

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

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Summary

Background Since dogs frequently develop allergic diseases, similar to those in humans, dogs represent a possible animal model for allergy in humans. In human atopic dermatitis (AD), CC chemokine receptor 4 (CCR4) has been shown to play an important role in the development of allergic inflammation of AD; however, the association between allergic reaction and CCR4 is not well understood in dogs.

Objective To examine CCR4 expression in peripheral blood CD4⁺ cells in dogs that had AD and were experimentally sensitized with Japanese cedar pollen.

Materials and methods Peripheral blood mononuclear cells (PBMCs) were isolated from 17 dogs with AD. The proportion of CCR4⁺ cells in peripheral blood CD4⁺ cells (CCR4/CD4) was evaluated by flow cytometry and compared with that in 10 healthy dogs. Similarly, in dogs that were experimentally sensitized to Japanese cedar pollen antigen, the proportion of CCR4/CD4 was examined pre- and post-sensitization.

Results The proportion of CCR4/CD4 in dogs with AD was $40.3 \pm 3.3\%$, which was significantly higher than that in normal dogs ($23.6 \pm 4.3\%$) ($P < 0.01$). In the experimentally sensitized dogs, the proportion of CCR4/CD4 was $25.4 \pm 2.6\%$ at pre-sensitization and it was significantly increased ($29.8 \pm 2.9\%$) at post-sensitization ($P < 0.01$).

Conclusion The proportion of CCR4⁺ cells in peripheral blood CD4⁺ cells was measured in dogs with allergic conditions. The present findings indicate that CCR4⁺ cells may be involved in the pathogenesis of allergy in dogs as in humans.

Keyword animal model, atopic dermatitis, canine, CC chemokine receptor 4

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Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease in humans that is mediated by allergic reactions. In the immunopathogenesis of human AD, T helper (Th)2-type cytokines such as IL-4, IL-5 and IL-13 have been shown to play essential roles in the initiation and maintenance of allergic inflammations [1]. In lesional skin of human AD, selective infiltration of allergen-specific Th2 cells is a characteristic finding [2]. Not only in lesional skin but also in peripheral blood, the number of Th2 cells increases in human AD [3]. These findings suggest that Th2 cells, which increase in peripheral blood in patients with AD, will migrate into inflammatory sites, finally resulting in the development of atopic lesions.

Chemokine receptors are expressed on various types of leucocytes and regulate specific or selective cell trafficking [4]. Specific expression profiles of chemokine receptors have been demonstrated in two different subsets of Th cells, Th1 and Th2 cells [5]. Regarding specific chemokine receptors, it has been shown that Th1 cells selectively express CXCR3 [6] and CC chemokine receptor 5 (CCR5) [7] whereas Th2 cells express CCR3 [8] and CCR4 [9]. Thus, the difference of patterns of the expression of chemokine receptors between Th1 and Th2 cells may cause selective infiltration or migration of the cells into inflammatory sites, depending on the chemokines produced in allergic lesions. In human AD, it was found that CCR4 expression on CD4⁺ cells was up-regulated [10] and correlated with the disease severity [11]. In lesional skin of human AD, CCR4⁺ cells were found to be markedly infiltrated [12], and thus may be involved in the production of a functional ligand for CCR4, thymus and activation-regulated chemokine (TARC/CCL17), in the lesion. The plasma TARC level is increased in human AD and correlates with disease severity [13]. In lesional skin of human AD, keratinocytes are the major cell source of TARC

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production [14]. In HaCaT cells, TARC production is up-regulated by stimulation with inflammatory cytokines including IL-1 β , IFN- γ and TNF- α [15]. These results suggest that CCR4 and TARC play an essential role in the immunopathogenesis of AD in humans in terms of the development of allergic lesion.

