

Developmental changes in interleukin-12-producing ability by monocytes and their relevance to allergic diseases

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Summary

Background The T helper type-2 (Th2)-dominated situation can be observed in allergic diseases such as asthma or atopic dermatitis. A reduced ability to produce IL-12, which is a key cytokine for the induction of Th1 responses, has been proposed to lead to aberrant Th2 development in these disease conditions.

Objective This study was intended to examine how IL-12-producing ability might associate with allergic diseases as a function of age.

Methods IL-12 production by monocytes at various ages was assessed in patients with bronchial asthma and/or atopic dermatitis ($n = 100$) in comparison with non-allergic control subjects ($n = 144$). Whole blood cells were stimulated with lipopolysaccharide (LPS) after priming with IFN- γ , then intracellular cytokine expression of IL-12 and IL-8 as a control cytokine of CD14-positive cells was assessed by flow cytometric analysis.

Results In the control subjects, the ability of monocytes to produce IL-12 was negligible at birth and gradually increased with advancing age, whereas IL-8 production was intense throughout the human life. At more than 7 years of age, IL-12 production of patients with allergic diseases was significantly lower compared with that of control subjects. The unexpected finding was that infants and children below 6 years of age with allergic diseases tended to produce more IL-12 compared with age-matched controls. In this young group, it was noted that enhanced IL-12 production by monocytes was especially observed in allergic patients with specific IgE antibodies against some food allergens. Significant inverse relationships between serum IgE levels and IL-12-producing ability were found in the teenage and adult groups, but not in the younger children.

Conclusion IL-12 appeared to play different roles in the pathogenesis of allergic diseases between younger and older ages.

Keywords asthma, atopic dermatitis, IFN- γ , IL-12, monocyte, Th1/Th2

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Introduction

It is well known that infants and young children show a lower capability to mount immune responses as compared with adults, resulting in an increased susceptibility to pathogens. Diminished ability to synthesize some cytokines seems to affect the immune status in the early period of human life [1]. One of these cytokines is IL-12, which is produced by dendritic cells, monocyte-macrophages and other antigen-presenting cells [2]. It induces cytokine secretion, activation of macrophages, and enhancement of cell-mediated cytotoxicity [3]. These diverse functions suggest that IL-12 may play an important role in facilitating cell-mediated immune responses. In addition to its role in the cell-mediated immune responses, the important

function of IL-12 is to control the balance between T helper type-1 (Th1) and Th2 cells by promoting Th1 cell development and enhancing IFN- γ production of those cells [4]. Seemingly supporting the current notion that the dominance of Th2 cells is responsible for the pathogenesis of allergic diseases, studies have demonstrated that a reduced ability to produce IL-12 may contribute to the Th2-dominated situation in allergic diseases such as asthma or atopic dermatitis [5–7]. Although IL-12 production in newborn infants has been observed to be markedly lower than that in adults [8, 9], there are no reports investigating developmental changes in IL-12 production and examining IL-12-producing ability of allergic individuals in the age-dependent manner. In this study, we employed the whole-blood cultures and flow cytometric analysis to compare IL-12-producing ability of monocytes between patients of various ages with allergic diseases and age-matched control subjects. We will show the age-dependent different roles of IL-12 in allergic diseases.

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Subjects and methods

Subjects

To evaluate developmental changes in IL-12-producing ability of monocytes, cord blood samples from 37 non-complicated delivery of full-term pregnancy and 107 control subjects were enrolled. Exclusion criteria included a history of bacterial or viral infection within the previous 2 weeks and other severe diseases. To compare the results of control subjects with those of patients with allergic diseases, 100 patients with atopic asthma and/or atopic dermatitis were enrolled (Table 1). In this study, diagnosis of asthma was empirically defined from recurrent asthmatic symptoms, such as cough, wheezing, or shortness of breath which improved by inhaled β_2 -agonists. Atopic dermatitis was diagnosed according to Hanifin and Rajka's criteria [10]. No patients had oral corticosteroid, and six were treated with inhaled steroid. All the patients were allergic to at least one of common allergens such as house dust mite, food, pet dander, or pollen. The study was conducted according to the ethical standards of Toyama Medical and Pharmaceutical University, which require informed consent from each subject.

