Article

The Influence of Age and Sex on the Cell Counts of Peripheral Blood Leukocyte Subpopulations in Chinese Rhesus Macaques

Hou-Jun Xia[1, 2], Gao-Hong Zhang[1], Rui-Rui Wang[1] and Yong-Tang Zheng[1, 3]

Non-human primates such as Chinese rhesus macaques are the favorable models for preclinical study of potential therapeutic drugs, vaccines and mechanisms of human diseases. Little is known about the normal levels of leukocyte subpopulations of Chinese rhesus macaques. To obtain these data, 100 blood samples from Chinese rhesus macaques were collected. The normal range of major leukocyte subpopulations, such as T lymphocytes, B lymphocytes, monocytes, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), were quantitatively analyzed by flow cytometry through BD trucount tubes. The influence of age and sex on the cell counts of leukocyte subpopulations was analyzed. The counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells decreased with age, but those of monocytes, mDCs and pDCs had no significant correlation with age. Significant differences existed in the cell counts of most leukocyte subpopulations between the male and female groups except pDCs. Furthermore the values of the females were higher than those of the males. The study provided basic information about the leukocyte subpopulations of Chinese rhesus macaques, and it may be valuable for immunobiological study of Chinese rhesus macaques. *Cellular & Molecular Immunology*. 2009;6(6):433-440.

Key Words: Chinese rhesus macaque, leukocyte subpopulation, absolute count, age, sex

Introduction

Non-human primates are the favorable models for testing vaccine strategies and studying human disease mechanisms. Rhesus macaques (*Macaca mulatta*) are widespread in wild and become most suitable animal models. Rhesus macaques have been divided into six subspecies by primatologists (1). Indian Rhesus macaques have been used in most studies. Chinese rhesus macaques had only been used extensively in recent years in order to resolve the shortage of India origin rhesus macaques (2). They are different in the morphometrics (3) and genetic background (4), which may cause different immunobiological characters of leukocytes.

Little is known about the basic immunologic features of Chinese rhesus macaques compared to Indian counterpart. However, with the increase of AIDS animal models using Chinese rhesus macaques, more and more studies focused their interests on the leukocytes of the species. The counts of $CD4^+$ T lymphocytes are important to monitor AIDS, so most studies had measured the changes of T lymphocytes subsets (5). Further, Qiu et al (6) used flow cytometry to detect the character of T lymphocyte subsets in the peripheral blood of Chinese rhesus macaques and analyzed its co-relation with age and sex. But other white blood cell subsets, like the neutrophils recently (7), were little depicted in the study of AIDS animal models. For there is not a systematic study about the basic information of these cells, we need more experiments to describe them.

The immune systems of rhesus macaque closely resemble those of humans. The major leukocyte subpopulations of rhesus macaques also include $CD3^+$ T lymphocytes, $CD20^+$ B lymphocytes, $CD14^+$ monocytes, $CD11c^+$ myeloid dendritic cells (mDCs) and $CD123^+$ plasmacytoid dendritic cells (pDCs). Age and sex play important roles in immune systems. Advancing age yields numerous immune system changes, in aggregate termed immunosenescence. Aged people are more susceptible to severe infections, take longer to recover from infections and are frequently less responsive to vaccination. This is in part a consequence of immunosenescence or the functional deterioration of the immune system with age (8). Sex and sex-related hormones also affect the prevalence, severity, and natural history of immunologic diseases. Females produce more vigorous cellular and humoral immune reactions, are more resistant to certain infections. Therefore they suffer a higher incidence of autoimmune diseases (9).

[1]Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China;

[2]Graduate School of the Chinese Academy of Sciences, Beijing 100039, China;

[3]Correspondence to: Dr. Yong-Tang Zheng, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China. Tel & Fax: +86-871-519-5684, E-mail: zhengyt@mail.kiz.ac.cn

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Table 1. Age and sex of the monkeys

Age (years)	Males		Females	
	N	Age (years)[a]	*N*	Age (years)[a]
1~	23	3.4 ± 1.3	14	2.4 ± 0.8
6~	18	7.6 ± 1.7	20	6.8 ± 1.2
11~	19	13.8 ± 4.2	6	12.7 ± 2.3
Total	60	8.0 ± 5.1	40	6.2 ± 3.7

[a]The data were shown as mean ± SD

In order to provide basic information about the normal immune system of Chinese rhesus macaques and analyze the influence of age and sex on the cell counts of leukocyte subpopulations, we detected 100 blood samples of Chinese rhesus macaques fed in Yunnan Province of China. The normal range of leukocyte subpopulations and the influence of age and sex on these parameters were reported in this study.

