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Longitudinal Follow-up of Lymphocyte Subsets during the First Year of Life

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SUMMARY **A longitudinal study of lymphocyte subsets during infancy was evaluated by using the flow cytometric immunophenotyping method. Two hundred and thirteen blood samples were obtained from 92 healthy, full-term infants of the following ages: 1-7 days old (n = 43), 3 months old (n = 55), 6 months old (n = 57) and 11 months old (n = 58). The absolute numbers of $CD3^+$ and $CD3^+/CD4^+$ T lymphocytes increased from birth to 3 months of age, and remained stable thereafter. The absolute number of $CD3^+/CD8^+$ T lymphocytes increased from birth to 11 months of age. The absolute number of $CD19^+$ B lymphocytes and NK cells increased rapidly (3 months) after birth and continued to increase throughout the study period. However, the changes in the relative counts of lymphocyte subsets did not always correspond with the changes in their absolute numbers. These results demonstrate the age-related changes in lymphocyte subpopulations and provide reference ranges for lymphocyte subsets during infancy.**

Analysis of lymphocyte subsets of peripheral blood can be used to evaluate the immunologic status or provide supportive evidence for the diagnosis of immunologic disorders such as DiGeorge syndrome, adenosine deaminase deficiency, X-linked agammaglobulinemia, and other forms of immunodeficiency. The immune system of the human newborns, while anatomically intact, is antigenically less experienced than that of adults.[1] During the first year of life, the immune system encounters many new antigens, which together induce massive activation, proliferation, and maturation of immune effector cells.[2,3] Therefore, evaluation of changes in the lymphocyte subsets during the first year of life is very important.

Several studies have examined lymphocyte subsets in healthy children.[2-12] However, limited numbers of blood samples from infants of a broad age range in the first year of life were analyzed in most of those reports,[2-6] and only one study determined the age-matched reference values for blood lymphocyte immunophenotyping during the first year of life.[2] Most of the previous reports used cord blood rather than blood samples from newborn infants to identify the lymphocyte subsets in the newborn period.[2,7,8,11] Only three studies reported the reference ranges for lymphocyte subsets in non-infected term infants during the first week of life.[4,13,14] To our best knowledge, there is only one prospectively longitudinal study of absolute counts of lymphocyte subpopulations in 10 children from birth to one year of age.[3]

In this study, we demonstrate the changes of blood lymphocyte subsets in infants during the first year of life, determined by phenotypic analysis of 213 blood samples from 92 healthy full-term infants, including 43 blood samples taken during the first week of life. We also establish the reference ranges of lymphocyte subpopulations during the first year of life.

SUBJECTS AND METHODS

Subjects

Full-term infants with a gestational age (GA) of 38-42 weeks at birth who were delivered at National Taiwan University Hospital, Taipei, from September 1996 through October 1997 were eligible for this study. Only those who were

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apparently healthy and free of major clinical diseases were included. Infants with evidence of infection, or immunological disorders, or who were taking medications were excluded. Informed consent was obtained from the parents of all participants.

Methods

Blood samples were drawn at the following ages: 1-7 days, 3 months, 6 months, and 11 months. Peripheral blood samples were collected in tubes containing EDTA and stored at room temperature for no longer than 8 hours before staining. Fifty microliter aliquots of blood from each sample were incubated with 10 µl of each monoclonal antibody for 30 minutes at room temperature in the dark. This was followed by lysis of red cells with FACS lysis buffer (Becton Dickinson, Mountain View, CA, USA), according to the manufacturers instructions. The samples were then washed thoroughly in phosphate-buffered saline and analyzed by two-color and three-color flow cytometry using the FACScan flow cytometer (Becton Dickinson), calibrated with CaliBRITE Beads and AutoCOMP software; the results of phenotypic analysis were obtained with SimulSET software (Becton Dickinson, Mountain View, CA). Background staining was carried out by using an unrelated fluorescein-labeled mouse monoclonal antibody of the same isotype. The fluorescein-labeled monoclonal antibodies were CD3, CD4, CD8, CD16-CD56, and CD19 (Becton Dickinson). The fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated monoclonal antibody pairs were as follows: CD3/CD19, CD3/CD16-CD56, CD45RA/CD4, CD45RO/CD4 and CD3/CD8, respectively. The results are expressed as percentages of total lymphocytes.

Complete blood counts, including automated differential counts, were determined with a cell counter (Sysmex NE9000 Cell Counter, Toa Medical Electronics, Inc. USA). The absolute counts were calculated with the following formula: absolute counts = WBC (cells/mm^3) x % lymphocytes x % antigen-positive lymphocytes.

