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ORIGINAL ARTICLE

The influences of age on T lymphocyte subsets in C57BL/6 mice

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KEYWORDS

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Abstract The aim of this study is to evaluate the age related changes of T lymphocyte subsets in C57BL/6 mice and immune function. Multi-color immunofluorescence techniques that were used to analyse relative numbers of T lymphocyte subsets include $CD4^+$, $CD8^+$, naive and memory $CD4^+$ and $CD8^+$, $CD8^+CD28^+$ T cells in peripheral blood of C57BL/6 mice from different age groups (Group I: 2 months old; Group II: 7 months old; Group III: 21 months old); Splenocytes isolated from different group mice were stimulated with Con A to evaluate the proliferative ability. Compared with group I, group II had a significant reduction in the percentage of $CD4^+$, naive $CD4^+$ and $CD8^+$ T cells and an increase in the percentage of $CD8^+$ T cells, while group III had a significant reduction in the percentage of $CD4^+$, naive $CD4^+$ and $CD8^+$ T cells and increase in the percentage of $CD8^+$, memory $CD4^+$ and $CD8^+$ T cells in peripheral blood. Compared with group II, group III had a significant reduction in the percentage of naive $CD8^+$ T cells and increase in the percentage of memory $CD4^+$ and $CD8^+$, $CD8^+CD28^+$ T cells in peripheral blood. The T lymphocyte proliferation in vitro showed that groups II and III had a lower proliferative capacity

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than group I, between groups II and III, there was not a significant difference. We provide relative values for the T lymphocyte subsets in the different age groups of C57BL/6 mice. The immune system began aging at 7 months old in C57BL/6 mice under a specific pathogen free environment.

1. Introduction

The human's average life span is continuously rising and leading to an ever increasing elderly population in the world, while, aging is a complex process that deeply affects the immune system. The decline of the immune system with age is reflected in the increased susceptibility to infectious diseases, poorer response to vaccination (Tonet and Nóbrega, 2008), increased prevalence of cancer (Derhovanessian et al., 2008), autoimmune and other chronic diseases characterized by a pro-inflammatory state (Krabbe et al., 2004).

Shortly after birth, thymic space designated for T cell development decreases by approximately 3% per year and continues to decline at a slightly lower rate after middle age (Steinmann et al., 1985). Due to the thymus shrinks, the number of T cells entering the peripheral T cell pool from the thymus is dramatically diminished. Therefore, to counteract reduced thymic output, the remaining peripheral T cells either undergo homeostatic proliferation or live longer-both of which contribute to diminished immunity in the aged (Aspinall and Andrew, 2000). Thus, the T cell compartment of aged individuals is smaller, less diverse, and less functional than that of a young individual.

In this study, we investigated age related changes of T lymphocyte subsets in peripheral blood of C57BL/6 mice and the proliferative ability of T lymphocyte in vitro, which would provide reference relative values for the T lymphocyte subsets and evaluate the immune function.

2. Materials and methods

2.1. Animals

Old C57BL/6 mice were purchased from SLAC (Shanghai Laboratory Animal Center) and housed for 21 months in the Tongji University animal center, young C57BL/6 mice were also purchased from SLAC and housed for 2 months in Shanghai Tongji hospital, 7 month old C57BL/6 mice were purchased from the Vital River Laboratory. All the mice were maintained under a specific pathogen free environment. All the experiments were reviewed and approved by the Tongji University animal center and Ethics Committee of Shanghai Tongji hospital.

2.2. T lymphocyte subsets

All mice were anesthesized by injecting with 1%(w/v) Pentobarbital sodium (cat.No. P8410, Solarbio) and about 0.9 ml blood was obtained in a Heparin sodium treated tube from the heart. Blood was stained with antibody (Percp/Cy5.5 anti-mouse CD3Antibody, FITC anti-mouse CD4 Antibody, PE anti-mouse CD8a Antibody, APC anti-mouse CD28 Antibody, PE anti-mouse CD44 Antibody, APC anti-mouse CD62L Antibody, BioLegend) and incubated for 30 min at room temperature. 500 μl lysing solution (349202, BD) was added in blood and incubated for 15 min at room temperature. Blood was washed twice and resuspended in 200 μl PBS. BD C6 FACS was used for cell analysis.

2.3. T lymphocyte proliferation in vitro

2.3.1. Cell preparation

Spleens from individual mice of each age group were processed separately, without pooling. Single cell suspensions of lymphocytes were prepared by pressing spleens through a 70 μm filter into PBS containing 5%(v/v) heat inactivated Fetal Bovine Serum (FBS) (10099-141, Gibco). Splenocytes were centrifuged at 300 g for 5 min at room temperature and the supernatant was discarded. Red blood cells were removed with ACK lysing buffer (A10492-01, Gibco). Cells were washed twice and resuspended in PBS containing 5%(v/v) FBS at a concentration of 1×10^7 cells ml^{-1}.

