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Potential Immunological Adjuvant of 'K'-Type CpG-Oligodeoxynucleotides Enhanced the Cell Proliferation and IL-6 mRNA Transcription in Canine B Cells

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ABSTRACT. CpG oligodeoxynucleotides (CpG-ODNs) are ligands for toll-like receptor 9 (TLR9), signaling of which plays a role in innate immunity by inducing T helper 1 (TH1)-cell responses and pro-inflammatory cytokine production. The activation of TLR9 signaling is considered to be effective for the therapy of cancer, infectious diseases, and allergies and preclinical studies using CpG-ODNs have been performed in dogs and humans. In order to investigate the precise mechanisms responsible for the effect of CpG-ODNs in dogs, we examined their role in cell proliferation and cytokine gene expression in canine B cells. Canine B cells were collected by a magnetic cell isolation method using anti-CD21 antibody. Flow cytometric analysis for the intracellular CD79α revealed the purity of canine B cells to be as high as 90.2 ± 2.1%. Transcription of TLR2, TLR4, and TLR9 mRNA on canine $CD21^+$ cells was confirmed by reverse-transcript polymerase chain reaction (RT-PCR). CpG-ODNs induced dose-dependent proliferation of canine $CD21^+$ cells ($P<0.05$ compared with control-ODNs) detected by BrdU incorporation. Quantification of IL-6, IL-10, and IL-12p40 mRNA transcription on canine $CD21^+$ cells revealed that CpG-ODNs enhanced IL-6 mRNA transcription but not IL-10 and IL-12p40 mRNA transcription ($P<0.05$ compared with control-ODNs). These responses to CpG-ODNs in the canine B cells indicated that CpG-ODNs would be useful as an immunological adjuvant for vaccine in dogs.

KEY WORDS: adjuvant, B cells, canine, CpG-oligodeoxynucleotides, IL-6.

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Activation of immune cells by bacterial DNA was first discovered through induction of interferon (IFN) secretion and tumor inhibition by the DNA fraction of *Mycobacterium bovis* strain BCG [30]. These effects depend on a hexamer-palindromic sequence with 5'-Cytosine-phosphodiester-Guanin (CpG)-3' motifs in the bacterial DNA [30]. CpG motifs are recognized by the cells expressing toll-like receptor 9 (TLR9), a pattern-recognition receptor (PRR) [9]. Signaling through the TLR9 pathway leads to innate immune responses from B cells and plasmacytoid dendritic cells (pDCs) [16, 18]. These cells induce the production of T helper 1 (TH1)-cell biased cytokines such as IFN-γ and interleukin (IL)-12 and pro-inflammatory cytokines such as IL-1, IL-6, IL-18, and tumor necrosis factor (TNF) and differentiate into plasma cells and/or professional antigen presenting cells (APCs) [16, 18, 30]. Because synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODNs) are useful as anti-allergic and otherwise immunoprotective agents and as vaccine adjuvants, TLR9 signaling has been used in cancer, infectious disease, and allergy therapy [13, 16, 18, 30].

Examination of homology of TLR9 amino acid sequences shows that the nucleotide binding domains of leucine-rich repeats (LRRs) are heterogenous among species [7, 9], such that the optimal sequence motifs of CpG-ODNs for stimulating human cells (GTCGTT) can differ from those for mouse cells (GACGTT) [1, 2, 6]. For therapeutic uses of CpG-ODNs in humans, at least three structurally distinct classes of CpG-ODNs have been developed: 'D'-type ODNs (also known as CpG-A), 'K'-type ODNs (also known as CpG-B), and 'C'-type ODNs [6, 20, 22, 31]. 'D'-type ODNs are constructed using a mixed phosphodiester (PO) and phosphorothionate (PS) backbone and contain a single CpG motif in palindromic sequence, the 3'end of which flanks poly-G tail. 'K'-type ODNs encode multiple CpG motifs and consist of only PS backbone. 'D'-type ODNs have potent ability to induce IFN-α secretion from pDCs, but have little stimulatory activity on B cells. In contrast, 'K'-type ODNs are strong stimulators for B cells, but only weakly induce IFN-α secretion from pDC. 'C'-type ODNs are intermediate in structure and activity between the 'K'- and 'D'-type ODNs [5, 24, 32]. Due to the diversity of immune responses to CpG-ODNs among species and immune cells, it is necessary to selectively employ the optimal sequence motifs of CpG-ODNs.