Lymphocyte subset identification

B cells were defined as those cells expressing the CD19 antigen, and T cells were defined as those cells expressing the CD3 antigen. Natural killer (NK) cells were defined as CD3$^-$ lymphocytes that express CD16-CD56. Cells expressing CD3 and CD4 were defined as helper T lymphocytes and those expressing CD3 and CD8 were defined as suppressor T lymphocytes. Cells expressing CD3, CD4, and CD45RA were defined as naïve T cells, and those expressing

Table 1 Absolute counts of lymphocyte subsets in blood

Lymphocyte subset	Age group			
	1-7 days (n = 43)	3 months (n = 55)	6 months (n = 57)	11 months (n = 58)
Total lymphocytes	2,782 (2,489-3,076)	5,146*** (4,674-5,619)	5,692*** (5,233-6,152)	5,169*** (4,659-5,680)
CD19$^+$ B lymphocytes	263 (217-308)	1,085*** (934-1,236)	1,400*** (1,243-1,558)	1,227*** (1,074-1,380)
CD3$^+$ T lymphocytes	2,372 (2,115-2,628)	3,743*** (3,400-4,086)	3,877*** (3,553-4,201)	3,510*** (3,138-3,883)
CD3$^+$/CD4$^+$ T lymphocytes	1,807 (1,584-2,031)	2,591*** (2,326-2,856)	2,751*** (2,488-3,013)	2,391* (2,060-2,722)
CD4$^+$/CD45RA$^+$	1,549 (1,364-1,734)	2,033* (1,795-2,271)	2,205** (1,972-2,437)	1,754 (1,543-1,965)
CD4$^+$/CD45RO$^+$	258 (193-323)	568*** (462-675)	546*** (490-602)	638* (458-818)
CD3$^+$/CD8$^+$ T lymphocytes	499 (419-580)	857*** (731-983)	952*** (844-1,061)	1,036*** (885-1,187)
CD4$^+$/CD8$^+$ ratio	6.5 (3.5-9.5)	3.9 (3.2-4.6)	3.6 (2.8-4.3)	2.6 (2.3-2.8)
CD3$^-$/CD16-56$^+$ (NK cells)	63 (49-77)	256*** (199-312)	291*** (239-342)	362*** (287-438)

Absolute counts (x 10^9/l) are expressed as mean (95% CI) as compared with the data at birth
*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$

CD3, CD4, and CD45 RO were defined as memory T cells.

Data analysis

Paired-t test was used to compare with the data at birth. To compare differences among different sex groups, statistical analysis was made by using the Kruskal-Wailis one-way analysis of variance. A $p < 0.05$ was determined as statistical significance.

RESULTS

A total of 213 peripheral blood samples were obtained from 92 infants during the study period. The mean GA of these 92 infants was 38.9 weeks, the mean birth weight was 3,248 g and the sex (F/M) ratio was 60/32. The lymphocyte counts and absolute counts of lymphocyte subsets during infancy are summarized in Table 1 and Figs. 1 and 2. The total lymphocyte counts were lowest at birth, increased by around twofold at month 3 ($p < 0.001$), and remained relatively stable thereafter. A similar trend was noted for $CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, and $CD3^+/CD4^+$ (helper) T lymphocytes: by 3 months of age, the $CD3^+$ T lymphocyte count had increased 1.6-fold relative to the value of 1-7 days ($p < 0.001$), the $CD19^+$ B lymphocyte count had increased fivefold ($p < 0.001$), and the $CD3^+/CD4^+$ T lymphocyte count had increased 1.5-fold ($p < 0.001$). The absolute numbers of $CD3^+CD4^+/CD45RA^+$ (naïve) T lymphocytes increased 1.4-fold from birth to 3 months of age ($p = 0.003$), but had decreased somewhat by 11 months of age ($p = 0.34$). The absolute counts of $CD3^+/CD8^+$ (1.7-fold) T lymphocytes ($p < 0.001$), $CD3^+CD4^+/CD45RO^+$ (2.2-fold) T lymphocytes ($p < 0.001$), and $CD3^-/CD16\text{-}56^+$ cells (fourfold) ($p < 0.001$) also increased rapidly from birth to 3 months of age. Thereafter, the increases were more gradual: 2.1-fold for $CD3^+/CD8^+$ T lymphocytes ($p < 0.001$), 2.5-fold for memory T lymphocytes ($p < 0.001$), and 5.7-fold for NK cells ($p < 0.001$) until 11 months of age.