It has been proposed that NC/Nga mice may be a useful animal model for human AD [16] because the mice develop skin lesions accompanied by overproduction of IgE when kept under conventional conditions [17]. The overproduction of IgE is most likely attributable to the presence of Th2-dominant immune responses, perhaps due to a deficiency of Th1 immune responses [18]. Interestingly, it was shown that the mice did not develop skin lesions under specific pathogen-free conditions [19], suggesting that some environmental factors may be important for the initiation of the allergic inflammation, although they have not been identified yet. As to the involvement of chemokines in the development of skin lesions in NC/Nga mice, a recent study showed that TARC was produced by keratinocytes in lesional skin together with CCR4 mRNA [20]. In this model, however, macrophage-derived chemokine (MDC/CCL22), another ligand for CCR4, was expressed not only in lesional skin but also in non-lesional skin, indicating that the pathogenesis of the skin lesions in the mice might be different from that in human AD in terms of the regulation of cell infiltration by chemotaxis [21]. Furthermore, expression of CCR4 in peripheral blood has not been investigated in NC/Nga mice.

Canine AD shares a number of clinical features with human AD [22]. House dust mite (HDM) allergen, which is the most common environmental allergen in humans, has been identified as the most important allergen in canine AD [23–29]. It has been speculated that a predisposing genetic factor may exist in dogs with AD, since there is a high incidence of the disease in certain breeds and families [30]. Histological analysis revealed that CD4⁺ cells were markedly infiltrated in lesional skin of canine AD [31]. Recent studies have indicated that IL-4 mRNA is preferentially expressed in lesional skin of dogs with AD [32, 33]. In lesional skin of canine AD, expression of TARC [34] and CCR4 mRNA [35] was detected in conjunction with the expression of inflammatory cytokines including IL-1 β , IFN- γ and TNF- α [34]. These studies indicate that the immunopathogenesis of canine AD may be similar to that of human AD, suggesting that canine AD can be used as an animal model for spontaneous allergy. Furthermore, dogs experimentally sensitized to allergens have been shown to have Th2-skewed responses, similar to those in humans with allergies [36, 37].

In this study, to clarify the possible association between allergic reaction and CCR4 expression, the proportion of CCR4⁺ cells in peripheral blood CD4⁺ cells was investigated in dogs that had AD and were experimentally sensitized to Japanese cedar pollen.

Materials and methods

Healthy dogs

Ten healthy dogs kept for experimental purposes were used to obtain control samples. All of the dogs were female and their

age ranged from 1 to 2 years. These dogs showed no clinical signs of allergic diseases and no physical signs of skin diseases.

Diagnosis of atopic dermatitis

Seventeen dogs referred to the Veterinary Medical Center of the University of Tokyo were diagnosed with AD according to Willemse's criteria for clinical diagnosis of canine AD [38]. All of the dogs with AD had seasonal or non-seasonal chronic pruritus with or without superficial pyoderma in the regions of the axilla, thigh and/or ventral abdomen. Dogs with other skin diseases causing pruritus such as infection with parasites, bacteria or fungi, were excluded based on routine dermatologic examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded when there was no history of change in clinical signs after change of diets. If food hypersensitivity was suspected from the clinical history, food elimination tests were performed with a commercial prescription diet using hydrolysed proteins (Hill's Z/d ultra, Hill's Pet Nutrition Inc., Topeka, KS, USA) or rice-based homemade diet with boiled chicken meat for a period of at least 8 weeks. Of the 17 dogs in this study, there was no dog showing improvement of clinical signs in response to the food elimination.