Materials and Methods

Animals

One hundred healthy Chinese rhesus macaques were gotten from Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). The age information about these monkeys was shown in Table 1: they are 2 to 25 years old (mean = 7.2 years), among which 60 were males, while 40 were females. Housing, maintenance and care of the animals were performed under the regulations and recommendations of the Animal Care Committee of KIZ, CAS.

Flow cytometry

Whole blood was collected from monkeys under anaesthesia in EDTA tubes. Undiluted blood (50 μl) was stained, in TruCount tubes (BD Biosciences), with mouse anti-human monoclonal antibodies selected for efficient cross-reaction with Chinese rhesus macaques. These antibodies were described in Table 2. Four stained samples were prepared for testing. The T lymphocytes and subsets were labelled with FITC-CD8, PE-CD3 and PerCP-CD4. The B lymphocytes and monocytes were prepared by labelling blood with mAbs for PerCP-CD20 and FITC-CD14 together. pDCs were labelled using Percp-HLA-DR and FITC-CD123. mDCs were stained with a cocktail of lineage-specific antibodies (FITC-CD3, CD14, CD20), Percp-HLA-DR and PE-CD11c. After 15-min incubation at room temperature, 500 μl FACS lysing solution (BD Biosciences) was added to each tube to lyse red blood cells and fix samples. Cells were acquired with a three-color flow cytometry, FACScalibur (Becton Dickinson), and analyzed through Cell Quest software.

Table 2. Monoclonal antibodies used for phenotyping in this study

Antigen	Source	Clone	Isotype
CD3-FITC	BD Biosciences	SP34	IgG3,λ
CD3-PE	Miltenyi Biotec	10D12	IgG1
CD4-PerCP	BD Biosciences	L200	IgG1,κ
CD8-FITC	Miltenyi Biotec	BW135/80	IgG2a
CD11c-PE	eBioscience	3.9	IgG1,κ
CD14-FITC	Miltenyi Biotec	TUK4	IgG2a
CD20-FITC	Miltenyi Biotec	LT20	IgG1
CD20-PerCP/Cy5.5	BioLegend	2H7	IgG2b,κ
CD123-FITC	BD Biosciences	7G3	IgG2a,κ
HLA-DR-PerCP	BD Biosciences	L243	IgG2a

Absolute quantification of peripheral blood leukocyte subpopulations

T lymphocytes (R1, Figure 1A) and TruCount beads (R2, Figure 1A) were directly gated using CD3 fluorescence intensity as threshold. $CD4^+$ T lymphocytes (R3, Figure 1A) and $CD8^+$ T lymphocytes (R4, Figure 1A) were quantified by gating on R1. Other leukocyte subpopulations were detected by another way. PBMC (R1, Figures 1B-D) and TruCount

Table 3. The cell counts of leukocyte subpopulations in different sex and age groups

