmbH, Strassberg, Germany with software AID). The number of spontaneously cytokine-producing cells was subtracted from all values shown.

Viral infections/serological methods

The IgG serostatus against CMV and EBV was investigated using in-house assays immunofluorescence and ELISA described in detail elsewhere [23, 24].

Specific immunoglobulin E

Specific IgE antibodies against the same allergens as for SPT were determined in plasma with Phadia ImmunoCAPTM (Phadia, Uppsala, Sweden). A positive test was defined as an IgE antibody level $\geq 0.35\text{ kU}_\text{A}/\text{L}$.

Classification of the immunoglobulin E-sensitized children

In accordance with Johansson et al. [25], the children were classified as IgE-sensitized if at least one or more SPT was $\geq 3\text{ mm}$ and/or if at least one or more specific IgE was $\geq 0.35\text{ kU}_\text{A}/\text{L}$. In order to optimize the classification in IgE-sensitized and non-IgE-sensitized children, the results of the *in vivo* and *in vitro* analysis were combined.

Statistical analyses

Descriptive statistics were used to characterize the data. Chi-squared analysis and the Student's *t*-test (two-tailed) were used for comparison between the groups of children where appropriate. The distributions of the cytokine-producing cells were not normally distributed and, therefore, they were transformed with natural logarithms by the statistical program Stata (7.0; Stata Corporation, College Station, TX, USA) to remove skewness, thus allowing us to model them as continuous measures and use them as the dependent variable in linear regression analysis. Linear regression was used to calculate the associations of seropositivity against CMV or EBV, or IgE-sensitization with the investigated cytokines (IFN- γ , IL-4, IL-10 and IL-12). Separate models were used for each cytokine (as dependent variable), but after unadjusted analysis, there was simultaneous mutual adjustment for the other cytokines as independent variables. When the main associations were altered notably by adjustment for the other cytokines, these were included in the model one by one to identify which factors were responsible. Additional adjustment for the other dependent measures, CMV seropositivity, EBV seropositivity and IgE-sensitization, were included as appropriate. The models were further adjusted for environmental factors associated with allergy in this population such as season of birth (winter = October–March or summer = April–September), number of older siblings (0, 1 or >1) and duration of exclusive breastfeeding (none, <4 , 4–5 or 5.1–10 months) [20].

Statistical significance was defined as 95% confidence intervals that do not include 0.00 and *P*-values >0.05 . The analyses were conducted using the Stata and SPSS statistical packages.

Results

Immunoglobulin E-sensitization and Epstein-Barr virus and cytomegalovirus serostatus

Fifteen (20.0%) of the children were classified as IgE-sensitized (one or more SPT $\geq 3\text{ mm}$ and/or one or more allergen-specific IgE $\geq 0.35\text{ kU}_\text{A}/\text{L}$) at 2 years of age. Twenty-two (29.3%) children were seropositive against EBV and 29 (38.7%) children were seropositive against CMV at 2 years of age. Boys were more often infected with CMV than girls (data not shown). EBV and CMV seropositivity was not associated with each other.

The association between IgE-sensitization and serostatus against CMV and EBV was evaluated. Seropositivity against EBV was significantly inversely associated with IgE-sensitization (Table 1). Among the EBV-seropositive children, one (4.5%) was sensitized. Seven (24.1%) children were sensitized among the CMV seropositives, and there were no statistically significant associations

Table 1. Seropositivity for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) in IgE-sensitized and non-sensitized children

	Non-sensitized	IgE-sensitized	<i>B</i> ; (95% CI)	<i>B</i> -adj [*] ; (95% CI)
<i>n</i>	60	15		
EBV; <i>n</i> (%)	21 (35)	1 (7)	−0.22; (−0.42 to −0.02) [#]	−0.26; (−0.46 to −0.06) ^{##}
CMV; <i>n</i> (%)	22 (37)	7 (47)	0.07; (−0.12 to 0.26)	0.11; (−0.10 to 0.32)

*Adjusted for the investigated cytokines and EBV and CMV, respectively. [#]*P* = 0.03, ^{##}*P* = 0.01.