Intradermal testing was performed in the dogs with AD as we reported previously [29]. Allergen extracts used in this study were purchased from a commercial supplier (Greer Laboratories, Lenoir, NC, USA). In the testing, eight groups of allergens were used: HDMs (*Dermatophagoides farinae* and *D. pteronyssinus*), arthropods (cockroach, housefly, mosquito, etc.), cat epithelia, foods (wheat, rice, beef, etc.), moulds (*Curvularia spicifera*, *Penicillium camemberti*, etc.), grasses (Kentucky blue, orchard, redtop, etc.), weeds (cocklebur, lambs quarter, rough pigweed, etc.), and trees (Japanese cedar, white ash, red birch, etc.). Most of allergens were used at a concentration of 1000 protein nitrogen units after being diluted with sterile diluent (0.9% sodium chloride and 0.4% phenol). The diluent was also used as a negative control. Histamine phosphate (0.05 mL of a 0.0275 mg/mL solution) was used as a positive control. The hair coat on the lateral thorax was clipped and 0.05 mL of each allergen solution was injected intradermally using a skin test syringe with a 26-Gauge needle. Diameter of weals were graded as follows: equal to or greater than the diameter of the positive control, +++; equal to or greater than the mean diameter of the positive and negative control, ++; larger than the diameter of the negative control but smaller than the mean diameter of the positive and negative control, +; equal to or smaller than the diameter of the negative control, –. Positive allergens were defined when the size of a reactive weal was graded as more than ++.

In vitro allergen-specific IgE testing was carried out using two commercially available kits (Topscreen test and Immunodot test kits, CMG Laboratories, Fribourg, Switzerland) as described previously [29]. Mixtures of allergens such as outdoor, indoor, two foods, and mould allergen groups, were used for the screening assay of five allergen groups (e.g., indoor, outdoor, foods, moulds), while the Immunodot test was used to detect IgE specific to 24 individual allergens in each allergen group (e.g. HDMs, storage mites, grasses, fleas). Amounts of allergen-specific IgE were expressed as labora-

tory units (LUs) ranging from 1 to 100 (0 being negative) based on standard titration curves of reference standards (pooled sera). Positive allergens were noted when measurable amounts of allergen-specific IgE were present in the sera.

Experimental sensitization with Japanese cedar (Cryptomeria japonica) pollen antigen

Seven healthy dogs kept for experimental purposes were used for experimental sensitization with Japanese cedar (*Cryptomeria japonica*, CJ) pollen antigen under a protocol approved by the Institutional Animal Care and Use Committee at the University of Tokyo. The dogs included five female and two male beagles and their age ranged from 1 to 3 years. These dogs did not have any clinical signs of allergic diseases and any physical signs of skin diseases. The sensitization was carried out as described previously [37]. Briefly, the dogs were injected subcutaneously with 100 µg of the CJ pollen antigen conjugated with 20 mg of alum for each injection. The injection was performed twice at 2-week intervals. Intradermal testing and *in vitro* allergen-specific IgE testing were then performed to confirm successful sensitization in these dogs. Blood samples were collected from the dogs before and 1 week after the second injection.

Detection of canine CC chemokine receptor 4 mRNA by RT-PCR

Total RNA was extracted from two canine lymphoid tumour cell lines, CL-1 [39] and GL-1 [40], using a commercially available kit (SV Total RNA Isolation System, Promega, Madison, WI, USA) according to the manufacturer's instructions. To examine CCR4 mRNA expression in the canine lymphoid cell lines, RT-PCR was carried out with primers specific for canine CCR4 (Forward, 5'-TACCTGGTGGGCTTTTACAGTGGCATCTTC-3', nucleotides (nt) 348–378 in canine CCR4 cDNA [35]; Reverse, 5'-GCCAGGGTCTCTGTGGCCTGAATAGCGTAG-3', nt 878–848) or canine GAPDH (Forward, 5'-CTCATGACCA CAGTCCATGC-3', nt 514–533 in canine GAPDH cDNA, GenBank/EMBL/DBJ accession number AB038240; Reverse, 5'-TGAGCTTGACAAAGTGGTCA-3', nt 925–906) sequences as previously described using a commercially available kit (RNA PCR Kit, Perkin Elmer, Branchburg, NJ, USA).