The percentages of lymphocyte subsets during the first year of life are shown in Table 2. These values had different trends than the absolute counts. The percentages of both $CD19^+$ B lymphocytes and NK cells increased rapidly after birth, and reached their highest levels at 11 months of age. However, the percentages of $CD3^+$, $CD3^+/CD4^+$, and naïve T lymphocytes dropped dramatically from birth to 3 months of age, and more gradually thereafter; the lowest values were reached at 11 months of age. The relative counts of memory and $CD3^+/CD8^+$ T lymphocytes were relatively stable during infancy. The fraction of naïve T lymphocytes decreased from 86% at birth to 75% at 11 months of age. The ratio of $CD4^+/CD8^+$ cells was very high at 6.6 at birth, but had decreased to around 2.5 at 11 months of age.

The data for lymphocyte subsets and results of statistical comparisons between sex groups are summarized in Table 3. Significant changes with sex were seen in all the NK cells and $CD4^+$ subsets, including naïve and memory T cells. In all age groups, male infants had a lower total lymphocyte

Table 2 Percentages of lymphocyte subsets in blood

Lymphocyte subset	Age group			
	1-7 days (n = 43)	3 months (n = 55)	6 months (n = 57)	11 months (n = 58)
$CD19^+$ B lymphocytes	9.6% (8.2-10.9)	20.8%*** (19.2-22.4)	24.5%*** (22.9-26.1)	24.3%*** (22.1-26.5)
$CD3^+$ T lymphocytes	85.3% (84.1-86.5)	72.8%*** (71.1-74.6)	69.1%*** (67.4-70.8)	67.4%*** (65.0-69.9)
$CD3^+/CD4^+$ T lymphocytes	64.4% (62.4-66.3)	51.1%*** (48.4-53.8)	48.8%*** (46.7-50.9)	44.7%*** (42.3-47.1)
$CD4^+/CD45RA^+$	55.3% (53.2-57.4)	40.6%*** (38.0-43.1)	39.0%*** (37.1-40.9)	33.7%*** (31.5-35.9)
$CD4^+/CD45RO^+$	9.1% (7.5-10.6)	10.5%* (9.3-12.1)	9.8% (9.0-10.6)	11.0% (9.3-12.8)
$CD3^+/CD8^+$ T lymphocytes	18.3% (16.0-20.6)	16.9% (15.0-18.8)	17.2% (15.6-18.8)	19.6% (17.9-21.2)
$CD3^-/CD16\text{-}56^+$ (NK cells)	2.3% (1.8-2.8)	4.9%*** (4.0-5.7)	5.1%*** (4.3-5.8)	6.9%*** (5.8-8.0)

Values are expressed as mean (95% CI) as compared with the data at birth.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

