

Age-related changes in blood lymphocyte subsets of Chinese children

Lin SC, Chou CC, Tsai MJ, Wu KH, Huang MT, Wang LH, Chiang BL.
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Pediatr Allergy Immunol: 1998; 9: 215–220. © Munksgaard, 1998.

Background. Flow cytometric analysis of major lymphocyte populations and their subsets reveals age-related changes in the human cellular immune system.

Subjects and methods. Immunophenotypic markers were evaluated in 136 healthy pediatric subjects divided into groups of newborn infants (cord blood), children aged 1 to 2 years, 2 to 5 years, and 6 to 15 years.

Results. The percentage of T cells increased gradually with age and the evolution of the percentage of B and NK cells was found to be variable. The percentage of CD4⁺ cells remains relatively unchanged from infancy to adolescence, but the percentage of CD8⁺ T cells was lowest at birth and reached maximal levels in the one to two year-old period. The percentage of naïve T cells declined with time, but the percentage of memory T cells increased with age. Similar trends were seen in T-cell receptor $\alpha\beta$ - and $\gamma\delta$ -bearing T cells. The percentage of CD11b⁺CD8⁺ T cells increased gradually from birth and reached maximal levels from 6 to 15 years old. The expression of the activation markers CD25 and HLA-DR on CD4⁺ T cells increased with age. The percentage of CD16⁺CD56⁺ NK cells declined with age, but the evolution of the percentage of CD16⁺CD56⁺ NK cells was variable. The fraction of B cells that expressed CD5 was high at birth (72.9%) and was highest in one to two year olds (73.1%), then declined steadily over time. The CD23 antigen was expressed on 41.9% of B cells at birth and 68.6% during the first to second year, then declined steadily with age.

Conclusion. These data may serve as a reference range for studies of Chinese pediatric subjects.

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Key words: flow cytometry; lymphocyte subpopulation, reference range; Chinese children

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Accepted the 1 October 1998

It has been suggested that the human immune system is functionally less mature at birth and within the first years of life undergoes a process of sequential development that is both programmed genetically and stimulated externally by antigen exposure (1, 2). The increasing interest in immunological studies accentuates the need for a more rigorous understanding of the normal sequence of development in the cellular components of the immune system. For example, in the management of pediatric human immunodeficiency virus disease and in the diagnosis of DiGeorge syndrome, T cell enumeration plays a vital role and extrapolation from adult normal range data has been misleading. The recent application of flow cytometric immunophenotyping analysis in human lymphocytes could provide a reliable data reference base in healthy children.

Few foreign studies have systematically examined lymphocytes to observe immunophenotypic changes from birth to adulthood (3–6). There is no detailed lymphocyte subset data for Chinese chil-

dren and the purpose of this study is to establish such a database for further study.

Subjects and Methods

Subjects

The study population consisted of 136 healthy newborns and children. Sixty samples were obtained from the cord blood of babies born in the National Taiwan University Hospital. All newborn infants were delivered normally at term and had no evidence of infection or congenital anomaly. Other pediatric blood samples were obtained from healthy volunteers; all samples were obtained with parental consent. Specifically, samples from children were excluded if they had evidence of an infection or immunological disorder, or were taking concomitant medications.

Subjects were grouped into four age categories: Group 1, newborn infants (cord blood) (n = 60);

Group 2, aged 1 to 2 years (n = 40); Group 3, aged 2 through 5 years (n = 22); Group 4, aged 6 through 15 years (n = 14). The sex ratio was 1:1 in each age category.

Sample preparation and analysis

Cord and peripheral venous blood samples were obtained in collection tubes containing ethylenediaminetetraacetic acid and stored at room temperature for no longer than 8 hours before staining. Two-color and three-color immunophenotyping of lymphocytes was performed on whole blood with matched combinations of murine monoclonal antibodies directly conjugated to fluorescein isothiocyanate, phycoerythrin, or cy-chrome. For each test sample, 50 µl of blood were stained with 10 µl of each monoclonal antibody for 30 minutes at 4°C. This was followed by the lysis of red blood cells with FACS® lysing buffer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) as directed by the manufacturer. All samples were then washed thoroughly in phosphate-buffered saline and analyzed using a FACScan flow cytometer, calibrated with CaliBRITE Beads and AutoCOMP software; results of phenotypic analysis were obtained with SimulSET software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). The panel of monoclonal antibodies, with their appropriate CD (clusters of differentiation) designations, is listed in Table 1.