Cross-reactivity of anti-human CC chemokine receptor 4 monoclonal antibody with canine CC chemokine receptor 4

Anti-human CCR4 monoclonal antibody (BD PharMingen, San Diego, CA, USA) was used to detect canine CCR4 using a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA, USA). At first, cross-reactivity of the anti-human CCR4 monoclonal antibody to canine CCR4 was evaluated using canine lymphoid cell lines (CL-1 and GL-1). The cells were incubated with anti-human CCR4 monoclonal antibody or an appropriate isotype (purified mouse IgG1, BD PharMingen) as a negative control in washing buffer (Phosphate-buffered saline with 2% fetal bovine serum) at 4 °C for 30 min. The cells were then stained with phycoerythrin (PE)-conjugated anti-mouse IgG1 monoclonal antibody

(BD PharMingen) in washing buffer at 4 °C for 30 min. The fluorescence intensities were examined using the flow cytometer.

Proportion of CC chemokine receptor 4/CD4 in peripheral blood mononuclear cells

Heparinized whole blood samples were obtained from the dogs. Each sample was diluted with an equal volume of PBS and then layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). Following centrifugation at 350 *g* at room temperature for 45 min, a layer of PBMCs was collected and washed twice with washing buffer. The PBMCs were incubated with anti-canine CD4 (COSMO BIO Co. Ltd, Tokyo, Japan) and anti-human CCR4 monoclonal antibodies in washing buffer at 4 °C for 30 min. Antibodies with the appropriate isotypes (purified rat IgG2a and mouse IgG1, BD PharMingen) were used as negative controls. The PBMCs were then stained with fluorescein isothiocyanate-conjugated anti-rat IgG2a and PE-conjugated anti-mouse IgG1 monoclonal antibodies (BD PharMingen) in washing buffer at 4 °C for 30 min. After gating the fraction of lymphocytes, the proportion of CCR4/CD4 was determined by flow cytometry.

Statistical analysis

The two-sample *t*-test was used to compare the proportions of CCR4/CD4 between healthy dogs and dogs with AD. The paired *t*-test was used to compare the proportions of CD4/CCR4 between pre- and post-sensitization. The level of significance was set at *P* < 0.01. All statistical analysis was performed using JMP Version 5 (SAS Institute Inc., Cary, NC, USA).

Results

Dogs with atopic dermatitis

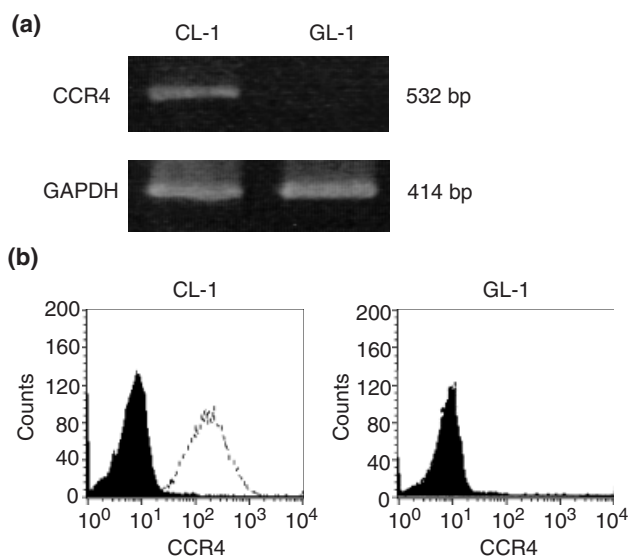
Seventeen dogs were diagnosed with AD based on the clinical diagnostic criteria proposed by Willemse [38]. The dogs with AD consisted of 11 males (five neutered) and six females (three neutered) whose age ranged from 1 to 9 years (5.0 ± 2.8 years old). All of the dogs with AD had various degrees of pruritic skin lesions with signs such as erythema, hair loss, papules and lichenification on the ventral abdomen and showed positive reactions to *D. farinae* and *D. pteronyssinus*, which are the most common environmental allergens for AD in Japan [29] in intradermal testing and *in vitro* allergen-specific IgE testing. Some dogs also showed positive reactions to other allergens such as storage mites, grasses and trees on these allergy tests.

