NOTE *Clinical Pathology*

Peripheral Blood CD4 and CD8 Double-Positive T Cells of Rhesus Macaques Become Vulnerable to Simian Immunodeficiency Virus by *In Vitro* Stimulation Due to the Induction of CCR5

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ABSTRACT. *In vivo* Simian Immunodeficiency Virus (SIV) challenge of macaques demonstrated the earlier disappearance of CD4 and CD8 double-positive (DP) T cells than CD4 single-positive T cells, although its mechanism remains unclear. Here we found that peripheral DP T cells were readily induced to express CCR5, a secondary receptor for SIV, by *in vitro* stimulation with either concanavalin A or anti-CD3/CD28 monoclonal antibodies. Activated DP T cells were more vulnerable to SIV infection, indicating that the ability of DP T cells to readily express CCR5 after activation may hasten DP T cell death by SIV infection *in vivo*.

KEY WORDS: CCR5, CD4 and CD8 double-positive T cells, Simian Immunodeficiency Virus.

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Simian immunodeficiency virus (SIV) uses the CD4 molecule as the major receptor for infection to target cells [4, 5]. The depletion of CD4-bearing T cells in the body of SIV-infected animals is the hallmark of this viral infection. CD4-bearing T cells include both CD4 single-positive (CD4SP) T cells and CD4 and CD8 double-positive (DP) T cells. DP T cells exist in the intestine and form the large proportion of intestinal T cells in macaque monkeys [10]. *In vivo* SIV challenge demonstrated that intestinal DP T cells are depleted faster and more profoundly than intestinal CD4SP T cells at the early stage of infection. Although this is thought to be caused by higher expression levels of CCR5, the coreceptor for SIV, which are mainly expressed on activated T cells [14, 15], it is still unclear why DP T cells are highly permissive to SIV infection.

Additionally, in macaque monkeys, DP T cells physiologically exist in the peripheral blood [2, 17], the significance of which has not been completely elucidated. However, several investigators have reported that DP T cells exhibit the following characteristics: they proportionally increase in an age-dependent manner [2, 8]; are phenotypically different from thymic DP T cells [1]; have dual immune functions, i.e., low helper and high cytotoxic activity and, freshly isolated, these cells express a high level of interferon γ mRNA [9], which is important for antiviral infection. Although studies have not yet determined whether DP T cells in peripheral blood and the intestine have a common origin, intestinal DP T cells are considered to be from extrathymic lineages [14, 15] and are suggested to have effector functions against viral infection [10]. Thus, both intestinal and peripheral blood DP T cells are considered to be in the same category of "extrathymic DP T cells", although their locations are different. In contrast to cells in the intestine, almost all CD4SP and DP T cells in peripheral blood are not in an activated state and have lower CCR5 expression [10]; therefore, these cells are not considered to be suitable target cells for SIV infection in the natural state of peripheral blood. We hypothesized that, upon activation, DP T cells in peripheral blood have the ability to express higher levels of CCR5 and are therefore more vulnerable to SIV infection than peripheral blood CD4SP T cells. To test this hypothesis, and to assess *in vivo* DP T cell vulnerability to SIV infection, we conducted *in vitro* activation of rhesus monkey peripheral blood mononuclear cells (PBMCs) and compared the expression of CCR5 and the vulnerability to SIV infection of DP T cells with those of CD4SP T cells.

We first examined CCR5 expression levels on *in vitro* activated peripheral blood CD4SP and DP T cells in rhesus monkeys. Acid citrate dextrose (ACD) anti-coagulated blood was taken from rhesus monkeys maintained at Kyoto University (*Macaca mulatta*: MM51, MM132, MM450, MM452, MM455, MM464; Samples were randomly selected depending on the availability of the animals). PBMCs were isolated from whole blood by the Ficoll-Paque gradient method. Concanavalin A (ConA) or anti-CD3/CD28mAbs were used to activate T cells *in vitro* [7, 11]. Freshly isolated PBMCs were stimulated with 12.5 μg/m*l* ConA (Wako, Osaka, Japan) (MM132, MM51 and MM455) or 100 *n*g/m*l* anti-CD3 mAb (clone SP34; BD PharMingen) and 1 μg/m*l* anti-CD28 mAb (clone 28.2; BD PharMingen) (MM132, MM455 and MM464), and cultured in RPMI growth medium (RPMI1640 medium supplemented with 10% fetal bovine serum, 50 U/m*l* penicillin, 50 μg/m*l* streptomycin, 50 μM 2-mercaptoethanol and 100 U/m*l* recombi-

