

Increased proportions of CCR4⁺ cells among peripheral blood CD4⁺ cells and serum levels of allergen-specific IgE antibody in canine chronic rhinitis and bronchitis

Yoshiki YAMAYA^{1)*} and Toshihiro WATARI¹⁾

¹⁾Laboratory of Comprehensive Veterinary Clinical Studies, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan

(Received 30 June 2014/Accepted 1 December 2014/Published online in J-STAGE 19 December 2014)

ABSTRACT. Canine chronic rhinitis (CR) and bronchitis (CB) are suspected to be allergic diseases. The present study tested whether dogs diagnosed with CR or CB present an atopic predisposition based on the ratio of CC chemokine receptor 4 (CCR4)-positive cells among peripheral blood CD4-positive cells (CCR4/CD4) and the serum levels of allergen-specific IgE antibodies. We found that most dogs with CR and CB have a possibility of atopic predisposition, and macrolide therapy constitutes an alternative to corticosteroid therapy in controlling the clinical signs.

KEY WORDS: bronchitis, canine, rhinitis

doi: 10.1292/jvms.14-0338; *J. Vet. Med. Sci.* 77(4): 421–425, 2015

In human medicine, the concept of “Atopic March” was proposed to describe the progression of disorders underlying an atopic predisposition, from atopic dermatitis in infants to allergic rhinitis and asthma in children and adults [22]. Based on the definition of atopy [12], an atopic predisposition identifies individuals who had allergic symptoms in the past, currently have allergic symptoms or may have allergic symptoms in the future, but not necessarily with increased serum IgE antibody levels. An atopic predisposition is a very important concept in human respiratory medicine to distinguish an atopic cough, which manifests as hypersensitivity to cough receptors and no response to bronchodilators, from a cough variant asthma, which manifests as airway hyper-reactivity well controlled with bronchodilators [7, 8]. There is little evidence that atopic cough and cough variant asthma occur in dogs. To our knowledge, only one case of canine reactive bronchopneumopathy has been reported, with histological features similar to those of asthmatic humans and felines [3].

Canine chronic rhinitis (CR) and bronchitis (CB) are also suspected to be allergic diseases, and their clinical signs of nasal water discharge, sneezing and cough may become incurable and intractable diseases due to an atopic predisposition [5, 14, 25]. However, it is usually difficult to diagnose allergic and atopic predispositions in dogs with naturally occurring CR and CB. There is only one report showing that three dogs with CR had specific IgE antibody for house dust

mite. In dogs with dermatitis, CD4-positive T lymphocyte levels are increased in atopic skin lesions compared with that in normal skin [18]. The ratio of CC chemokine receptor 4 (CCR4)-positive cells among peripheral blood CD4-positive cells (CCR4/CD4) was higher in dogs with experimentally-induced atopic dermatitis than in healthy dogs [16, 26]. This high ratio of CCR4/CD4 cells indicates an increased production of the thymus and activation-regulated chemokine (TARC), whose levels correlate with disease severity in human atopic dermatitis and are decreased by corticosteroid therapy [24]. In addition, TARC is likely to be produced by monocytes and dendritic cells and may facilitate the recruitment, activation and development of T helper type 2 (Th2)-polarized cells expressing CCR4, suggesting a role for TARC in Th2 responses [11]. Thus, recent screening tests to diagnose and treat canine atopic dermatitis combine the serum levels of IgE antibody and the ratio of CCR4/CD4 cells, because both measurements can be done in a veterinary diagnostic laboratory.

In this study, dogs with CR or CB were tested for the ratio of CCR4/CD4 cells and serum levels of allergen-specific IgE antibody to determine whether they have an atopic predisposition.