Age (years)	Sex	$CD3^+$ T cell (μl^{-1})[a]	$CD3^+CD4^+$ T cell (μl^{-1})[a]	$CD3^+CD8^+$ T cell (μl^{-1})[a]	B cell (μl^{-1})[a]	Monocyte (μl^{-1})[a]	mDC (μl^{-1})[a]	pDC (μl^{-1})[a]
1~	M	3509 ± 812	1822 ± 489	1411 ± 427	1629 ±790	335 ± 117	90 ± 72	6 ± 3
	F	4472 ± 1650	2399 ± 886	1727 ± 687	2170 ± 1348	532 ± 232	105 ± 57	9 ± 4
6~	M	2510 ± 1172	1206 ± 492	1068 ± 623	1175 ± 994	258 ± 135	62 ± 55	8 ± 3
	F	3783 ± 1468	1798 ± 717	1664 ± 819	1637 ± 1199	562 ± 182	87 ± 63	8 ± 4
11~	M	2116 ± 681	1043 ± 282	829 ± 314	747 ± 501	328 ± 166	66 ± 64	7 ± 4
	F	3691 ± 1599	1359 ± 287	1902 ± 1113	1036 ± 880	687 ± 330	138 ± 117	5 ± 3
Total	M	2768 ± 1074	1390 ± 552	1123 ± 538	1213 ± 855	310 ± 141	74 ± 65	7 ± 3
	F	4010 ± 1551	1942 ± 812	1722 ± 806	1733 ± 1246	570 ± 225	101 ± 71	8 ± 4
All		3265 ± 1417	1611 ± 718	1363 ± 718	1421 ± 1055	414 ± 220	85 ± 68	7 ± 4
Range		1171 ~ 8042	548 ~ 4539	384 ~ 3791	156 ~ 5328	69 ~ 1149	9 ~ 341	1 ~ 17

[a]The data were shown as mean ± SD

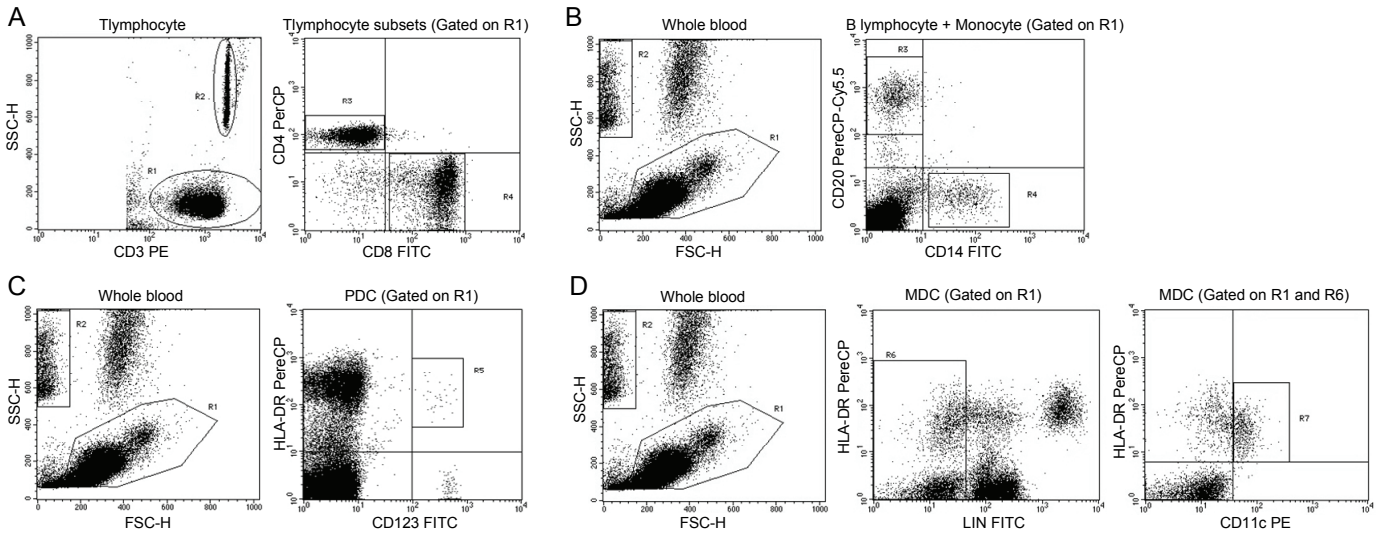

Figure 1. Gating strategies for leukocyte subpopulation counting in blood. (A) Gating strategies for T cells and subsets. Dot plots represent: (left) CD3/side-scatter scattergram used for gating T cells (R1) and TruCount beads (R2); (right) CD4/CD8 dot plot gated on R1 used to count $CD4^+$ (R3) and $CD8^+$ T cells (R4). PBMC (R1) and TruCount beads (R2) were first gated on Forward-scatter/side-scatter (FSC/SSC) scattergram in left dot plot of (B), (C) and (D). (B) Gating strategies for B cells and monocytes. Dot plots represent: (right) CD14/CD20 dot plot gated on R1 used to count B cells (R3) and monocytes (R4). (C) Gating strategies for pDC. Dot plots represent: (right) CD123/HLA-DR dot plot gated on R1 used to count pDC (R5). (D) Gating strategies for mDC. Dot plots represent: (middle) Linage/HLA-DR dot plot gated on R1 used to gate linage negative cells (R6); (right) CD11c/HLA-DR dot plot gated on R1 and R6 used to count mDC (R7).

