

CC Chemokine Receptor 4-Positive CD4⁺ Lymphocytes in Peripheral Blood Increases during Maturation in Healthy Beagles

Nobutaka YASUDA¹⁾, Kenichi MASUDA^{1)*} and Sadatoshi MAEDA²⁾

¹⁾Animal Allergy Clinical Laboratories Inc., SIC-1#206 5-4-21 Nishihashimoto, Sagamihara, Kanagawa 229-1131 and ²⁾Department of Veterinary Internal Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

(Received 28 January 2008/Accepted 8 May 2008)

ABSTRACT. In this study, percentages of CCR4⁺ cells in peripheral CD4⁺ T-lymphocytes were examined with flow cytometry in 46 healthy beagles between 3 months and 7 years of age. The percentage of CCR4⁺ cells varied from 9.9% to 33.5%. The mean percentage was significantly lower in the group with ages of less than 1 year than those with ages equal to or more than 1 year ($p < 0.05$), suggesting that maturation might increase the CCR4⁺ T-lymphocyte subset. No influence of aging on the percentages was detected among the groups with ages equal to or more than 1 year. The findings are useful for establishing a reference value for the percentage of peripheral CCR4⁺CD4⁺ lymphocytes in dogs.

KEY WORDS: canine, CCR4, lymphocyte.

J. Vet. Med. Sci. 70(9): 989–992, 2008

Leukocytes express various types of chemokine receptors and show specific trafficking via interaction between those receptors and chemokines produced at inflammatory sites [18]. Specific expression profiles of chemokine receptors have been used to classify specific cell types, for instance, two different subsets of helper T-lymphocytes: Th1 and Th2 cells [1]. Among the chemokine receptors, it has been shown that Th1 cells selectively express CXC chemokine receptor (CXCR) 3 [13] and CC chemokine receptor (CCR) 5 [5], whereas Th2 cells express CCR3 [14] and CCR4 [3]. In human atopic dermatitis (AD), it was found that the frequency of CCR4⁺ cells among CD4⁺ T-lymphocytes increases in the peripheral blood [12] and lesional skin [11]. Additionally, the plasma concentration of CC chemokine ligand (CCL) 17, a ligand of CCR4, was reported to increase in human AD and correlates with disease severity [4]. These results suggest that the interaction between CCR4 and CCL17 plays an essential role in the immunopathogenesis of AD in humans. Similarly, in canine AD, expression of CCL17 [6] and CCR4 mRNAs [8] was detected in conjunction with the expression of inflammatory cytokines including IL-1 β , IFN- γ , and TNF- α in lesional skin [6], and the number of CCR4⁺ cells also increased in peripheral CD4⁺ T-lymphocytes [7]. A recent study in dogs demonstrated that keratinocytes were the major CCL17-producing cells in lesional skin of dogs with canine AD [9]. These studies indicated that CCR4-CCL17 interactions might play important roles in canine AD, similar to those in human AD.

In humans, it was reported that CCR4 was specifically expressed on peripheral CD4⁺ T-lymphocytes from patients with AD but not from patients with psoriasis vulgaris [16]. Moreover, the percentages of CCR4⁺ cells in peripheral

CD4⁺ T-lymphocytes were reported to increase in correlation with disease severity and decreased with improvement by topical corticosteroid therapy [16]. CCR4 was preferentially expressed on peripheral CD4⁺ T-lymphocytes in dogs with canine AD compared with those in healthy dogs [7]. These indicated that CCR4 expression on CD4⁺ T-lymphocytes might be a useful marker to identify atopic diseases, monitor their disease severity, and evaluate effects of treatment in dogs as well as humans.