2.3.2. CFSE stain

1 ml volume of splenocytes was added in a fresh 15 ml tube and the tube was laid horizontally, 100 μl PBS was added to the non-wetted portion of the plastic at the top of the tube. 0.22 μl of the 5 mM stock of CFSE (C34554, life technology) was resuspended in the PBS, the tube was capped and quickly inverted several times and vortexed. The tube was incubated for 5 min at room temperature and washed twice to quench the unbound CFSE by diluting with ten volumes of PBS containing 5%(v/v) FBS and finally cells were resuspended in 1640 complete medium (Gibco) containing 10%(v/v) FBS and antibiotics at a concentration of 1×10^6 cells ml^{-1}.

2.3.3. Cell culture

Cell cultures were established in 96 well V-bottom plates with PRMI 1640 complemented with 10% FBS, mercaptoethanol and antibiotics. Each well was seeded with 200 μl volume splenocytes. Stimulation of cells with Concanavalin A (Con A) (C2010, Sigma) was achieved with an optimum concentration of 5 μg/ml. Cells were cultured for 72 h at 37 °C in a 5% CO_2-humidified environment.

2.3.4. Flow cytometry analysis

Cells were harvested into 1.5 ml tubes, centrifuged at 300 g for 3 min at room temperature and the supernatant was discarded. Cells were washed twice and resuspended in 100 μl PBS. 1 μl PerCP/Cy5.5 anti-mouse CD3 Antibody was added into PBS and mixed thoroughly. The tube was incubated for 30 min at room temperature avoiding exposure to excessive light, centrifuged at 300 g for 3 min at room temperature and the supernatant was discarded, and the cells were resuspended in 300 μl PBS. BD C6 FACS was used for cell analysis, Modfit

LT (verity) software was used for data analysis to obtain the proliferation index.

2.4. Statistical analysis

All results were expressed as mean ± standard deviation. The significance of differences between 3 groups was determined by Duncan's multiple range test. *P* values less than 0.05 were considered statistically significant. Flow cytometry gating scheme was performed by using Novo Express and statistical analysis was finished by using GraphPad Prism 6.

3. Results

3.1. Baseline characteristics of the subjects

Baseline characteristics of the mice were listed in Table 1. The group I had a age of 2 months, group II had a age of 7 months and group III had an age of 21 months. Flow cytometry gating scheme of the study was depicted in (Fig. 1).

3.2. The influences of age on T lymphocyte subsets' constitution in C57BL/6 mice

Compared with group I, the group II and group III had a significant reduction in the percentage of $CD4^+$ and an increase in the percentage of $CD8^+$ T cells in peripheral blood, while there was not a significant difference between the group II and group III (Fig. 2).

Compared with group I, the group II and group III had a significant reduction in the percentage of naive $CD4^+$ and $CD8^+$ T cells, while group III had a significant increase in the percentage of memory $CD4^+$ and $CD8^+$ T cells. Compared with group II, group III had a significant reduction in the percentage of naive $CD8^+$ T cells and an increase in the percentage of memory $CD4^+$ and $CD8^+$ T cells (Fig. 3).

Table 1 Characteristics of the subjects.

		Group I n = 6	Group II n = 8	Group III n = 6
Age (months)		2	7	21
Gender	Females	3	4	3
	Males	3	4	3