beads (R2, Figures 1B-D) were first gated using an appropriate gate in the forward-scatter/side-scatter (FSC/SSC) scattergram. B lymphocytes (R3, Figure 1B) and monocytes (R4, Figure 1B) were directly gated from PBMC. Lineage negative cells (R6, Figure 1D) were first gated from PBMC and $HLA\text{-}DR^+CD11c^+$ of these cells were mDCs (R7, Figure 1D), while pDCs (R5, Figure 1C) were characterized by gating on $CD123^{bright}$ $HLA\text{-}DR^+$ cells without gating on lineage negative cells, as previously described for rhesus pDCs in PBMC (10). Absolute cell count was calculated as follows: Cells concentration = (events in cells region × total number of beads in TruCount tube) / (events in beads region × sample volume)

Statistical analysis

Analyses were performed using the SPSS v13.0 software. The normal distributions were confirmed by the Kolmogorov-Smirnov test. The Mann-Whitney U test was used to compare the means between different sex groups and the Kruskal-Wallis test was used to compare the means among different age groups. The correlations between variables were evaluated by use of the Spearman rank correlation test. For all tests, two-sided $p < 0.05$ was considered to be significant.

Results

The cell counts of leukocyte subpopulations in whole blood of Chinese rhesus macaques

All animals had been divided into three groups based on the age: adolescent (1~5 years), adult (6~10 years) and aged (> 11 years). The cell counts of leukocyte subpopulations in the three age groups of each sex were listed in Table 3. The Kruskal-Wallis test was used to analyze the difference of age groups. Statistically significant difference in the counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells was observed between three age groups each other, while no significant difference in monocytes, mDCs and pDCs. The counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells were highest in the adolescent group, and lowest in the aged group.

Correlation analyses between age and the cell counts of leukocyte subpopulations

Correlation analysis between age and the cell counts of leukocyte subpopulations was shown in Figure 2. It showed a negative correlation between age and $CD3^+$ T cells ($r = -0.348$, $p < 0.001$), $CD3^+CD4^+$ T cells ($r = -0.461$, $p < 0.001$), $CD3^+CD8^+$ T cells ($r = -0.241$, $p = 0.016$) and B cell counts ($r = -0.395$, $p < 0.001$). However, the counts of monocytes, mDCs and pDCs showed no significant correlations with age.

Sex differences of the cell counts of leukocyte subpopulations in Chinese rhesus macaques

As shown in Table 3 and Figure 3, significant sex differences were observed in the counts of $CD3^+$ T cells ($p < 0.001$; male 2768 μl^{-1}, female 4010 μl^{-1}), $CD3^+CD4^+$ T cells ($p < 0.001$; male 1390 μl^{-1}, female 1942 μl^{-1}), $CD3^+CD8^+$ T cells ($p < 0.001$; male 1123 μl^{-1}, female 1722 μl^{-1}), B cells ($p = 0.041$; male 1213 μl^{-1}, female 1733 μl^{-1}), monocytes ($p < 0.001$;

