

## Crossreactivity of Antibodies to Canine CD25 and Foxp3 and Identification of Canine CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Cells in Canine Peripheral Blood

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**ABSTRACT.** The importance of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg cells) in immune response is increasingly being recognized. However, to date, only a few studies on these cells have been conducted in canine species, partly because of the unavailability of appropriate antibodies to detect this cell population. In this study, the crossreactivities of anti-human CD25 antibody (clone ACT-1) and anti-mouse Foxp3 antibody (clone FJK16s) to canine CD25 and Foxp3, respectively, were confirmed using cell lines overexpressing either of these genes. By using these antibodies, we determined if CD4<sup>+</sup>, CD25<sup>+</sup>, or Foxp3<sup>+</sup> cells were present in unstimulated canine peripheral blood mononuclear cells (PBMCs) and in concanavalin A (ConA)-stimulated PBMCs. CD25<sup>+</sup> cells were not detected in unstimulated canine PBMCs, unlike in human or mouse PBMCs. ConA stimulation of canine PBMCs induced Foxp3 and CD25 expression in CD4<sup>+</sup> and CD4<sup>-</sup> cells. These data indicate that the activation of canine lymphocytes might induce the production of adaptive Treg cells, as observed in humans.

**KEY WORDS:** canine, CD25, Foxp3, Treg.

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Naturally arising CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thymus, termed regulatory T cells (Treg cells), are found to be responsible for the peripheral immune tolerance to prevent the function of effector T cells [16]. In addition to expressing CD4 and CD25, Treg cells have a characteristic cell surface phenotype, such as the expression of CTLA-4, GITR, and folate receptor 4 (FR4) [19]. However, except for FR4, most cell surface antigens are not exclusively expressed in Treg cells, but they are also expressed in other types of cells. The transcriptional factor Forkhead box P3 (Foxp3) is found to be a Treg-specific marker that regulates the development of Treg cells in the thymus [7, 9]. Furthermore, mice carrying a natural mutation in the Foxp3 gene (*scurfy* mice) do not produce Treg cells, which leads to the development of autoimmune diseases [5]. Ectopic expression of Foxp3 causes CD4<sup>+</sup> T cells to become functional Treg-like cells in mice, indicating that Foxp3 is considered as a master gene in the development of Treg cells in mice [9].

On the other hand, some patients who were diagnosed with immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), a severe autoimmune disease, have defective functions of Treg cells and harbor Foxp3 mutations [2, 3, 18], suggesting that peripheral immune tolerance in humans is regulated by a mechanism similar to that in mice. However, some studies performed in humans also showed a slight difference in the Foxp3 status between mice and humans [7, 21]. Foxp3 and cell surface antigens such as CD25, CTLA-4, and GITR are induced in human CD4<sup>+</sup>CD25<sup>-</sup> T cells by *in vitro* stimulation with anti-

CD3 and antigen presenting cells (APCs) resulting in regulatory phenotypes of these cells [1, 14]. Yet, these phenotypic changes were transient. Induction of Foxp3 in human CD4<sup>+</sup>CD25<sup>-</sup> cells is not sufficient to change these cells to Treg-like cells in contrast to mice [1].

In canine peripheral blood mononuclear cells (PBMCs), Treg cells are identified by staining with anti-CD4 and anti-Foxp3 antibodies and by their ability to produce cytokines [4]. The percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells is altered in atopic dogs treated using allergen-specific immunotherapy [10]. The antibody used to detect canine Foxp3 in these two studies is commercially available, and was originally raised against mouse Foxp3. According to the manufacturer's information, the epitope of this antibody covers the region of exon 2, which is highly similar in its amino acid sequence in mice, humans, and dogs. However, specific crossreactivity of this antibody against canine Foxp3 has not been reported. In the study by Biller, CD4<sup>+</sup>Foxp3<sup>+</sup> cells are found to produce Treg-specific cytokines, namely, TGF- $\beta$  and IL-10, after appropriate stimulation [4]. However, it is not known whether these cells were positive for CD25, a marker constitutively expressed on human or mouse Treg cells. It is also shown that anti-human CD25 antibody (clone ACT-1) cross-reacted with PHA-activated canine PBMCs [8] and with the G2/M and G0/G1 populations in Concanavalin A (ConA)-stimulated canine PBMCs [12]. To the best of our knowledge, there are no studies showing that ACT-1 recognizes canine CD25 exclusively. Furthermore, no cell population analysis has showed that canine PBMCs are positive for CD4, CD25 or Foxp3 after stimulation. In the present study, we determined the crossreactivities of commercially available antibodies raised against human CD25 and mouse Foxp3 with canine CD25 and Foxp3, using cell lines overexpressing either of these genes.

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The populations of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in ConA-stimulated canine PBMCs were examined.

