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Short communication

Th17 cells increase during maturation in peripheral blood of healthy dogs

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ABSTRACT

Recent studies have indicated that T helper 17 (Th17) cells are involved in the pathogenesis of various inflammatory diseases in dogs. However, age-related changes in canine Th17 cells have not yet been investigated. In the present study, the proportion of Th17 cells was examined in the peripheral blood mononuclear cells (PBMCs) of healthy dogs at various ages: Group 1 (n = 16; less than 1 year of age), Group 2 (n = 25; 1–5 years), and Group 3 (n = 19; 6–9 years), using flow cytometry and an anti-human interleukin (IL)-17A monoclonal antibody that reacts with canine IL-17A. The proportion of circulating Th17 cells positively correlated with age. The age-related differences were observed in the proportion of Th17 cells among Group 1 (mean ± SD: 1.52 ± 1.18%), Group 2 (mean ± SD: 3.81 ± 1.94%) and Group 3 (mean ± SD: 7.49 ± 2.54%). Our results suggest that age-related changes in Th17 cells need to be considered in future research on Th17-related diseases in dogs.

1. Introduction

Interleukin (IL)-17 A is an important cytokine in host defenses against microbes due to its role in regulating local tissue inflammation through the induction of pro-inflammatory cytokines, such as IL-1β, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-alpha, and neutrophil-recruiting chemokines, including C-X-C motif ligand (CXCL)1, CXCL2, and CXCL8 (Veldhoen, 2017). IL-17A is mainly secreted by helper T cells (Harrington et al., 2005), but also by cytotoxic T cells (Happel et al., 2003), innate lymphoid cells (Buonocore et al., 2010), γδT cells (Lockhart et al., 2006), natural killer T cells (Lee et al., 2008), neutrophils and mast cells (Res et al., 2010). IL-17A-producing helper T cells, also called Th17 cells, have been implicated in the pathogenesis of various autoimmune and chronic inflammatory diseases in humans (Tesmer et al., 2008). A previous study demonstrated that the proportion of human Th17 cells in peripheral blood was significantly higher in patients with psoriasis than in healthy controls, and also that Th17 cells had infiltrated the lesions of psoriasis (Benham et al., 2013). Psoriatic lesional keratinocytes were shown to be activated by IL-17A-induced cytokines, chemokines, and antimicrobial peptides (Lowes et al., 2013; Zeichner and Armstrong, 2016). A recent study indicated the significant efficacy of anti- IL-17A biological agents in clinical trials for psoriasis (Wasilewska et al., 2016). Based on these findings, Th17 cells play an important role in the development of psoriasis.

IL-17A-producing cells were recently detected in the inflamed tissues of dogs with chronic dermatoses (Kol et al., 2016). Although there have been no other studies on IL-17A-producing cells in canine dermatological diseases, IL-17A is known to induce the expression of GM-CSF, S100 calcium-binding protein A8, IL-8, and IL-19 in canine keratinocytes (Asahina et al., 2015), the cytokine profile of which was similar to that in human psoriatic keratinocytes (Ryckman et al., 2003; Parsonage et al., 2008; Lee et al., 2012). Moreover, a previous study using an enzyme-linked immunospot assay revealed that the proportion of IL-17A-producing cells in peripheral blood mononuclear cells (PBMCs) was higher in dogs with steroid-responsive meningitis-arteritis (SRMA) than in healthy controls (Freundt-Revilla et al., 2017). These findings suggested that Th17 cell numbers are elevated in dogs with several autoimmune diseases and chronic inflammatory dermatoses.

The immune system is generally considered to develop with growth (Simon et al., 2015). In humans, age-related increases have been reported in helper T cells, including Th1 (Wiegering et al., 2009; Holcar et al., 2015; Valiathan et al., 2016), Th2 (Holcar et al., 2015), and Th17 cells (Holcar et al., 2015; Li et al., 2017). Therefore, age-matched

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healthy controls have been used to investigate helper T-cell-related diseases in human studies (Yamamoto et al., 2000; Benham et al., 2013; Ye et al., 2016). In dogs, previous studies reported that the proportions of Th1 and Th2 cells increased with age (Horiuchi et al., 2007; Yasuda et al., 2008). However, age-related changes in Th17 cells remain unclear. The aim of the present study was to investigate the proportion of circulating Th17 cells in healthy dogs at various ages for future research.