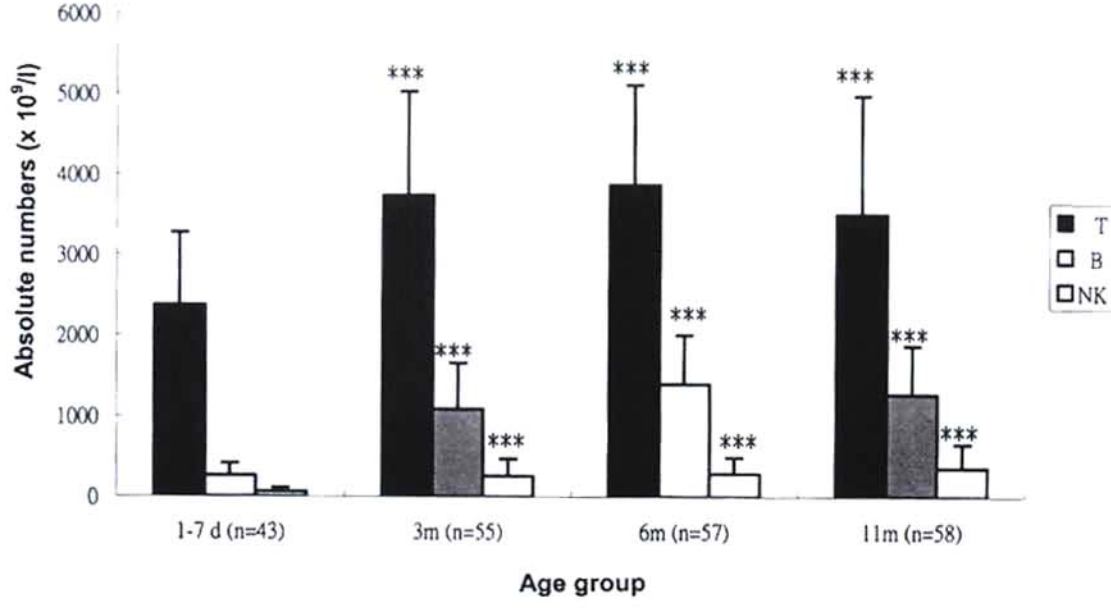

Fig. 1 The changes of T, B, and NK lymphocyte subsets during the first year of life. T, T lymphocytes; B, B lymphocytes; NK, nature killer cells; as compared with the data at birth: *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

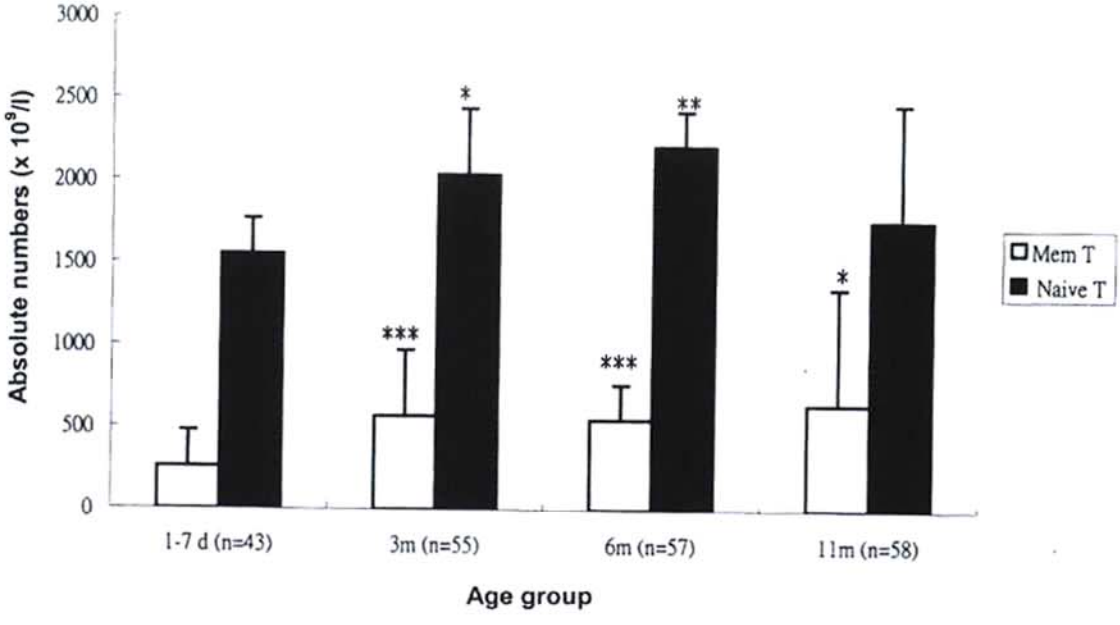

Fig. 2 The changes of naïve and memory T lymphocytes during the first year of life. Mem T, memory T lymphocytes; naïve T, naïve T lymphocytes; as compared with the data at birth: *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