## MATERIALS AND METHODS

**Cell cultures:** Human embryonic 293T cells and PLAT-A mouse amphotropic virus-packaging cells (kindly provided by Dr. Toshio Kitamura) were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin, and 50  $\mu$ M 2-mercaptoethanol (2-ME) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. A canine lymphoma cell line, GL-1 cells (kindly provided by Dr. Munekazu Nakaichi), were grown in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin, and 50  $\mu$ M 2-ME at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

PBMCs from four specific pathogen-free dogs were separated by Ficoll-Hypaque density-gradient centrifugation. The PBMCs (2  $\times$  10<sup>6</sup> cells/ml), suspended in RPMI1640 supplemented with 10% FCS, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin, and 50  $\mu$ M 2-ME, were cultured in the presence of 5  $\mu$ g/ml ConA (SIGMA, Tokyo, Japan) in a 24-well plate at 37°C for 3 days. The cells were then stained with antibodies for flowcytometric analysis.

**RNA isolation and RT-PCR:** Total RNA was extracted from the normal canine thymus or the cervical lymph node by the acid guanidium-phenol-chloroform (AGPC) method using TRIreagent (Molecular Research Center, Inc., Cincinnati, OH). After treatment with DNase I (Applied Biosystems, Tokyo, Japan), cDNA was synthesized from 0.5  $\mu$ g total RNA by using Superscript II (Invitrogen Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. Oligo dT primers were used to prime the first strand synthesis for each reaction. The full-length cDNA of canine Foxp3 and canine CD25 was obtained by PCR amplification using canine thymus and cervical lymph node cDNA as a template and the following primers. The primers YTM192 (5'-GGG TCC ACA TGG TTT AGG TG -3'; nt 42208951-42208932 in canine chromosome X, accession no. NW\_879562.1) and YTM208 (5'-CCT TCC TCC TTT CCT TTG CT -3'; nt 42202283-42202267 in canine chromosome X) were used to amplify canine Foxp3. They were synthesized on the basis of the genomic sequence of canine Foxp3. The primers YTM92 (5'-CAG CCT TCT GCA AAG AAA CA -3'; nt 11519026-11519045 in canine chromosome 2, accession no. NW\_876291.1) and YTM93 (5'-GGG TGA GCA TTT GAC TTC CT -3'; nt 11569579-11569598 in canine chromosome 2) were used to amplify canine CD25. They were synthesized on the basis of the genomic sequence of canine CD25. PCR amplifications were performed using a final volume of 25  $\mu$ l containing a pair of primers (0.3  $\mu$ M each), 1  $\mu$ l cDNA, 1  $\times$  PCR buffer for KOD Plus, 0.2  $\mu$ M dNTP, 1 mM MgSO<sub>4</sub> and 1.0 unit of KOD Plus (TOYOBO, Osaka, Japan). After incubation at 94°C for 2 min, PCR was carried out under the following conditions: denaturation at 98°C for 10 s, annealing at 56°C

for 30 s, and polymerization at 72°C for 1.5 min. After 40 cycles, the polymerization step was extended at 72°C for 10 min. The amplified PCR products were subcloned into pBluescript SK(-) vector or pCR2.1 vector and subjected to a sequencing reaction using the Big Dye Terminator v3.1 Cycle Sequencing kit (PerkinElmer Japan Co., Ltd., Kanagawa, Japan), followed by the run through the ABI PRISM3100-Avant sequencer (Applied Biosystems). The plasmid pBluescript SK(-) cloned with cFoxp3 and the plasmid pCR2.1 cloned with cCD25 were designated as pBS-cFoxp3 and pCR-cCD25, respectively.

**Plasmid construction:** The expression vectors, pMX-IRES-EGFP and pMXs-IP, were kindly provided by Dr. Kitamura [11]. A portion of EGFP in the vector pMX-IRES-EGFP was replaced with an extracellular and transmembrane domain of rat CD2 (pMX-IRES-rCD2) to achieve the expression of rat CD2 downstream of the internal ribosomal entry site (IRES). The vector pBS-cFoxp3 was digested with BamHI and XhoI and cloned into these sites of the vector pMX-IRES-rCD2 in order to construct a vector expressing canine Foxp3 (pMX-rCD2-cFoxp3). The vector pCR-cCD25 was used to generate a vector that expressed canine CD25. It was amplified by PCR, using the primers M13(-20) and YTM112 that carried a XhoI site. The PCR product was first subcloned into the EcoRI and XhoI sites of the pAPIG vectors (kindly provided by Dr. Yasuda, [20]) followed by subcloning into the EcoRI and NotI sites of the pMXs-IP retroviral expression vector (pMXs-IP-cCD25).

**Transfection:** 293T cells were transfected with pMX-IRES-rCD2 or pMX-rCD2-cFoxp3, using Transit-LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. After 48 hr, transfected cells were collected and stained as described below.