A complete blood count including automated differential counts was performed with a Technicon H2 (Technicon, Basingstoke, UK). Absolute counts were derived by using the following formula:

Absolute counts = WBC (cells/mm³) × % lymphocytes × % antigen positive.

Lymphocyte subset identification

T cells were defined as those cells expressing the CD3 antigen, and B cells were defined as those cells

Table 1. Combinations of monoclonal antibodies used in the two- and three-color immunofluorescence assays

	FITC	PE	Cy-chrome
T-cell subsets	TCR-αβ	TCR-γδ	CD3
	CD45RA	CD4	
	CD11b	CD8	
	CD45RA	HLA-DR	CD4
B-cell subsets	CD45RA	CD25	CD4
	CD5	CD20	
	CD23	CD20	
NK-cell subsets	CD16	CD56	CD3

FITC: fluorescein isothiocyanate, PE: phycoerythrin, TCR: T-cell receptor, NK: natural killer

Table 2. Changes in major lymphocyte populations

	Cord Blood (n = 60)	1–2 years (n = 40)	2–5 years (n = 22)	6–15 years (n = 14)
T cell (%)	62.1 (14.4)	64.9 (7.0)	65.4 (8.2)	67.1 (9.5)
Absolute count	3398 (760)	2661 (287)	2366 (294)	1593 (468)
B cell (%)	17.1 (9.0)	25.1 (6.9)	23.1 (5.3)	16.8 (2.9)
Absolute count	905 (470)	1291 (269)	803 (125)	386 (638)
NK cell (%)	19.2 (11.5)	12.8 (7.6)	14.7 (10.1)	20.3 (12.9)
Absolute count	1039 (615)	525 (317)	504 (371)	487 (271)

Values are expressed as mean (standard deviation)

expressing CD20. Natural killer (NK) cells are defined as CD3⁺ lymphocytes which express CD16, CD56, or both. In all cases, the sum of lymphocyte lineage percentages (%T + %B + %NK) was 100% ± 5% in each age group. The calculation of this “lymphosum” is an important quality control indicator for all lymphocyte subset determinations by flow cytometry.

Results

Major lymphocyte subpopulations

The absolute counts and percentage of T cells, B cells and NK cells are presented as Table 2 and Figure 1. Both the absolute T-cell and NK-cell counts decreased from infancy to adolescence; however, the percentage of T cells increased gradually with age and the evolution of the percentage of NK cells was variable. The absolute B-cell count increased from birth to one to two years old then declined with age. A similar condition was seen in B-cell percentage. The dissociation between trends in absolute counts and percentage of T and NK cells emphasizes the importance of lymphocyte subsets as a fraction of the total pool. This allows direct comparisons of the relative proportions of major lymphocyte

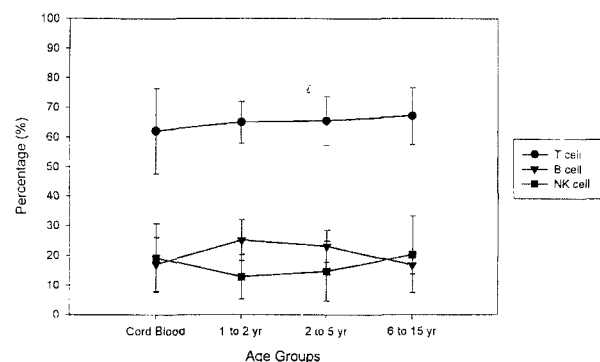


Fig. 1. Changes in major lymphocyte populations

Table 3. T-cell subset analysis in Chinese children

	Cord Blood (n = 60)	1–2 years (n = 40)	2–5 years (n = 22)	6–15 years (n = 14)
T cell (%)	62.1 (14.4)	64.9 (7.0)	65.4 (8.2)	67.1 (9.5)
TCR $\alpha\beta$ in CD3*	61.7 (14.2)	57.5 (7.1)	59.0 (12.1)	47.9 (18.1)
TCR $\gamma\delta$ in CD3*	1.4 (0.6)	6.7 (3.4)	6.4 (2.2)	8.4 (4.0)
CD4 T cell (%)	47.7 (12.6)	48.5 (7.2)	44.6 (10.1)	45.8 (15.2)
CD45RA ⁺ in CD4*	6.6 (3.4)	17.3 (6.0)	23.5 (8.1)	27.7 (9.2)
CD45RA ⁺ in CD4*	93.3 (3.4)	82.7 (6.1)	76.5 (7.4)	72.3 (9.2)
CD8 T cell (%)	15.3 (5.7)	29.8 (6.2)	21.1 (6.0)	23.2 (5.2)
CD11b ⁺ CD8 (%)	13.3 (5.6)	20.0 (6.2)	19.0 (5.8)	18.4 (6.8)
CD11b ⁺ CD8 (%)	1.4 (0.8)	1.3 (0.9)	2.4 (2.2)	4.9 (3.4)

Values are expressed as mean (standard deviation), *: proportion

lineages between age groups in which absolute counts may differ markedly.