2. Materials and methods

2.1. Animals and sample collection

For this study, a total of 60 dogs were recruited. They originated from a research colony (all apart from 1 Beagle, see below) or were privately owned. Written informed consent was obtained from all owners. Research dogs were kept for clinical training of veterinary students or experimental purposes under a protocol approved by the Institutional Animal Care and Use Committee and the Clinical Ethics Committees of Gifu university. Dogs were deemed to be healthy based on their medical history and physical examination. Recruitment occurred either in Gifu university hospital or 6 private veterinary hospitals. Breeds included Beagles (n = 23), toy poodles (n = 7), chihuahuas (n = 5), Border collies (n = 3), miniature dachshunds (n = 3), Pomeranians (n = 3), Golden retrievers (n = 2), Labrador retrievers (n = 2), Maltese terriers (n = 2), Pembroke welsh corgis (n = 2), Shiba inus (n = 2) and 1 each of the following breeds: American Staffordshire terrier, Great pyrenese mountain dog, miniature schnauzer, Shih tzu, Yorkshire terrier and 1 cross-breed dog. The study population included 20 male and 40 female dogs. Dogs were divided into 3 different age groups: “Group 1″ (less than 1 year), “Group 2″ (1–5 years old), and “Group 3″ (6–9 years old) (Patronek et al., 1997; Holcar et al., 2015). Five-to-ten-milliliter samples of heparinized whole blood were obtained from each dog. Each blood sample was diluted with an equal volume of Dulbecco’s phosphate-buffered saline (DPBS, Kohjin Bio Co., Ltd.; Saitama, Japan) then layered on Ficoll-Hypaque (Lymphoprep®, AXIS-SHIELD; Oslo, Norway). Following centrifugation at 1800 rpm at room temperature for 30 min, a layer of PBMCs was collected and washed with DPBS. Cells were stored in cell freezing medium (TC-Protector, DS Pharma Biomedical Co., Ltd.; Osaka, Japan) at −80 °C for later analyses.

2.2. Canine full-length IL-17A plasmid transfection

An encoding hexahistidine (His)-tagged canine full-length IL-17A cDNA was inserted into a pcDNA3.1 vector (Invitrogen; Carlsbad, CA, USA). Human embryonic kidney (HEK) 293 A cells (Invitrogen) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of air containing 5% CO_2. Cells were then cultured at a density of 1×10^6 cells/well in a 6-well plate. Four micrograms of a pcDNA3.1-IL-17A-His-tagged plasmid was transfected into HEK293 A cells using Lipofectamine™ 2000 (Life Technologies; Carlsbad, CA, USA) according to the manufacturer’s instructions.

2.3. Flow cytometry

Canine IL-17A-transfected HEK293 A cells were treated with 10 μg/ml Brefeldin-A (Sigma-Aldrich; St Louis, MO, USA) for 4 h. Non-transfected HEK293 A cells were used as negative controls. Cells were then washed with washing buffer (DPBS containing 1% fetal bovine serum) and resuspended in 100 μl blocking buffer (DPBS containing 1% normal rat serum). These samples were fixed with 4% paraformaldehyde and permeabilized with DPBS containing 0.2% Triton-X (Sigma-Aldrich). Cells were stained with anti-human IL-17A monoclonal antibody (mAb) (dilution 1:200, clone eBio64DEC17, eBioscience Inc.; San Diego, CA USA) and anti-His tag mAb (dilution 1:100, clone 3D5, Invitrogen) in washing buffer at room temperature for 30 min. Purified mouse IgG1 (dilution 1:200, clone MOPC-31C, BD Pharmingen; San Diego, CA, USA) and purified mouse IgG2b (dilution 1:100, clone MPC-11, BD Pharmingen) were used as isotype controls. Cells were then washed with washing buffer and stained with phycoerythrin (PE)-conjugated anti-mouse IgG1 (dilution 1:100, clone RMG1-1, BioLegend Inc.; San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG2a/2b (dilution 1:100, clone R2-40, BD Pharmingen) as secondary antibodies in washing buffer at room temperature for 30 min. Transfected cells were then used in flow cytometry to confirm the cross-reactivity of the anti-human IL-17A mAb to canine IL-17A at 48 h post-transfection.