Dogs experimentally sensitized to Cryptomeria japonica pollen antigen

The results of intradermal testing and *in vitro* allergen-specific IgE testing revealed that all of the dogs experimentally sensitized to CJ pollen antigen showed positive reactions only to CJ pollen antigen (Table 1). No clinical signs were induced in these dogs after the sensitization.

Table 1. Results of allergy tests in dogs experimentally sensitized to CJ pollen antigen

Dog number	Intradermal testing	<i>In vitro</i> allergen-specific IgE testing (LUs)
Dog 1	+++	40
Dog 2	+++	18
Dog 3	+++	33
Dog 4	+++	39
Dog 5	+++	51
Dog 6	+++	62
Dog 7	+++	48

LUs, laboratory units; CJ, *Cryptomeria japonica*.**Fig. 1.** Cross-reactivity of anti-human CC chemokine receptor (CCR)4 monoclonal antibody with canine CCR4. (a) RT-PCR analysis for expression of canine CCR4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs in canine lymphoid cell lines (CL-1 and GL-1). (b) Flowcytometric analysis of CCR4 expression in CL-1 and GL-1. The histogram shows the staining intensity of isotype (shaded area) and CCR4 (open area) monoclonal antibodies.

Cross-reactivity of anti-human CC chemokine receptor 4 monoclonal antibody with canine CC chemokine receptor 4

The results of RT-PCR analysis showed that CCR4 mRNA was expressed in CL-1 cells but not in GL-1 cells (Fig. 1a). The cross-reactivity of the anti-human CCR4 monoclonal antibody with canine CCR4 was then examined using these cell lines. The results of flowcytometric analysis using the anti-human CCR4 monoclonal antibody revealed that CL-1 cells expressing mRNA of CCR4 were positively stained, whereas GL-1 cells without expression of CCR4 mRNA were negative (Fig. 1b).

Proportion of CC chemokine receptor 4/CD4 in peripheral blood mononuclear cells

In canine PBMCs, most CCR4⁺ cells were found in CD4⁺ cells, as previously shown in human PBMCs [10] (Fig. 2a). In healthy dogs and dogs with AD, the proportions of CCR4/CD4 (average $\pm$ SEM) were $23.6 \pm 4.3\%$ and $40.3 \pm 3.3\%$,

respectively (Fig. 2b). The proportions of CCR4/CD4 in dogs with AD were significantly higher than those in healthy dogs ($P < 0.01$). The proportion of CCR4/CD4 increased after the experimental sensitization with CJ pollen antigen (Fig. 2c), the percentages were $25.4 \pm 2.6\%$ and $29.8 \pm 2.9\%$ at pre- and post-sensitization, respectively. The CCR4/CD4 ratio was significantly different between pre- and post-sensitization ($P < 0.01$).

Discussion

AD is characterized by a chronic inflammatory skin disease associated with cutaneous allergic reaction, therefore, it has been considered that the development of skin lesion is most likely initiated by immunological triggers such as encounter with foods or environmental allergens [41]. This has been experimentally demonstrated by the fact that epicutaneous or intradermal application of aeroallergens developed skin lesion in human [42] and canine [43] AD, histopathological findings of which were characterized by erythema, oedema and infiltration of inflammatory cells. Even in clinically unaffected AD skin, a sparse perivascular T cell infiltrate was found in humans [44] and dogs [31], suggesting that the AD skin may be in a stage that can easily and immediately form skin lesion by the immunological stimuli and consequent infiltration of inflammatory cells mediated by chemotaxis.