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nant interleukin-2). After a stimulation period of 24 hr, cultured cells were washed with RPMI1640, resuspended in RPMI growth medium, and seeded in 96-well cell culture plates (2×10^5 cells/200 μl/well). The experiments were performed in three monkeys for each treatment, with PBMCs from each animal measured in triplicate. To detect CCR5 expression levels on CD4SP and DP T cell populations, cultured cells were collected at day 2, 4, and 6 post-stimulation and subjected to flow cytometric analysis. Negative controls were prepared in the same manner without mitogen. Cell-surface expression of lymphocyte antigens was identified by flow cytometry using a FACS CantoII (BD PharMingen) and analyzed with BD FACSDiva software. The mAbs used in this study were as follows: anti-CD3 (PE-Cy7; clone SP34, BD PharMingen), anti-CD4 (FITC; clone L200, BD PharMingen), anti-CD8 (RPE-Cy5; clone DK25, Dako, Glostrup, Denmark) and anti-CCR5 (PE; clone 3A9, BD PharMingen). CCR5 expression levels were expressed as the percentage of CCR5-positive cells determined with a gate drawn from unstained controls. From days 4 to 6 post-ConA stimulation, CCR5 expression levels increased both on CD4SP T cells and DP T cells. Of note, DP T cells expressed higher levels of CCR5 (90.47 ± 3.40% at day 6) than CD4SP T cells (38.39 ± 13.98% at day 6) (Fig. 1A and 1B). In anti-CD3/CD28 mAbs-stimulated PBMC, almost identical results were obtained; DP T cells exhibited higher levels of CCR5 expression than CD4SP T cells, from days 4 to 6 post-stimulation (Fig. 1C and 1D). DP T cells exhibited higher levels of CCR5 expression than CD4SP T cells, although 4 to 6 days were required to observe a statistical difference. In previous reports on human peripheral blood lymphocytes, CCR5 expression levels increased by *in vitro* activation [12, 16]. In these reports, CCR5 was expressed on memory T cells, not on naïve T cells. Freshly isolated DP T cells from the peripheral blood of rhesus monkeys contain a large population of memory cells (CD95+) compared to CD4SP T cells [10]; therefore, it is possible that CCR5 is quickly induced on DP T cells because of the higher percentage of memory T cells in DP T cells than CD4SP T cells.

Next, we compared the vulnerability to SIV infection of *in vitro* activated DP T cells to that of CD4SP T cells. It was previously reported that CD4+CCR5+ T cells were selectively decreased in SIV-inoculated rhesus monkeys *in vivo* [14, 15]. Because *in vitro* activated DP T cells expressed CCR5 at higher levels than CD4SP T cells (Fig. 1), we