Intracellular expression of cytokines in monocytes

The method of whole-blood culture to induce cytokines in monocytes was modified from the previously described procedure [5]. Briefly, 500 μ L of whole blood were diluted 1/2 with RPMI-1640 (Nipro KK, Tokyo, Japan) supplemented with 2 mL L-glutamine, 10% fetal bovine serum (FBS; Equitech-Bio, Inc., Ingram, TX, USA), 2×10^{-5} M 2-mercaptoethanol, 10 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B, and 10 μ g/mL gentamycin (Gibco-BRL, Grand Island, NY, USA). Cells were primed with 1000 U/mL of IFN- γ (Shionogi Co., Tokyo, Japan) for 2 h, then stimulated with 100 ng/mL of lipopolysaccharide (LPS; Sigma, St Louis, MO, USA) in the presence of 3 μ g/mL of brefeldin A (Sigma) for 22 h at 37 °C in 5% CO₂ and 95% air. Intracellular cytokines were stained as previously described [11]. After washing, the stimulated cells were stained with phycoerythrin (PE)-conjugated anti-CD14 mAb (TÜK4, Dako, Kyoto, Japan), then fixed with 4% paraformaldehyde in phosphate-buffered saline

(PBS) at room temperature for 15 min, washed twice in PBS, and permeabilized with 0.1% Triton-X in Tris-buffered saline (TBS) with 1% fetal bovine serum (FBS) and 0.1% sodium azide for 15 min at room temperature. These fixed, permeabilized cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IL-12 mAb (p40/p70, C11.5, PharMingen, San Diego, CA, USA), anti-IL-8 mAb (G265-8, PharMingen), or control IgG (Dako) for 20 min on ice. CD14-positive cells were gated, then these positive cells were subsequently analyzed for the expression of IL-12 or IL-8 on a flow cytometer (EPICS® XL-MCL, Beckman Coulter KK, Tokyo, Japan). To validate the applicability of intracellular staining for cytokines, unstimulated cells from 10 healthy volunteers were stained, and demonstrated no staining for IL-12, when compared with isotypic control.

Statistical analysis

Results are shown as mean $\pm$ SD. Mann-Whitney test was used to determine the significance in differences among groups. Correlation between IL-12-producing ability and serum IgE levels was evaluated by linear regression analysis. *P*-values < 0.05 were considered significant.

Results

Developmental changes of IL-12- and IL-8-producing ability of monocytes

To study cytokine-producing ability of monocytes, we used an intracellular staining method detecting monocytes as CD14-positive cells. We chose the whole-blood culture, because production of IL-12 was low in isolated cells as reported previously [5]. Pre-incubation with IFN- γ was necessary to detect LPS-induced IL-12 synthesis as reported previously [12]. From our preliminary study regarding incubation period, the condition of 22 h with LPS after 2 h priming with IFN- γ was sufficient (data not shown). Figure 1 demonstrated representative examples

Table 1. Subject characteristics

	Age (years)						Total
	CB	0–2	3–6	7–12	13–19	20–40	
Controls							
Male	–	18	9	16	8	12	63
Female	–	8	11	9	5	11	44
Total	34	26	20	25	13	23	107
Patients							
Male	–	13	18	16	6	8	61
Female	–	8	10	5	6	10	39
AD	–	15	11	8	5	11	50
BA	–	2	8	9	4	1	24
AD+BA	–	4	9	4	3	6	26
Total	–	21	28	21	12	18	100

CB, cord blood; AD, atopic dermatitis; BA, bronchial asthma.