Whereas 'K'-type ODNs have a preferential effect on B cells, it is possible that when 'K'-type ODNs are artificially loaded on cationic microparticles or conjugated to cationic lipids, 'K'-type ODNs gain immune effects similar to those of 'D'-type ODNs [4, 10, 14]. Using 'K'-type ODNs, preclinical studies have already been performed in humans [13,

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16, 18]. In dogs, 'K'-type ODNs have also been studied *in vivo* as a potential vaccine adjuvant for rabies virus [28] and a potential cancer immunotherapy agent for malignant melanoma [25].

Selection of optimal CpG-ODN types for clinical trials must be preceded by extensive study of effects of various CpG motifs. However, few such studies of dogs have been reported. Wernette *et al.* [33] and Ren *et al.* [28] found that 'K'-type ODNs induced proliferation of canine spleen cells, lymph node cells, or both; however there are no reports that have shown the direct effects of CpG-ODNs on canine B cells. In our previous study, it was shown that 'D'-type ODNs induced canine PBMCs to secrete IFN-γ and to express IL-12p40 mRNA [21]. Im Hof *et al.* [12] examined the effects of novel synthetic agonists of TLR9, which are referred to as immune modulatory oligonucleotides (IMOs), on the proliferation and TH1 immune responses of T- and B-cells in canine PBMCs; however the effect of IMOs on immune cells needed co-stimulation by lipopolysaccharide (LPS) and/or concanavalin A.

For the purpose of developing an effective immunological adjuvant in dogs, we examined the direct effects of 'K'-type ODNs on the cell proliferation and induction of IL-6, IL-10, and IL-12p40 mRNA transcription in canine B cells. We focused on these cytokines because enhancements of the cytokine production by CpG-ODNs have previously reported in human and murine B cells [1, 6, 8, 17, 23, 24, 32]; both of the IL-6 and IL-10 are known to a B-cell growth and differentiation factor [8, 15, 26, 29]; and IL-12 has been shown to promote shifting the B-cell phenotype to TH1 in cooperation with CpG-ODN [23]. In the present study, we tried to make clear whether B cells could be identified as primary target cells for CpG-ODNs in dogs.

MATERIALS AND METHODS

Dogs: Six 2–7-year-old healthy beagle dogs (2 males, 3 females, and 1 neutered female) were kept according to the "Guidelines of Animal Welfare for Experimental Animals" issued by Azabu University. All dogs have been annually vaccinated against canine rabies virus every year and milbemycin oxime was administered monthly for prevention of heartworm diseases.

Reagents: All synthetic phosphorothioate oligodeoxynucleotides (ODNs) were purchased from Hokkaido System Science (Hokkaido, Tokyo, Japan). LPS contamination in ODNs was below the detection limit of 5 endotoxin units/mg ODN as determined by the manufacturer using the kinetic colorimetric method. Sequences of 'K'-type ODNs, 2006 [TCGTCGTTTTGTCGTTTTGTCGTT, molecular weight (MW)=7,697] and 1018 (TGACTGTGAACGTTCGAGATG, MW=7,150) used in this study were described in previous reports that showed effects of 'K'-type ODNs on human B cells and canine splenocytes [6, 24, 33]. Those of the negative control used in the present study were 2137, the GpC variant of 2006 (TGCTGC TTTTGTGCTTTTGTGCTT, MW=7,697) and 1040, the mutant CpG variant of 1018 (TGACTGTGAACCTTAGAGATGA, MW=7,134), respectively. LPS from *E.coli* (O55:B5, Sigma-Aldrich, St. Louis, MO, U.S.A.), which is known to be a TLR4 signaling-mediated mitogen for murine B cells, was used as a positive control.

Isolation of canine CD21-positive cells from venous blood: PBMCs were isolated from the venous blood of dogs by density gradient centrifugation using Lymphoprep (specific gravity 1.077) (AXIS-SHILD, Oslo, Norway) as directed by the manufacturer. The canine B cells were isolated from the PBMCs by positive selection using the magnetic cell isolation method (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). After blocking with canine sera at 4ºC for 30 min, the cells were incubated with R-phycoerythrin (RPE)-conjugated mouse anti-canine CD21 antibody (clone CA2.1D6) (AbD Serotec, Oxford, U.K.) at 4ºC for 10 min, and then reacted with anti-PE microbeads (Miltenyi Biotec) at 4ºC for 10 min. The CD21-positive ($CD21^+$) cells were collected using L/S column (Miltenyi Biotec) and cell viability (>95%) was determined by 0.5% trypan blue exclusion. The purity of canine B cells was assessed by morphology using Wright-Giemsa staining and flow cytometric analysis. The isolated canine $CD21^+$ cells were suspended in RPMI1640 medium containing 10% fetal bovine serum (FBS) and cultivated at 37ºC in a humidified atmosphere with 5% CO_2.