The protocol for cytokine production and the intracellular accumulation of PBMCs for flow cytometric detection was adopted from a previous study (Kol et al., 2016). PBMCs were adjusted to a final concentration of 2×10^6 cells/ml in RPMI 1640 medium (Sigma-Aldrich) with 10% FBS. Cells were treated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 500 ng/ml A23187 (Sigma-Aldrich). After a 3-hour incubation, 1 μg/ml Brefeldin-A (Sigma-Aldrich) was added and cells were cultured for an additional 3 h. Cells were then washed and resuspended in 100 μl blocking buffer (DPBS containing 0.02% sodium azide, 1% normal rat serum and 1% normal mouse serum). These samples were stained with viability dye in a total volume of 1 ml PBS at RT for 20 min in the dark. Washing was performed with washing buffer. Cells were fixed in 250 μl fixation/permeabilization working solution for 20 min at 4 °C. Thereafter, cells were washed twice in permeabilization buffer. Cells were stained with the following primary antibodies or appropriate isotype controls: anti-canine CD3 FITC-conjugated mAb (dilution 1:20, clone CA17.2A12, Bio-Rad; Hercules, USA), anti-canine CD4 Alexa Fluor 647-conjugated mAb (dilution 1:500, clone YKIX302.9, Bio-Rad), anti-human IL-17A PE-conjugated mAb (dilution 1:20, clone eBio64DEC17, eBioscience), FITC-conjugated mouse IgG1 (dilution 1:20, clone MCA928 F, Bio-Rad), Alexa Fluor 647-conjugated rat IgG2a (dilution 1:5000, clone RTK2758, Abcam; Cambridge, UK), and PE-conjugated mouse IgG1 (dilution 1:80, clone 400112, BioLegend). Fluorescence was detected using a flow cytometer (FACSCanto™II, BD Biosciences) and flow cytometry data were analyzed using FlowJo flow cytometry software (version 9.9.6, Tree Star Inc.; Ashland, OR, USA).

2.4. Statistical analysis

Spearman’s correlation coefficient by the rank test was used to analyze the relationship between the age and proportion of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes. The Steel-Dwass test was used to test for between-group differences. The statistical significance on the proportion between beagles and other breeds was evaluated by Mann-Whitney *U*-test. A value of $P < 0.05$ was considered to be significant. Statistical analyses were performed using the JMP 10.0 program (SAS Institute; Cary, NC, USA).

3. Results

The present results demonstrated that the antibody (eBio64DEC17) reacted with His-tagged canine IL-17A-transfected cells, but not with non-transfected cells (Fig. 1), validating the cross-reactivity of the antibody with canine IL-17A.

Age-distribution amongst the 3 groups was as follows: Group 1 ($n = 16$; 10 females, 6 males; 3–11 months of age), Group 2 ($n = 25$; 19 females, 6 males; 1.2–5.6 years of age), and Group 3 ($n = 19$; 11 females, 8 males; 6.5–9.9 years of age). The identification of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes was performed using the gating strategy shown in Fig. 2. We found that the proportion of $IL\text{-}17A^+$ cells in $CD3^+CD4^+$ lymphocytes positively correlated with age ($rs = 0.801$, $P < 0.001$; Fig. 3a). Significant differences were observed in the

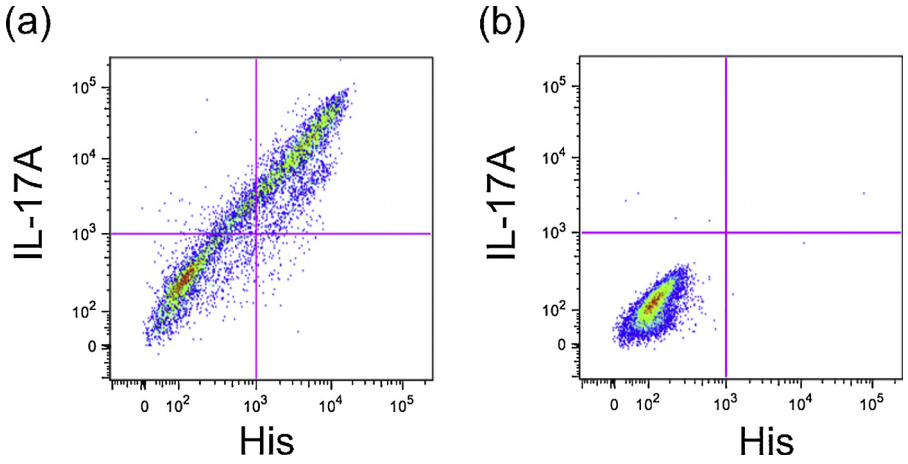

Fig. 1. Flow cytometry using an anti-human IL-17A antibody in canine IL-17A His-tagged transfected HEK293 A cells (a). Non-transfected cells were used as negative controls (b).