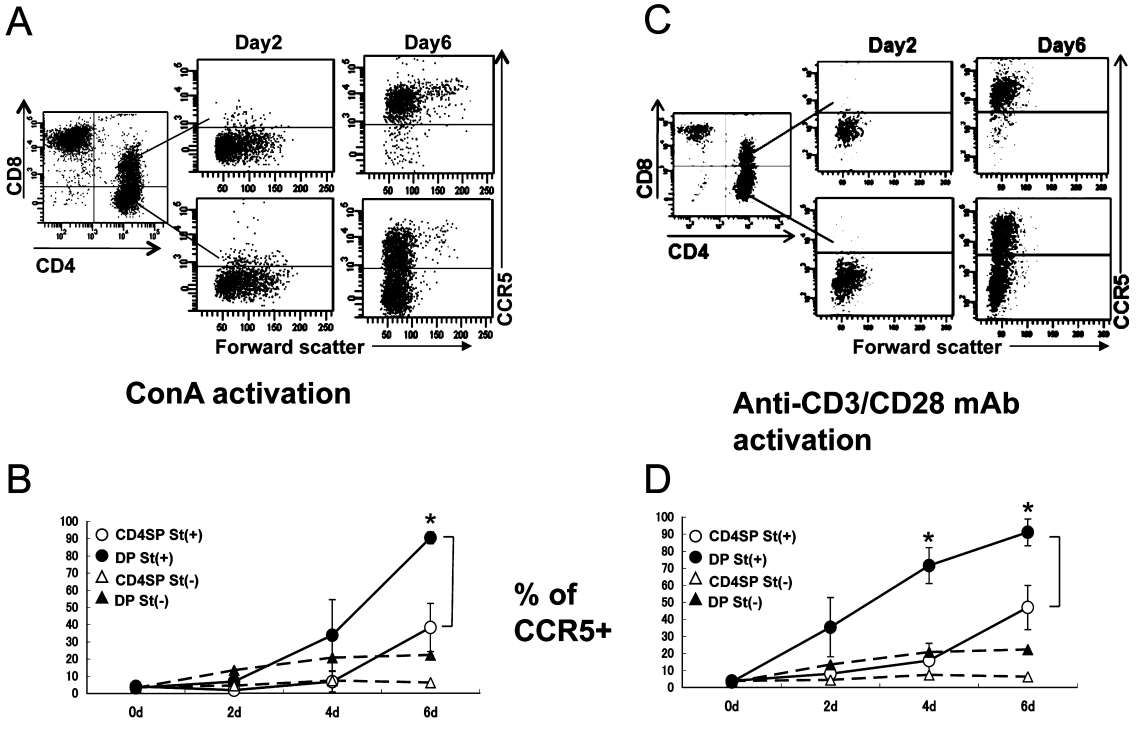

Fig. 1. CCR5 expression on CD4SP and DP T cells after ConA or anti-CD3/CD28mAb activation. (A) Flow cytometric analysis of CCR5 expression on CD4SP and DP T cells at days 2 and 6 after ConA activation, and (C) after anti-CD3/CD28 mAb activation. [(A) and (C) show representative data from one monkey, MM132.] (B) The kinetics of CCR5 expression levels on CD4SP and DP T cells post-ConA activation (circles). Vertical axis is the percentage of CCR5-positive cell population (CCR5 expression level). (D) Kinetics of CCR5 expression on both CD4SP and DP T cells after anti-CD3/CD28 mAb activation (circles). Triangles represent control cells (no mitogen stimulation). [(B) and (D) show the average and standard deviation (SD) of the data from three individual monkeys.] Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant ($P<0.05$).

hypothesized that DP T cells were more vulnerable to SIV infection than CD4SP T cells. PBMCs, activated by ConA for 24 hr as described above, were cultured in RPMI growth medium for a further 4 days. CD4-positive cells were then enriched from PBMCs using the CD4 microbead MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) to obtain a cell population containing only CD4SP and DP T cells. Flow cytometric analysis confirmed the purity of CD4-positive cells to be 98–99%. Soon after MACS separation, cells were infected with SIVmac239 [6] at a moi of 0.1. A mock control was also prepared in the same manner. At days 3, 5, 7, and 9 post-SIV infection, cultured cells were collected and subjected to flow cytometric analysis to detect a change in the cell population, and CCR5 expression on CD4SP and DP T cells. SIV growth was monitored by the measurement of SIV reverse transcriptase (RT) in SIV-infected cell supernatant. The supernatants were collected and stored at –80°C until used for RT assay. Virion-associated RT was measured by a commercial RT assay (Reverse Transcriptase Assay, colorimetric; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Experiments were performed independently with cells from three individual monkeys (MM132, MM450 and MM452). Results from the RT assay showed that SIV replicated well in cells from MM132 and MM450, and less effectively in those from MM452 (Fig. 2A). The percentage of CCR5-positive cells among the CD4-positive population was low in PBMC from MM452 (data not shown), which may have resulted in less effective SIV replication. On day 9 post-infection, the population of CD4-positive cells decreased in the SIV-infected group as compared to the mock control (Fig. 2B). To determine whether DP T cells were more vulnerable to SIV infection than CD4SP T cells, the percentage of either CD4SP or DP T cells in the SIV-infected group versus in the mock group was calculated at the times indicated in Fig. 2C. In experiments from all three animals, the percentage of residual DP T cells was significantly lower than that of CD4SP T cells at day 9 (Fig. 2C). The efficiency of viral replication was different in each monkey; however, it is important to note that even in the case of poor viral replication, the percentage of residual DP T cells was significantly lower than that of CD4SP T cells. These findings suggest that *in vitro* activated DP T cells were more vulnerable to SIV infection than CD4SP T cells. Although a previous study using herpesvirus saimiri transformed rhesus T cells showed similar results [13], this is the first report using primary PBMCs, and thereby more closely reflects the *in vivo* situation.

