Peripheral blood CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes in cynomolgus monkeys are of resting memory T lineage

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Abstract

In this study, we analyzed peripheral blood CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) lymphocytes in adult cynomolgus monkeys (Macaca fascicularis). Forty of 55 monkeys had >5% of the peripheral blood DP subpopulation (9.3 ± 5.9%; mean ± SD) in peripheral blood lymphocytes (PBL) in contrast to a low percentage of peripheral blood DP cells in humans and mice. In a cross-sectional study, the peripheral blood DP cells were found to increase in proportion with age. To clarify whether peripheral blood DP lymphocytes were immature precursors released from thymus without prior differentiation, the expressions of CD8 chains and CD1b on peripheral blood DP lymphocytes were compared with those on thymocytes. The peripheral blood DP lymphocytes were CD8α<sup>+</sup>β<sup>-</sup> and CD1b<sup>-</sup>, while thymic DP lymphocytes were CD8α<sup>+</sup>β<sup>+</sup> and CD1b<sup>-</sup>, suggesting that the peripheral blood DP cells are extrathymic T lymphocytes. Furthermore, the peripheral blood DP lymphocytes exhibited a resting memory T cell phenotype with CD2hi CD3<sup>-</sup>CD28<sup>-</sup>CD29<sup>hi</sup>CD49d<sup>hi</sup>CD69<sup>-</sup>CD80<sup>lo</sup>. Taken together, adult cynomolgus monkeys possess a unique peripheral blood DP T cell subpopulation which expresses a resting memory T cell phenotype. In addition, similar phenotypic properties of DP lymphocytes were distributed in the spleen and lymph nodes, although the proportion was less in the spleen and much less in lymph nodes than in PBL.

Introduction

There exist two major subsets of human T lymphocytes identified by the expression of the differentiation antigens, CD4 and CD8, which direct TCR contact with MHC class II and I respectively. In thymocytes, the major subset of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) T lymphocytes is differentiated into CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> single-positive (SP) T cells after positive and negative selection steps (1). Although common DP thymocytes are CD3<sup>+</sup>CD4<sup>+</sup>CD8α<sup>+</sup>β<sup>+</sup>, a subset that is CD3<sup>+</sup>CD4<sup>-</sup>CD8α<sup>+</sup>β<sup>-</sup> exists physiologically as an intermediate stage between triple-negative and general DP thymocytes (2).

In peripheral blood lymphocytes (PBL), most of the T lymphocytes express either CD4 or CD8 and a low percentage (1–3%) of DP T cells with blastoid morphology is usually found (3–5). Recently, some individuals with neoplastic or infectious diseases or without obvious diseases were shown to possess a much higher percentage of DP lymphocytes in blood (6–9), although such cases were rare; 18 of >15,000 subjects (>10% of PBL) (7,8) or 51 of 7500 subjects (>5% of PBL) (9).

It has been shown that a higher percentage (up to 15%) of peripheral blood DP lymphocytes was found in some rhesus monkeys (Macaca mulatta) (10,11) and cynomolgus monkeys (Macaca fascicularis) (12). However, no further characteristics of the peripheral blood DP subpopulation are known. Here, we first conducted a cross-sectional study for peripheral blood DP lymphocytes in cynomolgus monkeys and further analyzed their phenotypic properties.

Methods

Animals and tissues
Clinically healthy adult cynomolgus monkeys born and reared in our laboratory-bred colony at 10–27 years of age (14.0 years
old on average) were used for experiments. The monkeys originated from wild-caught parents imported from Indonesia, Philippine and Malaysia. The monkeys were free of simian immunodeficiency virus, varicella-zoster-like virus and measles virus. PBL were separated from blood by the Ficoll-Paque gradient method. The lymphoid tissues were minced to make a single-cell suspension. These cells were re-suspended in RPMI 1640 medium supplemented with 10% FCS at 4°C until use.

mAb
The mouse mAb used in this study were as follows: FITC-, phycoerythrin (PE)- or peridinin chlorophyll protein (PerCP)-conjugated mAb to monkey CD3 (FN18) (BioSource, Camarillo, CA) and to human CD4 (Leu-3a), CD8α (Leu-2a), CD16 (Leu-11a), CD49d (L25), CD69 (Leu-23) and CD80 (L307.4) (Becton Dickinson, Mountain View, CA), CD1b (NU-T2), CD2 (NU-TER), CD4 (Nu-Th/l) and CD28 (KOLT-2) (Nichirei, Tokyo, Japan), and CD29 (4B4) (Coulter, Tokyo, Japan). For detection of the CD8β chain, mouse mAb to human CD8β (5F2) (13) and the CD8αβ heterodimer (2ST8) (14), which were generously provided by Drs Neal Flomenberg and Ellis L. Reinherz respectively, were screened for reactivity to CD8 chains of cynomolgus monkeys. Only mAb 2ST8 was reactive but not 5F2; therefore, 2ST8 was used for further analysis. Cross-reactivity of 2ST8 mAb to monkey CD8αβ was confirmed by the expression of mRNAs for CD8α and β chains by RT-PCR (data not shown).