proportion of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes among Group 1 (mean ± SD: 1.52 ± 1.18%), Group 2 (mean ± SD: 3.81 ± 1.94%), and Group 3 (mean ± SD: 7.49 ± 2.54%) ($P < 0.001$; Fig. 3b). The statistical analysis indicated no significant difference on the proportion of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes between beagles and other breeds.

4. Discussion

Previous studies on dogs reported that the expression of IL-17A increased in the lesions of inflammatory colorectal polyps and chronic *Trypanosoma cruzi*-infection (Ohta et al., 2013; de Paula Costa et al., 2016). Additionally, IL-17A-producing cells were shown to infiltrate the lesions of inflammatory bowel disease, chronic idiopathic dermatitis, chronic ulcerative stomatitis, necrotizing meningoencephalitis, and chronic rhinitis (Kol et al., 2016; Anderson et al., 2017). These studies suggested that IL-17A-producing cells were involved in the pathogenesis of various diseases in dogs. Circulating IL-17A-producing cells in healthy dogs were identified by flow cytometry using anti-human or anti-mouse IL-17A mAbs (Costa-Pereira et al., 2015; Moreira et al., 2015; Ritt et al., 2015; Bonnefont-Rebeix et al., 2016; Kol et al., 2016), although the cross-reactivity of these mAbs to canine IL-17A was not confirmed. In the present study, we demonstrated the cross-reactivity of anti-human IL-17A (eBio64DEC17) mAb to canine IL-17A.

Previous studies reported age-related changes in canine Th1 or Th2 cells (Horiuchi et al., 2007; Yasuda et al., 2008), but not in canine Th17 cells. Thus, the proportion of Th17 cells in the PBMCs of healthy dogs at various ages was evaluated by flow cytometry. Stimulation of $CD4^+$ cells with PMA and ionomycin is necessary to induce the production of IL-17A for the evaluation of the proportion of Th17 cells by flow cytometric analysis. To sort $CD4^+$ cells before the stimulation, however, more than 10 ml of whole blood is required. Kol et al. described a method for identification of canine Th17 cells with a small volume of blood samples (Kol et al., 2016), which labeled lymphocytes by anti-CD3, anti-CD4 and anti-IL-17A antibodies after the stimulation. We could not collect the blood samples of more than 5 ml from private owned dogs. Thus, we employed the method of Kol et al. in the present study. The results indicated that the proportion of Th17 cells in the PBMCs of healthy dogs increased with age. A previous study reported an increase in canine Th1 cell numbers with age throughout life (Horiuchi et al., 2007). Canine Th2 cells also increased during the first year of life; however, further increases were not observed in the older age groups (Horiuchi et al., 2007; Yasuda et al., 2008). The present study demonstrated age-related increases in canine Th17 cells from the young to adult and elderly groups, similar to that in canine Th1 cells (Horiuchi et al., 2007). This study provided age-matched reference values for the population of circulating Th17 cells in healthy dogs, contributing to future research on Th17-related diseases in dogs.

Similar to our results, the proportion of human Th17 cells was shown to increase with age (Holcar et al., 2015; Li et al., 2017). Age-related changes in human Th17 cells were attributed to the