Flow cytometry: Intracellular staining of CD79α was performed on $CD21^+$ cells soon after isolation by MACS, using a commercial kit (IntraStain, DAKO, Copenhagen, Denmark) according to the manufacturer's instructions. Briefly, the $CD21^+$ cells were fixed with 100 *μl* of IntraStain reagent A. Then the cells were permeabilized with 100 *μl* of IntraStain reagent B and simultaneously stained with antibodies for 15 min at RT in the dark. Mouse anti-human CD79αcy antibody (clone HM-57, DAKO) or the isotype control of mouse IgG1 antibody (AbD Serotec) was used at the concentration of 1 *μ*g per 150 *μl*. The anti-human CD79αcy monoclonal antibody was previously shown to cross-react with canine B cells by immunohistochemistry and flow cytometry [3]. Prior to the staining of the cells, these antibodies were labeled with Alexa 488 using Zenon® Alexa Fluor® 488 mouse IgG labeling kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The fluorescence of RPE and Alexa 488 on the stained cells (1.0×10^4 cells) was analyzed by flow cytometry (Epics XL ADC system) (Beckman Coulter, Fullerton, CA, U.S.A.) using Expo32 software (Beckman Coulter).

Stimulation of the cells: Canine $CD21^+$ cells that were approximately 90% positive for CD79α by flow cytometric analysis were used. For the proliferation assay, the cells ($1–2 \times 10^5$ cells) were seeded in 96-well flat-bottom plates in a volume of 100 *μl* medium/well and stimulated with 8 *μ*g/m*l* of each of 2006, 1018, 2137, 1040, and LPS for 68 hr. To examine the dose-dependent effects of CpG-ODNs, a titrated dose range of 2006 and 2137 at 0.9, 2.7, 8, and 24 *μ*g/m*l* was used. For the quantification of cytokine gene expression, the cells (4×10^5 cells) were cultivated in 5 m*l*

polystyrene round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) in a volume of 500 μl per tube and stimulated with 24 μg/m*l* of 2006, 2137, and LPS for 2 , 4 and 6 hr. Cells cultivated in a similar manner but without stimulators were also used as negative controls.

Proliferation assay: A 5-bromo-2'-deoxyuridine (BrdU)-based cell proliferation ELISA kit (Roche Diagnostics, Tokyo, Japan) was used for the measurement of proliferation activity of the cells according to the manufacturer's protocol. Forty-eight hours from the start of cultivation, BrdU labeling reagent was added at 10 μl per well. The cells were incubated with the BrdU for an additional 20 hr (total 68 hr). After centrifugation, the plate was dried at 60ºC for 1 hr. BrdU-labeled DNA in the cells was fixed and denatured by incubation with FixDenat solution for 30 min at RT. After washing, the BrdU-labeled DNA was stained with peroxidase-conjugated anti-BrdU antibody for 90 min at RT. The plate was washed again, and TMB substrate solution was added. The reaction was stopped by adding 1 M H_2SO_4 solution. Absorbance was measured at 450 nm using a microplate reader (Multiskan JX, Thermo Labsystems, Helsinki, Finland). Stimulation index (SI) was calculated by dividing absorbance of the stimulated cells by that of the cells cultured without stimulators.

Reverse transcriptase (RT) assay: Total RNA was extracted from the cells using RNeasy plus micro kit, which was designed to purify total RNA from less than 5×10^5 of cells and remove genomic DNA in the same procedure (Qiagen, Tokyo, Japan). The cells were lysed with buffer RLT plus, then homogenized by QIAshredder homogenizer (Qiagen), and stored at –80ºC until use. First strand cDNA was generated from 50 and 25 *n*g of the total RNA by reverse transcription (RT) using the SuperScript® VILO cDNA synthesis kit (Invitrogen, Rockville, MD, U.S.A.).