Two- and three-color flow cytometric analysis
Surface markers on lymphocytes were analyzed as previously described (12). In brief, 2×10^5 cells were reacted with mAb described above at 4°C for 30 min. The cells were washed, then fixed with PBS containing 1% paraformaldehyde and kept at 4°C. In the case of indirect single-color staining, the cells pretreated with aggregated human Ig were stained with the first mAb at 4°C for 30 min. They were then washed and treated with FITC-labeled anti-mouse IgG F(ab)_2 followed by washing and fixation. For indirect two- and three-color staining, the labeled cells were treated with aggregated mouse Ig for 10 min at 4°C, then stained with PE/PerCP-labeled mouse mAb, followed by washing and fixation. The fluorescence of the stained cells was detected on a FACScaliber (Becton Dickinson). The cytometer was calibrated by FACSDiscrim software using CaliBRITE beads (Becton Dickinson). Analysis of the fluorescence intensity was performed using CellQuest software (Becton Dickinson).

Results
The proportion of peripheral blood DP lymphocytes
As we and others have described previously (10–12), macaque monkeys tend to have a higher proportion of peripheral blood DP lymphocytes. To further extend these observations, we analyzed the proportion of the DP cells in blood samples obtained from 55 randomly selected adult cynomolgus monkeys in our laboratory-bred colony. Figure 1(A) shows the histogram of cumulative numbers of monkeys for the percentage of peripheral blood DP subpopulation. The result indicated 15 monkeys of <5% (peripheral blood DP<sub>lo</sub>), 19 of 5–10% (peripheral blood DP<sub>mid</sub> and 21 of >10% peripheral blood DP cells (peripheral blood DP<sub>hi</sub>) respectively (9.3 ± 5.9%; average ± SD). Figure 2 indicates the representative two-color profiles of each group. The intensity of CD4 expression on peripheral blood DP cells was nearly comparable to CD4 SP lymphocytes, while that of CD8 expression was lower than CD8 SP cells, regardless of the proportion of DP cells. Figure 1(B) demonstrates the proportion of CD4 SP, CD8 SP and DP cells in PBL of 17 randomly selected adult monkeys. No significant correlation was seen in the proportions among these subpopulations.

The proportion of peripheral blood DP lymphocytes increases with age
To clarify the relationship between the proportion of the peripheral blood DP subpopulation and age, a cross-sectional study was conducted in the monkeys, which were classified into four groups by age. The results are summarized in Table 1. Interestingly, the proportions of peripheral blood DP cells in 0- to 4-year-old and 4- to 9-year-old monkeys were much lower (1.5 and 3.2% on average, respectively) than those in 10- to 14-year-old or >15-year-old monkeys. Thus, these data strongly suggested an age-dependent increase in the proportion of peripheral blood DP lymphocytes in cynomolgus monkeys.

The peripheral blood DP lymphocytes are independent of thymic DP T cells
As for the peripheral blood DP lymphocytes in cynomolgus monkeys, there was a possibility that thymic precursors might be released from the thymus to peripheral blood without further differentiation. To solve this question, we compared the expression of CD8 chains on peripheral and thymic T lymphocytes. Figure 3 indicates the result of two- and three-color analysis in a monkey as a representative of several experiments. In the case of this monkey, 20% of PBL co-expressed CD4 and CD8α; however, the subpopulation did not express the CD8αβ heterodimer (Fig. 3A, upper). Furthermore, three-color staining for CD4, CD8α and CD8αβ clearly showed that neither CD4 SP nor DP cells co-expressed the CD8αβ heterodimer, whereas 58% of CD8 SP cells expressed the CD8αβ heterodimer (Fig. 3A, lower). In the CD8 SP cells, the CD8αβ<sup>+</sup> subset was not found to be CD3<sup>+</sup> T cells, while the CD8αβ<sup>−</sup> subpopulation (42% in this case) belonged to the CD3<sup>−</sup>CD16<sup>+</sup> NK subset (Shibata et al., in preparation). By contrast, CD8 chains on thymic CD8 SP and DP cells consisted of the αβ<sup>+</sup> heterodimer, as has been shown in humans and mice (15) (Fig. 3B).