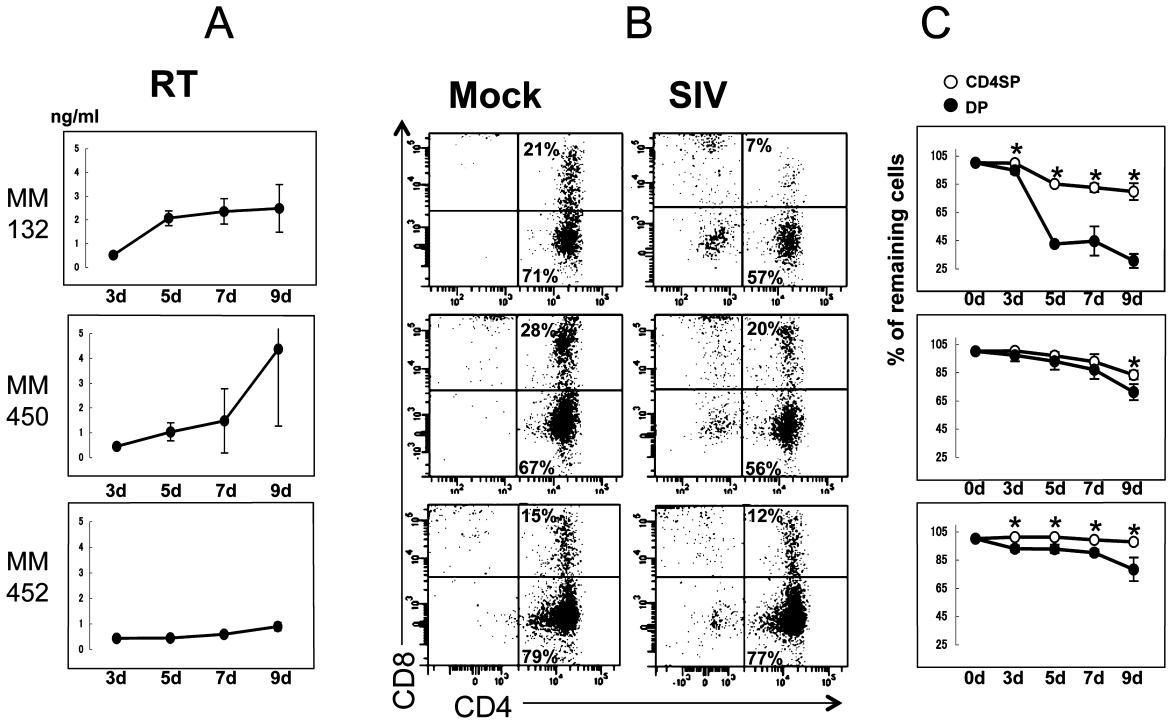

Fig. 2. SIV growth kinetics and populations of CD4SP and DP T cells post-SIV infection in experiments from three individual monkeys. (A) Viral replication was monitored by measurement of RT from the supernatant of SIV-infected cells. Average ± SD from triplicate experiments is shown. (B) Flow cytometric analysis of cell populations of mock and SIV-infected cells. These images show the CD4/CD8 pattern at day 9 after mock or SIV infection. (C) Kinetics of the remaining CD4SP and DP T cell populations post-SIV infection; shown as the percentage of cells in the mock experiment. Average ± SD from triplicate experiments is shown. Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant between DP and SP ($P<0.05$).