T-cell differentiation and activation (Tables 3 and 4)

The percentage of CD4⁺ T cells remained relatively unchanged from infancy to adolescence, but the percentage of CD8⁺ T cells was lowest at birth and reached maximal level in the one to two year-old period (Fig. 2).

At birth nearly all CD4 cells expressed CD45RA, a marker associated with virgin, non-antigen-primed T cells (CD3⁺CD4⁺CD45RA⁺ cell, thus designated as “naïve” T cells). The expression of CD45RA declined with time, but the percentage of CD45RA⁺CD4 cells (CD3⁺CD4⁺CD45RA⁺ cells, designated as “memory” T cells) increased with age (Fig. 3). The same trends were seen in T-cell receptor $\alpha\beta$ - and $\gamma\delta$ -bearing T cells (Fig. 4). The percentage of CD11b⁺CD8⁺ T cells increased gradually from birth and reached maximal level from 6 to 15 years old.

The percentage of CD4⁺ T cells expressing the activation markers HLA-DR and CD25 was observed to be lowest at birth and to increase with age. HLA-DR⁺CD4⁺ T cells reached maximal levels from one to two years old and CD25⁺CD4⁺ T cells likewise at adolescence.

Table 4. Memory T cell subsets analysis in Chinese children

	Cord Blood (n = 60)	1–2 years (n = 40)	2–5 years (n = 22)	6–15 years (n = 14)
CD4 T cell (%)	47.7 (12.6)	48.5 (7.2)	44.6 (10.1)	45.8 (15.2)
CD45RA ⁺ in CD4*	6.6 (3.4)	17.3 (6.0)	23.5 (8.1)	27.7 (9.2)
HLA-DR in CD4 (%)	2.1 (1.4)	14.5 (4.8)	13.2 (3.9)	11.5 (3.5)
HLA-DR in CD45RA ⁺ CD4*	3.7 (6.9)	29.2 (8.6)	25.2 (8.0)	24.8 (11.0)
HLA-DR in CD45RA ⁺ CD4*	1.9 (1.6)	11.8 (5.1)	9.4 (4.1)	7.7 (3.7)
CD25 in CD4 (%)	8.2 (8.2)	11.8 (2.3)	13.9 (3.1)	15.7 (4.1)
CD25 in CD45RA ⁺ CD4*	36.4 (35.8)	30.3 (8.3)	34.2 (7.7)	36.5 (9.3)
CD25 in CD45RA ⁺ CD4*	6.3 (6.3)	8.6 (2.0)	9.2 (3.1)	8.1 (3.3)

Values are expressed as mean (standard deviation), *: proportion

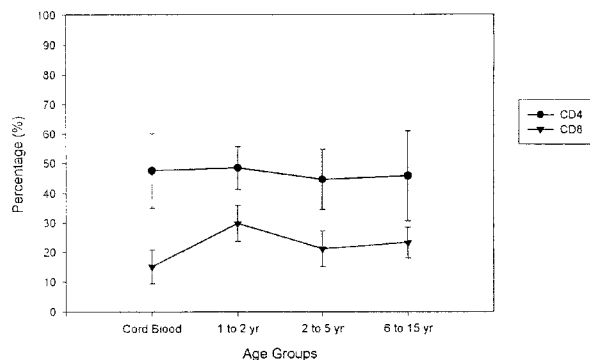


Fig. 2. CD4 and CD8 T cell development in Chinese children

B cell subsets (Table 5)

The fraction of B cells that expressed CD5 was high at birth (72.9%) and was highest from one to two years old (73.1%), then declined steadily over time. In contrast, the CD23 antigen was expressed on 41.9% of B cells at birth and 68.6% from one to two years old, then declined steadily with age.