Chemokines are important regulators of the selective recruitment of inflammatory cells into sites of inflammation [4]. It can be hypothesized that infiltration of Th2 cells from the peripheral blood into atopic lesions is regulated by a specific pair of chemokine and chemokine receptor in AD. Studies of human AD have demonstrated an increase of CCR4⁺ cells in peripheral blood [10, 11, 45] and lesional skin [12, 14]. TARC, a biological ligand of CCR4, was also found to be selectively expressed in lesional skin of humans with AD [13]. These results suggest that peripheral CCR4⁺ cells can be recruited into lesional skin by chemotaxis with lesional production of TARC in AD, finally resulting in the development of skin lesions in human AD. In this study, both spontaneously and experimentally induced allergic reactions were found to cause increased numbers of CCR4⁺ cells in peripheral CD4⁺ cells. This suggests that CCR4⁺ cells may increase in number as a consequence of a Th2-type reaction, and that these cells will be attracted to lesions, resulting in the development of clinical signs as shown in human AD. Thus, it could be considered that an association between allergic reaction and recruitment of cells by chemokines was further supported by our findings.

A canine lymphoid cell line, CL-1, expressing CCR4 mRNA was used to evaluate whether the anti-human CCR4 monoclonal antibody cross-reacted with canine CCR4. In our preliminary study, it had been confirmed that CL-1 cells expressed functional CCR4 on their cell surface, which was demonstrated by a transwell chemotaxis assay with recombinant canine TARC expressed by *Escherichia coli* (manuscript in submission). Therefore, it can be considered that CL-1 is a suitable cell line for examining the cross-reactivity of anti-human CCR4 monoclonal antibody with canine CCR4. In fact, the flowcytometric analysis in this