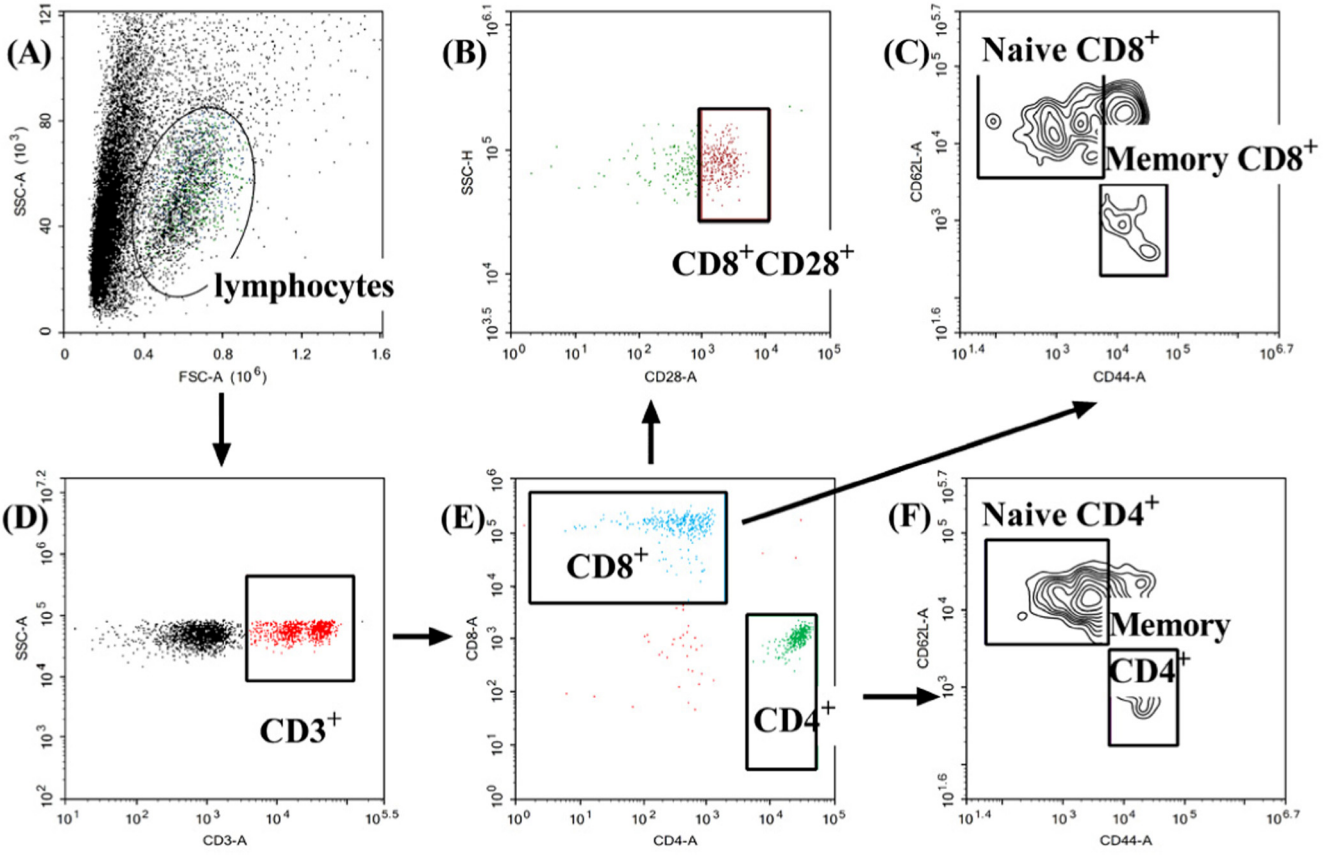

Figure 1 Flow cytometry gating scheme. (A) Lymphocytes were gated from the blood sample; (B) $CD8^+CD28^+$ T cells were gated from $CD8^+$ T cells; (C) naive and memory $CD8^+$ T cells were gated from $CD8^+$ T cells; (D) CD3+ T cells were gated from lymphocytes; (E) $CD4^+$ and $CD8^+$ T cells were gated from CD3+ T cells; (F) naive and memory $CD4^+$ T cells were gated from $CD4^+$ T cells.

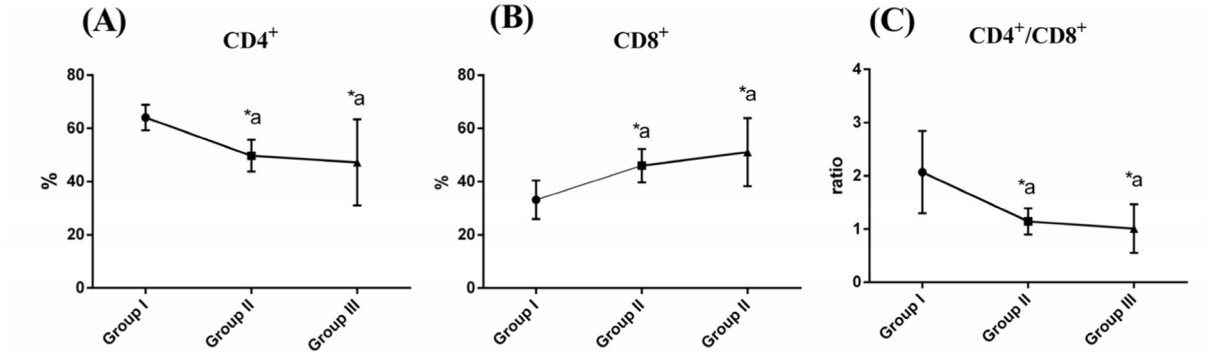

Figure 2 The percentages of $CD4^+$, $CD8^+$ T cells in peripheral blood of C57BL/6 mice from different age groups and $CD4^+/CD8^+$ ratio. Compared with Group I, $^{*a}P < 0.05$. (A) The percentages of $CD4^+$ T cells in T cells; (B) the percentages of $CD8^+$ T cells in T cells; (C) the ratios of $CD4^+/CD8^+$.