NK cell subsets (Table 6)

The NK cells were defined as CD3⁺ lymphocytes that expressed CD16, CD56 or both. The percentage of CD16⁺CD56⁺ NK cells declined with age but the evolution of the percentage of CD16⁺CD56⁺ NK cells was variable.

Discussion

It has been reported that the total T-cell, B-cell and NK cell counts decline with age. Moreover, despite the decline in absolute counts, the relative proportions of lymphocyte subsets vary, and T-cell percentages, both in CD4⁺ and CD8⁺ subsets, progressively increase (6, 7). In this study, similar declines were noted in T-cell and NK cell absolute counts but the absolute B-cell count increased from birth to

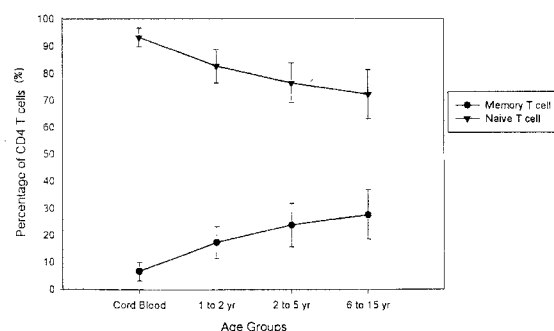


Fig. 3. Memory and naïve T cell development in Chinese children

Table 5. B-cell subsets analysis in Chinese children

	Cord Blood (n = 60)	1-2 years (n = 40)	2-5 years (n = 22)	6-15 years (n = 14)
CD 20 B cell (%)	17.1 (9.0)	25.1 (6.9)	23.1 (5.3)	16.8 (2.9)
CD5 in CD 20 B cell*	72.9 (10.6)	73.1 (12.2)	66.2 (8.4)	54.4 (9.9)
CD23 in CD 20 B cell*	41.9 (15.1)	68.6 (11.3)	57.4 (9.8)	52.2 (10.4)

Values are expressed as mean (standard deviation), *: proportion

age one to two, then declined with age. A similar increase was noted in the percentage of lymphocyte subsets in T cells but not in CD4⁺ and CD8⁺ subsets. Such variations may be the result of race difference, different grouping methods in age categories and other as yet undefined factors.

The CD45RA marker is expressed on 93.3% of CD4⁺ cord blood lymphocytes, but the fraction of CD45RA⁺/CD4⁺ cells decreased significantly with age, a finding confirmed by the observations of other groups (4, 8, 9). Also, the fraction of CD45RA⁺/CD4⁺ cells was noted to increase significantly with age. This is consistent with the hypothesis that CD45RA⁺/CD4⁺ cells are virgin, antigenically unprimed T cells which evolve into CD45RA⁻/CD4⁺ cells, presumably as the result of antigen exposure (10).

HLA-DR is a MHC class II antigen that is expressed as a lineage marker on B cells but as an activation marker on T cells. In the newborn infant, activated T cells, as measured by the percentages of CD25 and HLA-DR on CD4⁺ cord blood cells, were 8.2% and 2.1%, respectively. Both activation markers increased significantly across age groups, a finding consistent with other authors' reports (6, 7, 9). In HIV infection, fewer than 5% of CD4⁺ cells coexpress HLA-DR at any stage of HIV infection (11). The functional role of the HLA-DR⁺ T cells in both normal subjects and HIV patients is unclear, though there is some evidence that it marks a T cell primed for cytolytic activity (12).

CD11b is an adhesion molecule expressed mainly on monocytes, neutrophils, and NK cells and it con-

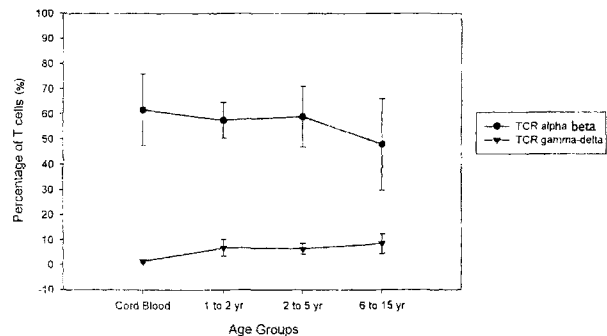


Fig. 4. T cell receptor development in Chinese children

tributes to cellular adhesion and phagocytosis. CD11b also plays a role as the type 3 complement receptor which binds to inactivated C3b. In addition, intercellular adhesion molecule-1 (ICAM-1), fibrinogen and coagulant factor X are also the natural ligands for CD11b. The function of CD11b⁺ T cells also has not been exactly defined. It was suggested that CD11b⁺CD8⁺ T-cell subset contained suppressor T cells, but not cytotoxic T cells (13). CD11b⁺ clones of either CD4⁺ or CD8⁺ T-cell subsets did show suppressor T-cell activity (14, 15). In our study, the percentage of CD11b⁺CD8⁺ T cells increased gradually from birth and reached maximal level from 6 to 15 years old. The result is consistent with an earlier report showing an age-dependent increase in the CD11b⁺CD8⁺ T-cell population (16).