In mice, age-associated changes in chemokine receptor expression on peripheral T-lymphocytes have been reported [10], suggesting that the normal age-related range of the CCR4 expression should be investigated, before it has a clinical application as one of possible markers for canine atopic dermatitis. In this study, 46 healthy beagles (22 males and 24 females), which had been kept for experimental purposes in a pharmaceutical company (Nihon Zenyaku Kogyo, Koriyama, Japan), were enrolled and divided into 8 groups according to their age, which ranged from less than 1 year (3–6 months, 0 year) to 7 years (Table 1). These dogs were clinically normal and without any skin diseases.

Peripheral blood was collected from each dog in an ethylenediamine tetraacetic acid (EDTA)-containing sample tube and stored at 4°C until analysis. The blood samples from the 46 dogs were used for examination of CCR4-positive T lymphocytes with flow cytometry, and 10 of the 46 samples were also kept at 4°C for up to 3 days to investigate the repeatability of the assay. Fifty μ l of each blood sample was resuspended with 25 μ l of blocking buffer (phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), 2 mM EDTA, 4% normal mouse serum, 4% normal rat serum, and 4% normal goat serum), and then incubated for 30 min at 4°C. These samples were then stained with Alexa647-conjugated anti-canine CD4 antibody (Serotec, Oxford, U.K.) and R-phycoerythrin (PE)-conjugated anti-human CCR4 antibody (BD Biosciences, San Jose, CA,

* CORRESPONDENCE TO: MASUDA, K., Animal Allergy Clinical Laboratories, Inc., SIC-1#206 5-4-21 Nishihashimoto, Sagamihara, Kanagawa 229-1131, Japan.
e-mail: Masuda@aacl.co.jp

U.S.A.) in PBS containing 0.5% BSA and 2 mM EDTA for 30 min at 4°C. After staining of the blood samples, erythrocytes were removed by adding 2 ml of lysing buffer (ACK lysing buffer, Invitrogen, Carlsbad, CA, U.S.A.) and washed three times with PBS with 0.5% BSA and 2 mM EDTA.

As a control sample for the assay, peripheral blood mononuclear cells (PBMCs) were collected from a dog using density gradient centrifugation with a commercial density buffer (Lymphoprep, Axis-shield, Oslo, Norway), according to the manufacturer's instructions, and aliquots were stored at -80°C in a cryoprotectant (CellBanker, Nihon Zenyaku Kogyo) until the analysis. The PBMCs were stained with antibodies in the same manner as described above and were also stained with isotype antibodies such as Alexa647-conjugated rat IgG2a and PE-conjugated mouse IgG1 (Sero-tec).

Finally, the cells were suspended in 500 μ l of PBS with 0.15 μ g of propidium iodide (BD Biosciences) for dead cell exclusion and filtered through a 40 μ m nylon cell-strainer (BD Biosciences) before the analysis of flow cytometry. The percentage of CCR4⁺ cells in CD4⁺ T-lymphocytes was examined with a FACSCantoII flow cytometer using FACSDiva software (BD Biosciences). A lymphocyte fraction was gated based on front and side scattered light signals with the exclusion of dead cells stained with propidium iodide. The number of CCR4 and CD4 double-positive lymphocytes was counted in 5000 CD4⁺ lymphocytes.

Using samples from 10 dogs between 3 to 6 months of age, the repeatability of the assay system was validated in advance. The measurement of CCR4⁺ cells in the samples was repeated after storage, at 4°C for up to 3 days, in order to investigate whether the storage of samples would affect the results. Figure 1 shows the CCR4⁺ T-lymphocyte percentages in each sample among the sequential assays. Standard deviation (SD) of the percentages was very small with a range from 0.9% to 3.2% (average: 1.1%), indicating the high repeatability of the assay system (Fig. 1).

