

Age-related changes in intracellular cytokine profiles and Th2 dominance in allergic children

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The unbalanced T helper response has been pointed out in allergic diseases. Especially in childhood, it is important to consider the development of acquired immunity. We investigated the relationship between age and Th1, Th2, Tc1 or Tc2 cells. In addition, Th1, Th2, Tc1 or Tc2 cells in allergic diseases were compared with control subjects. Thirty-four healthy controls (0–40 years old), 200 samples of cord blood, nine patients with atopic dermatitis (AD) (1–3 years old) and five patients with bronchial asthma (BA) (2–6 years old) were studied. Surface staining with CD4, CD8 and intracellular staining with anti-interferon- γ (IFN- γ) and anti-interleukin (IL)-4 were carried out, and analyzed by using flow cytometry. In the healthy controls, the percentages of Th1, Tc1 or Th2 showed positive correlation with age. The absolute numbers of Th1 or Tc1 also correlated with age. Cord blood with a family history of allergic disease showed no significant difference compared to that without a family history. The percentage of Th2 in AD and BA patients was significantly higher than in the age-matched healthy controls. The increase in Th1, Th2 and Tc1 with age might reflect on the development of acquired immunity. Age matching is important when evaluating the cytokine profiles of T cells. In allergic diseases, although cord blood showed a Th1-dominant pattern, it changed to Th2 dominance in childhood, and this may reflect on some genetic background.

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The concepts of Th1 and Th2 have stemmed from the report that the mouse CD4⁺ cell can be divided into two subsets on the basis of their patterns of cytokine production (1). Consequently, the human CD4⁺ cell can also be divided into two subsets (2, 3). Similar subpopulations exist in the CD8⁺ T cell population, and were designated as Tc1 and Tc2 (4, 5). From various subsequent reports, the paradigm of Th1/Th2 came to be applied to allergic diseases such as bronchial asthma (BA) and atopic dermatitis (AD) (6–8).

Furthermore, by using flow cytometry, the profiles of cytokines were studied at the single-

cell level (9–11). To date, various measurements of Th1 and Th2 cells using flow cytometry have been reported, and such measurements have also been performed for allergic diseases (12–18).

Several studies of intracellular cytokine-producing cells in adult allergic diseases have been reported. Jung et al. (12) and Wong et al. (13) reported that Th1 was lower in adult AD patients than in healthy controls, and that Th2 exhibited no significant difference between the two groups.

There are some reports on AD and BA. In the adult type of AD, Nakazawa et al. (14) and Farrell et al. (15) reported that Th1 was lower and Th2 was higher in AD patients than in

healthy controls. In BA of adults, Brightling et al. (16) reported that Th1 and Tc1 were higher in BA patients than in healthy controls, and that Th2 and Tc2 displayed no significant difference between the two groups. In contrast, Schuerwegh et al. (17) reported that the percentages of Th1, Th2, Tc1 and Tc2 showed no significant difference between AD patients and healthy controls or between BA patients and healthy controls.

In children, there is only one study of intracellular cytokine-producing cells (19). In this report only CD3+IL4+ and CD3+IL5+ cells were examined. Especially in childhood, there are only a few reports of intracellular cytokine profiles, and the effect of aging on intracellular cytokine profiles is unclear. In this report we first investigated the relationship between age and Th1, Th2, Tc1 and Tc2 cells. Secondly, Th1, Th2, Tc1 and Tc2 cells in allergic diseases were compared with control subjects.

Patients and methods

Subjects

Thirty-four healthy individuals (0–40 years old) were enrolled as the healthy controls. Nine children (1–3 years old) were selected from 46 AD patients. Five age-matched healthy children were selected as the control group. Five children with BA (2–6 years old) were selected from 30 patients with that allergy. Five age-matched healthy children were selected as the control group. All patients of AD were in mild or moderate condition and had no infectious diseases of the skin. All of them had atopic eczema at the time of blood drawing. All patients of BA were in moderate condition.

To avoid influence by other diseases, the patients with AD were selected as a group that had only AD, without any other allergic disease such as BA or food allergy. Similarly, the BA patients had only BA and were chosen as a group without other allergic diseases such as AD or food allergy.

The diagnosis of AD was made according to Hanifin's and Rajka's minor criteria (20), and that of BA according to the criteria of the American Thoracic Society. The healthy controls had a negative history of atopic disease and their serum immunoglobulin E levels were within normal limits for their age. They were healthy and free of acute infection at the time of testing.

In this study, 151 samples of cord blood were enrolled. The cord blood samples were collected from the umbilical vein. The family histories of doctor-diagnosed allergic diseases (AD, BA,

allergic rhinitis, conjunctivitis, and others) were confirmed by questionnaire. We considered a person who was diagnosed by a doctor as having a positive family history of allergy.