Table 3 Lymphocyte subsets for male (M) and Female (F) infants

	1-7 days		3 months		6 months		11 months	
	M (n = 18)	F (n = 25)	M (n = 15)	F (n = 40)	M (n = 21)	F (n = 36)	M (n = 21)	F (n = 37)
Lymphocyte count	2.6 (2.1-3.0)	2.9 (2.6-3.3)	4.9 (4.1-5.7)	5.2 (4.7-5.8)	2.6 (2.1-3.0)	2.9 (2.6-3.3)	2.6 (2.1-3.0)	2.9 (2.6-3.3)
B lymphocytes								
Percentage	9.3 (7.4-11.3)	9.8 (7.9-11.4)	21 (18.6-23.4)	20.8 (18.7-22.9)	25.2 (23.2-27.2)	24.1 (21.8-26.4)	24.8 (20.1-29.5)	23.9 (21.7-26.1)
Absolute count	0.24 (0.17-0.31)	0.28 (0.22-0.34)	1.0 (0.8-1.2)	1.1 (0.92-1.3)	1.3 (1.1-1.6)	1.4 (1.2-1.6)	1.1 (0.9-1.3)	1.3 (1.1-1.5)
T lymphocytes								
Percentage	85.6 (83.8-87.4)	85.4 (83.6-87.2)	71.6 (69.1-74.1)	73.3 (71.0-75.6)	67.1 (64.8-69.4)	70.2 (67.9-72.5)	66.0 (61.2-70.8)	63.4 (60.8-66.0)
Absolute count	2.2 (1.8-2.6)	2.5 (2.2-2.8)	3.6 (2.9-4.2)	3.8 (3.4-4.2)	3.5 (2.9-4.0)	4.1 (3.7-4.5)	3.1 (2.5-3.7)	3.8 (3.3-4.3)
$CD3^+/CD4^+$ T lymphocytes								
Percentage	63.8 (60.5-67.1)	64.8 (62.4-67.2)	47.6 (44.5-50.7)	52.5 (49.0-50.0)*	45.1 (41.9-48.3)	51.0 (48.4-53.6)**	38.5 (34.1-42.9)	46.3 (43.7-48.9)**
Absolute count	1.6 (1.3-2.0)	1.9 (1.6-2.2)	2.3 (1.9-2.8)	2.7 (2.4-3.0)	2.4 (2.0-2.8)	3.0 (2.6-3.3)*	2.0 (1.4-2.5)	2.6 (2.2-3.0)**
$CD4^+/CD45RA^+$								
Percentage	54.0 (50.4-57.6)	56.2 (53.6-58.8)	36.8 (33.2-40.4)	42.0 (38.8-45.2)*	36.3 (33.6-39.0)	40.6 (38.1-43.1)*	28.8 (25.8-31.8)	36.0 (33.3-38.7)**
Absolute count	1.4 (1.1-1.7)	1.7 (1.4-1.9)	1.8 (1.5-2.1)	2.1 (1.8-2.4)	1.9 (1.6-2.3)	2.4 (2.1-2.7)	1.3 (1.1-1.6)	2.0 (1.7-2.3)**
$CD4^+/CD45RO^+$								
Percentage	9.8 (7.1-12.5)	8.5 (6.7-10.3)	10.7 (6.8-14.6)	10.5 (9.1-11.9)	8.8 (7.4-10.2)	10.3 (9.3-11.3)*	12.3 (8.3-16.3)	10.3 (8.8-11.8)
Absolute count	0.25 (0.16-0.34)	0.27 (1.8-3.6)	0.56 (0.25-0.86)	0.56 (0.46-0.65)	0.46 (0.37-0.54)	0.60 (0.53-0.67)**	0.63 (0.28-1.0)	0.64 (0.44-0.84)
$CD3^+/CD8^+$ T lymphocytes								
Percentage	18.4 (14.1-22.7)	18.2 (15.6-20.8)	18.7 (14.7-22.7)	16.2 (14.1-18.3)	19.0 (16.2-21.8)	16.1 (14.3-17.9)	21.4 (18.0-24.8)	18.8 (17.0-20.6)
Absolute count	0.46 (0.32-0.59)	0.53 (0.43-0.63)	0.92 (0.67-1.2)	0.83 (0.69-0.98)	1.0 (0.8-1.3)	0.9 (0.8-1.0)	1.0 (0.8-1.3)	1.1 (0.9-1.3)
$CD4^+/CD8^+$ ratio	6.8 (2.6-11.0)	6.3 (2.0-10.6)	3.4 (2.1-4.7)	4.2 (3.4-5.0)	3.1 (2.0-4.2)	3.8 (2.8-4.8)*	2.2 (1.8-2.6)	2.7 (2.4-3.0)*
$CD3^-/CD16\text{-}56^+$ (NK cells)								
Percentage	2.1 (2.8-1.4)	2.6 (1.7-3.4)	6.6 (4.9-8.3)	4.2 (3.3-5.1)**	5.7 (4.3-7.1)	4.7 (3.8-5.6)	7.5 (5.3-9.7)	6.5 (5.3-7.7)
Absolute count	0.06 (0.04-0.08)	0.06 (0.04-0.08)	0.32 (0.22-0.42)	0.23 (0.16-0.30)	0.30 (0.21-0.39)	0.28 (0.22-0.35)	0.36 (0.23-0.48)	0.36 (0.26-0.45)

Absolute counts ($x10^9/l$) are expressed as mean (95% CI) as compared with the data of male infants
*: $p < 0.05$; **: $p < 0.01$

counts, lower percentages of $CD4^+$ T lymphocytes and a lower ratio of $CD4^+/CD8^+$ cells. In contrast, the percentage of NK cells was generally higher in males than in females throughout the age groups.

