Possible Role of Genetic Factor(s) on Age-related Increase of Peripheral CD4⁺CD8⁺ Double Positive T Cells in Cynomolgus Monkeys

Won-Woo LEE1, 2), Ki-Hoan NAM1, 3), Keiji TERAO1), and Yasuhiro YOSHIKAWA2)

1) Tsukuba Primate Center, National Institute of Infectious diseases, 1 Hachimandai, Tsukuba, Ibaraki, 305-0843, 2) Department of Biomedical Science, The Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1, Yayoi, Bunkyo-ku, Tokyo, 113-8657, 3) Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Yusong P.O. 115, Daejon, 305-333, South Korea

Abstract: Mature TCR αβ T cells in peripheral blood are generally classified into either CD4 single positive (sp) T cells or CD8sp T cells. Several studies demonstrated that considerable amounts of CD4⁺CD8⁺ double positive (DP) T cells exist in peripheral blood of human and several animals. In particular, we previously reported that peripheral DP T cells increase in an age-related manner in cynomolgus monkeys (Macaca fascicularis), but the finding that DP T cells in some aged monkeys were maintained at a low proportion (under 5%), suggests that the increase in peripheral DP T cells might be genetically controlled in cynomolgus monkeys. To test this hypothesis, 24 families were randomly selected and used in a formal genetic analysis of the proportion of DP T cells. Parents and offspring in selected families were classified into DP-High and DP-Low groups based on a 5% cutoff level of DP T cells. The cutoff value was set by analysis of the distribution of the proportion of DP T cells. Nine out of 13 offspring (69.2%) with DP-High × DP-High parents belonged to the DP-High group, whereas three out of nine offspring (33.3%) belonged to DP-High group in the case of DP-High × DP-Low mating pairs. No offspring (0%) of two offspring with DP-Low × DP-Low parents belonged to the DP-High group. In addition, heritability (h²: narrow sense) obtained from the regression coefficient of offspring on mid-parent values was 0.54 ± 0.19. Both findings suggest that increases in DP T cells in cynomolgus monkeys may be genetically controlled.

Key words: cutoff value, cynomolgus monkeys (Macaca fascicularis), Double positive (DP) T cells, heritability, mid-parent values

Introduction

Mature circulating T cells are generally divided into two subsets: CD4 single positive (sp) and CD8sp T cells. The CD4sp T cells interact with MHC class II/antigen complex and then function as helper/inducer T cells, whereas CD8sp T cells recognize antigen in combination with MHC class I and have cytotoxic functions.

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Address corresponding: K. Terao, Tsukuba Primate Center, National Institute of Infectious Diseases, 1-Hachimandai, Tsukuba, Ibaraki, 305-0843, Japan
In addition to the two major subsets, a low proportion (under 5%) of CD4+CD8– double negative (DN) and CD4+CD8+ double positive (DP) T cells are also observed in the peripheral blood of healthy individuals [3, 14].

A number of studies have recently reported on transient and/or persistent increases of circulating peripheral DP T cells in chicken, swine, macaque monkeys and humans [1, 5, 6–8, 10, 16, 17, 20, 24–26, 29, 30]. In contrast to the immature CD4+CD8αβ+ DP subset in the thymus [3, 14, 19, 28], these peripheral DP T cells are a mature memory T cell subset with strong cytotoxic activity and are considered to be of extrathymic T cell lineage having a distinct origin and maturation pathway due to their phenotype with CD8αα homodimers [21, 25, 29]. The origin and functional significance of this subset has remained largely unclear.

Our previous studies demonstrated that CD4+CD8αα+ DP T cells in cynomolgus monkeys: 1) significantly increase at over 11 years of age; 2) have a phenotype of resting memory T cell and distribute mainly in peripheral blood and spleen but rarely in lymph nodes; and 3) have both helper and cytotoxic functions, and some of their clones share the same TCR Vβ with CD4sp T cells, suggesting that they are derived from the same origin [1, 20, 21].