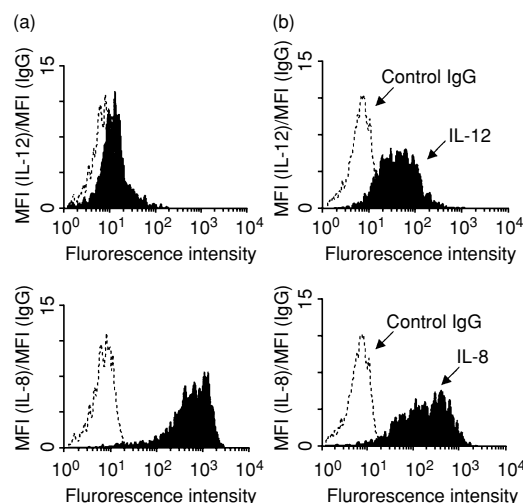


Fig. 1. Histograms of fluorescence intensities for expression of IL-12 and IL-8 in monocytes. Whole-blood cells were stimulated with LPS after 2 h priming with IFN- γ in the presence of brefeldin A for 22 h. Intracellular cytokine expression was analyzed for monocytes gated by CD14-positive on flow cytometer. Representative stainings in a cord blood sample (a) and a 25-year-old male (b) were shown.

of fluorescence intensities of IL-12 and IL-8 production in monocytes from a cord blood sample and an adult healthy subject. IL-12-producing ability of cord blood monocytes seemed to be lower than that of adult ones, whereas IL-8 levels of cord blood samples were rather higher compared with adults. To evaluate if there were developmental changes in cytokine-producing ability, the subjects were divided into five subgroups: infants (0–2 years old), pre-school children (3–6 years old), school-aged children (7–12 years old), teenagers (13–19 years old), and adults (20–40 years old). Cytokine production was expressed as the ratio of mean fluorescence intensity (MFI) with anticytokine antibody to the MFI with control IgG. In the control children, IL-12-producing ability tended to increase gradually with age, and there was a significant correlation between ages of the subjects and their capability to produce IL-12 (Fig. 2a). On the contrary, IL-8 levels of cord blood samples were significantly higher compared with those of other age groups, and there was no significant correlation between IL-8 levels and ages of the subjects except cord blood samples and infant group (Fig. 2b).

Ability of monocytes to produce IL-12 in patients with allergic diseases

We evaluated IL-12-producing ability of patients with allergic diseases in which Th1/Th2 balance skewed toward a Th2-dominant state. There were age-related differences in the capability to synthesize IL-12 between allergic and control groups (Fig. 3). In the school-aged children, teenagers and adults, IL-12-producing ability of the allergic patients were significantly lower than that of age-matched controls ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively). On the contrary, children below 6 years of age with allergic diseases tended to produce more IL-12 compared with age-matched controls. In the infant group, a significantly higher ability to produce IL-12 was observed in patients with allergic diseases compared with controls ($P < 0.01$). According to their IgE CAP-RAST scores against food allergens (egg white, cow milk, soy bean, or wheat), these patients were divided into two subgroups: high (score ≥ 3)

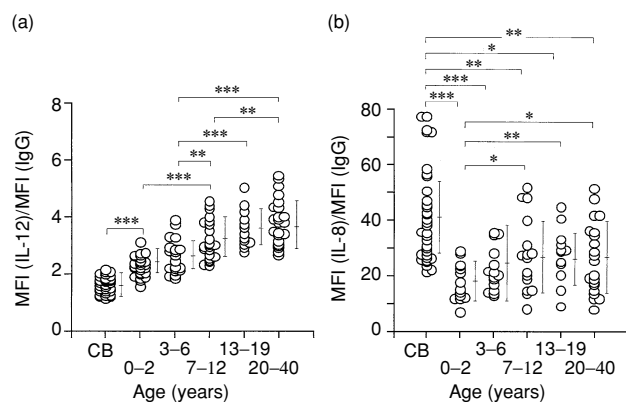


Fig. 2. Developmental changes in monocyte capability to produce IL-12 and IL-8 in control subjects. Cytokine production was expressed as a ratio of MFI with anticytokine Ab to MFI with control IgG. IL-12 (a) and IL-8 (b) production was evaluated in cord blood samples (CB), infants (0–2 years old), pre-school children (3–6 years old), school-aged children (7–12 years old), teenagers (13–19 years old), and adults (20–40 years old). Data points were from individual subjects. Horizontal thick bars represent average of each group, and error bars indicate standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and low (score ≤ 2) responders. IL-12-producing ability of high responders was significantly increased compared with that of low responders and controls (Fig. 4, $P < 0.05$ and $P < 0.001$, respectively). It has been known that serum IgE level is one of markers for Th2-dominated condition. We therefore evaluated correlation between serum IgE levels and IL-12-producing ability in each age group (Fig. 5). A significant inverse relationship was found in the teenage and adult groups ($r = 0.626$, $P < 0.001$), meanwhile there were no significant relationships between serum IgE levels and IL-12-producing ability in the children younger than 13 years old.