MATERIALS AND METHODS

Animals: This study included 46 dogs separated into the CR group (n=27) and the CB group (n=19). The mean age was 7 years for dogs with CR (range: 2–14 years) as well as for dogs with CB (range: 3–15 years). These dogs were referred to the Animal Medical Center of Nihon University from April 2009 to December 2012 for diagnostic purposes and consultations on long-term therapy for nasal discharge or chronic cough. The common breeds were Miniature Dachs-hund (n=8), Shiba (n=3), Toy Poodle (n=3), Shih Tzu (n=2), Pembroke Welsh Corgi (n=2), Pomeranian (n=2) and Maltese (n=2). The dogs did not have dermatitis, digestive com-

*CORRESPONDENCE TO: YAMAYA, Y., Laboratory of Comprehensive Veterinary Clinical Studies, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan.
e-mail: yamaya@brs.nihon-u.ac.jp

©2015 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

Table 1. Diagnostic criteria for canine chronic rhinitis (CR) and bronchitis (CB)

CR:	1) Nasal water discharge and/or sneezing for over one month, with normal complete blood counts and no skin or gastrointestinal disease.
	2) Swelling and enlargement of nasal membrane, and no tumor, foreign body or parasite in the nasal cavities by computed tomography (CT) and rhinoscopy.
	3) Lymphocytic- and/or plasmacytic-dominant inflammation in biopsies of nasal mucosa.
CB:	1) Productive cough for over one month, with normal complete blood counts and no skin or gastrointestinal disease.
	2) Thickening of bronchial wall on chest radiography and CT imaging.
	3) Exclusion parameters: cardiac diseases and any other respiratory diseases of the nose or pharyngeal, laryngeal, tracheal and lung parenchyma determined by chest radiography, CT imaging and endoscopic examination.
	4) Elevated total cell number with macrophage dominance or normal total cell number with neutrophil dominance, in bronchoalveolar lavage fluid.

plications (i.e., vomiting or diarrhea) and the corticosteroid therapy. The diagnosis of CR was confirmed by computed tomography (CT) to rule out nasal tumors, lymphoplasmatic infiltration with or without neutrophils in nasal lavage and biopsy, and fungal infections, such as the aspergillosis. All biopsies were re-evaluated by NORTH LAB Inc. (Sapporo, Japan). The diagnosis of CB was confirmed by CT to rule out pneumonia and tumors, by slightly increased numbers of total cells, macrophages and neutrophils in bronchoalveolar lavage collected by bronchoscopy. Table 1 presents a summary of the diagnostic criteria of CR and CB.

Sample collection and analysis: Blood samples were collected from the jugular vein and stored immediately at 4°C until analysis. The proportions of CCR4/CD4 cells were determined by flow cytometry using an Alexa 647-conjugated anti-canine CD4 antibody (Serotec, Oxford, U.K.) and R-phycoerythrin (PE)-conjugated anti-human CCR4 antibody (BD Biosciences, San Jose, CA, U.S.A.), as previously reported [16, 26]. These determinations were made by Animal Allergy Clinical Laboratories, Inc. (Sagamihara, Japan). Serum levels of each antigen-specific IgE antibody were measured by fluorometric enzyme-linked immunosorbent assay, as previously described [6, 17]. Briefly, microwells were coated with 100 µl of 1 µl/ml allergens, including arthropods (*Dermatophagoides pteronyssinus*, *D. farina*, fleas, mosquitoes and cockroaches), pollens (mugwort, ragweed, goldenrod, dandelion, daisy, orchard, sweet vernal, timothy, rye, Bermuda grass, Japanese cedar, birch and alder), molds (*Aspergillus fumigatus*, *Alternaria alternaria*, *Cladosporium herbarum* and *Penicillium notatum*), meat and dairies (beef, pork, chicken, egg white, egg yolk, milk, lamb, turkey and duck), fish (salmon, codfish, catfish and capelin) and vegetables and grain (wheat, soybean, corn, potato and rice). The wells were blocked with 1% gelatin phosphate buffered saline (PBS) for 2 hr at room temperature and then washed with phosphate buffered saline with Tween 20 (PBST). Sera were diluted 1:1,000, and 100 µl was applied to each microwell. After incubating overnight at 4°C, the wells were washed, and 100 µl of biotinylated CRE-DM was used as an anti-canine IgE monoclonal antibody. The serum levels of each antigen-specific IgE antibody were calculated using a standard curve. These determinations were made by Animal Allergy Clinical Laboratories, Inc. Sensitization was defined by antigen-specific IgE concentrations >100 ng/ml [6].