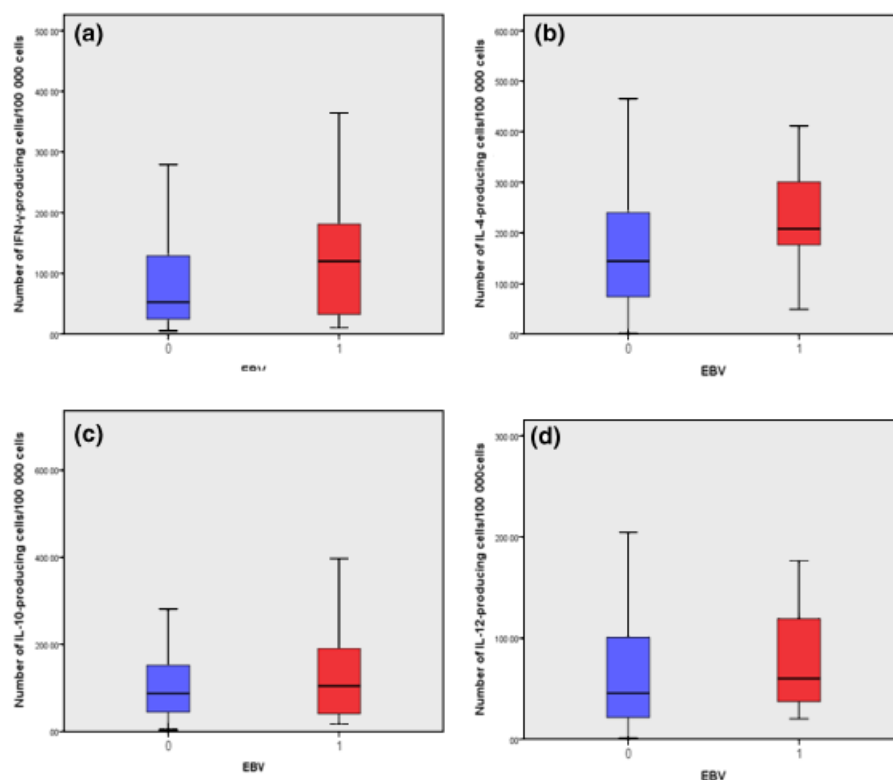


Fig. 1. Frequency of cytokine-producing cells/100 000 peripheral blood mononuclear cells (PBMC) after stimulation with phytohaemagglutinin (PHA) among infants seropositive (red box, *n* = 22) and seronegative (blue box, *n* = 53) against Epstein-Barr virus (EBV) during the first 2 years of age: (a) IFN- γ -producing cells, (b) IL-4-producing cells, (c) IL-10-producing cells, (d) IL-12-producing cells. Results are illustrated using the box plot model. Boxes cover the middle 50% of the data values, between the 25th (*q*₁) and 75th (*q*₃) percentiles, the central line being the median.

between seropositivity against CMV and IgE-sensitization (Table 1). None of the seven children (9%) who were seropositive against both EBV and CMV were sensitized.

Epstein-Barr virus seropositivity and the number of cytokine-producing cells

The associations between the EBV serostatus and the number of cytokine-producing cells, before they were transformed with natural logarithms, are presented in Fig. 1. There was a non-significant tendency to a positive association between high number of all individual cytokine-producing cells and EBV seropositivity.

After transformation with natural logarithms, there were no notable differences in the linear regression coefficient (*B*) for IFN- γ , IL-4 and IL-12-producing cells

Table 2. Linear regression coefficient (*B*) for IFN- γ , IL-4, IL-10 and IL-12-producing cells after phytohaemagglutinin (PHA) stimulation among Epstein-Barr virus (EBV)-seropositive compared with EBV-seronegative children

	<i>B</i> ; (95% CI)	<i>B</i> -adj [*] ; (95% CI)
IFN- γ	0.37; (−0.07 to 0.80)	0.25; (−0.17 to 0.68)
IL-4	0.15; (−0.03 to 0.33)	0.07; (−0.05 to 0.19)
IL-10	0.20; (−0.24 to 0.63)	−0.10; (−0.37 to 1.16)
IL-12	0.22; (−0.13 to 0.58)	0.05; (−0.22 to 0.32)

*Adjusted for IFN- γ , IL-4, IL-10 and IL-12.

and EBV neither before nor after adjustment for the other cytokines (Table 2). However, for IL-10, *B* decreased after adjustment of the other cytokines (Table 2). This was almost entirely due to adjustment for IL-4 alone.