We divided the samples of cord blood into four groups according to the family history of allergic disease. Babies whose both parents had no allergic disease were enrolled as group 1. Babies who had at least one parent with an allergic disease were enrolled as group 2. In addition, for strict comparison, two more groups were selected based on other conditions. Babies in whose family there was no history of allergic disease within the third degree of relationship by blood were enrolled as group 3. Babies whose both parents had allergic diseases were enrolled as group 4.

All samples from healthy controls, allergic patients and cord blood were taken after informed consent was obtained from all subjects or their parents.

Intracellular cytokine analysis

A total of 0.5 ml of heparinized whole blood was added to 0.5 ml of RPMI-1640 medium and incubated with a combination of 25 ng/ml of phorbol myristate acetate and 1 μ g/ml of ionomycin in the presence of 10 μ g/ml of brefeldin A (Sigma, St Louis, MO, USA) and cultured for 4 h at 37°C, in a humidified atmosphere containing 5% CO₂. Surface staining (CD4, CD8) was then carried out at room temperature (RT) for 20 min in the dark. Blood samples were then lysed and fixed in FACS Lysing Solution (BD Biosciences, San Diego, CA, USA) for 10 min at RT. The cells were subsequently resuspended in FACS Permeabilizing Solution (BD Biosciences) for 10 min at RT. Finally, the cells were incubated with monoclonal antibodies of anti- γ -interferon (IFN- γ)-fluorescein isothiocyanate (FITC) (BD Biosciences) or anti-interleukin (IL)-4-phycoerythrin (PE) (BD Biosciences) for 40 min in the dark, and then analyzed by using flow cytometry. The CD8+ cells were divided into CD8^{bright} and CD8^{dull}. CD8^{dull} cells expressed CD56 and CD16 (data not shown), and were considered to be natural killer cells. In contrast, CD8^{bright} cells expressed CD3, but not CD16 or CD56 (data not shown). So, we defined CD8^{bright} cells as CD8-positive T cells.

Percentages and absolute numbers of the T-cell subpopulation

The percentage of IFN- γ -producing cells in a CD4 or CD8-positive cell was considered as Th1% or Tc1%, respectively. The percentage of

IL-4-producing cells in a CD4 or CD8 positive cell was considered as Th2% or Tc2%, respectively. The value of Th1 divided by Th2 is expressed as the Th1/Th2 ratio and that of Tc1 divided by Tc2 is expressed as the Tc1/Tc2 ratio.

The number of leukocytes and the percentage of lymphocytes were measured simultaneously when collecting blood samples for flow cytometry. The absolute numbers of Th1, Th2, Tc1, and Tc2 were calculated from these numbers.

Statistical analysis

The correlations between age and Th1, Th2, Tc1 and Tc2 were determined by using Spearman's rank correlation. The Mann-Whitney *U*-test was used for statistical analysis of allergic patients. The number of days after birth was used as the age for all statistical analysis in order to make strict evaluations. $p < 0.05$ was considered to be statistically significant.

Results

Healthy controls

The representative results of cord blood with negative family history of allergic disease within the third degree of relationship by blood, healthy child, and healthy adult are shown in Fig. 1. Although there is no significant difference between the child and adult in regard to Th2 and Tc2, Th1 and Tc1 of the child and the cord blood clearly showed a low value compared with Th1 and Tc1 of the adult.

The percentage of Th1, Th2, Tc1, and Tc2 for every age is shown in Fig. 2a–d. Thirty-four healthy volunteers were examined. The percent-

age of Th1 and Tc1 showed a strong correlation with age ($r = 0.880$, $p < 0.001$ and $r = 0.799$, $p < 0.001$, respectively). The percentage of Th2 indicated a weak correlation with age ($r = 0.571$, $p < 0.001$). The percentage of Tc2 did not show any correlation with age ($r = 0.233$, $p = 0.191$).

The Th1/Th2 ratio is shown in Fig. 2e, and had a strong correlation with age ($r = 0.555$, $p < 0.001$). In contrast, Tc1/Tc2 (as shown in Fig. 2f) did not correlate with age ($r = 0.134$, $p = 0.456$).

We also examined the absolute numbers of Th1, Tc1, Th2, and Tc2 of 27 healthy individuals (Fig. 3). The absolute numbers of Th1 and Tc1 showed a strong correlation with age ($r = 0.708$, $p < 0.001$ and $r = 0.458$, $p = 0.016$, respectively). The absolute numbers of Th2 and Tc2 showed no correlation with age ($r = -0.004$, $p = 0.983$ and $r = -0.087$, $p = 0.667$, respectively).

Cord blood analysis

The percentage of Th1, Th2, Tc1, and Tc2 was examined from 151 samples. The average [$\pm$ standard deviation (s.d.)] of Th1%, Th2%, Tc1% and Tc2% in cord blood were 0.264 (± 0.266), 0.203 (± 0.299), 0.542 (± 0.666) and 0.025 (± 0.052), respectively.