Treatment protocol: All dogs were treated according to our therapeutic guidelines for CR and CB. When a diagnosis of CR or CB was made, macrolide therapy was initiated with azithromycin (5 mg/kg, SID) for the first 2 weeks. If macrolide therapy failed to control the clinical signs, an anti-allergic therapy was initiated, with montelukast (0.1 mg/kg, SID) [2] and ozagrel (5–10 mg/kg, BID) [9], for the second 2 weeks. If this anti-allergic therapy failed to control the clinical signs, corticosteroid therapy was provided with prednisolone (0.5 mg/kg, BID) for the third 2 weeks. The definitive therapy for each dog was based on the treatment responses.

Statistical analysis: All data were expressed as mean ± standard deviation (SD). The mean ratios of CCR4/CD4 cells in dogs with CR and CB were compared with the mean value ($21.2 \pm 3.4\%$) obtained for five healthy 7-year-old dogs of similar breeds [26], which value is not statistically different in 39 client-owned healthy dogs ($19.1 \pm 6.9\%$) [19]. The analysis was conducted by unpaired *t*-test after a Shapiro–Wilks test of normality. $P < 0.05$ was considered statistically significant. All analyses were performed with SigmaPlot for Windows, vers12.0 (SYSTAT SOFTWARE, San Jose, CA, U.S.A.).

RESULTS

The ratios of CCR4/CD4 cells were $31.9 \pm 8.4\%$ for dogs with CR and $30.2 \pm 6.8\%$ for dogs with CB. Both values were significantly higher than the average value previously reported for 7-year-old healthy dogs ($21.2 \pm 3.4\%$) [26]. In addition, ratios of CCR4/CD4 cells >28.0% were detected in 77% (20/27) of the dogs with CR and in 63% (12/19) of the dogs with CB, which was the upper limit of the range found in healthy dogs (mean ± 2SD) (Fig. 1).

The sensitization rates for a single allergen were 77% (20/27) and 79% (15/19) for dogs with CR and CB, respectively. In addition, multi-sensitization rates were 80% (16/20) and 67% (10/15) for dogs with CR and CB, respectively. Figure 2 compares the sensitization rates of each allergen compiled in each group. Over 25% of the dogs with CR were sensitized for *Penicillium notatum* [median: 150, range: 102–317 ng/ml], beef [128, 104–335 ng/ml] and soybean [165, 112–226 ng/ml]. In contrast, over 25% of the dogs with CB were sensitized for *Aspergillus fumigatus*

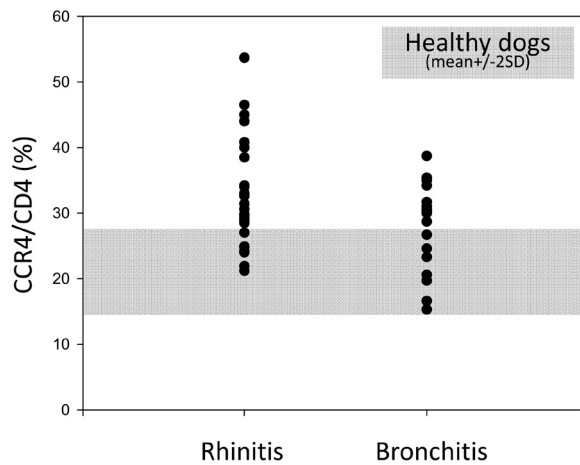


Fig. 1. Ratios of CCR4/CD4 cells (mean \pm 2SD) for 7-year-old healthy dogs [26] and dogs with chronic rhinitis or bronchitis.

[149, 127–452 ng/ml], *Alternaria alternaria* [134, 102–311 ng/ml], *Penicillium notatum* [122, 115–424 ng/ml], beef [249, 123–1054 ng/ml] and rice [142, 118–306 ng/ml].