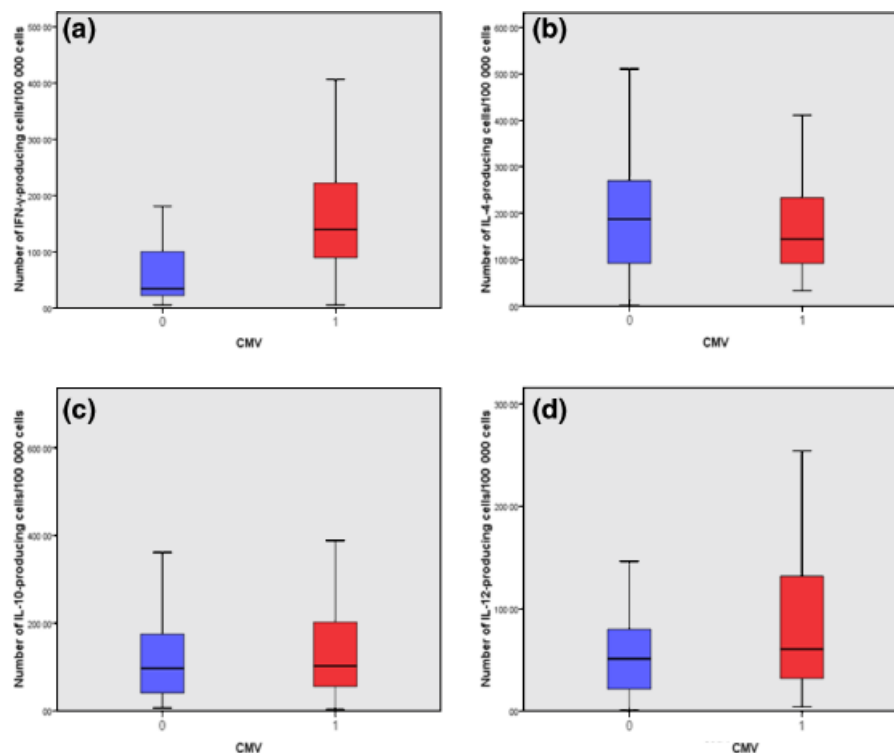


Fig. 2. Frequency of cytokine-producing cells/100 000 peripheral blood mononuclear cells (PBMC) after stimulation with phytohaemagglutinin (PHA) among infants seropositive (red box, $n = 29$) and seronegative (blue box, $n = 46$) against cytomegalovirus (CMV) during the first 2 years of age: (a) IFN- γ -producing cells, (b) IL-4-producing cells, (c) IL-10-producing cells, (d) IL-12-producing cells. Results are illustrated using the box plot model. Boxes cover the middle 50% of the data values, between the 25th (q_1) and 75th (q_3) percentiles, the central line being the median.

Further adjustment for CMV and sensitization did not alter the associations (data not shown). Adjustments for environmental factors did not change the associations between EBV-seropositive children and the different cytokines.

Cytomegalovirus seropositivity and the number of cytokine-producing cells

The associations between the serostatus against CMV and the number of cytokine-producing cells, before they were transformed with natural logarithms, are presented in Fig. 2. After transformation with natural logarithms, the CMV-seropositive children had significantly higher numbers of IFN- γ -producing cells compared with CMV-seronegative children with a linear regression coefficient (B) of 0.83 (95% CI 0.47–1.20). Adjustment for the other cytokines did not alter the association (Table 3). Adjustment for the cytokines and EBV serostatus increased the association between CMV seropositivity and the number of IFN- γ -producing cells to a B of 0.85 (95% CI 0.50–1.20) ($P = <0.001$). Adding sensitization to the adjusted model did not result in further notable changes (data not shown).