GL-1 cells were retrovirally transduced with pMXs-IP or pMXs-IP-cCD25 to obtain cells that stably express canine CD25. In brief, PLAT-A cells were transfected with either the vector pMXs-IP or pMXs-IP-cCD25 by using Transit-LT1. The supernatant containing the virus was collected 48 hr after transfection. GL-1 cells were suspended in the virus-containing supernatant (1  $\times$  10<sup>6</sup> cells/ml) containing 2.5  $\mu$ g/ml of polybrene (SIGMA) and incubated for 12 hr. The virus-containing medium was replaced, and GL-1 cells were cultured in the presence of 2  $\mu$ g/ml puromycin (SIGMA) to select CD25-expressing cells. Once the colonies were obtained, we cloned each colony by applying the limiting dilution method and used flow cytometry analysis to confirm the expression.

**FACS analysis:** 293T cells transfected with pMX-rCD2-cFoxp3 were resuspended in FACS buffer (PBS containing 2%FCS and 0.1%NaN<sub>3</sub>) and stained with biotin-conjugated anti-rat CD2 antibody (CEDARLANE Laboratories Ltd., Burlington, Ontario), followed by incubation with PE-Cy5-conjugated streptavidin (Becton Dickinson, Tokyo, Japan) in order to analyze the cell surface expression of rat CD2 and the intracellular expression of canine Foxp3. The cells were incubated for 30 min on ice and washed twice with

FACS buffer. They were stained with PE-conjugated anti-Foxp3 antibody (clone FJK016s; eBioscience, Inc., San Diego, CA), fixed, and permeabilized according to the manufacturer's instructions, and analyzed by flow cytometry. For staining with anti-CD25 antibody, the cells were incubated with PE-conjugated anti-CD25 antibody (clone ACT-1; Dako Japan, Tokyo, Japan) for 30 min on ice and washed with FACS buffer.

The unstimulated and stimulated PBMCs were washed with FACS buffer and stained with anti-CD4-FITC antibody (Serotec), followed by staining with anti-CD25-PE antibody. The cells were fixed and permeabilized as described above. The cells were then stained with PE-Cy5-conjugated anti-Foxp3 antibody (eBioscience) and analyzed by flow cytometry. Appropriate isotype controls were used for each sample. Flow cytometry analyses were performed on EPIX-XL (Beckman Coulter, Tokyo, Japan), and the results obtained were analyzed using the FlowJo software (Treestar, Inc., San Carlos, CA, U.S.A.). Samples from experiments involving readouts on different days were run on the same instrument with identical compensation settings to ensure comparability.

## RESULTS

**Nucleotide and deduced amino acid sequences of canine Foxp3 cDNA:** PCR amplification of the canine Foxp3 cDNA generated a band with an expected size of approximately 1.5 kbp. The nucleotide sequence of this product revealed a cDNA clone covering 1424 bp, containing an open reading frame of 1293 nucleotides that coded for 430 amino acids (Fig. 1A). The nucleotide sequence of Foxp3 cDNA showed 89%, 84%, and 96% homology with that of humans, mice, and cats, respectively, and was completely comparable with the corresponding region found in the canine genome database.

The alignments of the predicted amino acid sequence of the canine Foxp3 cDNA clone with those of the cat, human, and mouse Foxp3 cDNA are shown in Fig. 1B. The deduced amino acid sequences of the canine Foxp3 cDNA showed 86%, 86%, and 95% homology with that of the human, mouse, and cat Foxp3 polypeptides, respectively. In particular, the forkhead domain, the characteristic features of the forkhead/winged-helix family of transcriptional regulators, and the zinc-finger domain were highly conserved between these species.

**Expression of canine Foxp3 and crossreactivity of anti-mouse Foxp3 antibody to canine Foxp3:** The canine Foxp3 protein was transiently expressed in 293T cells and analyzed by flow cytometry to confirm the crossreactivity of anti-mouse Foxp3 antibody to canine Foxp3. Approximately 50% of mock-transfected cells were positive for anti-rat CD2 antibody, indicating a good transduction efficiency of the vector pMX-IRES-rCD2. In addition, none of the cells were positive for anti-Foxp3 antibody (Fig. 2). An almost similar number of pMX-rCD2-Foxp3-expressing cells were positive when stained with anti-rat CD2 antibody. Further-

more, the cells positive for anti-rat CD2 antibody were also stained with anti-Foxp3 antibody, and a good correlation was found between the expression level of rat CD2 and Foxp3. This result indicates that anti-mouse Foxp3 antibody crossreacts with the canine Foxp3 protein when overexpressed in the 293T cells.

**Expression of canine CD25 and crossreactivity of anti-human CD25 antibody to canine CD25:** We cloned the CD25 cDNA, which was generated using RNA from the canine thymus, and expressed it stably in the canine lymphoma cell line GL-1 in order to investigate whether anti-human CD25 antibody (clone ACT-1) crossreacts with the canine CD25 protein. The nucleotide sequence of the canine CD25 cDNA revealed that the cDNA clone was 920 bp long containing an open reading frame of 807 nucleotides that coded for 268 amino acids (data not shown, DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank, accession number AB501356). The nucleotide sequence of the coding region was compared with the canine CD25 sequence previously registered at the NCBI database (AF056491), and a difference was found in four nucleotides. The guanine at positions 233, 244, and 699 was replaced by adenine, and the cytosine at position 708 was replaced by thymine. Comparison of the amino acid sequences between CD25 cloned in this study and CD25 cloned previously revealed that two mutations were nonsense mutations. Arginine at position 233 and valine at position 244 were replaced by glutamine and isoleucine, respectively. Yet, the nucleotide sequence of the CD25 cDNA used in this study was completely comparable with the corresponding region found in the canine genome database.