Seven dogs with CR and one dog with CB were excluded from the treatment course for financial reasons and time constraints faced by the owners. The remaining dogs with high ratios of CCR4/CD4 cells ($>28.0\%$) or sensitized levels

of specific-antigen IgE (>100 ng/ml) received macrolide therapy (59% vs 50%), anti-allergic therapy (24% vs 13%) or corticosteroid therapy (18% vs 38%; Fig. 3). Macrolide therapy reduced clinical signs in 50% of the 36 dogs with CR and CB (Fig. 3).

DISCUSSION

It has been reported that serum TARC levels increase in eosinophilic inflammatory nasal and respiratory diseases, such as allergic rhinitis and asthma [15, 23]. In addition, the number of CCR4-positive lymphocytes also increases in airway epithelial cells of human patients with allergic CR and asthma, but not in those with non-allergic rhinitis and other lung diseases, such as chronic obstructive pulmonary disease [1, 20, 21]. These phenomena indicate that atopic inflammatory respiratory diseases are characterized by late allergic responses in both the upper and lower airways. Recently, the concept of “one airway, one disease” was proposed, because 60–78% of asthma patients have co-existing allergic rhinitis, which is better described as a continuum of inflammation involving one common airway [10]. In our clinical experience, dogs and cats sometimes show signs of both upper and lower respiratory diseases. In this study, the proportions of CCR4/CD4 cells were elevated in most dogs with CR and CB, although no eosinophil infiltration was detected in nasal tissue samples or bronchoalveolar lavage fluid. In addition,