When comparing the numbers of IL-4-producing cells and seropositivity against CMV, there was an inverse,

Table 3. Linear regression coefficient (B) for IFN- γ , IL-4, IL-10 and IL-12-producing cells after phytohaemagglutinin (PHA) stimulation among cytomegalovirus (CMV)-seropositive compared with CMV-seronegative children

	B ; (95% CI)	B -adj [*] ; (95% CI)
IFN- γ	0.83; (0.47 to 1.20) ^{##}	0.83; (0.47 to 1.18) ^{##}
IL-4	−0.05; (−0.22 to 0.12)	−0.14; (−0.26 to −0.02) [#]
IL-10	0.08; (−0.33 to 0.49)	0.08; (−0.21 to 0.37)
IL-12	0.23; (−0.10 to 0.55)	0.14; (−0.15 to 0.42)

^{*}Adjusted for IFN- γ , IL-4, IL-10 and IL-12. [#] $P = 0.024$, ^{##} $P = <0.001$.

non-significant association (Table 3), which became statistically significant after adjustment for IFN- γ , IL-10 and IL-12. No single cytokine was responsible for this change; it was rather an additive effect. This was also true after adjustment for EBV and sensitization (data not shown).

No statistically significant associations between the number of IL-10 and IL-12-producing cells and CMV serostatus were observed. Adjustments for environmental factors associated with allergy revealed no changes in the association between CMV-seropositive children and the different cytokines.

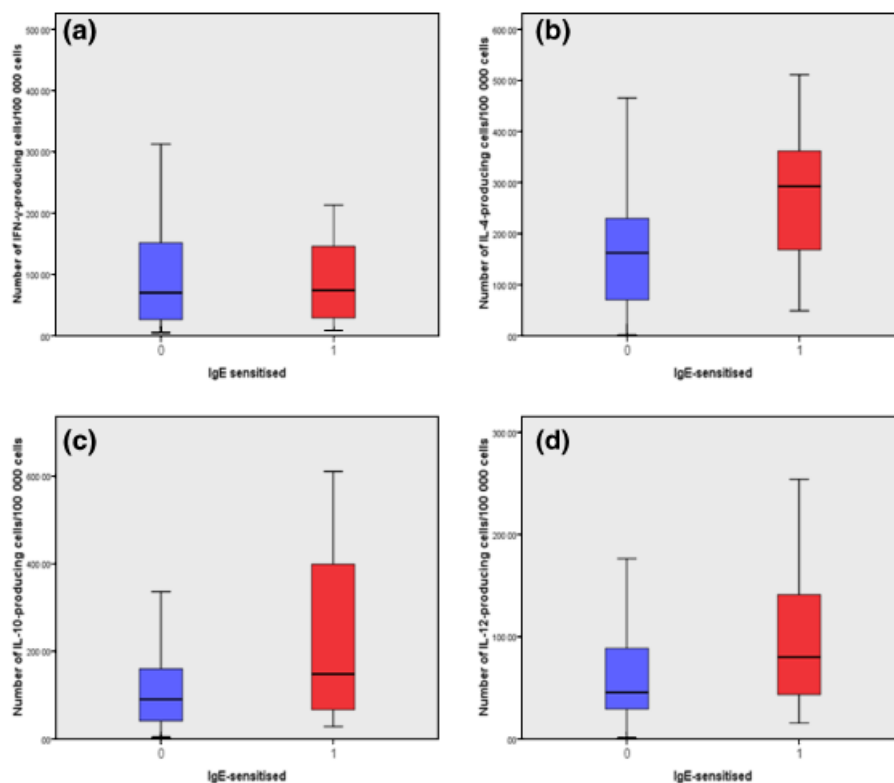


Fig. 3. Frequency of cytokine-producing cells/100 000 peripheral blood mononuclear cells (PBMC) after stimulation with phytohaemagglutinin (PHA) among infants IgE-sensitized (red box, $n = 15$) and non-sensitized (blue box, $n = 60$) at 2 years of age: (a) IFN- γ -producing cells, (b) IL-4-producing cells, (c) IL-10-producing cells, (d) IL-12-producing cells. Results are illustrated using the box plot model. Boxes cover the middle 50% of the data values, between the 25th (q_1) and 75th (q_3) percentiles, the central line being the median.