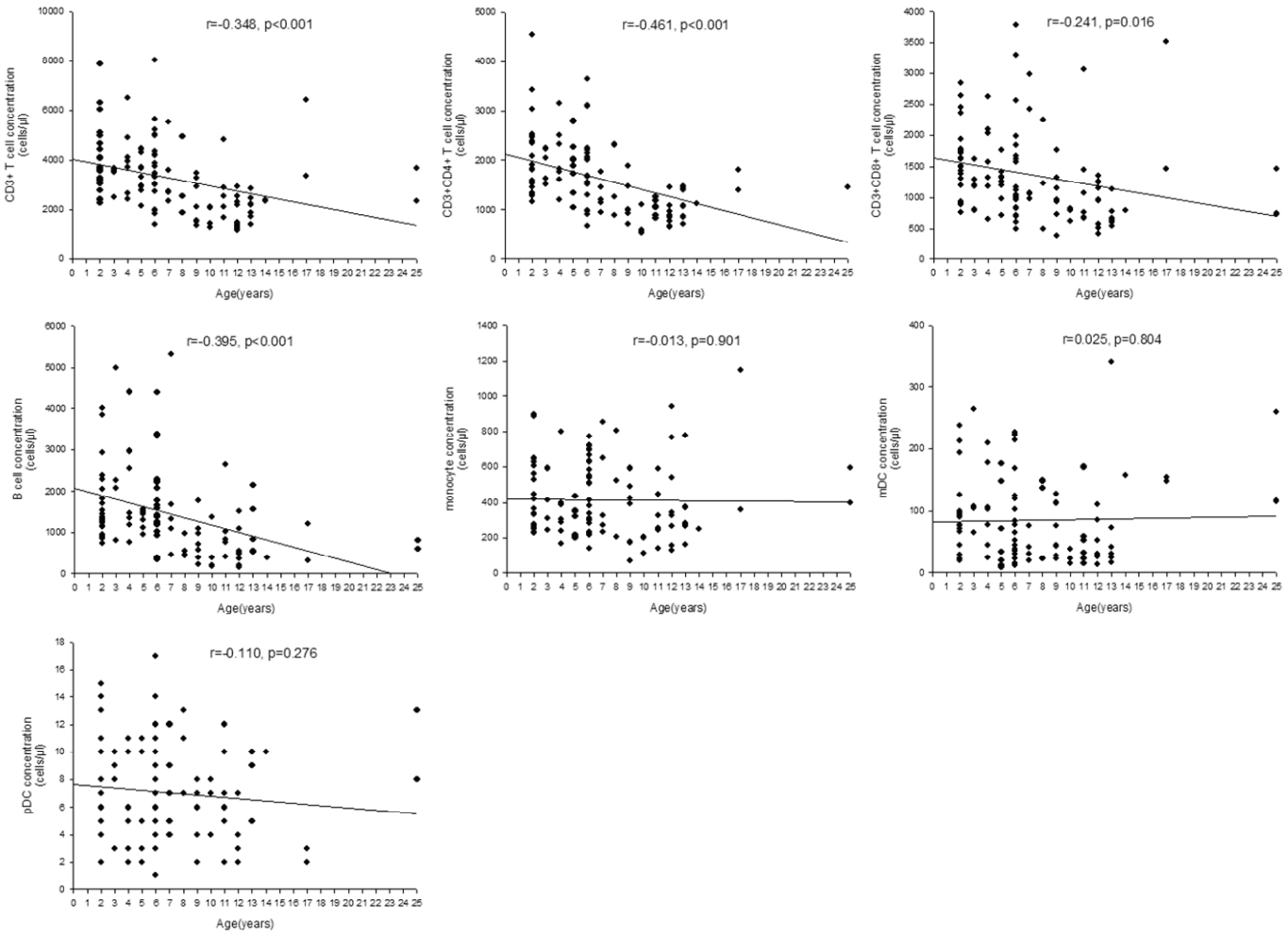

Figure 2. Effects of aging on the counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, B cell, monocytes, mDC and pDC in the peripheral blood of Chinese rhesus macaques. The absolute numbers of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells decreased with age, but monocytes, mDCs and pDCs were not found significant changes with age.

male 310 μl^{-1}, female 570 μl^{-1}) and mDC ($p = 0.021$; male 74 μl^{-1}, female 101 μl^{-1}). All of the values of the females were higher than that those of the males in these leukocyte subpopulations. But there was no significant sex difference in the counts of pDCs between the male and female groups.