Interestingly, our cross-sectional data on 195 cynomolgus monkeys also showed that in some aged monkeys (over 11 years of age), the DP T cell subset was maintained at a low proportion [under 5% in the peripheral blood lymphocytes (PBL)] [20]. Some investigators have reported that genetic factor(s) have an influence on the sizes of T cell subsets in humans and mice [2, 4, 13, 27]. From these findings, it occurred to us that levels of peripheral DP T cells might be genetically controlled in cynomolgus monkeys. With regard to this, it is important to note that the appearance of DP T cells in chicken was found to be an inherited dominant trait [16, 17]. Like cynomolgus monkeys, CD4+CD8αα+ DP T cells in chicken also increases in an age-dependent manner [8].

To examine the effect of genetic background on the increase in peripheral DP T cells, a familial study on 24 cynomolgus monkey families was carried out and heritability (h²) was estimated.

In the present study, we report that the increase in peripheral DP T cells is genetically controlled in cynomolgus monkeys and the heritability (h²) obtained by regression of offspring on mid-parent values is 0.54 ± 0.19 (± standard error).

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**Materials and Methods**

**Animals**

All cynomolgus monkeys used in this study were bred and reared in our indoor facilities at the Tsukuba Primate Center (TPC), National Institute of Infectious Diseases. The indoor facilities were maintained under conditions of 25 ± 2°C (temperature), 60 ± 5% (relative humidity), and 14 h light per day (from 05:00 to 19:00) [12]. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Infectious Diseases.

In the present study, offspring over 11 years of age were selected because the proportion of DP T cells becomes stable after 10 years of age [20]. To select a suitable family, information on the parentage and breeding group was obtained from the database that is maintained at the TPC. The 24 selected families underwent random mating and all forty-one monkeys in these selected families were born in the TPC. There were no infectious outbreaks that they had been exposed to and all monkeys had no apparent clinical symptoms at the time of blood collection. They were anesthetized with 10 mg/kg ketamine HCl and 2 ml of heparinized blood was collected from the femoral vein at around 10:00 a.m. To establish the cutoff value for classification, data on 195 healthy cynomolgus monkeys from our previous cross-sectional study [20] was re-assessed.

**Preparation of cells**

The leukocytes were isolated as previously described [20]. Briefly, heparinized blood was treated with ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM of Na₂EDTA, pH 7.2) to lyse red blood cells for 5 min at 37°C. After lysis, leukocytes were washed and resuspended with RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS; Sigma, St. Louis, MO), 50 IU/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma), and 2 mM L-glutamine (Sigma)[hereafter RPMI-10%]. The leukocytes were stored at 4°C until use.
Monoclonal Antibodies (mAbs) and Flow Cytometry Analysis

Cell surface antigens were analyzed by three-color flow cytometry as previously described [1]. Typically, \(2 \times 10^5\) leukocytes were stained with the following antibodies: FITC (Fluorescein isothiocyanate)-labeled anti-CD3 (clone FN18; Biosource, Camarillo, CA), phycoerythrin (PE)-labeled anti-CD4 (NU-TH/1; Nichirei, Tokyo, Japan), anti-CD8\(\beta\) (2ST8.5H7; Immunotech, Westbrook, ME), and R-phycoerythrin (RPE)-Cy5-labeled anti-CD8 (DK25; DAKO, Glostrup, Denmark). For negative controls, fluochrome-labeled isotype-matched mouse Abs were used. After staining for 1 h at 4°C, leukocytes were washed with RPMI-10%, fixed with CellFIX (Becton Dickinson, Mountain View, CA), and then were kept at 4°C. The fluorescence of the stained samples was analyzed by a FACSCalibur (Becton Dickinson). Lymphocytes were gated on the forward and side scatter pattern and 10,000–20,000 events were collected. The analysis of the fluorescence intensity was performed with CellQuest software (Becton Dickinson) and markers were based on negative controls.

Statistics

The relationship between variables was tested by Student’s \(t\)-test and differences were accepted as significant at \(P<0.05\). Estimate of heritability (h\(^2\)) was obtained from the regression coefficient of offspring on mid-parent (average of the two parents) values. The slope of this regression line was deemed to be the heritability (h\(^2\)), especially, the narrow-sense heritability [9, 18, 23].

Results

Distribution of peripheral DP T cell subset in selected families

To examine whether the proportion of peripheral DP T cells is genetically controlled, we selected 24 families from our cynomolgus monkey colony. Because our previous study demonstrated that peripheral DP T cells significantly increased over 11 years of age [20], only families in which offspring were over 11 years of age were selected so the number of selected families was very limited. The DP T cell proportion in peripheral blood was measured for all members of the families by three-color flow cytometry analysis as described in materials and methods.