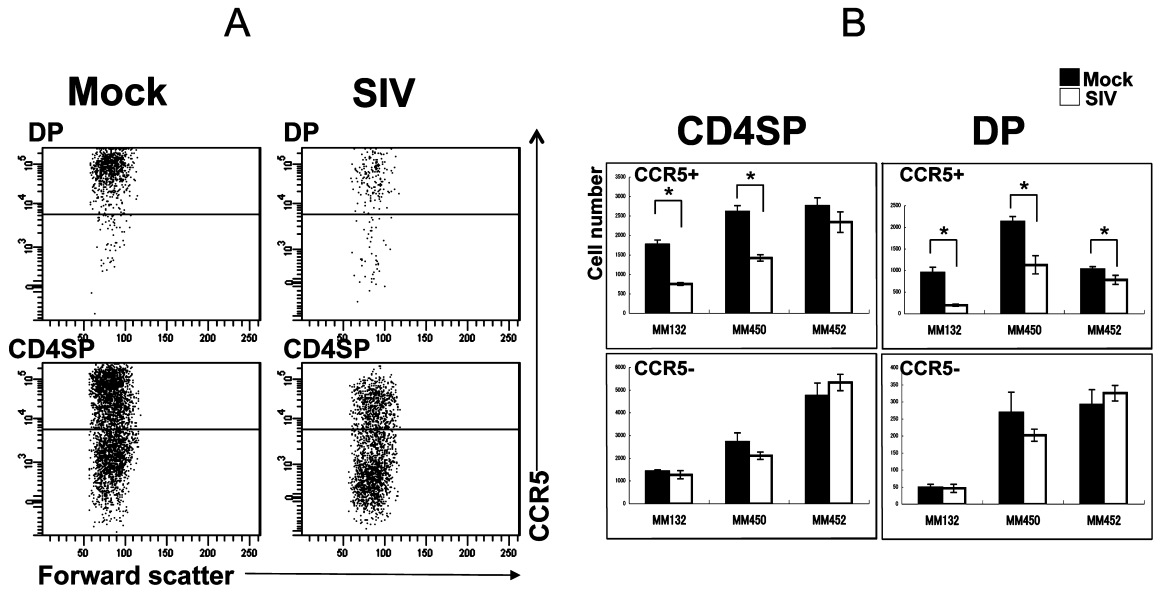

Fig. 3. Change in CCR5-positive cell numbers in CD4SP and DP T cells post-SIV infection. (A) Flow cytometric analysis of CCR5-positive cell populations of mock and SIV-infected cells. The figure shows the pattern of CCR5 expression at day 9 after mock or SIV infection (data from MM132). (B) Comparison of the number of CCR5-positive and CCR5-negative cells in CD4SP and DP T cells between mock and SIV-infected cells. A total of 30,000 events per sample were collected from flow cytometric analysis. Vertical axis is the cell number of each phenotype among all 30,000 events. Average ± SD from triplicate experiments is shown. Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant ($P<0.05$).

To examine the relationship between CCR5 expression and decreased cell counts following *in vitro* SIV infection, the population of cells expressing CCR5 was quantified, in both CD4SP and DP T cells, by comparing SIV-infected cells with the mock control. By flow cytometric analysis, the decrease in CCR5+ cell populations was observed both in CD4SP and DP T cells at day 9 post-SIV infection (Fig. 3A: one of the replicates from MM132). The number of CCR5+ and CCR5- cells was examined in both CD4SP and DP T cell populations by comparing the SIV-infected group to that of the mock group. As for CCR5- cells, no significant decrease was observed in the experiments from all three animals. In contrast, a significant decrease in CCR5+ cell numbers was observed both in CD4SP and DP T cells from two monkeys (Fig. 3B; MM132 and MM450). A similar tendency was observed in the cells from the other animal (MM452). It is possible that CCR5+ cells were selectively depleted both in CD4SP and in DP T cells after SIV infection. While the release of viruses into the cultured fluids from CD4+ selected lymphocytes provides indirect evidence of SIV replication, identification of the actual infected cells in the population would be preferred. Further study is required to clarify this point.

Our present data may indicate that the ability of DP T cells to readily express CCR5 after activation hastens DP T cell death by SIV infection *in vivo*. Naïve lymphocytes recirculate through secondary lymphoid organs where priming occurs, followed by homing to effector sites [3]. Lymphocytes in the bloodstream are activated when they are passing through these lymphoid organs. Thus, our data suggest that DP T cells were more vulnerable to SIV infection than CD4SP T cells, not only in the intestine [14, 15] but also in other tissues containing lymphocytes. In other words, all extrathymic DP T cells in the body were possibly more vulnerable to SIV infection than CD4SP T cells. In fact, an *in vivo* SIV inoculation study showed an abrupt decline in peripheral blood DP T cells at the early stage of infection [1]. Furthermore, the decline in DP T cells was not restored at any point during the infection study, whereas restoration of CD4SP T cells occurred in some monkeys [1]. This may indicate that the deletion of DP T cells in several lymphoid organs is more severe than that of CD4SP T cells after SIV infection.