Group 1 (babies with no allergic disease in either parent) consisted of 32 subjects. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% were 0.247 (± 0.282), 0.243 (± 0.305), 0.700 (± 0.789) and 0.013 (± 0.018), respectively. Group 2 (babies who had at least one parent with an allergic disease) consisted of 80 subjects. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and

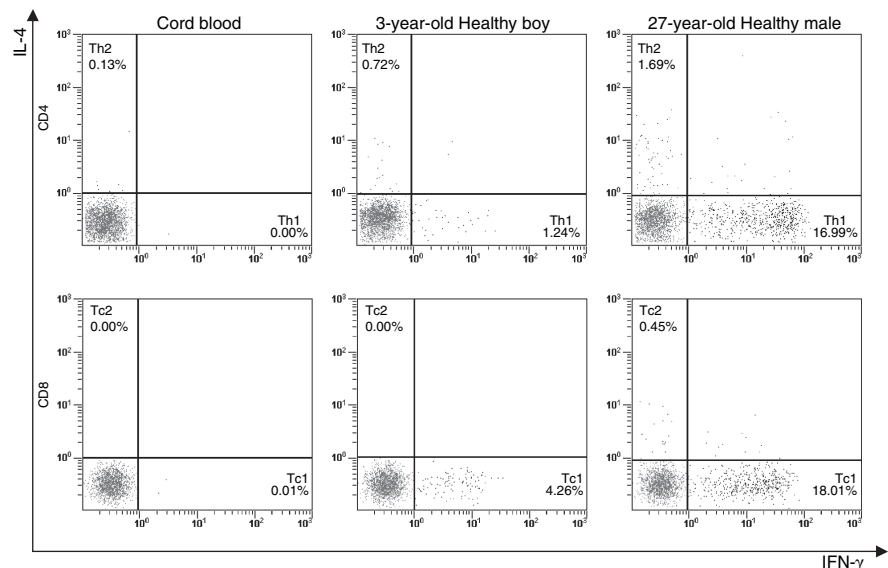


Fig. 1. Representative results of Th1, Th2, Tc1 and Tc2 cells in cord blood, healthy boy and healthy male. This cord blood was taken from a baby in whose family there was no history of allergic disease (atopic dermatitis, bronchial asthma, allergic rhinitis, or other allergic disease) within the third degree of relationship by blood. The Th1, Th2, and Tc1 cells of the cord blood were the lowest in the three samples, and the Th1, Th2, and Tc1 cells of the healthy male were the highest in the three samples.