We next examined the expression of the CD1b marker, which is known to be expressed on thymic lymphocytes but not on PBL in other species (16–18). The result indicated that CD1b was clearly positive on thymic DP cells but was not expressed on peripheral blood DP cells (Fig. 4). Consistent results as for CD8 chains and CD1b expression were obtained from several monkeys (data not shown). It should be noted that aged monkeys were depleted of the thymus, although these monkeys also had peripheral blood DP cells, of which the proportion differed individually (Fig. 1A). These findings
Peripheral blood CD4$^{+}$CD8$^{+}$ lymphocytes in monkeys

**Fig. 1.** Analysis of peripheral blood DP lymphocytes in adult cynomolgus monkeys. (A) The histogram of cumulative numbers of monkeys for the percentage of peripheral blood DP lymphocytes. (B) Variation in the proportions of CD4 SP, CD8 SP and DP lymphocyte subpopulations in PBL of 17 randomly selected monkeys.

DP, 26% of CD4 SP and 45% of CD8 SP cells respectively. The peripheral blood DP cells were found to be positive for CD3, which confirmed that the peripheral blood DP cells were of the T cell lineage (Fig. 5A). The peripheral blood DP cells expressed high levels of CD2, CD29 (integrin $\alpha_1$) and CD49d (integrin $\alpha_4$) (Fig. 5A and B). These expression patterns of co-stimulatory molecules on peripheral blood DP T cells suggested that the cells may play a role as memory T lymphocytes. Surprisingly, most peripheral blood DP cells were CD28$^-$ and CD80lo (Fig. 5A and B). In addition, peripheral blood DP T cells did not express CD16 (Fig. 5A).

**Fig. 2.** Two-color flow cytometric analysis of CD4 and CD8 expressions on PBL. Representatives of three groups of cynomolgus monkeys with peripheral blood DPlo (<5%), peripheral blood DPmid (5–10%) and peripheral blood DPhi (>10%) are shown. The percentages of cells in the respective quadrants are indicated in the corners.

**Table 1.** Comparison of the proportion of peripheral blood DP lymphocytes in different age groups

<table>
<thead>
<tr>
<th>Age Group</th>
<th>DPlo</th>
<th>DPmid</th>
<th>Dphi</th>
</tr>
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<tbody>
<tr>
<td>0–4 years</td>
<td>1.2</td>
<td>2.7</td>
<td>9.4</td>
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<tr>
<td>5–9 years</td>
<td>1.58</td>
<td>9.4</td>
<td>8.7</td>
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<tr>
<td>10–14 years</td>
<td>1.58</td>
<td>9.4</td>
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<tr>
<td>&gt;15 years</td>
<td>1.58</td>
<td>9.4</td>
<td>8.7</td>
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Values are expressed as mean percentage of peripheral blood DP lymphocytes ± SD in respective age groups (n = 20).

indicate that peripheral blood DP cells are independent of thymic DP T cells.

**Phenotypic properties of peripheral blood DP lymphocytes**

We examined the phenotypic properties of the peripheral blood DP lymphocytes and compared them with those of the SP cells by three-color flow cytometric analysis. Figure 5 shows representative histograms of the fluorescence intensity of the CD4 SP, CD8 SP and DP subpopulations stained with mAb to third markers. In this case, PBL consisted of 17% of DP, 26% of CD4 SP and 45% of CD8 SP cells respectively. The peripheral blood DP cells were found to be positive for CD3, which confirmed that the peripheral blood DP cells were of the T cell lineage (Fig. 5A). The peripheral blood DP cells expressed high levels of CD2, CD29 (integrin $\alpha_1$) and CD49d (integrin $\alpha_4$) (Fig. 5A and B). These expression patterns of co-stimulatory molecules on peripheral blood DP T cells suggested that the cells may play a role as memory T lymphocytes. Surprisingly, most peripheral blood DP cells were CD28$^-$ and CD80lo (Fig. 5A and B). In addition, peripheral blood DP T cells did not express CD16 (Fig. 5A).