Immunoglobulin E-sensitization and the number of cytokine-producing cells

The associations between IgE-sensitization and the number of cytokine-producing cells, before they were transformed with natural logarithms, are presented in Fig. 3. Among the sensitized children, after transformation with natural logarithms, there was a statistically significant positive association with the number of IL-4-producing cells (Table 4). Adjustment for the other cytokines notably altered the association (Table 4) and this was entirely due to adjustment for IL-10. After adjustment for the cytokines, as well as CMV and EBV seropositivity, the linear regression coefficient (B) increased and the positive association between the number of IL-4-producing cells and IgE-sensitization was statistically significant ($P = 0.006$). No single infection was responsible for this change; it was rather an additive effect.

A statistically significant, positive association was found for IgE-sensitization and the number of IL-10-producing cells before adjustment with the other cytokines, CMV and EBV. IgE-sensitization was also positively associated with a larger number of IL-12-producing cells, but this was not statistically significant (Table 4).

Table 4. Linear regression coefficient (B) for IFN- γ , IL-4, IL-10 and IL-12-producing cells after phytohaemagglutinin (PHA) stimulation among IgE-sensitized and non-sensitized children

	B ; (95% CI)	B -adj [*] ; (95% CI)
IFN- γ	0.03; (−0.47 to 0.54)	−0.22; (−0.72 to 0.28)
IL-4	0.29; (0.09 to 0.49) ^{##}	0.11; (−0.03 to 0.24)
IL-10	0.58; (0.10 to 1.06) [#]	0.03; (−0.28 to 0.35)
IL-12	0.39; (0.00 to 0.79)	0.66; (−0.25 to 0.38)

*Adjusted for IFN- γ , IL-4, IL-10 and IL-12. [#] $P = 0.018$, ^{##} $P = < 0.001$.

Discussion

In this study, IgE-sensitization, the EBV/CMV serostatus and the association of these characteristics with the numbers of IFN- γ , IL-4, IL-10 and IL-12-producing cells were examined. We did not detect any statistically significant associations between children with IgG antibodies against EBV at 2 years of age and the investigated cytokines. However, there was a non-statistically significant tendency to enhanced numbers of all cytokine-producing cells investigated. In contrast, CMV-seropositive children had higher numbers of IFN- γ and lower numbers of IL-4-producing cells than their

CMV-seronegative counterparts. There was an inverse association between IgE-sensitization and EBV seropositivity and but not with CMV seropositivity.

Among the EBV-seropositive children, there was a non-statistically significant indication of higher numbers of IFN- γ , IL-4, IL-10 and IL-12-producing cells than in their seronegative counterparts. This is indicative of a mixed cytokine response without clear dominance of either Th1-type or Th2-type of cytokines (Th0). These findings are consistent with those seen in young adults with IM [10]. We do not know when the seropositive children were infected because primary EBV infections are normally asymptomatic in young children. However, the suggestion of a similar cytokine pattern in EBV-seropositive children as observed in IM is intriguing. These data support the idea that persistent viral infections may affect the immune system for a considerable period.

Our finding that CMV-seropositive children had significantly more IFN- γ -producing cells is compatible with previous reports showing that CMV seems to induce Th1-type cytokine responses [11]. Important sources of IFN- γ production are T-helper cells (CD4⁺) and NK cells, both implicated in the defence against CMV [26]. In our study we did not phenotype the cytokine-producing cells, so we do not know the cellular source of the IFN- γ . Previous studies have shown that age is a very important variable regarding lymphocyte population distribution, both for absolute numbers and percentages [27], and that young children in general have a larger pool of naïve cells [28]. This might be beneficial in handling early primary virus infections.