Reverse transcriptase polymerase chain reaction: Using the primers listed in Table 1, cDNA was amplified with a TAKARA Ex Taq hot start version (TAKARA, Shiga, Japan) using a thermal cycler (iCycler, Bio-Rad Laboratories, Hercules, CA, U.S.A.). The PCR amplification consisted of 35 cycles of denaturation (98ºC, 10 sec), annealing [60ºC for TLR2, TLR4, and TLR9, and 62ºC for Glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 30 sec], and extension (72ºC, 30 sec). Absence of contamination by genomic DNA in total RNA was confirmed by amplification of non-RT controls (data not shown). The PCR products were analyzed by ethidium bromide-electrophoresis in a 2% agarose gel and visualized using UV transilluminator (BioDoc-It® Imaging System, UVP, Upland, CA, U.S.A.).

Quantitative polymerase chain reaction: Taqman gene expression assay (Applied Biosystems, Foster, CA, U.S.A.) was used for measurement of IL-6, IL-10, and IL-12p40 mRNA transcription. Eukaryotic 18S ribosomal RNA was used as an internal control. The assay ID of primer probe-mix of each gene is shown in Table 2. The PCR amplification was carried out using Taqman universal PCR master mix (Applied Biosystems) on ABI 7500 amplification system (Applied Biosystems) according to the manufacturer's instructions. Data were analyzed by the comparative threshold cycle (CT) method described in the previous report [21]. Fold change in expression was calculated by dividing the value of the stimulated cells by that of the non-stimulated cells. Statistically significant differences were not observed between CT values of the internal control in the cells before and after the cultivation with stimulator (Dunnet test) (data not shown).

Statistical analysis: Kruskal-Wallis ANOVA was applied for comparison of data among groups using the different stimulators in the cell proliferation assay. Post hoc analysis (Turkey-Kramer HSD test) was performed to determine the groups showing significant differences in cell proliferation.

Table 1. Canine oligonucleotide primers for reverse transcriptase-polymerase chain reaction

Target	Primer type	Oligonucleotides (5'–3')	Amplicon size (bp)	Seaquence reference
TLR2	Forward	GGAGAACCTTCTGGTCAGAAGC	310	In this study
	Reverse	GGTGTTCATTATCTTCCGCAGC		NM_001005264
TLR4	Forward	GCTTCTCGCTTGGCTTGCAAAAGG	419	In this study
	Reverse	CCCATTCCAGGTAAGTGTTCCTGC		NM_001002950
TLR9	Forward	GGTGCTGGACCTGAGTGAGAACTTCC	510	In this study
	Reverse	GGTCCAAGGTGAAGTTGAGGGTCC		NM_001002998
GAPDH	Forward	CTCATGACCACAGTCCATGC	412	Ohmori *et al.* (2004) [27]
	Reverse	TGAGCTTGACAAAGTGGTCA		AB_038240

Table 2. Canine oligonucleotide primer probe-mix for quantitative-polymerase chain reaction

Target	Assay ID in Taqman gene expression assay	Amplicon size (bp)	Seaquence reference
IL-6	Cf02624282_m1	105	NM_001003301.1
IL-10	Cf02624265_m1	89	NM_001003077.1
IL-12p40	Cf02623323_m1	96	NM_001003292.1
18s rRNA*	Hs99999901_s1	187	X03205

*The primers and probe are constructed at high homology region on nucleotide sequence between dog and human 18s rRNA.

Multivariate ANOVA (MANOVA) was used to analyze the dose-dependent effect of 2006 on cell proliferation. Wilcoxon rank-sum test was performed to compare the ratio of fold change in the cytokine mRNA transcription between the cells stimulated with 2006 and 2137. *P*-values lower than 0.05 were considered significant. All statistical tests were performed using JMP software version 8 (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Isolation of canine B cells from whole blood: Between 10^7 and 2×10^7 canine PBMC were obtained from 10 m*l* of whole blood. The mean (± SD) of canine CD21$^+$ cells isolated from 1×10^7 canine PBMC by MACS was $4.5 \pm 2.0 \times 10^5$ cells (n=5 individual dogs). The uniform population of canine CD21$^+$ cells was verified by the dot plots of forward scatter (FSC) vs. side scatter (SSC) produced by flow cytometric analysis (Fig. 1a). Wright-Giemsa staining showed that in contrast to the canine PBMC, which were composed of a variety of cell types such as band neutrophil, monocyte, and lymphocyte, the isolated canine CD21$^+$ cells were composed of a uniform population showing morphology characteristic of lymphocytes (Fig. 1b). Differential leukocyte counts evaluated from the preparation of canine CD21$^+$ cells showed the following mean (± SD) percentages for the different leukocyte types: 0.7 ± 0.8% band neutrophils, 0.2 ± 0.3% segmented neutrophils, 0.1 ± 0.1% eosinophils, 0% basophils, 3.0 ± 2.7% monocytes, and 96.0 ± 3.2% lymphocytes (n=5). Flow cytometric analysis using anti-CD79α antibody showed that the purity of canine B cells in the canine CD21$^+$ cells was as high as 90.2 ± 2.1% (n=5) (Fig. 1a).