Similar to many traits expressed in an out-bred mating colony, such as non-human primates, the proportion of peripheral DP T cells in cynomolgus monkeys didn’t show a simple discontinuous distribution. To evaluate the contribution of genetic background to the proportion of DP T cells in selected families, we first needed to set a cutoff level for classification. For this purpose, data on 195 healthy cynomolgus monkeys from our previous cross-sectional study [20] was re-plotted.

As shown in Fig. 1, the proportion of peripheral DP T cells in cynomolgus monkeys shows an obvious bimodal distribution in both males (Fig. 1A) and females (Fig. 1B). When the cutoff value was considered as about 5%, the cynomolgus monkeys tested could be classified into two populations: high and low populations. The percentages of monkeys, which belonged to the high population, were very similar between male and female monkeys (34.3% and 32.3%, respectively). These findings suggest that gender differences have no relation to the distribution of DP T cell proportions in cynomolgus monkeys.

We previously reported that the proportion of peripheral DP T cells in cynomolgus monkeys significantly increased in an age-dependent manner [20]. To examine the age-related change in distribution of peripheral DP T cell proportions, the data of the 195 monkeys shown in Fig. 1 were divided into two age groups, young (under 10 years of age) and aged (over 11 years of age) groups, and were plotted according to their peripheral DP T cell proportions. As seen in Fig. 2B, most monkeys (111/119) in the young group exhibited low DP T cell proportions (under 5% in the PBL), whereas monkeys in the aged group showed two populations similar to Fig. 1 (Fig. 2A). By setting 5% as a cutoff value, only 25% (19/76) of monkeys in the aged group belonged to the low population (under 5%). However, this bimodal distribution in the aged group was not attributable to age-related increases in DP T cells because there was no significant difference in the mean age of monkeys between the low (17.65 ± 5.48 years, \(n=19\)) and high populations (16.59 ± 4.69 years, \(n=57\)). On the basis of these results, the cutoff value of peripheral DP T cell proportion was set at 5% and all monkeys tested were classified into DP-High (DP T cells over 5%) or DP-Low (under 5%) groups by this.
Next, the familial study was performed by comparing DP T cell proportions between parents and offspring in the 24 families selected. Consistent with published reports [1], most of increased DP T cells in this familial study exhibited CD4+CD8\textsuperscript{dim} phenotype and multicolor flow cytometry analysis clearly showed that they exclusively expressed the CD8αα homodimer, but not the CD8αβ heterodimer (data not shown). Figure 3 shows the distribution of peripheral DP T cells in the offspring of 24 families in which parents were classified into DP-High or DP-Low group by the 5% cutoff value. In the case of families with DP-High × DP-High (Fig. 3A), 69% of offspring (9/13) belonged to the DP-High group, whereas only 33% offspring (3/9) (Fig. 3B) and none (0/2) (Fig. 3C) from DP High ×
DP-Low parents and DP-Low × DP-Low parents, respectively, belonged to the DP-High group.

Heritability

Many traits in out-bred populations, such as humans and monkeys, are controlled by single or multiple genes. However, environmental factors also frequently influence them [9, 18, 23]. Therefore, these traits show quantitative or continuous properties rather than qualitative or discontinuous ones [2, 23]. To analyze the relative contributions of genes and environment to a variation in a specific trait, the concept of heritability is generally used. Particularly, narrow-sense heritability ($h^2$) indicates the proportion of observed phenotypic variation that is attributable to additive or average genetic effects [2, 9, 18, 23].

Figure 4 represents a regression plot of the offspring phenotype and mid-parent phenotype (average of the two parents). The magnitude of heritability is defined as the slope of the regression line. The heritability in the present study, analyzed by parent-offspring regression, was estimated as $0.54 \pm 0.19$.

Contrary to current immunological dogma stating that mature T cells are generally classified into CD4sp or CD8sp T cells [19, 28], evidence is accumulating of transient and/or persistent increases in peripheral DP T cells in humans and several species of animals [1, 5, 6–8, 10, 16, 17, 20, 24–26, 29, 30]. Although the origin and functional significance of this population subset...
are controversial, a number of studies on human DP T cells suggest that the CD8 molecule, particularly the CD8α chain, is expressed on the surface of activated memory CD4sp T cells, rarely vice versa, and subsequently the CD4+CD8+ phenotype is maintained permanently in certain physiological circumstances such as chronic viral infection, autoimmune diseases, leukemia and lymphoma, and even in apparently healthy individuals [5, 10, 15, 24–26].