There was a positive association between IL-4 and IgE-sensitization which is consistent with previous studies, for example, from Koning et al. who reported that children with allergic asthma had a high production of IL-4 compared with non-sensitized children [29]. The sensitized children had a statistically positive association to the number of IL-10-producing cells and a non-statistically significant indication of higher numbers of IFN- γ and IL-12-producing cells than their non-sensitized counterparts. These findings are in agreement with what have been reported by Aktas et al. in adults with IgE-mediated allergy [30].

The inverse association between seropositivity against EBV and IgE-sensitization is consistent with our previous study [20]. The findings that EBV seropositivity has a protective effect against IgE-sensitization while the cytokine profile in those seropositive against EBV was rather similar to that seen in the sensitized children may seem paradoxical. However, it fits well with the recent theory that children who will develop allergy have an immature immune response at birth, and may need stimulants at an early age to induce immune maturation [31, 32]. EBV could well be one such stimulant. In contrast the high IFN- γ and low IL-4 seen in CMV-infected children should be

associated with reduced sensitization according to the theory that high Th1 responses are protective against IgE-mediated allergy. Despite this, CMV alone was not inversely associated with IgE-sensitization. However, in our previous study, we noted that the reduced risk of being IgE-sensitized after acquisition of EBV was enhanced by CMV co-infection. In this study we observed that among the few children (9%) who were seropositive to both EBV and CMV none were sensitized. When EBV and CMV act together, there is a tendency that the cytokine profile changes in direction to a theoretical protection against sensitizations. In a study of 4 years old children, it was shown that an increase in IgE against some allergens seen in children who were positive against CMV alone was not found in children positive to both EBV and CMV [14]. In combination with the findings in our study, it indicates that there are complex interactions, but the EBV infection is the main protective force.

To explain the protective effect of EBV infection, we thus speculate that it may be mediated by a general activation, possibly followed by maturation, of the immune system. It is likely that the protective effect acts through some other mechanisms than the cytokines tested in this study. One possibility is that EBV drives B cells to mature more rapidly, and that a rapid maturation transforms the B cells to produce IgG4 instead of IgE in small children. IgG4 would then act as a blocking antibody preventing the cross-linking of the IgE-sensitized mast cells [33].

In developing countries, antibodies against EBV and CMV are found in a majority of small children [5, 6, 34]. In our study cohort, the prevalence of EBV and CMV was 29% and 39%, respectively. Although low in a global perspective, it is similar to what is seen in other studies in other westernized countries [13, 14, 35]. The cytokine data presented here are based on *in vitro* PHA-activated cells. Indeed, it would have been interesting to evaluate the effects of other types of stimulation, but due to shortage of cells this was not possible. Furthermore, it is important to remember that measurements of cytokine production following *in vitro* stimulation of PBMC might differ from the actual *in vivo* situation. Our study was designed for different pattern of family history of allergy and was not a general-population-based study. As the cytokine distributions were highly skewed, the measures were transformed to produce non-skewed distributions, so that they could be modelled as continuous variables before statistical analysis. We were interested in the cytokine profiles associated with EBV and CMV serostatus, rather than environmental exposures that might influence the risk of these infections. Adjustment for factors, intermediate in the causal process, may mask rather than clarify associations between infections and cytokine profiles. However, we adjusted for environmental factors associated with allergy in this population, and observed no associations.

The production of one cytokine is not independent of the other cytokines and different children have different levels of cytokines. To adjust for the investigated cytokines is to standardize for each patient and therefore we adjusted for multiple cytokines. However, we cannot entirely exclude the possibility that being allergic affects the propensity for being infected with, or seropositive against, EBV and CMV.

Our earlier published data on an inverse association between EBV-seropositivity and sensitization [20] could not only be explained by reduced numbers of IL-4 and increased number of IFN- γ secreting cells. Taken together our data suggest that the putative protective effect of an early EBV exposure does not only operate through conventional Th1–Th2 concept while CMV induced a Th1-type of cytokine profile with significantly higher numbers of IFN- γ -producing cells in CMV-seropositive subjects. Taken together our results indicate that co-infection with EBV and CMV in different ways will affect the immune system, and EBV alone and CMV and EBV together can induce an immunological state which may be protective against early atopy.

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