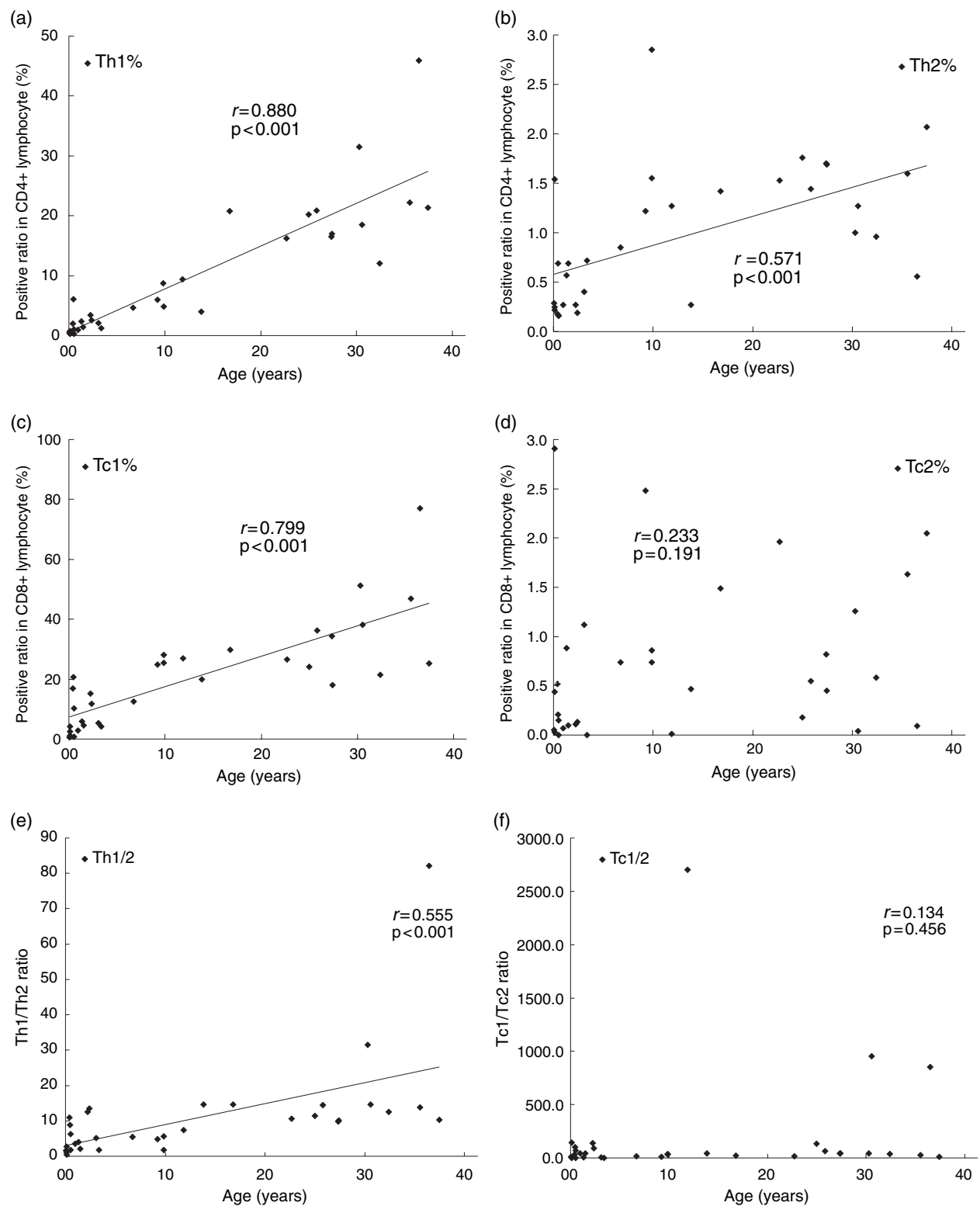


Fig. 2. (a–d) The relationship between age and Th1% (a), Th2% (b), Tc1% (c), and Tc2% (d). The scatter plot of Th1%, Tc1%, and Th2% showed a significant correlation (Th1: $r = 0.880$, $p < 0.001$; Th2: $r = 0.571$, $p < 0.001$; Tc1: $r = 0.799$, $p < 0.001$). The scatter plot of Tc2% showed no significant correlation ($r = 0.233$, $p = 0.191$). (e, f) The relationship between age and the ratio of Th1/Th2 (e) or the ratio of Tc1/Tc2 (f). The scatter plot of Th1/2 showed a significant correlation with age ($r = 0.55485$, $p < 0.001$). The scatter plot of Tc1/Tc2 showed no correlation with age ($r = 0.134$, $p = 0.456$).