Transcription of TLR2, TLR4, and TLR9 mRNA in canine B cells: In order to evaluate the ability of canine B cells to recognize the LPS and CpG-ODNs, the transcriptions of TLR2, TLR4 and TLR9 mRNA on canine CD21$^+$ cells were examined. RT-PCR analysis showed that the isolated CD21$^+$ cells expressed all of three TLRs. The intensities of the amplified bands of TLR2, TLR4 and TLR9 on agarose gel were as high as those of GAPDH (Fig. 2).

Proliferation of canine B cells induced by CpG-ODNs: To extend the study of the effects of 'K'-type ODNs in dogs, BrdU incorporation in the DNA of proliferating canine CD21$^+$ cells was assessed in cells stimulated with 2006, 2137, 1018, 1040, and LPS. The SI values for the proliferation were significantly different among groups (P<0.05) (Fig.3a). To determine whether the enhanced proliferative

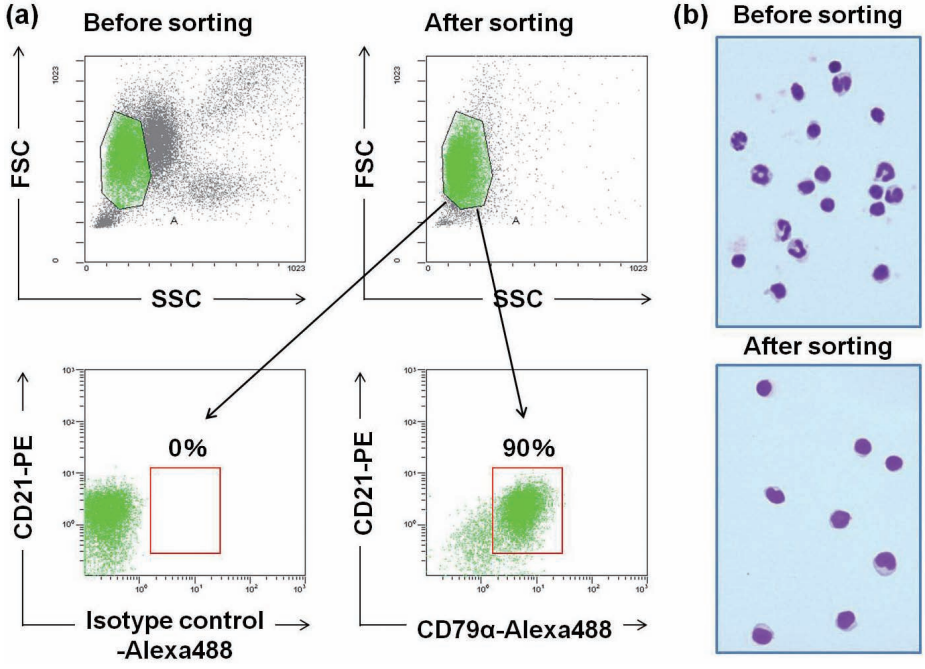

Fig. 1. Isolation of canine B cells. (a) The cells are shown in the dot plots of forward scatter (FSC) vs. side scatter (SSC) before and after the isolation by MACS using anti-CD21 antibody. Gates shown in the dot plots indicate a lymphocyte region. The isolated canine CD21$^+$ cells stained with anti-CD79α antibody or isotype control labeled with Alexa 488 are shown in dot plots of fluorescent intensity for CD21-RPE vs. CD79α-Alexa 488. The figures shown in the dot plots indicate the percentage of CD21 and CD79α double-positive cells in the square. The percentage of isotype control was set at smaller than 3% and 1.0×10^4 cells are shown in the dot plots. (b) Light microscopic observations of the cytocentrifuged preparations stained with Wright-Giemsa solution (magnification, × 400). The cells are shown before and after the isolation by MACS. Data were representative of two independent experiments from 5 individual dogs.