GL-1 cells were infected with a retrovirus carrying the canine CD25 genes and were cultured in a medium containing puromycin. The clones that were resistant to puromycin were selected by the limiting dilution method. One puromycin-resistant GL-1 clone was stained with PE-conjugated ACT-1 antibody to analyze the crossreactivity of anti-human CD25 antibody (ACT-1) to canine CD25. As shown in Fig. 3, GL-1 cells transduced with an empty vector were negative for ACT-1. However, puromycin-resistant GL-1 cells were positive for ACT-1. This result indicates that the canine CD25 protein is recognized by the monoclonal antibody against human CD25. At the same time, anti-feline CD25 monoclonal antibody (clone 9F23) did not crossreact with the canine CD25 protein overexpressed in GL-1 cells (data not shown).

**Expression of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> lymphocytes in unstimulated and ConA-stimulated canine PBMCs:** To analyze the expression of CD25 in canine PBMCs, firstly, we examined the expression of canine CD25 in unstimulated PBMCs. Triple staining of canine PBMCs with FITC-conjugated anti-canine CD4 antibody, PE-conjugated anti-human CD25 antibody, and PE-Cy5-conjugated anti-mouse Foxp3 antibody showed a small number of Foxp3<sup>+</sup> cells (1.08% to 2.65%) in the CD4<sup>+</sup> populations. This amount was less than those shown in a previous study [4] and was

A

GGGTCCACATGGT

TTAGGTGGTTGAGTAGTTCATGCACCTGCTGGGGACATGTGAACCTCTCTCTCTATGTCCTCCACAGCTGCCCTCGGACAGGACCG

1 ATGCCCAACCCAGGCCAGCCCTCAGCCCTTCCTTGGACCTGGCCCATCCAGGAGCCTTGCCAGCTGGAGGCTGCACCC  
 1 M P N P R P A K P S A P S L A P G P S P G A L P S W R A A P

93 AAGGCTTCAGACCTGCTTGGGCCAAGGGCCGGGGTGACCTTCCAGGCCGGGACCTCCGAGCGGGACCATGCCTCTCTCTT  
 31 K A S D L L G A K G P G V T F Q G R D L R G G T H A S S S L

183 AACCCATGCCACCATGCAGCTGCAGCTGCCACAGTGCCCTCTGGTCATGGTGGACCTCTGGGGCAGGCTGGGCCCCCTGCCCCAC  
 61 N P M P P S Q L Q L P T V P L V M V A P S G A R L G P S P H

273 TTGAGCGCTCTCCAGGACAGGCCACATTCATGCACAGCTCTCCACGGTGGTACCCACACCCGGACCCGGTCTGCAGGTGCGC  
 91 L Q A L L Q D R P H F M H Q L S T V G T H T R T P V L Q V R

363 CCATGACAGCCAGCTATGATGACCTGCCACACCACTGCTGCCACAGCTCTCTCTCTCAAGGCCGGCCAGGCTGCACCT  
 121 F L D S P A M I S L P P P T A A T S V F S L K A R P F G L P P

453 GGGATCAACGTAGCCAGCTGGAGTGGGTGCCAGGAGCCAGCAGCTCTGCACCTTCCCAAGCCACAGCACCCAGGAAGACAGC  
 151 G I N V A S L E W V S R E P A L L C T F F P S P S T P R K D S

543 ACCCTCCGACCGTCCCGAGGCTCTACTGCTGCTGGCAATGGTGTCTGCAAGTGGCTGGATGTGAGAAGCTCTCGAGGAGCA  
 181 T L P T V P Q G S Y S L L A N G V C K W P G C E K V F E E P

633 GAGGATTCTCAAGCACTGCCAGGCGGACCATCTCTAGATGAGAAGGCGAGCAGTGTCTCTCCAGAGGGAGGTGGTGCAGTCT  
 211 E D F L K H C Q A D H L L D E K G R A Q C L L Q R E V Q S

723 CTGGACAGCAGCTGGTCTGGAGAAGGAGAGCTGGGTCTATGCAGGCACACTGGCTGGGAAGATGACCTGACCAAGCTCCATCC  
 91 L Q A L L Q D R P H F M H Q L S T V G T H T R T P V L Q V R

813 ACAGCATCATCTGACAAGGCTCTGCTGATGCTGCTGGCACTCTGCCACACGGGCCGGCTGGTCCAGCCCGAGGAGCC  
 271 T A S S D K G S C I V A A G T P A T T G P A W S S P Q L P P