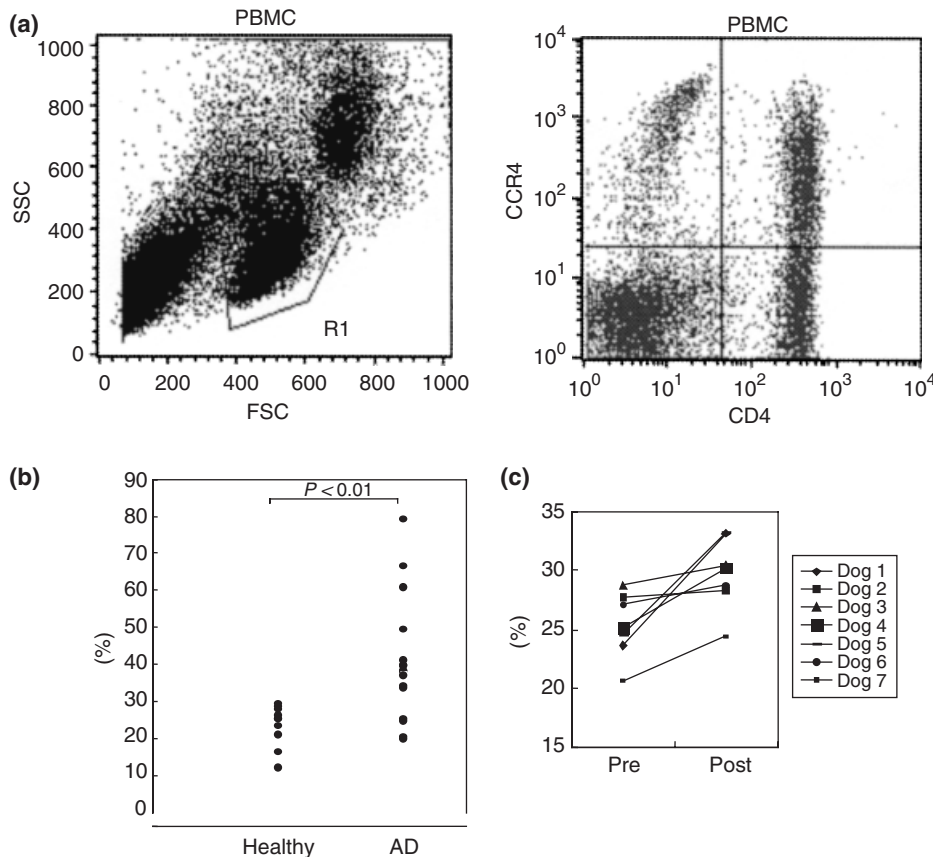


Fig. 2. CC chemokine receptor (CCR)4 expression in peripheral blood mononuclear cells (PBMCs) from dogs. (a) A representative result for the expression of CCR4 in CD4⁺ cells in the PBMCs from dogs with atopic dermatitis (AD). Expression of CCR4 in the lymphocyte fraction as gated (left) was examined by double staining with anti-CD4 and anti-CCR4 monoclonal antibodies (right). (b) Proportions of CCR4⁺ cells in CD4⁺ cells in the PBMCs from healthy dogs and dogs with AD. (c) Changes (pre- vs. post-sensitization) of the proportions of CCR4⁺ cells in CD4⁺ cells in dogs experimentally sensitized to *cryptomeria japonica* pollen antigen.

study demonstrated that the anti-human CCR4 monoclonal antibody bound to CL-1 cells, but not to GL-1 cells, which are negative for CCR4 mRNA. The high level of sequence homology of CCR4 among humans [46], dogs [35], mice [47] and guinea-pigs [48] also supports the possible cross-reactivity of the monoclonal antibody to CCR4 in these species. As reported previously, anti-human CCR4 monoclonal antibodies cross-reacted with stable transfectant cells expressing guinea-pig CCR4 [48]. The transfectants also showed chemotactic activity toward the human and murine forms of CCR4 ligands, MDC and TARC [48]. These results further emphasize that CCR4 is highly conserved among different species in terms of the antigenicity and biological function, indicating that the anti-human CCR4 monoclonal antibody used in this study reacted with canine CCR4.

Since canine AD shares many clinical and pathological features with human AD [22], dogs can be considered as an appropriate animal model for human AD. In this study, therefore, CCR4 expression on CD4⁺ cells was evaluated in dogs with AD in order to further demonstrate similarities in the involvement of chemokines in the immunopathogenesis of AD between dogs and humans. The present results clearly indicated that the proportion of CCR4/CD4 in dogs with AD was higher than that in normal dogs. In addition, we further demonstrated that CCR4 expression was induced by allergic stimuli, using an experimental allergy model employing dogs sensitized to CJ pollen antigen. Our previous studies indicated that the dogs experimentally sensitized to CJ pollen antigen had increased serum allergen-specific IgE [37] and IL-4 mRNA expression in PBMCs [36]. These results also support

the idea that the dogs experimentally sensitized to CJ pollen antigen can be used for an animal model of allergic inflammation.

Recent studies have led to the proposal that regulation of chemotaxis can be a new therapeutic strategy for allergic disease [49–51]. In a study of a murine model of bacteria-induced hepatic failure, it was shown that CCR4⁺ cells were infiltrated as a consequence of chemotaxis mediated by TARC produced in hepatic lesions and that the cell infiltration was inhibited by the administration of anti-TARC monoclonal antibody [52]. Another study also indicated that a neutralizing monoclonal antibody to TARC suppressed the allergic inflammation in a murine model with allergen-induced asthma [53]. No clinical studies on suppression of chemotaxis have been carried out in humans with allergic diseases; however, these results strongly suggest that intervention to inhibit chemotaxis may be effective for reducing allergic inflammation in AD or bronchial asthma. To evaluate the clinical efficacy of chemokine-targeted therapy in human AD, therefore, a suitable animal model with spontaneously occurring AD should be useful. From the results of the present study we propose that it should be useful to employ dogs as an animal model for allergic diseases such as AD in order to evaluate the clinical efficacy of chemokine-targeting therapy.

In conclusion, together with the findings of our previous studies showing lesional expression of TARC [34] and CCR4 mRNA [35], the results of the present study demonstrate that the involvement of chemokines in the immunopathogenesis of canine AD is similar to that of human AD.

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