Although the physiological significance of DP T cells in the body is not fully elucidated, DP T cells are thought to have certain immune functions, including helper and cytotoxic activities [9]. Thus, the systemic depletion of DP T cells in infected animals may correlate to the pathogenicity of SIV.

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REFERENCES

1. Akari, H., Nam, K. H., Mori, K., Otani, I., Shibata, H., Adachi, A., Terao, K. and Yoshikawa, Y. 1999. Effects of SIVmac infection on peripheral blood CD4+CD8+ T lymphocytes in cynomolgus macaques. *Clin. Immunol.* **91**: 321–329.
2. Akari, H., Terao, K., Murayama, Y., Nam, K. H. and Yoshikawa, Y. 1997. Peripheral blood CD4+CD8+ lymphocytes in cynomolgus monkeys are of resting memory T lineage. *Int. Immunol.* **9**: 591–597.
3. Bimczok, D. and Rothkotter, H. J. 2006. Lymphocyte migration studies. *Vet. Res.* **37**: 325–338.
4. Gallatin, W. M., Gale, M. J. Jr., Hoffman, P. A., Willerford, D. M., Draves, K. E., Benveniste, R. E., Morton, W. R. and Clark, E. A. 1989. Selective replication of simian immunodeficiency virus in a subset of CD4+ lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 3301–3305.
5. Hoxie, J. A., Haggarty, B. S., Bonser, S. E., Rackowski, J. L., Shan, H. and Kanki, P. J. 1988. Biological characterization of a simian immunodeficiency virus-like retrovirus (HTLV-IV): evidence for CD4-associated molecules required for infection. *J. Virol.* **62**: 2557–2568.
6. Kestler, H., Kodama, T., Ringler, D., Marthas, M., Pedersen, N., Lackner, A., Regier, D., Sehgal, P., Daniel, M., King, N. and Desrosiers, R. 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. *Science* **248**: 1109–1112.
7. Loetscher, P., Seitz, M., Baggiolini, M. and Moser, B. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* **184**: 569–577.
8. Nam, K. H., Akari, H., Terao, K., Ohto, H., Itagaki, S. and Yoshikawa, Y. 1998. Age-dependent remodeling of peripheral blood CD4+ CD8+ T lymphocytes in cynomolgus monkeys. *Dev. Comp. Immunol.* **22**: 239–248.
9. Nam, K. H., Akari, H., Terao, K., Shibata, H., Kawamur, S. and Yoshikawa, Y. 2000. Peripheral blood extrathymic CD4(+)CD8(+) T cells with high cytotoxic activity are from the same lineage as CD4(+)CD8(–) T cells in cynomolgus monkeys. *Int. Immunol.* **12**: 1095–1103.
10. Pahar, B., Lackner, A. A. and Veazey, R. S. 2006. Intestinal double-positive CD4+CD8+ T cells are highly activated memory cells with an increased capacity to produce cytokines. *Eur. J. Immunol.* **36**: 583–592.
11. Pongracz, J., Parnell, S., Anderson, G., Jaffrezou, J. P. and Jenkinson, E. 2003. Con A activates an Akt/PKB dependent survival mechanism to modulate TCR induced cell death in double positive thymocytes. *Mol. Immunol.* **39**: 1013–1023.
12. Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E., Moser, B. and Mackay, C. R. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* **101**: 746–754.
13. Takahashi, M., Ido, E., Uesaka, H., Fukushima, T., Ibuki, K., Miura, T., Hayami, M. and Takahashi, H. 2005. Comparison of susceptibility to SIVmac239 infection between CD4(+) and CD4(+)8(+) T cells. *Arch. Virol.* **150**: 1517–1528.
14. Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E. and Lackner, A. A. 2000. Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. *J. Virol.* **74**: 11001–11007.
15. Veazey, R. S., Tham, I. C., Mansfield, K. G., DeMaria, M., Forand, A. E., Shvetz, D. E., Chalifoux, L. V., Sehgal, P. K. and Lackner, A. A. 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. *J. Virol.* **74**: 57–64.
16. Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R. A. and Mackay, C. R. 1997. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J. Exp. Med.* **185**: 1681–1691.
17. Zuckermann, F. A. 1999. Extrathymic CD4/CD8 double positive T cells. *Vet. Immunol. Immunopathol.* **72**: 55–66.