The percentage of CCR4⁺ cells in CD4⁺ T-lymphocytes ranged from 9.9% to 33.5% in the healthy dogs examined (0 year, 9.9–14.3%; 1 year, 12.0–23.2%; 2 years, 11.9–33.5%; 3 years, 14.5–27.6%; 4 years, 17.5–22.1%; 5 years, 13.8–23.0%; 6 years, 20.6–27.0%; 7 years, 15.4–24.2%), and the mean values \pm SD were $1.6 \pm 1.4\%$, $15.8 \pm 4.6\%$, $22.4 \pm 10.0\%$, $21.7 \pm 4.4\%$, $20.3 \pm 2.2\%$, $19.8 \pm 4.0\%$, $24.6 \pm 2.8\%$,

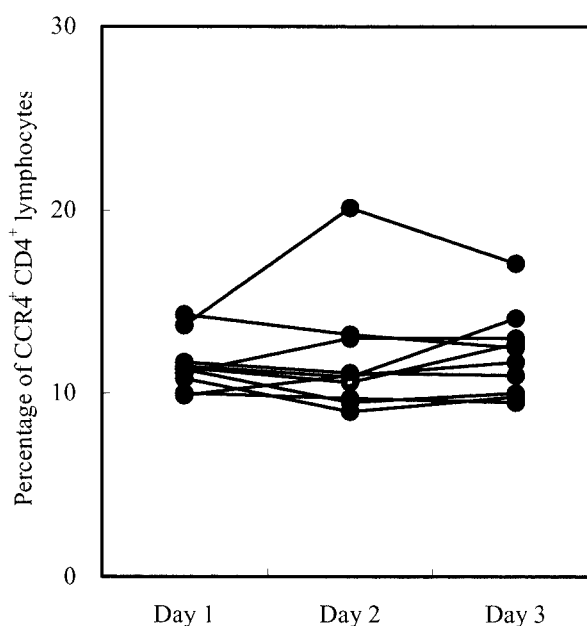


Fig. 1. The change of percentage of peripheral CCR4⁺ cells in peripheral T-lymphocytes after storage at 4°C for up to 3 days.

and $21.2 \pm 3.4\%$ in the age groups of 0, 1, 2, 3, 4, 5, 6, and 7 years old, respectively (Table 1). The percentages of CCR4⁺ cells were compared among different age groups using the Kruskal-Wallis test (JMP IN 5.1.2, SAS Institute, NC, U.S.A.). If a significant difference was found, a Tukey-Kramer test was performed to determine which pair was responsible for the difference (JMP IN 5.1.2, SAS Institute). Statistical analysis indicated that the mean value in the age group of 0 year was significantly lower than those in the other age groups ($p < 0.05$, Fig. 2).

Similar to the results observed in this study, it has been observed that CCR4 expression in CD4⁺ cells in the peripheral blood was lower in mice with age of 3–4 months than mice with age of 20–22 months [10]. These age-associated differences in the proportion of peripheral CCR4⁺ T-lymphocytes may be due to a shift in immunological status. A memory T-lymphocyte subset is known to increase in human with aging, presumably due to lifelong antigenic stimulation [2, 15]. CCR4 expression was reported to

Table 1. Range and mean \pm standard deviation (SD) of percentage of CCR4⁺ cells in peripheral CD4⁺ T-lymphocytes in healthy dogs. Note that 0 year of age indicates 3–6 months

Age (year)	Male	Female	Total	Range (%)	mean \pm SD (%)
0	5	5	10	9.9–14.3	1.6 ± 1.4
1	2	3	5	12.0–23.2	15.8 ± 4.6
2	3	2	5	11.9–33.5	22.4 ± 10.0
3	3	3	6	14.5–27.6	21.7 ± 4.4
4	2	3	5	17.5–22.1	20.3 ± 2.2
5	3	3	6	13.8–23.0	19.8 ± 4.0
6	2	2	4	20.6–27.0	24.6 ± 2.8
7	2	3	5	15.4–24.2	21.2 ± 3.4