903 CCTGATGGCTGTTTGTCTGTGAGGAGCACCTCTGGGGCAGCCATGGAAACAGCACATCCAGAGTCTTCCACAACATGGACTACTTC  
 301 F D G L F A V R R H L W G S H G N S T F F E F F H N M D Y F

993 AAATCCACAACATGCCCGCCCTTACCTACGCCACCTCATCGCTGGGCACTCGAGGAGTCTGAGAAGCAGGCGACATCAAT  
 331 K F H N M R P P P F T Y A T L I R W A I L E A P E K Q R T L N

1083 GAGATCTACCACTGGTTCACAGCATGTTGCTCTTTCAGGAACACCTGCCACCTGGAAGATGCCATCCGCCACATCTGAGCCTG  
 361 E I Y H W F T R M F A F F R N H P A T W K N A I R H N L S L

1173 CACAAGTGTCTGCTGCGGGTGGAGAGTGAAGGGGCGCTGTGGACCTGGAGCAATTCGAGTTCGCAAGAAGAGGAGCAAGGCGCT  
 391 H K C F V R V E S E K G A V W T V D E F E F R K K R S Q R P

1263 AGCAGGAGTTCACCCACACCTGGCCCTAAACTCAAGCAAGGAAGGAGGAGG  
 421 S R S S N P T P G P \*

B

canine	MFNRPAPKPSAPSLAPQPSGALPSWRAAPKASDLLGAKPGVTFQGRDLRGTHASS-	59
feline	*****L*****S*****G*T*P*****A*****_	59
human	*****G*****L*****S*****R*****G*****A*****S	60
mouse	*****M*****L*****V*****K*****G*****E*****T*****S*****G*****S*****A*****T*****	59
.....		
canine	LNPMPPSQLQLPTVPLVMVAPSGARLGPSPHLQALLQDRPHMHQLSTVGTHTRTPVLQV	119
feline	*****T*****D*****A*****	119
human	*****L*****D*****A*****	120
mouse	**L*****D*****A*****AQ*****	119
.....		
canine	RPLDSPAMISLPPTAATSVFSLKARPLPGINVASLEWVSREPALLCTFPSPSTPRKD	179
feline	*****G*****	179
human	H*E*****T*****G*****	180
mouse	***N*****S*****G*****R*****SG*****	179
Zinc finger domain		
canine	SLPTVPQGSYSLLANGVCKWPGCEKVFEEPEFLRHCOADHLLDEKGRAQC11QREVQ	239
feline	*S*X*****	239
human	*SA**S**P*****M**	240
mouse	*N*LAA**P*****E*****K*****	239
.....		
canine	SLEQQLVLEKEKLGAMQAHLAGKMTLTAPSTASSDKGSCCIVAAGTPATTGPAWSSPQE	299
feline	*****A*****T*****A*****P*****	299
human	*****S*****A*****S*****V*****SQGFVV*****G*****R*****	300
mouse	*****E*****A*****V*****M*****S*****TS*****QGSVL*****A*****R*****	299
.....		
canine	APD-GLFAVRRLWGSNGSTFPEFFHNMDYFKFHNMRPPTATYATLIRWAILEAPEKQRT	358
feline	*****L*****	358
human	**S*****L*****	359
mouse	**G*****S*****Y*****R*****	359
.....		
canine	LNIEYHWFTRMFAFRNHPATWKNNAIRHNLHLKCFVRVESEKGAHVTVDEFERKKRSQ	418
feline	*****L*****	418
human	*****L*****	419
mouse	*****Y*****	419
Forkhead domain		
canine	RPSRSSNPTFGP	430
feline	***S*****	430
human	***C*****	431
mouse	**NKC**C*	429

Fig. 1. Molecular cloning of canine Foxp3. (A) The nucleotide sequence of the canine Foxp3 cDNA and the predicted amino acid sequence of canine Foxp3. The nucleotide sequences of canine Foxp3 are shown along with the predicted amino acid sequences (shown below the nucleotide sequences). The numbers on the left refer to the nucleotide position in the canine Foxp3 cDNA or the amino acid position in the canine Foxp3 sequence. The underlined sequences indicate the primers. The nucleotide sequence data reported in this paper were submitted to the DNA Data Bank of Japan (DBJ)/European Molecular Biology Laboratory (EMBL)/GenBank (accession number AB501357). (B) Comparison of the predicted amino acid sequences of canine Foxp3 with counterparts from different species. The amino acid sequence of the canine Foxp3 was aligned with its feline, human, and mouse counterparts. The asterisks indicate similarities with the amino acids of the canine Foxp3 sequence and the dashes indicate gaps introduced for maximal alignment. The numbers on the right refer to the amino acid positions. Putative exon/intron boundaries are indicated by a dotted line. The forkhead domain and zinc-finger domain are shaded.