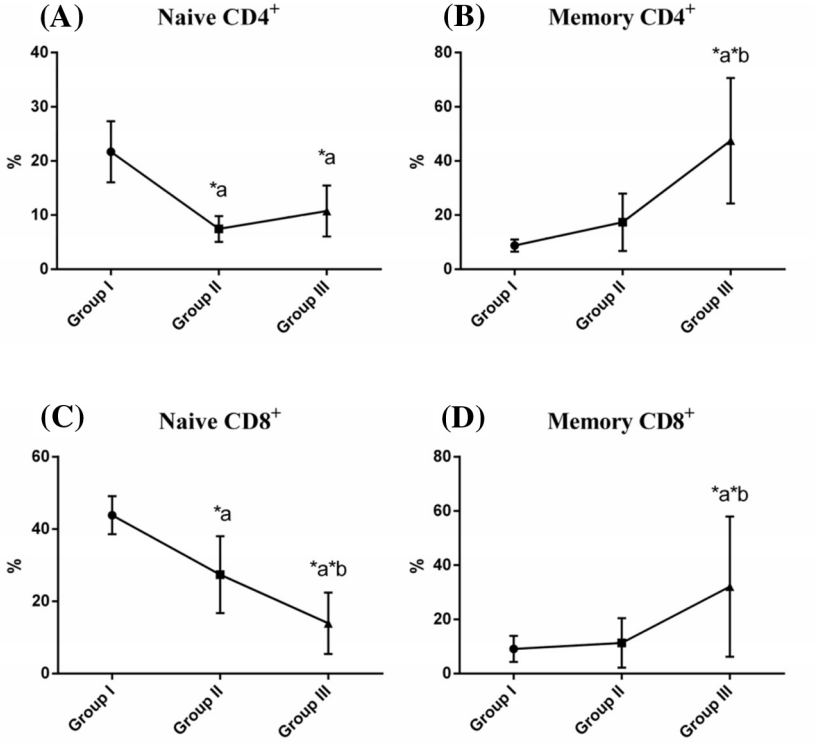

Figure 3 The percentages of naive and memory $CD4^+$ and $CD8^+$ T cells in peripheral blood of C57BL/6 mice from different age groups. Compared with Group I, $^{*a}P < 0.05$; compared with group II, $^{*b}P < 0.05$. (A) The percentages of naive $CD4^+$ T cells in $CD4^+$ T cells; (B) the percentages of memory $CD4^+$ T cells in $CD4^+$ T cells; (C) the percentages of naive $CD8^+$ T cells in $CD8^+$ T cells; (D) the percentages of memory $CD8^+$ T cells in $CD8^+$ T cells.

The group III had a significant increase in the percentage of $CD8^+CD28^+$ T cells compared with group I and group II (Fig. 4).

3.3. The influences of age on T lymphocyte subsets' viability in C57BL/6 mice

The T lymphocyte proliferation in vitro was shown, compared with group I, group II and group III had a decrease in proliferative capacity. Between group II and group III, there was not a significant difference (Fig. 5).

4. Discussion

In this study, we sought to determine the change of age on relative numbers of T lymphocyte subsets in C57BL/6 mice. Our study demonstrated that there was a reduction in $CD4^+$ T cell and an increase in $CD8^+$ T cell of peripheral blood happened at the age of 7 months in C57BL/6 mice, which led to an inverted $CD4^+/CD8^+$ ratio. This finding is agreement with a study done on peripheral blood of aging BALB/c mice, reported a drop in $CD4^+/CD8^+$ ratio with aging (Demir et al., 2008). $CD4^+$ T cells are mainly regulatory cells

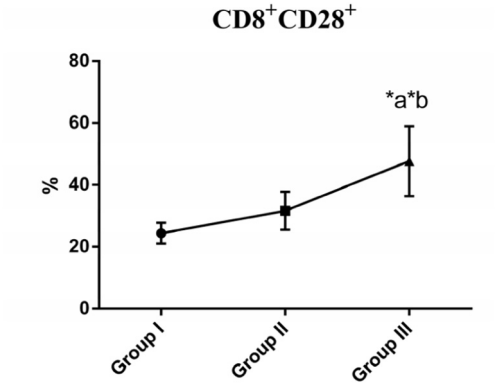

Figure 4 The percentages of $CD8^+CD28^+$ T cells in $CD8^+$ T cells. Compared with Group I, $^{*a}P < 0.05$; compared with group II, $^{*b}P < 0.05$.