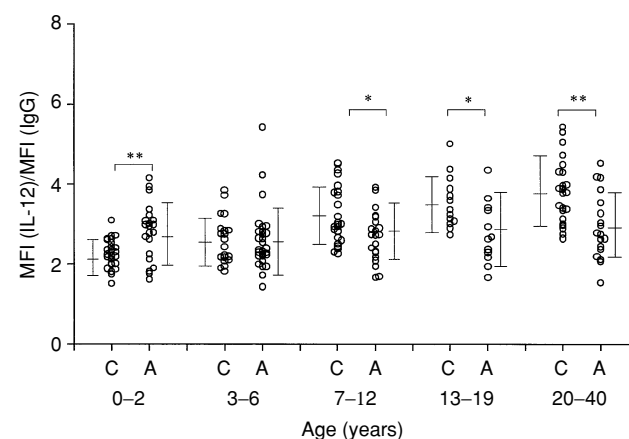


Fig. 3. Difference in IL-12-producing ability between allergic patients (A) and control subjects (C). Whole-blood cells were stimulated with LPS for 22 h after 2 h priming with INF- γ . Production in CD14-positive cells was expressed as ratio of MFI with anti-IL-12 Ab to MFI with control IgG. Data points were from individual subjects. Horizontal thick bars represent average of each group, and error bars indicate standard deviation. * $P < 0.05$; ** $P < 0.01$.

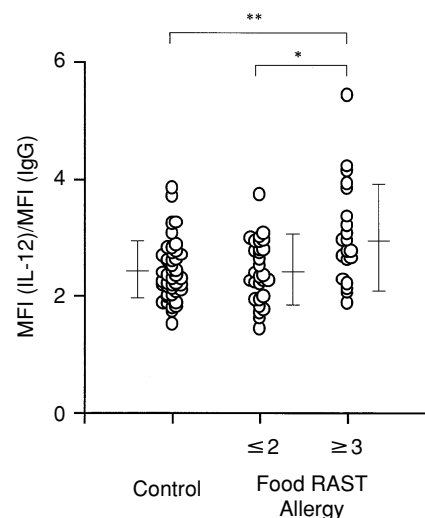


Fig. 4. Association of food allergy with IL-12-producing ability in younger children. The younger children aged 6 years or less with allergic diseases were divided into two subgroups according to their CAP-RAST scores against food allergens (egg white, cow milk, soy bean, or wheat). IL-12 production was expressed as ratio of MFI with anti-IL-12 Ab to MFI with control IgG. Data points were from individual subjects. Horizontal thick bars represent average of each group, and error bars indicate standard deviation. * $P < 0.05$; ** $P < 0.01$.