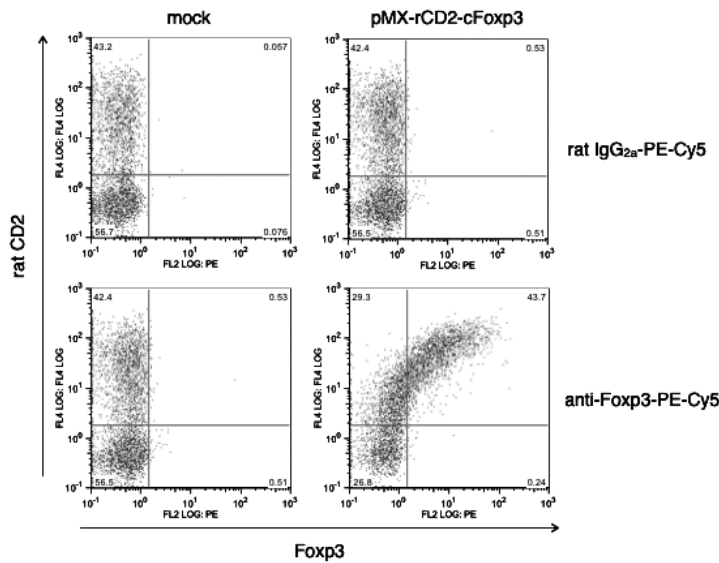


Fig. 2. Crossreactivity of anti-mouse Foxp3 antibody to canine Foxp3. 293T cells were transiently transfected with pMXs-IP (mock control) or pMXs-IP-cFoxp3 and stained with anti-rat CD2-Bio and streptavidin-PE-Cy5, and anti-mouse Foxp3-PE antibodies. They were analyzed by flow cytometry. One of two comparable experiments is shown.

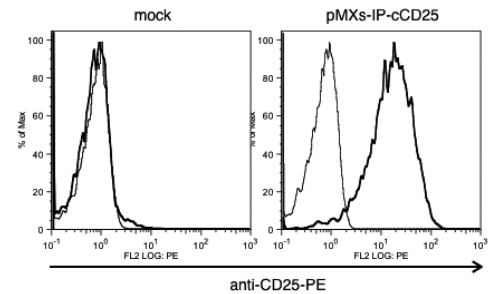


Fig. 3. Crossreactivity of anti-human CD25 antibody to canine CD25. GL-1 cells that stably expressed canine CD25 were stained with a PE-conjugated anti-human CD25 antibody and analyzed by flow cytometry. One of two comparable experiments is shown.

similar in all four dogs used in this study. In addition, none of these cells were stained with anti-CD25 antibody in the lymphocyte population (data not shown) indicating that unstimulated canine PBMCs are CD25 negative.

Next, to determine the expressions of CD25 and Foxp3 in mitogen-stimulated canine PBMCs, canine PBMCs from four dogs were stimulated with ConA for 3 days, followed by triple staining. Stimulation of canine PBMCs induced the expression of CD25 both in the CD4<sup>+</sup> and CD4<sup>-</sup> populations (Fig. 4A). More than 50% of the CD4<sup>+</sup> and CD4<sup>-</sup> lymphocytes of all dogs (except CD4<sup>-</sup> lymphocytes from Dog1) were positive for CD25 after stimulation. More than 30% of CD4<sup>+</sup>CD25<sup>+</sup> cells were weakly positive for Foxp3 (all dogs) and 2–4% were highly positive for Foxp3 (boxed in each plot in Fig. 4A). The induction rate of CD25 and Foxp3 in stimulated cells was variable in individual dogs. The lymphocytes from Dog4 showed a high induction rate of Foxp3 and CD25. This result indicates that Foxp3 and CD25 expressions were induced in ConA-stimulated canine PBMCs parallel to the activation state of lymphocytes. Finally, to examine the correlation between CD25 and Foxp3 expressions in CD4<sup>+</sup> cells after stimulation, the expression level of Foxp3 was analyzed in the CD25<sup>-</sup>, CD25<sup>low</sup> and CD25<sup>high</sup> populations, using the same samples. As shown in Fig. 4B, CD4<sup>+</sup>CD25<sup>high</sup> cells (Gate A) expressed more Foxp3 than did CD4<sup>+</sup>CD25<sup>low</sup> cells (Gate B) and CD4<sup>+</sup>CD25<sup>-</sup> cells (Gate C).

## DISCUSSION

Antibody raised against mouse Foxp3 (FJK16s) have been used to detect Foxp3<sup>+</sup> cells in canine PBMC [4, 10], but there have been no reports confirming the crossreactivity of this antibody to canine Foxp3. In this study we cloned and expressed the canine Foxp3 protein and confirmed the crossreactivity of this antibody to canine Foxp3. The amino acid sequence putatively obtained in this study from the nucleotide sequence of canine Foxp3 is highly similar to that of mouse Foxp3, especially in the region of exon2, which is indicated to be the epitope of FJK16s by the manufacture.