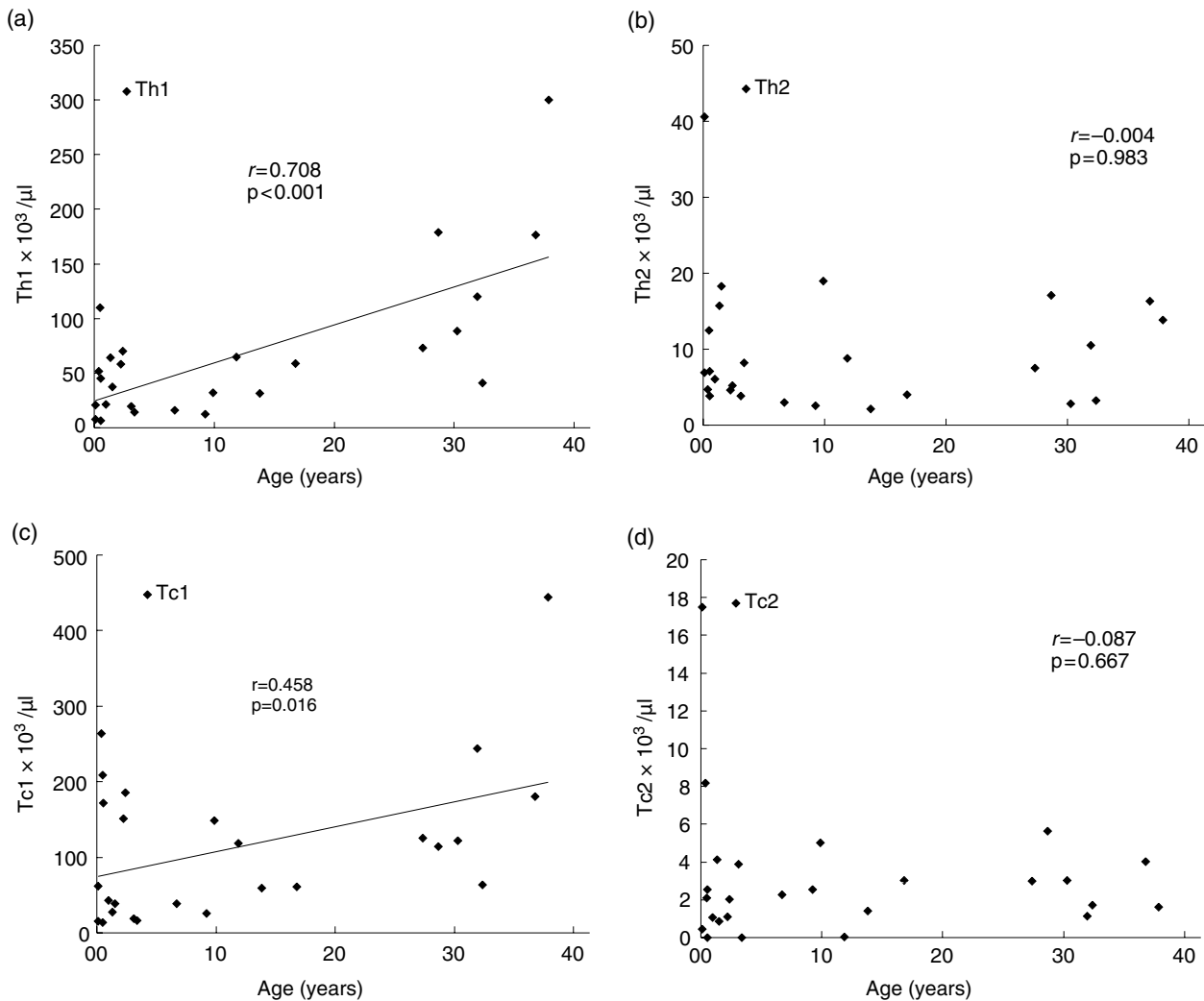


Fig. 3. The relationship between age and the absolute numbers of Th1 (a), Th2 (b), Tc1 (c), and Tc2 (d). The scatter plot of Th1 and Tc1 showed a significant correlation with age (Th1: $r = 0.708$, $p < 0.001$; Tc1: $r = 0.458$, $p = 0.016$). The scatter plot of Th2 and Tc2 showed no significant correlation with age (Th2: $r = -0.004$, $p = 0.983$; Tc2: $r = -0.087$, $p = 0.667$).

Tc2% were $0.286 (\pm 0.239)$, $0.180 (\pm 0.233)$, $0.539 (\pm 0.639)$ and $0.020 (\pm 0.034)$, respectively. Between group 1 and group 2, there were no significant differences in Th1% ($p = 0.208$), Th2% ($p = 0.249$), Tc1% ($p = 0.738$), and Tc2% ($p = 0.750$) (Fig. 4a–d).

Group 3 (babies in whose family there was no history of allergic disease within the third degree of relationship by blood) consisted of 18 subjects. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% were $0.278 (\pm 0.346)$, $0.293 (\pm 0.340)$, $0.714 (\pm 0.842)$ and $0.016 (\pm 0.020)$, respectively. Group 4 (babies whose parents both had allergic diseases) consisted of 21 subjects. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% were $0.330 (\pm 0.282)$, $0.137 (\pm 0.163)$, $0.595 (\pm 0.730)$, and $0.020 (\pm 0.049)$, respectively. Between group 3 and group 4, there were

no significant differences in Th1% ($p = 0.338$), Th2% ($p = 0.067$), Tc1% ($p = 0.888$) or Tc2% ($p = 0.464$) (Fig. 4e–h).

Atopic dermatitis

From the results of the healthy controls, it is thought that age influences Th1, Th2, Tc1, and Tc2. When the percentages of Th1, Th2, Tc1, and Tc2 are compared, groups with a restricted age range should be compared with groups of the same age.

The CD4 or CD8 cells producing cytokines (Th1, Th2, Tc1, and Tc2) in children (1–3 years old) with AD are shown in Fig. 5a–d. Patients with AD consisted of nine subjects, and the healthy controls (controls 1–3) consisted of five patients. The average number of days after birth