Discussion

Chinese rhesus macaques have been increasingly used as a substitute for Indian rhesus macaques in the research of AIDS and other human diseases (11). However, several researches of Chinese rhesus macaques focused on the morphometrics (3) and genetic background (4) differences comparing with their Indian counterparts. Compared with Indian rhesus macaques, SIV(mac) pathogenesis in Chinese rhesus macaques was closer to HIV-1 infections in untreated adult humans (12). So Chinese rhesus macaques may be a better model used in AIDS research. The cell counts of major blood white cells decreased through AIDS progression and $CD4^+$ T lymphocyte count has been an important parameter in HIV/AIDS clinical tests. If we choose Chinese rhesus macaques as AIDS animal models, it is necessary to know more basic immunological features of blood white cells of these monkeys. So we quantitatively analyzed the leukocyte subpopulations, including T lymphocytes, T lymphocyte subsets, B lymphocytes, monocytes, mDCs and pDCs. We had used flow cytometry to gate and count these cells. T lymphocytes and TruCount beads were easily to be defined with CD3 antibody fluorescence intensity as threshold, and their subsets could be directly quadrantal-divided using CD4 and CD8 antibodies gated on CD3 positive cells. But other leukocyte subpopulations were chosen different gating strategy, using SSC as threshold to gate PBMC and TruCount beads. Leukocyte subpopulations were identified on their special markers according to common methods.

Age is an important factor in influencing the counts of white blood cells. Old people always increased incidence of