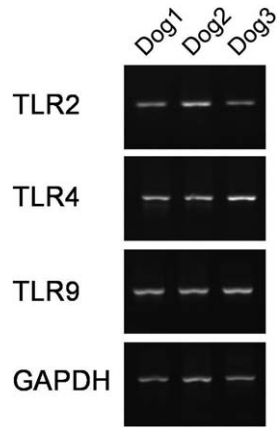

Fig. 2. Transcription of TLR2, TLR4, and TLR9 mRNA on canine CD21$^+$ cells. Total RNA was extracted from canine CD21$^+$ cells and subjected to RT-PCR analysis using the specific primers for TLR2, TLR4, TLR9 and GAPDH. The data shown here are representative of the results in three individual dogs (Dogs 1–3).

responses were CpG-motif dependent, the SI values of 'K'-type ODNs were compared with those of control-ODNs. As compared with the response to the negative controls of 2137 and 1040, 'K'-type ODNs 2006 and 1018 induced significantly greater proliferation of canine CD21$^+$ cells, 2.1-fold (P<0.05) and 1.5-fold (P<0.05), respectively (Fig. 3a). Furthermore, the response to 2006 was significantly higher than that to 1018 (1.3-fold, P<0.05) (Fig. 3a). Based on the ability to induce high-level cell proliferation, 2006 was used for the following assays. Dose-dependent proliferation was observed in response to 2006 (P<0.05) and the dose-response curve peaked at the concentration of 2.7 μg/m*l* (Fig. 3b).

Cytokine gene expression in canine B cells induced by CpG-ODNs: To determine the effects of 'K'-type ODNs on the production of cytokines in dogs, the transcription levels of IL-6, IL-10, and IL-12p40 mRNA on canine PBMC and isolated canine CD21$^+$ cells were measured by qPCR. After 4 hr of stimulation of canine PBMC and CD21$^+$ cells with 2006 and 2137, IL-6 mRNA transcription in canine CD21$^+$ cells stimulated with 2006 was 9.3-folds higher than that in the cells subjected to 2137 (P<0.05) (Fig. 4a). Although the enhanced IL-10 and IL-12p40 mRNA transcription were not observed, time course-dependent increase was observed in the transcription of IL-6 mRNA in the canine CD21$^+$ cells after stimulation with 2006 (P<0.05) (Fig. 4b).

DISCUSSION

We revealed that 'K'-type ODNs affected the proliferation of, and IL-6 gene expression in, canine B cells isolated from PBMCs. IL-6, which was originally identified as B cell stimulating factor 2 (BSF2), has been shown to have various biological activities such as stimulation of the growth of B cells and differentiation of naïve B cells into antibody-secreting plasma cells [15]. It has been reported that over expression of IL-6 gene in transgenic mice induces polyclonal increase in IgG1 in serum and generation of massive plasmacytosis in thymus, lymph node, and spleen [29]. Klinman *et al.* [17] revealed that CpG-induced IgM production was regulated by IL-6. Ren *et al.* [28] reported that the CpG-ODN called YW07 induced antigen-specfic IgG antibody responses in dogs with immunization against the rabies virus. It may be that 'K'-type ODN-induced B-cell proliferation is associated with IL-6 secreted by B cells in an autocrine manner. 'K'-type ODNs might have the potential to induce IL-6 mediated-antigen response in canine B cells.