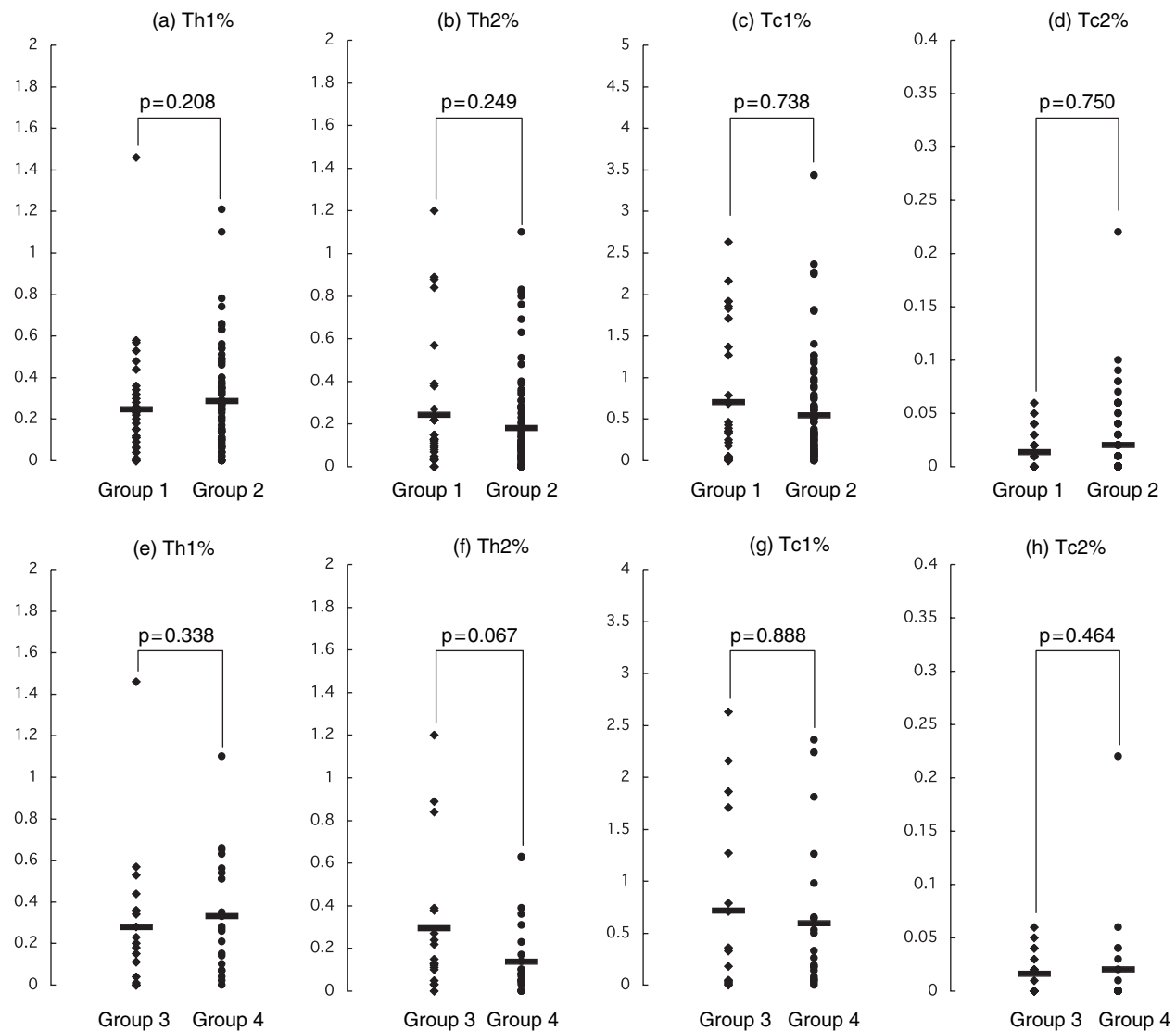


Fig. 4. Th1%, Th2%, Tc1% and Tc2% in cord blood with or without allergic background. Black bar shows the mean value. (a–d) Group 1: Cord blood was taken from babies in whose parents there was no history of allergic disease within the first degree of relationship by blood ($n = 32$). Group 2: Cord blood from babies in whose family there was a history of allergic disease within the first degree of relationship by blood ($n = 80$). In Th1%, Th2%, Tc1%, or Tc2%, no significant differences were shown between group 1 and group 2. (e–h) Group 3: Cord blood was taken from babies in whose parents there was no history of allergic disease within the third degree of relationship by blood ($n = 18$). Group 4: Cord blood from babies whose both parents have allergic disease ($n = 21$). In Th1%, Th2%, Tc1%, or Tc2%, no significant differences were shown between group 3 and group 4.

in AD and controls 1–3 was 809 days (2 years and 2 months old), and 780 days (2 years and 2 months old), respectively. The s.d. values of days after birth in AD and controls 1–3 was 301.96 and 263.31, respectively.

The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% in patients with AD were 3.83 ($\pm$ 2.24), 0.96 ($\pm$ 0.32), 15.14 ($\pm$ 9.00), and 1.24 ($\pm$ 2.00), respectively. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1%, and Tc2% in control subjects were 2.35 ($\pm$ 0.72), 0.42 ($\pm$ 0.21), 8.54 ($\pm$ 4.70), and 0.47 ($\pm$ 0.49), respectively. Th2%

was significantly high in the AD patients compared with the healthy controls ($p = 0.009$). Th1%, Tc1%, and Tc2% showed no significant difference between the healthy controls and the AD patients ($p = 0.125$, 0.125 , and 0.463 , respectively).

The Th1/Th2 ratio and Tc1/Tc2 ratio were also examined. The mean values ($\pm$ s.d.) of the Th1/Th2 ratio and Tc1/Tc2 ratio in patients with AD were 4.50 ($\pm$ 3.73) and 55.37 ($\pm$ 53.92), respectively. The mean values ($\pm$ s.d.) of the Th1/Th2 ratio and Tc1/Tc2 ratio in control