The first T cells appearing during embryonic development carry $\gamma\delta$ T-cell receptors. Later in development, T cells are produced continuously and T cell receptor $\alpha\beta$ -bearing T cells predominate. It has been reported that $\gamma\delta$ T cells increase with age in the peripheral blood, peaking at around 10 years of age (17). Another study pointed out that $\gamma\delta$ T cells continued to increase with age until at least 25 years old (9) and a similar trend was noted here.

The CD5 antigen was detected on the majority of B cells in cord blood, a finding that agrees with previous reports (6, 9, 18). Further, CD5⁺ B cells account for the major B cell subset in fetal lymph nodes, spleen, and liver (19, 20). This population predominates in B-cell chronic lymphocytic leukemia (21) and in certain autoimmune diseases, especially rheumatoid arthritis (18). Results of functional studies suggest that the CD5⁺ population is responsible for the production of many autoantibodies and low affinity polyreactive antibodies (22). The decline of CD5 expression appears to be an important maturational characteristic of circulating peripheral human B cells.

CD23 appears in the early phase of B-cell activation and is considered to be a B-cell differentiation antigen (23). It also serves as an IgE receptor that traps antigens on B cells for internalization, process-

Table 6. NK-cell subset analysis in Chinese children

	Cord Blood (n = 60)	1-2 years (n = 40)	2-5 years (n = 22)	6-15 years (n = 14)
CD16 ⁺ CD56 ⁺ CD3 ⁺ -NK cells (%)	19.2 (11.5)	12.8 (7.6)	14.7 (10.1)	20.3 (12.9)
CD16 ⁺ CD56 ⁺ in NK cells*	12.7 (10.1)	8.7 (5.0)	8.1 (6.4)	5.2 (3.3)
CD16 ⁺ CD56 ⁺ in NK cells*	33.2 (25.2)	21.7 (12.7)	22.1 (14.1)	35.3 (29.1)
CD16 ⁺ CD56 ⁺ in NK cells*	54.1 (21.9)	69.6 (11.6)	63.2 (16.4)	59.4 (29.8)

Values are expressed as mean (standard deviation), *: proportion

ing and presentation to T cells. Unprotected CD23 is cleaved from the surface of cells by endogenous protease, and the soluble fragments induce the growth of not only B cells but also T cells and myeloid cells (24). Reports about the age-related changes of CD23+ B cells percentage are variable (6, 9), but all showed a trend toward increase across age groups. Our study result also shows the same pattern. The CD23 antigen was expressed on 41.9% of B cells at birth and 68.6% from one to two years old, then declined steadily with age. It is likely that its increase results from continuous antigenic exposure during aging (23).

Little is known about the ontogenic development and maturation pathways of human NK cells. It has been postulated that CD16⁺CD56⁺ cells are precursors of CD16⁺CD56⁺ NK cells (25–27). However, Gaddy and Broxmeyer reported that cord blood CD16⁺CD56⁺ cells with low lytic activity acquired adult-like lytic activity and surface antigen phenotype (CD16⁺CD56⁺ or CD16⁺CD56⁺ NK cells) upon culture with certain cytokines. Thus, CD16⁺CD56⁺ NK cells are implicated as possible precursors of mature NK cells (28). In our study, the decline in percentage of CD16⁺CD56⁺ NK cells with age may reflect the process of maturation.

The data showed that the percentage of NK cells in peripheral blood increased over time, except that the percentage was as high as 19.2% at birth. An increase in NK percentages from infancy to adulthood was absent in studies involving Caucasian populations (6, 9) but has been reported by a study of Asian subjects (29). This finding is consistent with the report of higher NK cell percentages found in Chinese blood donors than in Caucasian blood donors (30).

In conclusion, it was shown that there are age-related changes in the lymphocyte subsets in normal Chinese children. When evaluating the immune status of children, consideration of age-related changes is essential and the findings of this study are important for interpreting changes in various diseases.

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