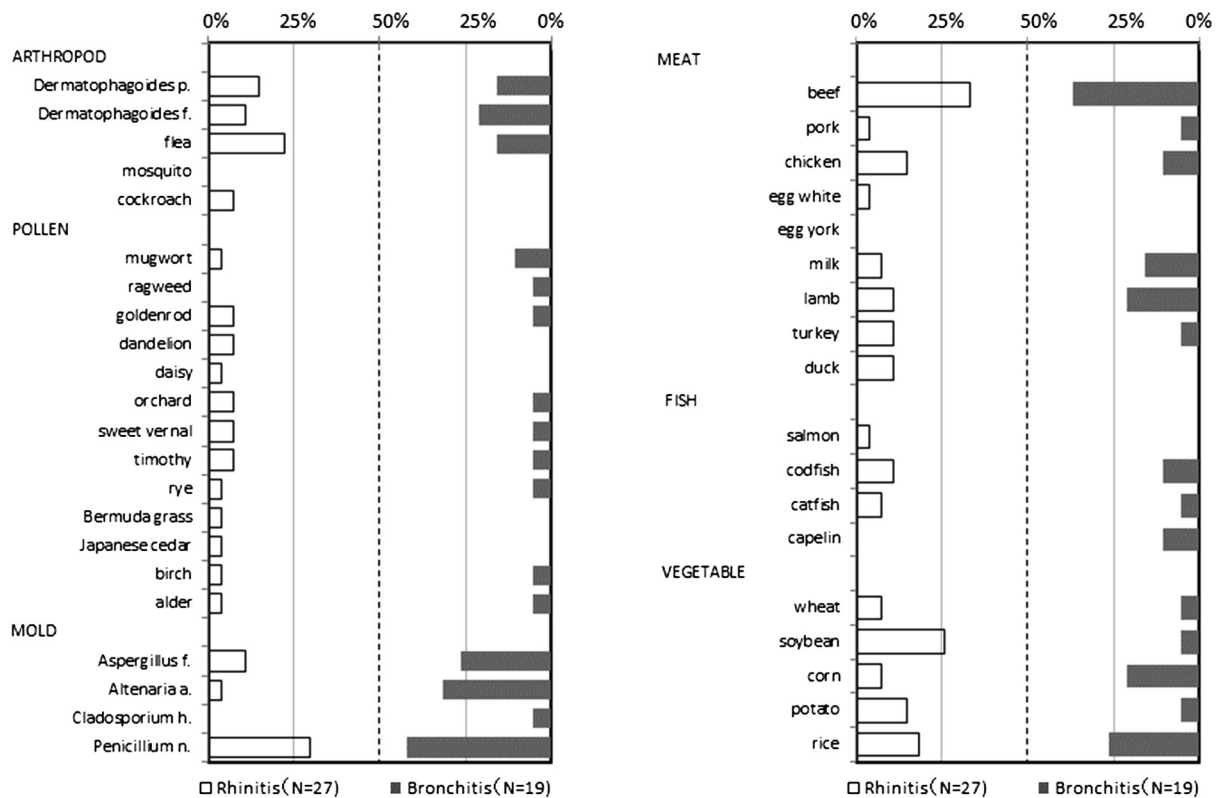


Fig. 2. Sensitization rates of dogs with chronic rhinitis or bronchitis, determined by measurements of serum-specific IgE antibodies (>100 ng/ml).

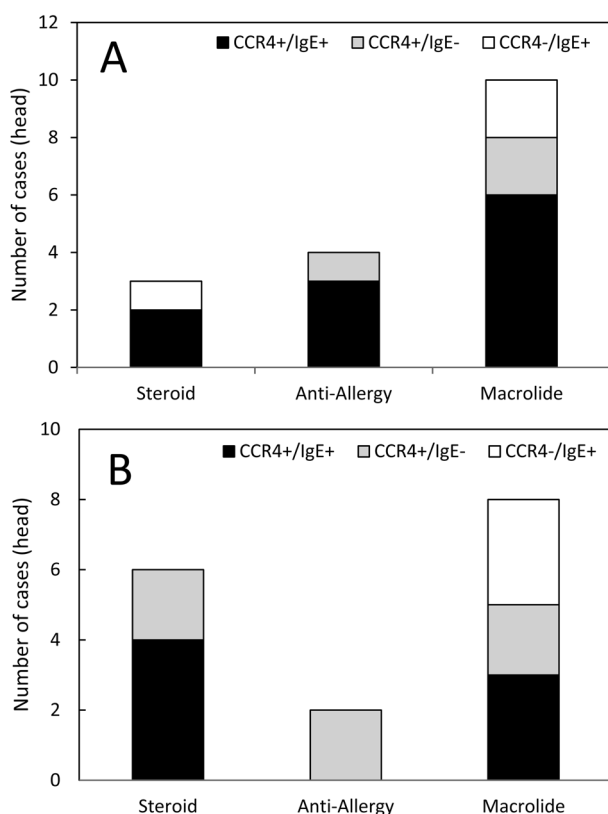


Fig. 3. Frequency distribution of the definitive therapies effective for dogs with (A) chronic rhinitis or (B) chronic bronchitis, based on high ratios of CCR4/CD4 cells (>28.0%) or high sensitized levels of specific-antigen IgE (>100 ng/ml).

they were sensitized to a broad range of aeroallergen and not only house dust mite.

A major limitation of this study is that we are unaware how these dogs became sensitized to such a broad range of allergens because there is no proof that CCR4+ lymphocytes increase locally in the nasal mucosa and bronchoalveolar lavage fluid. Moreover, it was not possible to include a healthy control group, because of the referral population of our hospital. It remains unclear, therefore, how many dogs with atopic predisposition were in a subclinical state, because our study could not measure the proportions of CCR4/CD4 cells and sensitized levels of antigen-specific IgE in client-owned healthy dogs. However, we hypothesize that exposure to arthropods, molds and dry powder inhalation of food materials may be one of the triggers for canine airway diseases. Thus, our data suggest that most dogs with CR and CB have a tendency toward atopic predisposition, necessitating a reduction in their exposure to environmental allergens. In addition, they may exhibit hypersensitivity of sensory receptors in the airway, similar to atopic cough in humans [7, 8].

Regarding the allergic disease, corticosteroid therapy is commonly selected in clinical settings. However, azithromycin (5 mg/kg SID for 5 days and then twice weekly) was introduced as an effective macrolide for dogs with CR [25].