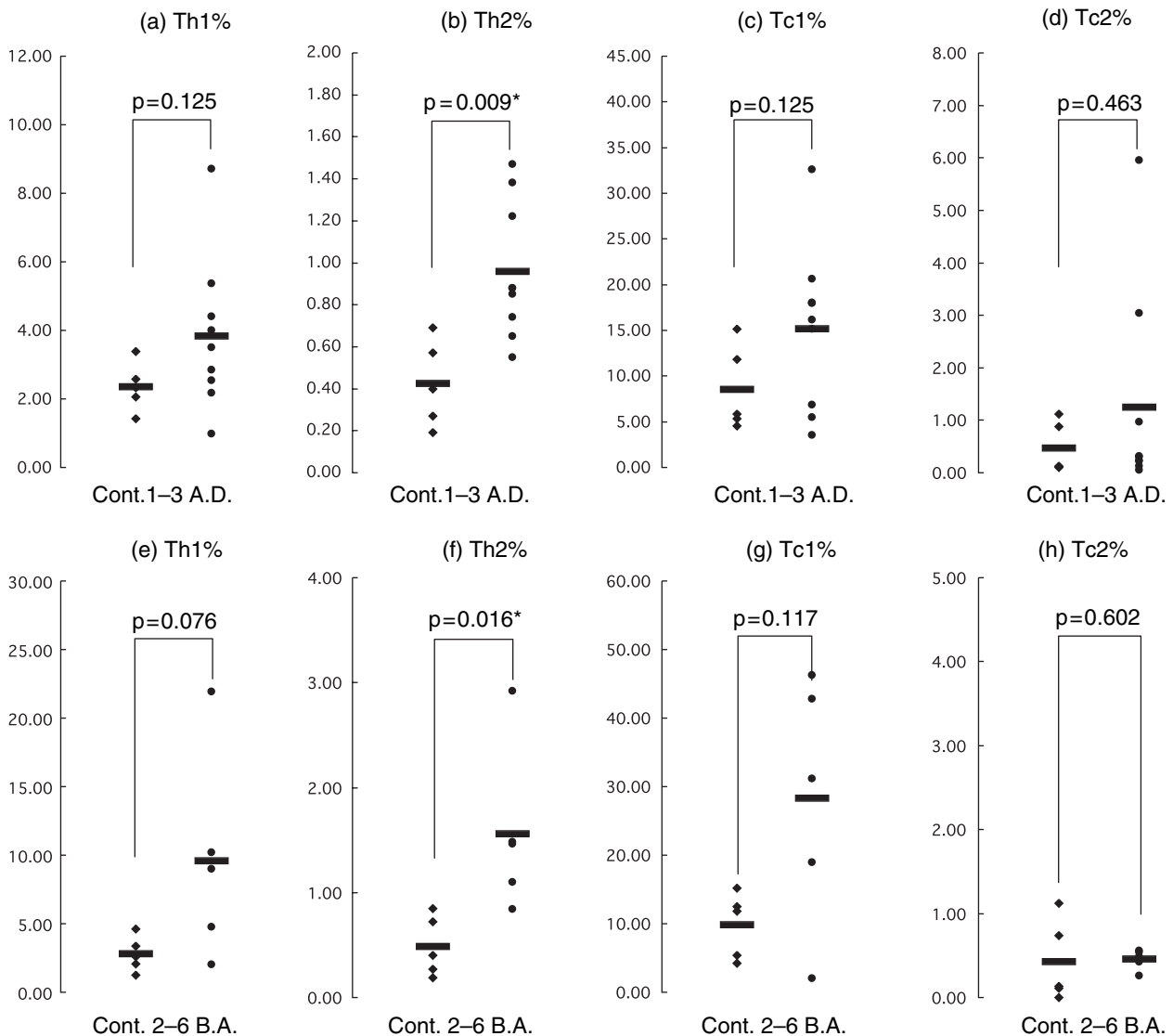


Fig. 5. (a–d) Comparison of Th1%, Th2%, Tc1%, and Tc2% between atopic dermatitis (AD; 1–3 years old) and healthy controls (controls 1–3; 1–3 years old). Black bar shows the mean value. Th2% is significantly higher in patients with AD than in healthy controls ($p = 0.009$). Th1%, Tc1%, and Tc2% showed no significant difference between patients with AD and healthy controls. (e, h) Comparison of Th1%, Th2%, Tc1%, and Tc2% between bronchial asthma (BA) (2–6 years old) patients and healthy controls (controls 2–6; 2–6 years old). Black bar shows the mean value. Th2% is significantly higher in patients with BA than in healthy controls ($p = 0.016$). Th1%, Tc1%, and Tc2% showed no significant difference between BA patients and healthy controls.

subjects were $7.48 (\pm 5.21)$ and $57.08 (\pm 57.21)$, respectively. The Th1/Th2 ratio and Tc1/Tc2 ratio showed no significant difference between the AD patients and the healthy controls ($p = 0.205$ and 0.947 , respectively) (scatter plots are not shown).

Bronchial asthma

The CD4 or CD8 cells producing cytokines in BA patients of later childhood (2–6 years old) are shown in Fig. 5e–h. Five patients with BA were considered as subjects and five as healthy

controls (controls 2–6). The average number of days after birth in BA and controls 2–6 was 1715 days (4 years and 9 months old), and 1316 days (3 years and 7 months old), respectively. The s.d. values of days after birth in BA and controls 2–6 were 403.70 and 675.24, respectively.