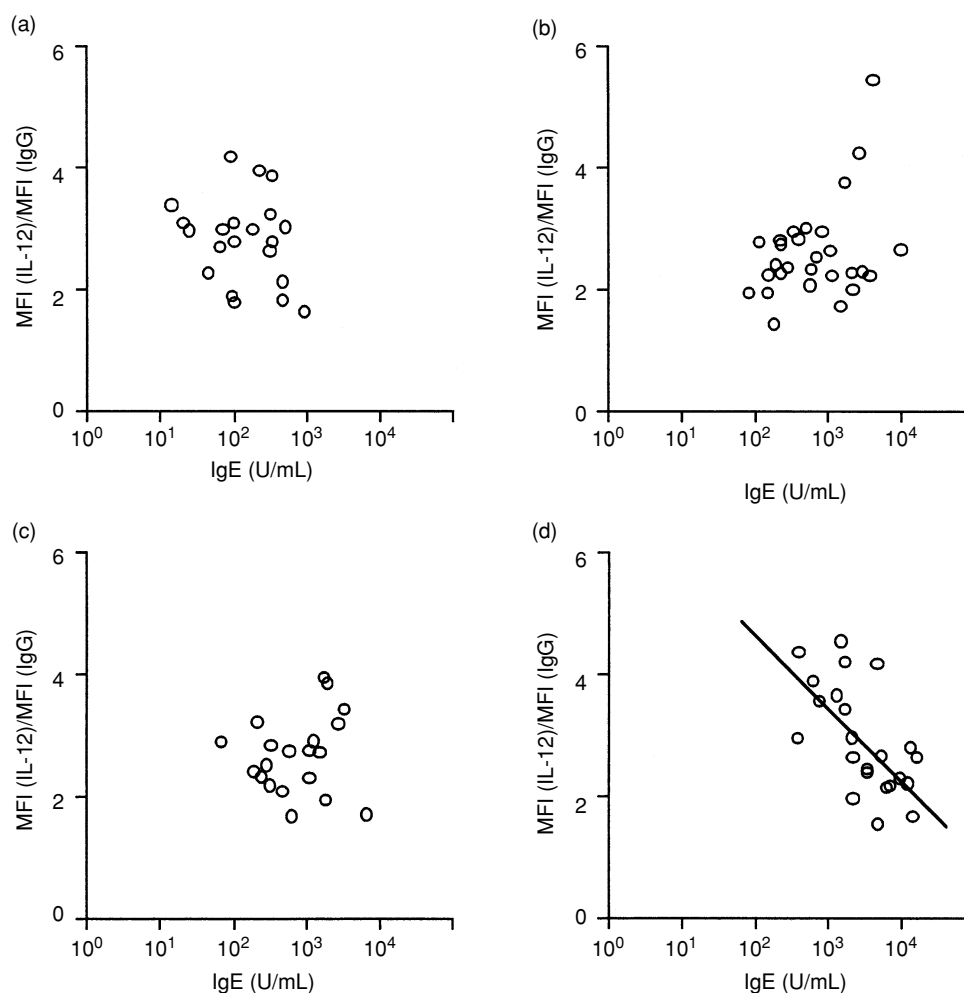


Fig. 5. Correlation between IL-12-producing ability and serum IgE levels. (a) Infants ($n = 21$), (b) pre-school children ($n = 28$), (c) school-aged children ($n = 20$), and (d) teenagers and adults ($n = 23$). Serum IgE level was measured at the same time when IL-12-producing ability was assayed. IL-12 production was expressed as ratio of MFI with anti-IL-12 Ab to MFI with control IgG. Data points were from individual subjects.

Discussion

In the present report, we showed that IL-12-producing ability of monocytes increased with age by using a flow cytometric analysis in whole-blood culture stimulated with LPS. We chose the whole-blood culture because low production of IL-12 in isolated cells was reported [5]. This method might have the disadvantage that a mixture of several kinds of cells obscures the results, especially when culture supernatants were used for assaying cytokine concentration. We therefore analyzed IL-12 production at a single cell level in CD14-positive monocytes. More than 95% of monocytes gated by the forward and right scatters on a flow cytometer were CD14-positive, and there was no significant age-related differences in the frequency and fluorescence intensity of CD14-positive cells (data not shown). These results suggest that the lower ability to produce IL-12 in younger children was due to neither the reduced expression of CD14, the receptor for LPS, nor insufficient number of monocytes. In this study, IL-8 production at a single cell level was also assayed simultaneously. From the result of no age-related difference in IL-8 production, reduced IL-12 synthesis in younger children was not due to a general failure of monocytes to produce cytokines.