We compared the nucleotide and amino acid sequences of the canine CD25 cloned in this study to the cDNA sequence registered previously by another group, and found differences in four nucleotides. These differences resulted in the change of 2 amino acids. However no differences were detected when compared to the corresponding sequences from the canine genome database. The identified differences might indicate a single nucleotide polymorphism between individual dogs. However, these findings need to be further investigated.

The result of our study showed the crossreactivity of ACT-1 with the canine CD25 protein. Furthermore, our data support previous results in which this antibody recognized the canine CD25 protein in activated PBMCs [8, 12] thereby justifying the use of this antibody to detect the canine CD25 protein. Using ACT-1 antibody, we did not find any CD25<sup>+</sup> cells in unstimulated canine CD4<sup>+</sup>Foxp3<sup>+</sup>

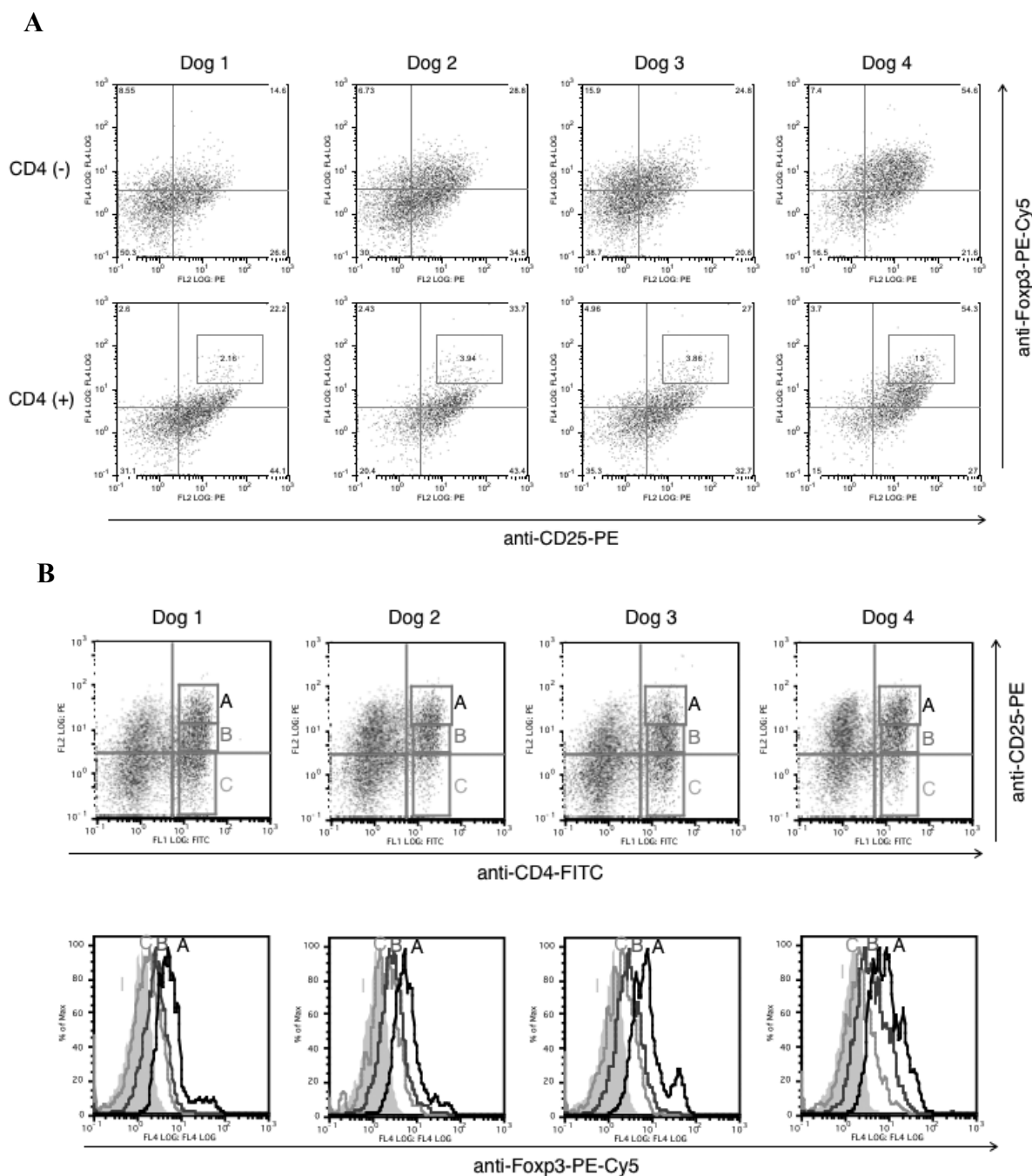


Fig. 4. Staining of ConA-stimulated canine PBMCs with anti-CD4, anti-CD25 and anti-Foxp3 antibodies. Canine PBMCs were isolated from four dogs and cultured in the presence of ConA ( $5 \mu\text{g/ml}$ ) for 3 days. After stimulation, PBMCs were collected and stained with anti-CD4, anti-CD25, and anti-Foxp3 antibodies and analyzed by flow cytometry. Quadrants were set on the basis of isotype control (data not shown). The numbers indicate the percentage of positive cells in each quadrant. One of two comparable experiments is shown. (A)  $\text{CD4}^+$  gated lymphocytes and  $\text{CD4}^-$  gated lymphocytes are shown. (B)  $\text{CD4}^+$  gated lymphocytes were further gated on the basis of the expression level of CD25 (Regions A, B, and C). Foxp3 expression is shown in histograms; gates for analysis were set on  $\text{CD4}^+\text{CD25}^-$  cells (region C),  $\text{CD4}^+\text{CD25}^{\text{low}}$  (region B), or  $\text{CD4}^+\text{CD25}^{\text{high}}$  (region A) cells, as shown in the quadrant. Shaded region in the histograms indicates staining with isotype control antibody (rat IgG<sub>2a</sub>-PE-Cy5).

PBMCs, while unstimulated human and mouse CD4<sup>+</sup>Foxp3<sup>+</sup> PBMCs have been shown to be positive for CD25. In humans and mice, CD25 is expressed in Treg populations and represents a cell surface marker characteristic of Treg cells. The reason why we did not detect CD25 in the CD4<sup>+</sup>Foxp3<sup>+</sup> cells before stimulation remains to be elucidated, but there are two possible explanations. First, the ACT-1 antibody is not sensitive enough to detect less profound expression of CD25 in unstimulated canine PBMCs since canine CD25-overexpressing 293T cells and ConA-stimulated PBMCs were positive for CD25. Another explanation could be that canine CD4<sup>+</sup>Foxp3<sup>+</sup> cells present an entirely different phenotype compared to that of human and mouse CD4<sup>+</sup>Foxp3<sup>+</sup> cells with regard to their cell surface molecules. It is necessary to use a monoclonal antibody against the canine CD25 protein to confirm one of these possibilities. The GL-1 cells stably expressing canine CD25 that were established in this study might be very useful for producing monoclonal antibodies.

The percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in unstimulated PBMCs were slightly lower in this study compared to those in the previous study [4]; however, the reason for this is unknown. Differences in the staining procedure for the detection of intracellular Foxp3 could be the reason for this because even though the manufacturer's protocol was used, the recommended incubation times for fixation/permeabilization and for staining with anti-Foxp3 antibody ranges from 30 min to overnight.