The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% in patients with BA were $9.58 (\pm 7.64)$, $1.56 (\pm 0.81)$, $28.26 (\pm 18.14)$ and $0.45 (\pm 0.12)$, respectively. The mean values ($\pm$ s.d.) of Th1%, Th2%, Tc1% and Tc2% in the control subjects were $2.78 (\pm 1.29)$, $0.49 (\pm 0.29)$, 9.82

(± 4.75) and $0.42 (\pm 0.49)$, respectively. Th2% was significantly high in the BA patients compared with the healthy controls ($p = 0.016$). Th1%, Tc1% and Tc2% showed no significant difference between the healthy controls and the BA patients ($p = 0.076$, 0.117 , and 0.602 , respectively).

The Th1/Th2 ratio and Tc1/Tc2 ratio were also examined. The mean values ($\pm$ s.d.) of the Th1/Th2 ratio and Tc1/Tc2 ratio in patients with BA were $7.31 (\pm 7.309)$ and $66.17 (\pm 46.13)$, respectively. The mean values of the Th1/Th2 ratio and Tc1/Tc2 ratio in the control subjects were $7.68 (\pm 5.13)$ and $62.63 (\pm 62.97)$, respectively. The Th1/Th2 ratio and Tc1/Tc2 ratio showed no significant difference between the BA patients and the healthy controls ($p = 0.917$ and 0.807 , respectively) (scatter plots are not shown).

Discussion

One of the major findings in the present study was that the CD4 or CD8 cells producing cytokines increased with age. Especially, the percentages of Th1, Th2 and Tc1 showed a strong correlation with age. It was reported that Th1 and Tc1 cell populations progressively increase with age in healthy controls (21), consistent with our results. In that report, Chipeta et al. showed that Th1 and Tc1 increase with age, and have a strong correlation with the CD45RO surface antigen expression. Further, they showed Th2 and Tc2 in minimal percentages across all age groups. However, our result is that Th2 shows a significant correlation with age though Tc2 shows no correlation with age. Because the percentage of Tc2 is very low, it might be that considerable variation of Tc2 was high, and it may be the reason why Tc2 did not show a correlation with age.

In another report of type 1 and type 2 cytokines in adults, healthy subjects in the 81-year-old age group were significantly higher in Th1, Th2, Tc1, and Tc2 than healthy people in the 21- to 30-year-old age group (22). Our results showing that the ratio of Th1, Th2, Tc1, and Tc2 increases with age might be applicable even in people who are older than those in our study.

The proportion of CD4 and CD8 in lymphocytes changes according to age (23). Therefore, there is a possibility that an increase in Th1% is influenced by the percentage of CD4. The absolute numbers of Th1 and Tc1 correlate with age, whereas the absolute numbers of Th2 and Tc2 do not correlate with age. It is paradoxical that the

absolute number of Th2 does not correlate with age, whereas the percentage of Th2 significantly correlates with age, which may suggest that the Th2 correlation percentage is influenced by the percentage of CD4.

As shown in the healthy controls, cytokine-producing T cells were influenced by age. We investigated the T-cell subpopulation of cord blood, as the immune feature of cord blood is assumed to be minimally influenced by the environment. The averages of the percentages of Th1, Th2, Tc1, and Tc2 of cord blood were very low and were plotted at the point where the percentages of Th1, Th2, and Tc1 correlated with age. In previous reports (21, 24, 25), the CD4 or CD8 cells producing cytokines of cord blood showed a similar tendency. To investigate the influence of the allergic background, we compared the percentages of cytokine-producing T cells with or without a family history of allergy. There is no significant difference between group 3 and group 4, which suggests that the percentages of the CD4 or CD8 cells producing cytokines were unrelated to the allergic background. In one report, only CD4+IL-13+ cells in cord blood showed a relation with the allergic background, not CD4+IFN- γ + or CD4+IL-4+ cells (24).

Our results of healthy controls and cord blood suggest that age-matching is very important when comparing intracellular cytokine-producing cells. Therefore, we compared healthy people and those with allergic diseases. In addition, the peak prevalence of childhood AD is lower than that of childhood BA (26). Therefore, we carefully selected some groups with a restricted age range and compared them. First, we compared AD and healthy controls, and we limited the age from 1 to 3 years. The percentage of Th2 was the only factor that was significantly different between the two groups. Secondly, we compared BA patients and healthy controls, and we limited the age from 2 to 6 years. Here too the percentage of Th2 was the only value which was different between the two groups.

Several studies of intracellular cytokine-producing cells in adult allergic diseases have been reported. However, in a study of children, Krug et al. (19) reported that CD3+IL4+ cells and CD3+IL5+ cells were significantly higher in atopic asthmatic children (mean age: 9.9 years old) than in healthy controls (mean age: 9.4 years old). There was no significant difference between atopic asthmatic adults (mean age: 36 years old) and healthy controls (mean age: 33 years old). In their report, IFN- γ -producing cells were not studied.

In summary, intracellular cytokine profiles of T cells, especially IFN- γ -producing cells, increase according to age. In cord blood, there was no significant difference between babies with and without an allergic background. In children with allergic diseases, the percentage of Th2 was significantly higher than in healthy people when we carefully selected age-matched groups.

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