Accordingly, we observed that macrolide therapy reduced the clinical signs in 50% of the dogs with CR and CB. Macrolide therapy suppresses inflammatory responses by reducing the levels of pro-inflammatory mediators, neutrophil and eosinophil chemotaxis, leukocyte adhesion and oxidative burst, bacterial adherence, biofilm formation and bacterial virulence, and by enhancing neutrophil apoptosis, mucociliary clearance and mucus hypersecretion in the airways [4]. In addition, roxithromycin, a 14-membered ring macrolide, inhibited the production of TARC by suppressing p38 in the mitogen-activated protein kinase pathway and the NF- κ B signaling, to maintain immune homeostasis in barrier tissues [13]. The macrolide therapy also has a benefit to control the respiratory bacterial infection, such as *Mycoplasma*, though we were not able to detect that by the cultivation test in this study. Altogether, these data suggest that macrolide therapy represents an effective alternative to corticosteroid therapy to control the clinical signs of canine CR and CB with an atopic predisposition.

In conclusion, most dogs with CR and CB have a possibility of atopic predisposition, and those with an atopic predisposition may require improvements in their environmental conditions to reduce allergens, as well as macrolide therapy to control their clinical signs.

ACKNOWLEDGMENTS. I would like to express the deepest appreciation to the staff of Animal Medical Center in Nihon University for following up and helping our research. Part of this study was supported by the Nihon University College of Bioresource Sciences Research Foundation for 2009.

REFERENCES

- Banfield, G., Watanabe, H., Scadding, G., Jacobson, M. R., Till, S. J., Hall, D. A., Robinson, D. S., Lloyd, C. M., Nouri-Aria, K. T. and Durham, S. R. 2010. CC chemokine receptor 4 (CCR4) in human allergen-induced late nasal responses. *Allergy* **65**: 1126–1133. [Medline]
- Booth, D. M. 2004. Drugs affecting the respiratory system. pp. 229–253. In: *Textbook of Respiratory Disease in Dogs and Cats* (King, L. G. ed.), Saunders, St. Louis.
- Boudreau, B., Nelson, L. L., Carey, S. A. and Williams, K. J. 2013. Spontaneous pneumothorax secondary to reactive bronchopneumopathy in a dog. *J. Am. Vet. Med. Assoc.* **242**: 658–662. [Medline] [CrossRef]
- Cervin, A. and Wallwork, B. 2007. Macrolide therapy of chronic rhinosinusitis. *Rhinology* **45**: 259–267. [Medline]
- Chapman, R. W. 2008. Canine models of asthma and COPD. *Pulm. Pharmacol. Ther.* **21**: 731–742. [Medline] [CrossRef]
- Fujimura, M., Masuda, K., Hayashiya, M. and Okayama, T. 2011. Flow cytometric analysis of lymphocyte proliferative responses to food allergens in dogs with food allergy. *J. Vet. Med. Sci.* **73**: 1309–1317. [Medline] [CrossRef]
- Fujimura, M., Nishi, K., Ohka, T., Yasui, M. and Kasahara, K. 2000. Bronchial biopsy and sequential bronchoalveolar lavage in atopic cough: In view of the effect of histamine H1-receptor antagonists. *Allergol. Int.* **49**: 135–142. [CrossRef]
- Fujimura, M., Ogawa, H., Yasui, M. and Matsuda, T. 2000. Eosinophilic tracheobronchitis and airway cough hypersensitivity