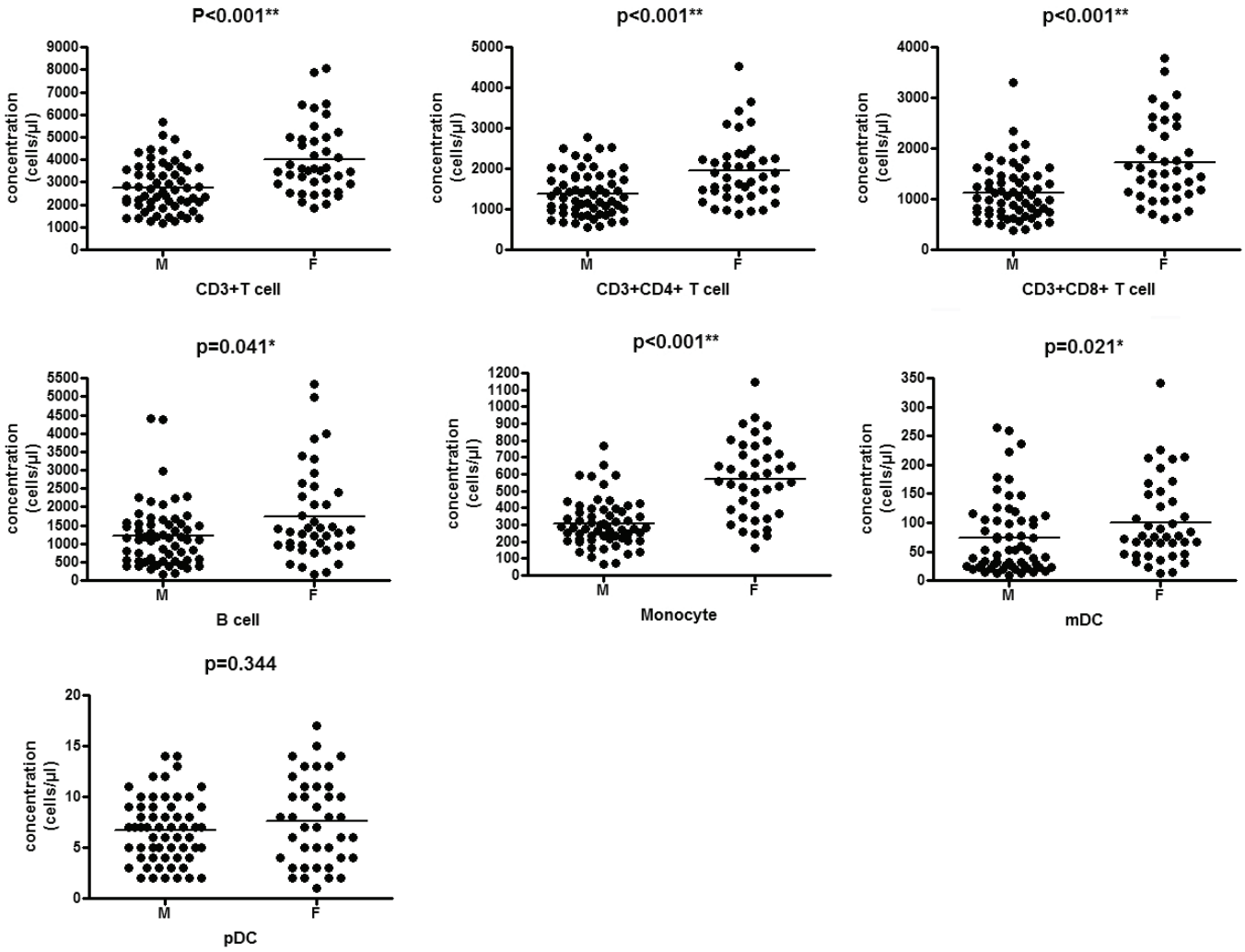

Figure 3. The counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, B cells, monocytes, mDC and pDC in different sex groups. There were significantly differences in the counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, B cells, monocytes and mDC between the male and female groups whereas pDC was not.

malignancies and predisposition to both bacterial and viral infections. The age range influences the character of immune system. Klose et al divided adult into four age groups (18~20/20~29/30~39/40~78) and showed that no statistically significant influence of age on the distribution of lymphocyte subpopulations, either in terms of absolute or of relative cell numbers (13). Jiao et al also set three groups of adult: healthy young (19-44 years), middle-aged (45-64 years) and elderly adults (≥65). Their results showed that a statistically significant increase in $CD16^+CD56^+$ NK cells was observed between the middle-aged and elder cohorts, whereas for the majority of the parameters, a significant decline was observed between the young and the middle-aged cohorts (14). Qiu et al divided Chinese rhesus macaques into four age groups (1~5/6~10/10~15/≥16), showed that statistically significant difference in the percentage of T lymphocytes was observed between the adolescent group (1~5 years) and the other three age groups, and between the adult (6~10 years) and the aged (10~15 years) groups. It was the highest in the aged group (6). In our study, we showed statistically significant difference between the counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells observed between three age groups, while no significant difference between that of monocytes, mDCs and pDCs. Young rhesus macaques had more cells count than old ones. That was different from Qiu' result, which might be due to the choice of percentage or count as a judgment.

The correlation of age and circulating white blood cell counts has been documented in both of the human and animals, however, the results were not consistent and some conclusions were even opposite to each other. It was reported that old people showed significant reduction in the total number of lymphocytes compared with those in normal adult controls, 18 to 51 years of age as early as in 1975. There was a significant increase in the relative proportions of peripheral blood B lymphocytes in the elderly although the absolute

numbers of B lymphocytes in the elderly did not differ from those in younger controls (15). Wiener et al also pointed the absolute number and percentage of total lymphocytes, the percentage and absolute number of $CD19^+$ lymphocytes, and the absolute number of $CD3^+$ lymphocytes decreased with age (16). Shahabuddin et al (17) found absolute and percentage values for most lymphocyte populations differed substantially not only between children and adults but also among children from different age groups. The same result was reported that absolute numbers of all the lymphocyte subsets, including T cells, B cells, NK cells, $CD4^+$ T cells and $CD8^+$ T cells, decreased with age from 1 month to 13 years. However, the different result was the lymphocyte percentage increased in all the subsets except B cells and $CD4^+$ T cells with time. There are conflictive reports about monocytes and DCs with age. Tollerud et al (18) investigated 266 nonsmokers and found no significant age effects were observed for $CD14^+$ cells, but Della Bella et al (19) showed an increase of circulating monocytes with age. As DC subsets, mDCs and pDCs always have been studied together. A phenomenon has been reported in children during the first decade of life that their pDCs numbers decline rapidly whereas the numbers of mDCs remain relatively stable (20, 21). There are at least three studies demonstrated an age-related decline of pDC (22-24) but two other studies showed no changes (19, 25). Conflicting findings have also been reported with regard to the age-related changes in mDCs. The BDCA-1^+ mDC kept unaltered (23) while the $Lin^-HLA\text{-}DR^+CD11c^+$ mDC progressively declines with age (19).