DISCUSSION

Several reports have shown that changes in the absolute counts of lymphocyte subsets are not always correlated with changes in the relative counts.[2,5,7,8,11,12] Our findings are consistent with this observation. For example, the relative count of $CD3^+$ T lymphocytes was as high as 85% after birth and decreased gradually to 67% at 11 months of age, while the absolute count was relatively low at birth and increased 1.5-fold in the same age range. The relative count of memory T lymphocytes remained relatively constant during the first year of life, but the absolute count increased gradually, by around 2.5-fold during the same age period. This phenomenon is due to the increasing numbers of total lymphocytes during the first year of life. Therefore, the percentages of lymphocyte subsets do not accurately reflect their absolute numbers.

To the best of our knowledge, only three reports have defined the normal range for lymphocyte subsets in term newborn infants during the first week of life.[4,13,14] In comparison with their reports, our percentage of NK cells and total lymphocytes are nearly half of theirs.[13,14] This is the first report of reference ranges for lymphocyte subsets during the first week of life in a non-Caucasian population.

Raes *et al.*[13] demonstrated significant differences in lymphocyte subsets both in cord blood and in venous blood samples obtained at day 5 from the same healthy infants.[13] They found that both the percentages and absolute numbers of $CD19^+$ B lymphocytes and NK cells were lower, whereas those of $CD3^+$ and $CD3^+/CD4^+$ T lymphocytes were higher in venous blood on day 5. In comparison with our previous report, we found similar changes.[7] These data showed significant changes in relative and absolute counts of lymphocyte subsets immediately after birth.

The trends of the relative $CD19^+$ B lymphocyte, $CD3^+$ T lymphocyte, $CD3^+/CD4^+$ T lymphocyte, and $CD3^+/CD8^+$ T lymphocyte counts in our study were similar to those reported by Comans-Bitter *et al.*[2] However, the relative NK cell counts increased from 2.3% to 6.9% during the first year of life in our study, but were relatively stable, around 5-8%, in Comans-Bitter *et al.*'s study. This difference may be due to the small sample size (n = 13) and the relatively broad age range (1 weeks-2 months) in their first age group.

The absolute counts of all lymphocyte subsets were the lowest in the first week of life, which corresponds with the low total lymphocyte counts during this period. The absolute counts of $CD19^+$ B lymphocytes, $CD3^+$ T lymphocytes, and $CD4^+$ T lymphocytes increased markedly after birth, with a peak at 6 months of age. This is consistent with the changes in the total lymphocyte counts and the relatively stable percentages of these lymphocyte subsets.

The absolute counts of $CD3^+/CD8^+$ lymphocytes and NK cells showed similar upward trends in our study, both reaching their highest levels at the end of the first year of life. Comans-Bitter *et al.*[2] on the other hand, found the absolute counts of these two subsets to be relatively stable during this period. This discrepancy may reflect ethnic, dietary, environmental, or other differences between the study groups. While the downward trend of the $CD4^+/CD8^+$ ratio was similar in Comans-Bitter *et al.*'s study[2] and ours, the ratio at birth was much higher in our subjects (6.5 *vs* 3.8). This difference may due to the small sample size (n = 13) and wide age range (1 week-2 months) in the former study and in our study (n = 43 and age range = 1-7 days old).

The percentages of naïve and memory T lymphocytes showed opposite trends, with memory T cells accounting for an increasingly large proportion of $CD3^+/CD4^+$ lymphocytes during the first year of life. This result supports the hypothesis that $CD3^+CD4^+/CD45RA^+$ naïve T lymphocytes are antigenically untriggered T cells which evolve into $CD3^+CD4^+/CD45RO^+$ memory T lymphocytes, presumably as the result of antigen exposure.[7,12,15] Furthermore, we found that males generally had a lower total lymphocyte count, percentages of $CD4^+$ T lymphocytes, and ratio of $CD4^+/CD8^+$ cells, but a higher percentage of NK cells throughout the age groups. Similar findings were reported by Lee *et al.*[8] and Webster *et al.*[16]

In summary, we evaluated blood samples of Chinese infants during the first year of life. The reference ranges of lymphocyte subsets from birth to 1 year of age and the trends in the relative and absolute counts of lymphocyte subsets demonstrated in this study should be useful in evaluating the quantity of immune effector cells during infancy.

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