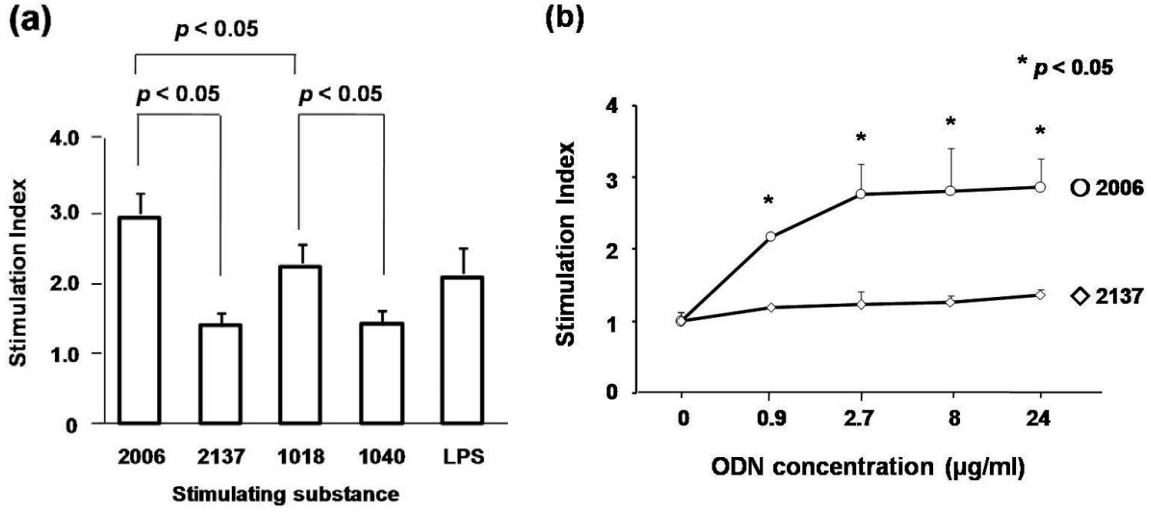

Fig. 3. Cell proliferation responses of canine CD21$^+$ cells to CpG-ODNs. (a) Stimulation index of cell proliferation for the responses to 2006, 2137, 1018, 1040, and LPS. (b) Stimulation index of cell proliferation for the responses to 2006 and 2137. Each bar represents mean ± SD of stimulation index. The P-values were shown in figures. These experiments were performed on 5 individual dogs in duplicate.