There are few reports about to age-related change of rhesus macaques. From birth to 44 wk of age, the white cell counts and absolute lymphocyte counts of Indian rhesus macaques were both elevated compared to adult-hood. $CD4^+$ T cells constituted more than 80% of all T cells at birth, and declined gradually over the first 12 wk of life, while the percentages of $CD8^+$ T cells and $CD20^+$ B cells increased in the same period (26). Gould et al (27) examined changes in circulating lymphocyte subsets from the neonatal period until adulthood (4 months until 5.5 years of age) in male Indian rhesus macaques and found absolute numbers of B lymphocytes, T lymphocytes and $CD4^+$ T cells decreased, but $CD8^+$ T cells did not change with age. Qiu et al (6) studied the blood lymphocytes and found that the counts of total white blood cells and lymphocytes decreased with age in Chinese rhesus macaques. Phenotypic analysis of CD4 and CD8 expression on $CD3^+$ T lymphocytes showed that the percentage of $CD4^+$ T cells, $CD4^-CD8^-$ T cells decreased with age; and the percentage of $CD8^+$ T cells, $CD4^+CD8^+$ T cells and $CD3^+$ lymphocytes increased with age. There are no direct studies about monocytes, mDC and pDC with age. Most studies focused on the changes of these cells in disease. Our results showed absolute numbers of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells decreased with age, but monocytes, mDCs and pDCs were not found significant changes with age.

The influence of sex on white blood cells has been reported in human and rhesus macaques. In human, female had a higher frequency of $CD4^+$ T cells but no significant gender effects were observed for $CD14^+$ cells (18) and it was also found that the Indian adult females had significantly higher $CD4^+$ T cell counts (28). It was the same with Chinese females who had a higher $CD4^+/CD8^+$ T cell ratio than the males (29). In infant cynomolgus monkeys (*Macaca fascicularis*), statistically significant differences were observed between male and female monkeys for the mean percentage levels of CD4 (females > males) and for the CD4/CD8 ratio (females > males) (30). However, no statistically significant difference was observed between the male and female groups in most parameters in these monkeys except for the percentage of $CD4^+CD8^+$ T cells in Chinese rhesus macaques (6). In this study, we found there were significant differences in the counts of leukocyte subpopulations between the male Chinese rhesus macaques and the females except pDCs (females > males).

There are reports about the biological and genetic differences between Chinese and Indian rhesus macaques. For example, the DC-SIGN expressed on monocyte-derived DCs of Chinese rhesus macaques (31) but not expressed on that of Indian (32). So there maybe exist difference in leukocyte subpopulations counts of these two animals. The baseline levels of $CD4^+$ T cells of Indian rhesus macaques were 1369 cells/μl (median number) in Reimann et al.'s study (33) and 1300 cells/μl in George et al.'s study (34). Chinese rhesus macaques analyzed in Qiu et al.'s (1661 ± 777 cells/μl) and our study (1611 ± 718 cells/μl) showed a higher $CD4^+$ T cells counts. A higher mDC and pDC counts also existed in Chinese rhesus macaques when compared with Indian rhesus macaques. Brown and Barratt-Boyes (35) showed the median number of mDC and pDC in blood for all nine Indian rhesus macaques was 50 and 3 cells/μl respectively, lower than our result (85 and 7 cells/μl, respectively). Low cell counts have been reported in other Indian rhesus macaque studies (36, 37). It seems geographic distribution of rhesus macaques caused differences in the counts of each leukocyte subpopulations. However, we need a direct comparative study using age and sex matched Indian rhesus macaques to get a reliable result.

In conclusion, the leukocyte subpopulations cell counts of 100 Chinese rhesus macaques blood samples were detected and quantified, including T cells, B cells, monocytes, mDC and pDC. The counts of $CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and B cells decreased with age, but monocytes, mDCs and pDCs were not significantly correlated with age. There were significant differences in the cell counts of most leukocyte subpopulations between the male and female groups except pDCs, and the values of all the females were higher than those of the males. This study provided some basic information about major white blood cells in the peripheral blood of Chinese rhesus macaques, which is useful for future disease study or comparative study.

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