It was reported that human peripheral blood DP lymphocytes were characterized as larger, blastoid cells (3), while those with a small lymphocyte morphology were also found in some cases (6,8). Thus, we analyzed the expression of CD69, an immediate activation marker, on peripheral blood DP cells. Uniquely, they did not express CD69 (Fig. 5A), which concordant with the result that they did not express MHC-II DR or CD25 (data not shown). Furthermore, they exhibited predominantly a small agranular morphology like CD4 SP or CD8 SP cells as examined by scatter analysis (Fig. 5C). Taken together, these results strongly suggest that peripheral blood DP lymphocytes are resting memory T cells.

**Distribution of DP lymphocytes in lymphoid tissues**

We studied the distribution of DP cells in lymphoid tissues, i.e. spleen, inguinal and mesenteric lymph nodes. The results are shown in Fig. 6. In the case of one cynomolgus monkey as a representative, the proportion of peripheral blood DP cells was 19.5%. The proportions of DP cells in splenocytes, inguinal and mesenteric lymph node cells were 10.7, 2.5 and 4.5% respectively. Essentially similar results were obtained from several other monkeys (data not shown). The phenotypic properties of the DP cells in these lymphoid tissues were examined. The result was essentially similar to those of peripheral blood DP T cells, i.e. CD2$^+$CD3$^+$CD4$^+$CD8$^-$ of CD28$^+$CD29$^+$CD49d$^+$CD69$^-$CD80$^+$.
Peripheral blood CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes in monkeys

Fig. 3. The expression of the CD8αβ heterodimer on CD4 SP, DP and CD8 SP subpopulations in PBL (A) and thymocytes (B). The expression level of CD8αβ on each subpopulation displayed by respective quadrants on a dot-plot (upper, left) is indicated as histograms (lower). Alternatively, two-color flow cytometric analysis of CD4 and CD8αβ expressions in PBL is shown as a dot-plot (upper, right). The percentages of cells in the respective quadrants are indicated in the corners.

Fig. 4. Histograms of the expression levels of CD1b on peripheral blood DP and thymic DP cells. Three-color staining of CD4, CD8 and CD1b was performed, and fluorescence intensity of CD1b (open) and background controls (closed) on the DP subpopulation gated on the basis of quadrants is indicated.

Discussion

In this report, we describe for the first time the characteristics of peripheral blood DP lymphocytes in cynomolgus monkeys. The findings of our study were as follows. (i) Forty of 55 adult monkeys had >5% of the peripheral blood DP subpopulation, which was in contrast to previous reports in humans (>1% of individuals) (7–9). (ii) The proportion of the peripheral blood DP subpopulation remarkably increased with age, especially between two groups of 5- to 9- and 10- to 14-year-old monkeys. (iii) The peripheral blood DP lymphocytes were independent of thymic DP T cells because of CD4<sup>+</sup>CD8α<sup>+</sup>β<sup>-</sup> and CD1b<sup>-</sup>. (iv) The peripheral blood DP lymphocytes exhibited the phenotype of small resting T cells with CD2<sup>+</sup>CD3<sup>-</sup>CD28<sup>-</sup>CD49d<sup>-</sup>CD69<sup>-</sup>CD80<sup>-</sup>, which suggested memory T cells. (v) The similar characteristics of DP lymphocytes were distributed not only in PBL but also among lymphoid tissues, mainly in the spleen, while the proportion was less than in PBL. From these results, it was concluded that cynomolgus monkey DP lymphocytes in blood and lymphoid tissues possess properties of the T lineage with a phenotype of resting memory T cells which are independent of the thymus.

In macaque monkeys, a higher proportion of DP lymphocytes was found in PBL than in humans (10–12). The results in this study demonstrated that 40 of 55 adult monkeys (73%) had >5% of the peripheral blood DP subpopulation (Fig. 1A) in contrast to a low percentage in humans (>1% of individuals). Interestingly, the peripheral blood DP cells increased in proportion around before or after 10 years old (Table 1). These monkeys were healthy without any clinical or hematological abnormality. Furthermore, peripheral blood DP lymphocytes possessed small cell morphology and were CD69<sup>-</sup> (Fig. 5) and CD25<sup>-</sup> MHC-IIDR<sup>-</sup> (data not shown). Thus, it is likely that peripheral blood DP T lymphocytes were not activated blastoid cells which were related to neoplasia or some infectious diseases as previously described in humans (7–9), but rather they existed physiologically in adult cynomolgus monkeys.