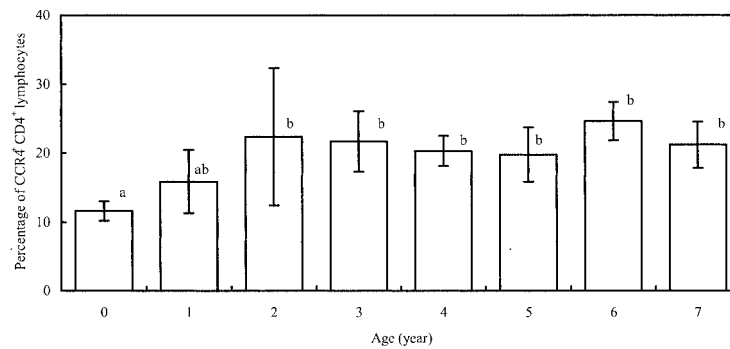


Fig. 2. The percentage of CCR4⁺ cells in CD4⁺ lymphocytes from healthy dogs with different ages. Bars with different letters are significantly different (Tukey-Kramer method, $p < 0.05$).

increase in memory T-lymphocytes compared with naïve T-lymphocytes [17]. Taken together, a shift of T-lymphocyte phenotype from naïve to memory cells during the first year of life may cause an increased proportion of CCR4⁺ T-lymphocytes in peripheral blood of dogs, although canine memory T-lymphocytes have not ever been examined in detail. Additionally, the percentages of CCR4⁺ T-lymphocytes were lowest in the youngest dogs of the less than 1 year age group, and there was no further increase of the percentages in the older age groups. In dogs, populations of memory T-lymphocytes in the peripheral blood may only increase during the period of maturation, and aging itself may not affect the population.

In mice, it was reported that CCR4 expression in CD4⁺ T-lymphocytes was higher in females, suggesting that gender could affect immune responses mediated by CCR4. However, in this study, there was no statistical difference in the percentages of CCR4⁺ T-lymphocytes between male and female beagles (student's two-tailed t test using Microsoft Excel software, $p = 0.70$). Therefore, it is suggested that gender difference does not affect CCR4-mediated immune responses in healthy dogs.

Since cross-reactivity of the anti-human CCR4 antibody with canine CCR4 was confirmed in a previous report using a canine lymphoid cell line expressing CCR4 mRNA [7], CCR4⁺ cells in dogs can be measured. From this study, normal reference values can be proposed, in terms of percentages of CCR4⁺ cells in peripheral CD4⁺ T-lymphocytes, in healthy dogs aged up to 7 years. Using these values, abnormalities in populations of CCR4⁺CD4⁺ lymphocytes in the peripheral blood can be precisely detected in dogs. In approximately 80% of the dogs with AD, the percentages of CCR4⁺ cells in peripheral CD4⁺ T-lymphocytes were reported to be higher than those in the healthy control dogs, however, these controls were not age-matched [7]. The data in this study can be utilized as an age-matched reference value to identify abnormalities of peripheral CCR4⁺ T-lymphocytes in dogs.

In summary, we examined the percentages of CCR4⁺ cells in peripheral CD4⁺ T-lymphocytes among healthy

dogs with different ages. The percentages gradually increased during the first 1 year of life. These data can be used to investigate CCR4⁺ T-lymphocyte populations in the peripheral blood of dogs with various diseases including allergy, bacterial infection, parasitic infestation, and cancer.