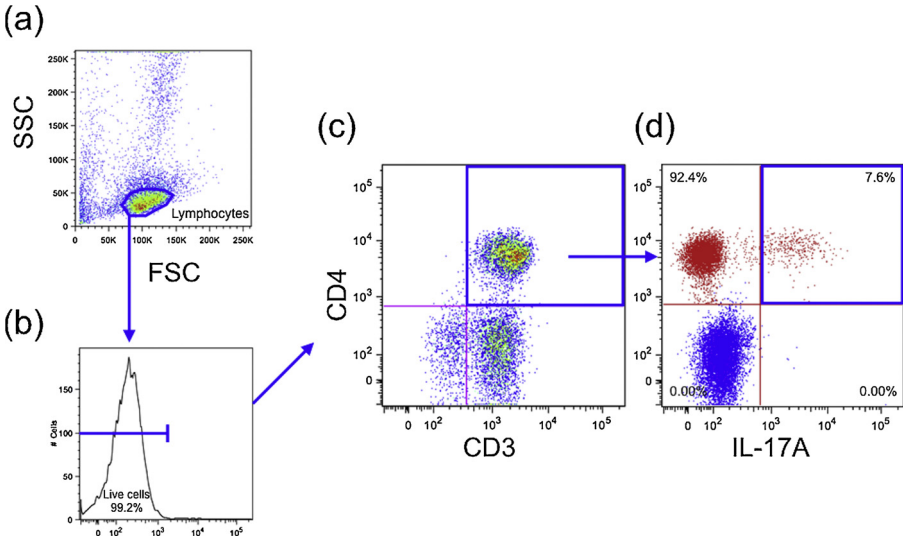

Fig. 2. Gating strategy to detect circulating $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes. (a) Lymphocytes were gated based on the size and granularity of cells. (b) Viability dye-negative cells were gated to exclude dead cells. Helper T cells were gated by the expression of CD3 and CD4 (c), followed by a division into $IL\text{-}17A^+$ and $IL\text{-}17A^-$ cells (d). Samples and isotype controls were indicated in red and blue, respectively. SSC, side scatter; FSC, forward scatter (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

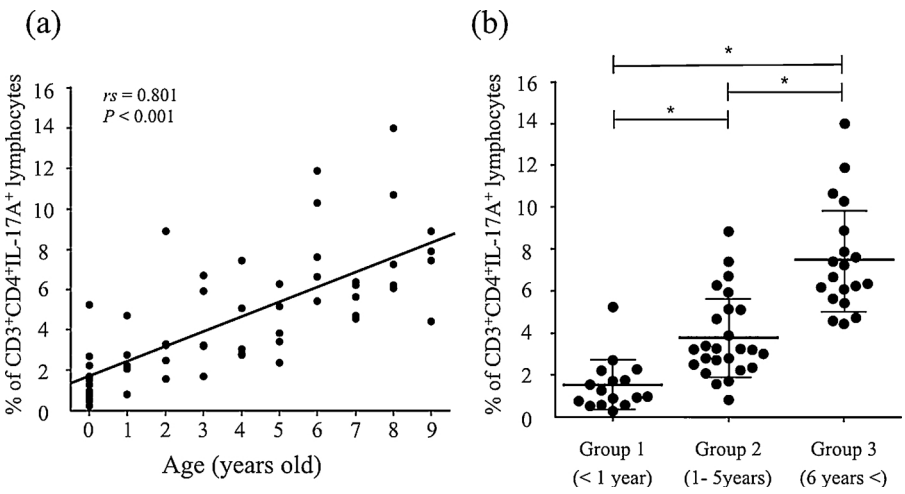

Fig. 3. Effects of age on the proportion of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes. (a) Correlations were assessed by Spearman's correlation coefficient. *rs*- and *P*-values were calculated by a linear regression analysis. (b) The proportion of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes in 3 different age groups. The Steel-Dwass test was used for multiple comparisons. Black lines indicated the mean value with standard deviations. $^*P < 0.001$.

accumulation of memory T cells stimulated by microorganisms (Mubarak et al., 2016). Previous studies using mice demonstrated that differentiation into Th17 cells from naïve T cells was induced by *Staphylococcus aureus* or *Salmonella typhimurium* via an interaction with macrophages or dendritic cells (Uematsu et al., 2008; Uematsu and Akira, 2009). Further studies are required to elucidate the mechanisms responsible for age-related changes in canine Th17 cells.

The main cellular source of human IL-17A was not only Th17 cells, but also Th1/Th17 cells, which produced interferon-gamma and IL-17A (Brenchley et al., 2008; Gosselin et al., 2010). The proportion of human Th1/Th17 cells increased with age (Holcar et al., 2015). However, Th1/Th17 cells were not identified in dogs. The subset of canine Th1/Th17 cells needs to be identified for future research.

In conclusion, we demonstrated that Th17 cells in the PBMCs of healthy dogs increased with age. Our results suggest that age-related changes in Th17 cells need to be considered in future research on Th17-related diseases in dogs.

Conflict of interest

The authors confirm that there are no conflicts of interest concerning the contents of this publication.

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