- in chronic non-productive cough. *Clin. Exp. Allergy* **30**: 41–47. [Medline] [CrossRef]
9. Funayama, M., Terasaki, E., Komiyama, E. and Uechi, M. 2013. Thrombosis in Left Ventricle of a Dog Remains with Anticoagulant Therapy. *J. Jpn. Vet. Med. Assoc.* **66**: 52–56 [in Japanese]. [CrossRef]
 10. Grossman, J. 1997. One airway, one disease. *Chest* **111** Suppl: 11S–16S. [Medline] [CrossRef]
 11. 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. [Medline] [CrossRef]
 12. Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P. and Williams, H. C. 2004. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J. Allergy Clin. Immunol.* **113**: 832–836. [Medline] [CrossRef]
 13. Komine, M., Kakinuma, T., Kagami, S., Hanakawa, Y., Hashimoto, K. and Tamaki, K. 2005. Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin. *J. Invest. Dermatol.* **125**: 491–498. [Medline] [CrossRef]
 14. Kurata, K., Maeda, S., Yasunaga, S., Masuda, K., Sakaguchi, M., Ohno, K. and Tsujimoto, H. 2004. Immunological findings in 3 dogs clinically diagnosed with allergic rhinitis. *J. Vet. Med. Sci.* **66**: 25–29. [Medline] [CrossRef]
 15. Leung, T. F., Wong, G. W., Ko, F. W., Lam, C. W. and Fok, T. F. 2005. Clinical and atopic parameters and airway inflammatory markers in childhood asthma: a factor analysis. *Thorax* **60**: 822–826. [Medline] [CrossRef]
 16. 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. [Medline] [CrossRef]
 17. Okayama, T., Matsuno, Y., Yasuda, N., Tsukui, T., Suzuta, Y., Koyanagi, M., Sakaguchi, M., Ishii, Y., Olivry, T. and Masuda, K. 2011. Establishment of a quantitative ELISA for the measurement of allergen-specific IgE in dogs using anti-IgE antibody cross-reactive to mouse and dog IgE. *Vet. Immunol. Immunopathol.* **139**: 99–106. [Medline] [CrossRef]
 18. Olivry, T., Naydan, D. K. and Moore, P. F. 1997. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. *Am. J. Dermatopathol.* **19**: 477–486. [Medline] [CrossRef]
 19. Ozaki, E. and Maeda, S. 2009. Clinical usage of allergic reaction intensity test (CCR4/CD4) in canine allergic dermatitis. *Proceeding of Annual Meeting of the Japanese Society of Veterinary Dermatology* **12**: 38. (in Japanese).
 20. Panina-Bordignon, P., Papi, A., Mariani, M., Di Lucia, P., Casoni, G., Bellettato, C., Buonsanti, C., Miotto, D., Mapp, C., Villa, A., Arrigoni, G., Fabbri, L. M. and Sinigaglia, F. 2001. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J. Clin. Invest.* **107**: 1357–1364. [Medline] [CrossRef]
 21. Sin, B. and Togias, A. 2011. Pathophysiology of allergic and nonallergic rhinitis. *Proc. Am. Thorac. Soc.* **8**: 106–114. [Medline] [CrossRef]
 22. Spergel, J. M. and Paller, A. S. 2003. Atopic dermatitis and the atopic march. *J. Allergy Clin. Immunol.* **112** Suppl: S118–S127. [Medline] [CrossRef]
 23. Takeuchi, H., Yamamoto, Y., Kitano, H. and Enomoto, T. 2005. Changes in thymus- and activation-regulated chemokine (TARC) associated with allergen immunotherapy in patients with perennial allergic rhinitis. *J. Investig. Allergol. Clin. Immunol.* **15**: 172–176. [Medline]
 24. Wakugawa, M., Nakamura, K., Kakinuma, T. and Tamaki, K. 2002. CC Chemokine Receptor 4 as a Possible Target for Therapy of Atopic Dermatitis. *Drug News Perspect.* **15**: 175–179. [Medline] [CrossRef]
 25. Windsor, R. C. and Johnson, L. R. 2006. Canine chronic inflammatory rhinitis. *Clin. Tech. Small Anim. Pract.* **21**: 76–81. [Medline] [CrossRef]
 26. Yasuda, N., Masuda, K. and Maeda, S. 2008. CC chemokine receptor 4-positive CD4(+) lymphocytes in peripheral blood increases during maturation in healthy beagles. *J. Vet. Med. Sci.* **70**: 989–992. [Medline] [CrossRef]