Compared with the frequency of the DP T cell subset in humans, considerable proportions of peripheral DP T cells were observed in chicken, swine, and macaque monkeys [1, 6–8, 16, 17, 20, 29, 30]. In particular, features of DP T cells in cynomolgus monkeys are very similar to those in humans, that is, peripheral DP T cells in the two species: 1) increase in older individuals [1, 5, 15, 20]; 2) exhibit a CD4+CD8αα+ phenotype known as a characteristic of extrathymic T cell lineage [1, 20, 25]; 3) provide help for B cell differentiation and have strong cytolytic activity [21, 22, 25, 29]; and 4) some of their clones may be derived from CD4sp T cells by clonal expansion [5, 21, 25]. Therefore, research on peripheral DP T cells in monkeys may have implications of biological significance for human DP T cells.

In a previous cross-sectional study, we described that in the process of age-dependent remodeling of peripheral DP T cells in cynomolgus monkeys, individual differences in proportions of this subset became broader. Furthermore, some aged monkeys (over 11 years of age) still had a DP T cell subset with a low proportion (under 5%) [20]. Because all monkeys tested in this cross-sectional study were born and reared in the same indoor facility, it is reasonable to assume that age-related individual differences in DP T cell proportion are attributable to factor(s), other than environmental factors. With regard to this, it should be noted that the appearance of DP T cells in chicken was found to be an inherited dominant trait [16, 17]. These findings led us to examine whether the increase in peripheral DP T cells is genetically controlled in cynomolgus monkeys.

As shown in Figs. 1 and 2, re-assessment of data published in our previous cross-sectional study allowed us to decide 5% as the proportion of DP T cells for the cutoff value for classification. At this cutoff value, the proportion of DP T cells was characterized by a bimodal distribution comprised of DP-High (over 5%) and DP-Low (under 5%) groups. Furthermore, the 3:1 ratio of DP-High to DP-Low group in the aged group (Fig. 2A) suggested that the increase in peripheral DP T cells might be simply controlled by one locus system with two alleles in which dominance is complete. In many cases, the pattern of genetic transmission, such as polygene, single-gene recessive or dominant and co-dominant effects, can be demonstrated by complex segregation analysis [2, 4]. Although the number of selected families in the present study was small, familial studies suggest a genetic transmission pattern. It is clear Fig. 3 that the proportion of DP T cells in offspring was affected by genetic factor(s), and the fact that there were no offspring with over 5% of peripheral blood DP T cells from DP-Low × DP-Low parent mating pairs, suggests that allele(s) contributing to the DP-Low phenotype are recessive. Additionally, a high level of heritability (h²=0.54 ± 0.19), as shown in Fig. 4, supports the conclusion that the DP T cell proportion is affected by genetic factor(s).

In the present study, this genetic control is unlikely to be caused by modulation and/or block of CD8α chain expression on memory CD4sp T cells after activation due to normal CD8α chain expression on CD8sp T cells. It is more easily conceived that this finding is related to certain MHC types associated with resistance and susceptibility to infectious agents that may become recall antigen to DP T cells.

Several studies reported genetic control on the proportions of T cell subsets in humans, inbred mice, and chickens [2, 4, 11, 13, 16, 17, 27]. In particular, it was evident from statistical analysis based on the large-scale familial studies that the numbers of CD4 and CD8 T cells, and consequent CD4/CD8 ratio, were controlled by an autosomal recessive gene in humans [2, 4]. By accumulating a number of proper families, the pattern of genetic transmission of DP T cell phenotype can be made clear in cynomolgus monkeys.

This is the first report to describe the genetic factor(s) affecting the increase of peripheral DP T cell proportions in cynomolgus monkeys. We propose that the proportion of peripheral DP T cells is genetically controlled with high heritability [h²: 0.54 ± 0.19 (± standard error)]. Considering the similarity of characteristics between human and monkey DP T cells, it is possible that increase of peripheral DP T cells in humans is also under genetic control.
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References