IL-12 stimulates proliferation of activated T cells, and plays a pivotal role in the development of Th1 cells by augmenting secretion of IFN- γ , a key cytokine in initiating the defense against extracellular pathogens. IFN- γ has a direct antiviral effect and is particularly important in stimulating the cytolytic activity of natural killer cells and CD8⁺ cytotoxic T cells. Aberle et al. reported that reduced IFN- γ expression is an important factor in the pathogenesis of severe RS virus-induced bronchiolitis in infancy [13]. Consistent with the previous report [7], we found a good correlation between IL-12 production of monocytes and IFN- γ synthesis of CD4⁺ T cells in control subjects (data not shown). Monocytes could regulate helper T cell differentiation toward Th1-type cells through IL-12 production. There have been several reports shown that IL-12-producing ability of cord blood monocytes was decreased compared with adult peripheral monocytes [8, 9]. We found that IL-12-producing ability of monocytes is impaired not only in infancy, but also over the whole childhood compared with adulthood. In control subjects, increasing capability to synthesize IL-12 with age, as demonstrated in this study, may contribute to the maturation of protective immunity.

In addition to its role in the defense against viral infection, IFN- γ inhibits the synthesis of IgE and the differentiation of

precursor cells to Th2 cells. In the context of Th1/Th2 balance, allergic diseases have been well known as a Th2-dominant disorder. When we evaluated IL-12-producing ability of patients with allergic diseases across all age groups, the patients aged 7 years or older showed a significant reduction in monocyte capability to produce IL-12 compared with control subjects. These results are consistent with previously reported data [5–7]. Because it is well documented that IL-12 not only promotes the differentiation toward Th1 cells but also inhibits allergen-induced Th2 cell development [14], the reduced ability to produce IL-12 in the allergic patients might have direct and indirect effects on their Th2-dominated conditions. Consistent with these results, we found a good negative correlation between serum IgE level and IL-12-producing ability in teenagers and adults. In older children, impaired ability of IL-12 production might be associated with persistence of allergic symptoms.

The unexpected finding was that infants and children below 6 years of age with allergic diseases tended to produce more IL-12 compared with age-matched controls. Blanco Quiros et al. reported that serum IL-12 levels from patients with food allergy were higher than those of controls in children younger than 4 years old [15]. Most patients in our infant group were allergic to food antigens. When the subjects were divided into two groups according to their CAP-RAST scores against food allergens, IL-12-producing ability of high responders was increased compared with that of low responders or control subjects. Most of the high responders also had a clear past history of immediate reaction after oral food intake. There is a possibility that food allergy might contribute to this high capability to produce IL-12. It is well known that most children with food allergy outgrow their sensitivity to food during several years, while sensitivity to inhaled allergens like dust mites remains much longer [16]. Human Peyer's patches contain large amounts of IL-12, and T cells isolated from Peyer's patches expressed a Th1-associated chemokine receptor CCR5 in response to dietary antigen [17]. In a murine model, oral IL-12 treatment had therapeutic as well as preventive effect on peanut allergy [18]. Our result of higher capability to produce IL-12 in infants with food allergy suggests that pathogenesis of food allergy might be different from that of allergy to inhaled allergens, and that higher producing ability of IL-12 in this period of age might lead tolerance to food allergens.

Recent findings that p40, one isomeric form of IL-12, is induced without p70 production suggest two molecules might have separate functions [19]. In this study, we used a monoclonal antibody against IL-12 (p40/p70) to detect intracellular IL-12. Therefore we have assayed IL-12 (p40) and IL-12 (p70) separately by using corresponding enzyme-linked immunosorbent assay (ELISA) kits in the supernatants of whole-blood cultured with the same condition of this study. Data from 24 control volunteers demonstrated good correlation between p40 and p70 levels ($r = 0.617$, data not shown). From this result, IL-12 production shown in this study could represent p70 synthesis.

In conclusion, we showed that, in control children, IL-12-producing ability of monocytes was increased with age, and that there were age-dependent differences in IL-12 production between allergic subjects and controls. The recently conducted therapeutic trial of human recombinant IL-12 to asthmatic patients failed to show any significant effects on airway hyper-reactivity or the late asthmatic response [20]. Further studies

regarding regulatory roles of IL-12 in Th1/Th2 balance among various age groups might shed light on future management of allergic diseases.

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