Stimulation with ConA resulted in the production of a high percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> cells. The percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> cells were slightly higher than those reported by Biller [4]. This may be attributed to the shorter incubation time applied in this study than in Biller's study, since the other conditions seem to be similar in both studies, including the amount of ConA used as a stimulant. The origin of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells is unknown, but they were possibly generated from CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> cells or CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> cells. The possibility that CD4<sup>+</sup>Foxp3<sup>+</sup> cells converted to CD25<sup>+</sup> and expressed more of Foxp3 after stimulation of CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> cells with ConA has not been ruled out in this study. CD4<sup>+</sup>Foxp3<sup>+</sup> cells could not be eliminated at the start of culturing because of the lack of the cell surface receptor CD25 for cell sorting. Results from human studies showed that this is not uncommon since the expression of CD25 and Foxp3 has been shown to be upregulated after CD3/CD28 stimulation in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg populations [1, 17]. CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> cells (naïve T cells) might generate CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after stimulation; however, this possibility needs to be confirmed.

The upregulation of Foxp3 was also detected in CD4<sup>+</sup>CD25<sup>+</sup> cells. We did not identify the phenotype of these cells, but our preliminary results showed the presence of CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the ConA-stimulated culture (data not shown). Human studies showed the upregulation of Foxp3 in CD4<sup>+</sup> and CD8<sup>+</sup> populations after activation [13, 14]. These activated cells that expressed Foxp3 showed

the classic phenotypes of Treg cells in terms of their function and cell surface markers. These data indicated that Foxp3 is expressed in peripheral and functional PBMCs, causing some cells to function as Treg cells. We did not perform a functional assay to prove that Foxp3<sup>+</sup> cells in the ConA-stimulated culture became functional Treg cells. Therefore, this result needs to be confirmed because it is an important pathway to suppress the excess activation of T cells in the periphery as a response to pathogens.

In the CD4<sup>+</sup>CD25<sup>+</sup> T cells after stimulation, the level of Foxp3 expression was correlated with that of CD25 expression, as shown in Fig. 4B. In human studies, the intensity of the activation-induced expression of Foxp3 and CD25 was significantly higher in Treg cells than in activated effector T cells [1]. This suggests the possibility that CD25<sup>high</sup>Foxp3<sup>high</sup> and CD25<sup>int</sup>Foxp3<sup>int</sup> populations in this study were derived from the activated Treg and naïve T cells, respectively. In addition, these adaptive Treg cells have only been identified under certain conditions in a mouse model [6, 15] that is often used to study human Treg systems, suggesting that dogs are promising alternative good models to analyze human adaptive Treg cells.

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