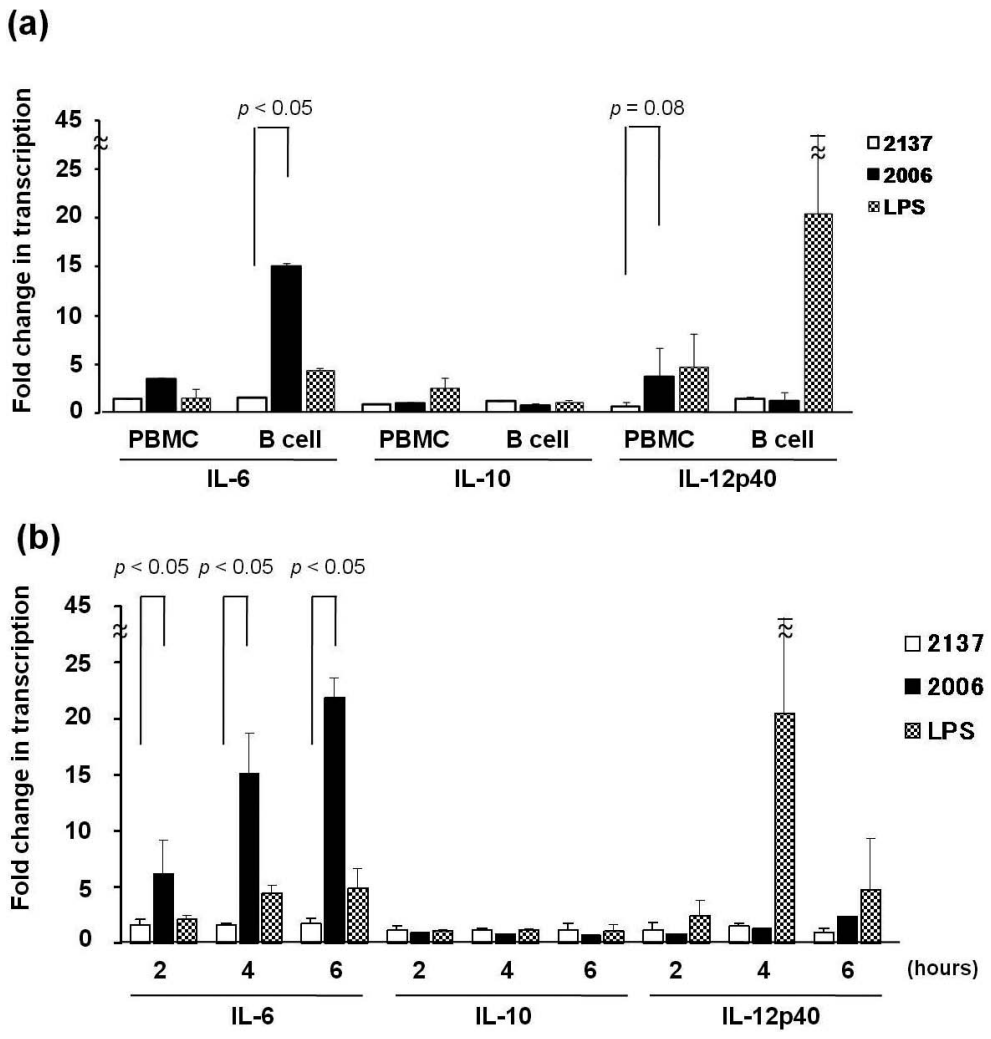

Fig. 4. Transcription of the IL-6, IL-10, and IL-12p40 mRNA induced by CpG-ODNs. (a) Fold change in cytokine mRNA transcription on canine $CD21^+$ cells and PBMC for the responses to 2006, 2137, and LPS after 4 hr cultivation. (b) Fold change in cytokine mRNA transcription on canine $CD21^+$ cells for the responses to 2006, 2137, and LPS after 2, 4, and 6 hr of cultivation. Each bar represents mean ± SD of fold change in transcription. The *P*-values compared 2006 with 2137 are shown in figures. These experiments were performed on 3 to 4 individual dogs.

Further researches are needed to examine the antibody response of canine B cells stimulated by 'K'-type ODNs.

The proliferation of canine $CD21^+$ cells in response to 2006 was shown to be significantly higher than in response to 1018. Both 2006 and 1018 have often been used as typical 'K'-type ODNs which potently activate human B cells. 2006 and 1018 have different immune stimulatory CpG motifs; 2006 contains TCGTCG at the 5' end and multiple GTCGTT motifs with no palindromes while 1018 contains the palindromic motif of AACGTTCG [24]. It has been reported that the optimal CpG motifs for activating human B cells contain $(TCG)_n$ [6]. The 'C'-type ODN called C274, which is generated by substitution of TCGTCG for 5' base flanking AACGTTCG in 1018, resulting in the sequence 5'-TCGTCGAACGTTCGAGATGAT-3', induces more proliferation, maturation, and cytokine production in human B cells than 1018 [24]. In the present study, canine $CD21^+$ cells responded little to control ODN 2137, while a dose-dependent response to 2006 was shown. These results could indicate that effects of 2006 were CpG-specific and the motif of $(TCG)_n$ was suitable for the activation of canine B cells.

We previously showed that 'D'-type ODNs induced the production and mRNA transcription of IL-12p40 from canine PBMCs [21]. In the present study, although the significant difference was not reached (P=0.08), the transcription of IL-12p40 mRNA in canine PBMCs was enhanced after 4-hr stimulation with 'K'-type ODNs (5.2-fold higher than control-ODNs) (Fig. 4a). In human PBMCs, B cells and pDCs highly express TLR9 and are directly reactive to

CpG-ODNs, whereas T cells, NK cells, and monocytes express much lower level of TLR9 than B cells and pDCs and they do not respond to CpG-ODNs [2, 11]. In our study, the proliferation of canine $CD21^+$ cells by (K'-type ODNs was observed; however that of non-$CD21^+$ cells, which consisted largely of T cells and monocytes, was not (data not shown). In contrast to the cellular patterns of TLR9 expression in human PBMCs, murine monocytes have been known responding to CpG-ODNs [18]. Comparison of the cell sensitivity to CpG-ODNs among the human, mouse and dog suggest that canine T cells may be less sensitive to CpG-ODNs. Since 'K'-type ODNs could not induce the IL-12p40 mRNA transcription in canine $CD21^+$ cells, it might be considered that the cellular source of the IL-12 mRNA transcription in canine PBMCs was not B and T cells but monocytes or pDCs.

B cells are considered an attractive target for immunotherapy due to their relative abundance in peripheral blood, ability to produce antibodies and present antigens to naïve T cells, and potential for expansion and long-lived humoral immunity. Accordingly, human clinical trials using 'K'-type ODNs have been performed for the therapy of cancers such as non-Hodgkins lymphoma and colorectal cancer, infectious diseases such as hepatitis B, and allergies such as asthma and allergic rhinitis [13, 16, 18, 30]. In the present study, we demonstrated that 'K'-type ODNs acted as an immunological B cell adjuvant in vitro. Further studies of biological activities of CpG-ODNs in immune cells are needed to extend the usefulness of CpG-ODNs as immunological adjuvants in dogs.

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