Recently, several groups have reported the presence of a
Peripheral blood CD4⁺CD8⁺ lymphocytes in monkeys

Fig. 5. Phenotypic analysis of CD4 SP, DP and CD8 SP subpopulations in PBL. The fluorescence intensity of CD2, CD3, CD16, CD28, CD29 and CD69 labeled with FITC in combination with CD4–PE and CD8–PerCP (A) and CD49d and CD80 labeled with PE in combination with CD4–FITC and CD8–PerCP (B) on each subpopulation gated on the basis of the quadrant is indicated as histograms. Background fluorescence intensity on each subpopulation is shown as a control. (C) The flow cytometric dot-plot profiles based on FSC and SSC for each subpopulation are indicated.

Similar DP subpopulation in swine (17–24). The proportion of DP cells in swine PBL was comparable or rather higher than that of cynomolgus monkeys. Thus, peripheral blood DP T cells might be found in a broad range of animal species.

The proportions of CD4 SP or CD8 SP cells in PBL were not correlated with that of peripheral blood DP T cells (Fig. 1B). Thus, our results suggest that peripheral blood DP T cells are an independent subpopulation from both CD4 SP and CD8 SP cells. In swine, however, there was an inverse correlation between CD4 SP and peripheral blood DP cells (20). So far, we have no clear answer for the difference between two species.

It has been known that thymic DP T lymphocytes express the CD8αβ heterodimer (25) and the β chain is required for positive selection during T cell differentiation (26–28). Actually, thymic DP T cells in cynomolgus monkeys expressed the CD8αβ heterodimer (Fig. 3B) and CD1b (Fig. 4) as previously described in other species (16–18). By contrast, peripheral blood DP T cells expressed only the α chain, although the CD8 SP T cells had the αβ heterodimer (Fig. 3A), as has been described in humans (25,29,30). In addition, aged monkeys were depleted of the thymus, although these monkeys also had peripheral blood DP cells, of which the

Fig. 6. Proportion of DP subpopulations in the lymphocytes of peripheral blood, spleen, inguinal and mesenteric lymph nodes. The percentages of DP subpopulations are indicated.
proportion differed individually (Fig. 1A). These findings and the lack of CD1b expression (Fig. 4) suggest that peripheral blood DP T cells may be extrathymic T lymphocytes. It is notable that most peripheral blood DP T cells in humans expressed a low level of CD8 composed only of the α chain (8) and that the peripheral blood DP cells in swine also lacked CD1 expression (17,18). Thus, the peripheral blood DP T cells in these reports may have a similar origin.

One major representative of lymphocytes differentiated extrathymically is intraepithelial lymphocytes (IEL); a subpopulation of them are thymus-independent and express the CD8αα homodimer but lack CD28 (15). Interestingly, an age-related increase of CD28 – T cells Expansion of a lymphocytes population co-expressing T4 (CD4) and T8 (CD8) antigens in the peripheral blood of a normal adult with a resting memory phenotype was reported in humans (36). We also found an age-related increase of the CD28 – peripheral blood T subpopulation (K.-H. Nam et al., submitted). Therefore, both CD28 – T cells in humans and monkeys might have similar implications in ageing.

CD80 is a ligand for CD28 co-stimulatory molecules and is expressed on antigen-presenting cells such as dendritic cells, monocytes, and activated B and T lymphocytes (35). A low level of CD80 expression was found on peripheral blood DP T lymphocytes. CD28 is expressed from the early stages of differentiation of T lymphocytes in the thymus (15). It is suggested that CD28 – T lymphocytes represent a memory subpopulation which has been recently stimulated in vivo (35). Interestingly, an age-related increase of CD28 – T cells with a resting memory phenotype was reported in humans (36). We also found an age-related increase of the CD28 – peripheral blood DP T subpopulation (K.-H. Nam et al., submitted). Therefore, both CD28 – T cells in humans and monkeys might have similar implications in ageing.

(35). It still remains to be elucidated whether the expression of CD80 on peripheral blood DP T cells is effective for a role as antigen-presenting cells on T–T interaction.

Taken together, these results strongly suggest that the peripheral blood DP T cells have a resting memory T phenotype. Recently, swine peripheral blood DP cells have been shown to possess memory T helper function inducible by stimulation with recall antigens (21,23). We are now trying to evaluate the functional properties of peripheral blood DP T cells.

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Abbreviations
- DP: double-positive
- IEL: intraepithelial lymphocyte
- PBL: peripheral blood lymphocyte
- PE: phycoerythrin
- PerCP: peridinin chlorophyll protein
- SP: single-positive

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