REFERENCES

1. Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A. and Sinigaglia, F. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* **187**: 129–134.
2. Gabriel, H., Schmitt, B. and Kindermann, W. 1993. Age-related increase of CD45RO⁺ lymphocytes in physically active adults. *Eur. J. Immunol.* **23**: 2704–2706.
3. Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P.W., Matsushima, K. and Yoshie, O. 1999. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* **11**: 81–88.
4. Kakinuma, T., Nakamura, K., Wakugawa, M., Mitsui, H., Tada, Y., Saeki, H., Torii, H., Asahina, A., Onai, N., Matsushima, K. and Tamaki, K. 2001. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J. Allergy Clin. Immunol.* **107**: 535–541.
5. Loetscher, P., Uguccioni, M., Bordoli, L., Baggiolini, M., Moser, B., Chizzolini, C. and Dayer, J.M. 1998. CCR5 is characteristic of Th1 lymphocytes. *Nature* **391**: 344–345.
6. Maeda, S., Fujiwara, S., Omori, K., Kawano, K., Kurata, K., Masuda, K., Ohno, K. and Tsujimoto, H. 2002. Lesional expression of thymus and activation-regulated chemokine in canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **88**: 79–87.
7. Maeda, S., Ohmori, K., Yasuda, N., Kurata, K., Sakaguchi, M., Masuda, K., Ohno, K. and Tsujimoto, H. 2004. Increase of CC chemokine receptor 4-positive cells in the peripheral CD4 cells in dogs with atopic dermatitis or experimentally sensitized to Japanese cedar pollen. *Clin. Exp. Allergy* **34**: 1467–1473.
8. Maeda, S., Okayama, T., Omori, K., Masuda, K., Sakaguchi,

- M., Ohno, K. and Tsujimoto, H. 2002. Expression of CC chemokine receptor 4 (CCR4) mRNA in canine atopic skin lesion. *Vet. Immunol. Immunopathol.* **90**: 145–154.
9. Maeda, S., Tsukui, T., Saze, K., Masuda, K., Ohno, K., Tsujimoto, H. and Iwabuchi, S. 2005. Production of a monoclonal antibody to canine thymus and activation-regulated chemokine (TARC) and detection of TARC in lesional skin from dogs with atopic dermatitis. *Vet. Immunol. Immunopathol.* **103**: 83–92.
 10. Mo, R., Chen, J., Han, Y., Bueno-Cannizares, C., Misek, D.E., Lescure, P.A., Hanash, S. and Yung, R.L. 2003. T cell chemokine receptor expression in aging. *J. Immunol.* **170**: 895–904.
 11. Nakatani, T., Kaburagi, Y., Shimada, Y., Inaoki, M., Takehara, K., Mukaida, N. and Sato, S. 2001. CCR4 memory CD4⁺ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **107**: 353–358.
 12. Okazaki, H., Kakurai, M., Hirata, D., Sato, H., Kamimura, T., Onai, N., Matsushima, K., Nakagawa, H., Kano, S. and Minota, S. 2002. Characterization of chemokine receptor expression and cytokine production in circulating CD4⁺ T cells from patients with atopic dermatitis: up-regulation of C-C chemokine receptor 4 in atopic dermatitis. *Clin. Exp. Allergy* **32**: 1236–1242.
 13. Sallusto, F., Lenig, D., Mackay, C.R. and Lanzavecchia, A. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* **187**: 875–883.
 14. Sallusto, F., Mackay, C.R. and Lanzavecchia, A. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* **277**: 2005–2007.
 15. Utsuyama, M., Hirokawa, K., Kurashima, C., Fukayama, M., Inamatsu, T., Suzuki, K., Hashimoto, W. and Sato, K. 1992. Differential age-change in the numbers of CD4⁺CD45RA⁺ and CD4⁺CD29⁺ T cell subsets in human peripheral blood. *Mech. Ageing. Dev.* **63**: 57–68.
 16. Wakugawa, M., Nakamura, K., Kakinuma, T., Onai, N., Matsushima, K. and Tamaki, K. 2001. CC chemokine receptor 4 expression on peripheral blood CD4⁺ T cells reflects disease activity of atopic dermatitis. *J. Invest. Dermatol.* **117**: 188–196.
 17. Yamamoto, J., Adachi, Y., Onoue, Y., Adachi, Y.S., Okabe, Y., Itazawa, T., Toyoda, M., Seki, T., Morohashi, M., Matsushima, K. and Miyawaki, T. 2000. Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4⁺ T cells. *J. Leukoc. Biol.* **68**: 568–574.
 18. Zlotnik, A. and Yoshie, O. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* **12**: 121–127.