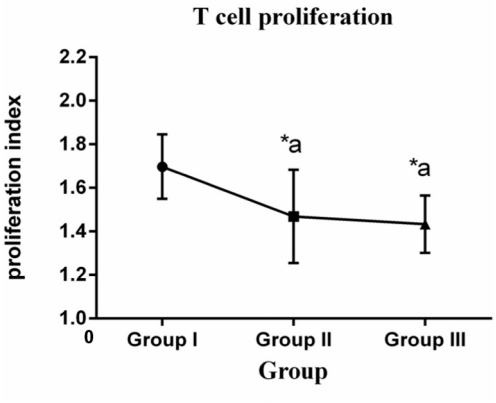

Figure 5 T lymphocyte proliferation in vitro. Splenocytes isolated from different group mice were stimulated with Con A, harvested, and stained with PerCP/Cy5.5 anti-mouse CD3 antibody, and then 10,000 cells were analyzed by flow cytometry. Compared with Group I, $^{*a}P < 0.05$.

and recognize antigens in the context of Class II major histocompatibility complex (MHC), whereas $CD8^+$ T cells are mainly cytotoxic cells and recognize antigen presented within Class I MHC molecules. Both functions are of vital importance in adaptive and innate immune responses (Castelo-Branco and Soveral, 2013).

T cell differentiation takes place in the thymus and results in the production of $CD4^+$ or $CD8^+$ naive cells, which are then exported to the periphery (Moro-García et al., 2012). Both $CD4^+$ and $CD8^+$ T cells can be further subdivided based on expression of other surface molecules. Naive T cells can be identified by expression of the high-molecular weight isoform of the CD45 molecule known as CD45RA, while the memory T cells express the low-molecular weight isoform of CD45 known as CD45RO. In our study, naive and memory T cells were subdivided by CD44 and CD62L which was shown in Fig. 1. The absolute number of T cells decrease with age and this decrease affects more importantly the naive subset (Koch et al., 2008). A significant decline in both $CD4^+$ and $CD8^+$ naive T cells was observed when they were compared between group I and group II, group III. From Fig. 2, we can know that change emerged at the age of 7 months old of the mice, while, $CD4^+$ and $CD8^+$ memory T cells were increased when they were compared between group I, group II and group III. An earlier observation had demonstrated an age-related shift in the representation of naive and memory phenotypes with a decrease in naive T cells and an accumulation of memory lymphocytes in human (Provinciali et al., 2009).

An interesting result of the study is that the expression of $CD8^+$ $CD28^+$ showed a significant increase with age, which was different from human. Activation of both naive and memory T cells is a complex process that requires the intervention of co-stimulatory molecules after the binding of TCR to MHC molecules (Moro-García et al., 2012). CD28 is an important co-stimulatory molecule present in T cells and the binding of CD28 to its co-receptor results in potent activation stimuli for T cells (Finney et al., 2004). However, with age, CD28 expression decreases in both $CD4^+$ and $CD8^+$ T cells (Nociari et al., 1999; Weyand et al., 1998), consistent with a decreased naive cell pool and the accumulation of highly differentiated T cells. CD28 presence in T cells decreases with cell differentiation from naive to central memory to effect memory cells as a result of persistent antigenic stimulation and repeated proliferation cycles (Vallejo, 2005). While, our different age group mice were all housed under a specific pathogen free environment, further study showed be conducted to clarify the reason of this difference between human and C57BL/6 mice.

When T lymphocytes were stimulated with Con A, the group II and group III had a significant lower proliferative ability than group I and there was not a significant difference between group II and group III. T lymphocyte proliferation with Con A stimulation in vitro can perform the immune function well and most of the T lymphocyte subsets parameters were changed at the age of 7 months in mice, which indicated that C57BL/6 mice entered the old age period at 7 months and had a long time old age period.

In conclusion, age related changes in the immune system are a complex process. The relative numbers of $CD4^+$, naive $CD4^+$ and $CD8^+$ T cells decreased with age and $CD8^+$, memory $CD4^+$ and $CD8^+$ T cells increased with age. While the percentage of $CD8^+CD28^+$ T cells increased with age which were different from human, this needs further study. We thought the immune system began aging at 7 months in C57BL/6 mice under a specific pathogen free environment. In our study, we provide relative values for the T lymphocyte subsets in the different age groups of C57BL/6 mice.

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