



Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvj

The immunostimulatory effect of CpG oligodeoxynucleotides on peripheral blood mononuclear cells of healthy dogs and dogs with atopic dermatitis

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ARTICLE INFO

Article history:

Accepted 14 December 2013

Available online xxx

Keywords:

Canine atopic dermatitis

CpG-motif

Interferon

Interleukin-10

Toll-like receptor 9

ABSTRACT

Synthetic oligodeoxynucleotides containing cytosine phosphatidyl guanine-rich DNA sequences (CpG ODN) can promote T-helper type 1 (Th1) responses, reduce T-helper type 2 (Th2) responses and/or favour regulatory T cell (Treg) responses in vitro and in vivo in humans and animals, by acting via Toll-like receptor 9 (TLR9). Since CpG ODN can be used as immune-modulators for canine atopic dermatitis (AD), the aim of the current study was to investigate their immunostimulatory potential on peripheral blood mononuclear cells (PBMC) and their subsets, from AD and healthy dogs. Expression of TLR9 and cytokine mRNA in CpG ODN-stimulated and unstimulated cells was assessed by real-time quantitative PCR.

Stimulation of PBMC with CpG class C ODN upregulated mRNA expression of interleukin (IL)-6, interferon (IFN)- γ and IL-12p40 in AD dogs ($P < 0.05$). It also stimulated IFN- γ protein secretion by PBMC of atopic and healthy dogs as measured by ELISA. In healthy dogs only, CpG class C ODN stimulated IFN- α mRNA production by CD21⁺ cells, and IL-10, IL-13 and IFN- γ mRNA production by CD3⁺ cells. Increased expression of TLR9 mRNA was only observed in CD3⁺ cells from AD dogs. No significantly increased gene expression was found in the CD11c⁺ subset upon stimulation, for those genes evaluated. The results indicate that PBMC of healthy and atopic dogs are sensitive to stimulation with CpG ODN class C, with a resulting Th1 cytokine response in AD dogs and a mixed Th1/Th2/Treg cytokine response in healthy dogs. From this study, little evidence was found to support the use of CpG ODN class C for therapeutic purposes in dogs affected with AD.

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Introduction

Canine atopic dermatitis (AD) is a common allergic skin disease that shares many similarities with human atopic eczema/dermatitis. Immunologically, acute lesions typically demonstrate a predominantly T-helper (Th) type 2 cell profile, with production of cytokines such as interleukin (IL)-4, IL-5 and IL-13. When these develop into a more chronic dermatitis, Th1-related cytokines such as interferon (IFN)- γ , IL-12 and IL-18 can also be detected (Marsella et al., 2006; Schlotter et al., 2011). Lower Th1 and higher Th2 cytokine concentrations have been found to be produced by peripheral blood mononuclear cells (PBMC) of atopic dogs, compared to those from healthy dogs (Hayashiya et al., 2002).

Canine AD is a chronic disease and immunomodulatory treatment is available in the form of allergen-specific immunotherapy (ASIT). The exact mechanism of action for this type of treatment is unclear; however, increased IL-10 expression and decreased circulating allergen-specific IgE has been reported to occur during ASIT in dogs affected with AD (Keppel et al., 2008). Studies in humans with similar Th2-biased atopic allergies and treated with ASIT, have shown reduced Th2:Th1 cytokine ratios and allergen-specific IgE, together with increased allergen-specific IgG4, regulatory cytokines, such as IL-10 and transforming growth factor (TGF)- β , as well as antigen-specific suppressive activity of CD4⁺CD25⁺ T-cells (Gleich et al., 1982; Ebner et al., 1997; Jutel et al., 2003). In atopic dogs, ASIT is only successful in around 50–80% of cases (Loewenstein and Mueller, 2009) therefore, potentiation of ASIT, to improve its efficacy, would be beneficial for future therapeutic interventions.

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Synthetic oligodeoxynucleotides, containing cytosine phosphatidyl guanine-rich DNA sequences (CpG ODN), possess potent immunostimulatory activity, mimicking native bacterial and viral unmethylated CpG motifs and initiating cell signalling pathways, through Toll-like receptor 9 (TLR9), which leads to activation of nuclear factor- κ B (Yi et al., 1998). In general, CpG ODN promote development of Th1 cell responses in vitro and in vivo and can prevent induction of cytokine production by allergen-specific Th2 cells (Hessel et al., 2005).

In a murine model of AD, increased allergen-specific IgG2a, decreased allergen-specific IgE, increased numbers of CD4⁺CD25⁺ FoxP3⁺ regulatory T cells (Treg) and enhanced cutaneous expression of IL-12, IL-10 and TGF- β were detected after topical application of CpG ODN (Inoue et al., 2005, 2006; Inoue and Aramaki, 2007). Furthermore, in humans with allergic rhinitis, improved clinical signs, combined with increased Th1 and decreased Th2 cytokine profiles, were found in a Phase II clinical trial of pre-seasonal short-term CpG ODN-conjugated ASIT (Tulic et al., 2004). Long-term beneficial clinical and immunological effects were also found when ASIT was combined with administration of virus-like particles (VLP)-conjugated CpG motifs in a Phase I/IIa open clinical trial in human atopic patients (Senti et al., 2009). Interestingly, even in the absence of a specific allergen, VLP-CpG ODN complex alone was capable of significantly improving symptom medication scores in human allergic rhinoconjunctivitis and asthma patients (Klimek et al., 2011; Beeh et al., 2013).

In humans, CpG ODN exert their effect primarily on plasmacytoid dendritic cells and B cells (Kadowaki et al., 2001; Hornung et al., 2002). Furthermore, three classes of CpG ODN (class-A, -B and -C) exist, which elicit different effects on immune cells, due to their distinct structural characteristics (Marshall et al., 2003, 2005). In previous studies, stimulatory effects of CpG ODN class A and B have been found, in terms of increased proliferation of canine lymphocytes from spleen, lymph node and blood (Wernet et al., 2002; Kurata et al., 2004). However, it is currently unclear as to which types of canine immune cells are specifically responsive to CpG ODN.

The aim of the current study was to investigate the immunostimulatory potential of CpG ODN on PBMC and their subsets from AD-affected and healthy dogs, as assessed by TLR9 mRNA expression and expression and secretion of a selection of signature cytokines produced by Th1, Th2 and Treg cells.

Materials and methods

Study population

To evaluate the immunomodulatory effects of different CpG ODN classes on PBMC, three healthy dogs (two neutered females and one neutered male; aged 2, 4 and 8 years) were used as controls, with informed owner consent. The group consisted of one Rhodesian ridgeback and two crossbreed dogs. The immunomodulatory effect of CpG ODN class C was also assessed on PBMC obtained from 10 dogs affected with AD. Five of these dogs were also selected for determination of CpG ODN class C effects on PBMC subtypes as described below.

AD was diagnosed according to previously described criteria (Willemse, 1986; Favrot et al., 2010). The group consisted of six females, two males and two neutered males (2–10 years old, median 5 years). Breeds represented were Beagle ($n=4$), Beagle crossbreed ($n=3$), Labrador retriever ($n=2$) and Dogue de Bordeaux ($n=1$). Seven of these dogs were owned by the Department of Clinical Sciences of Companion Animals (DCSCA), Faculty of Veterinary Medicine, Utrecht University, the other three were client-owned and all were enrolled in the study with informed owner consent.

To measure PBMC cytokine secretion and to identify PBMC subsets responsive to CpG ODN class C stimulation, five healthy and five AD dogs, all owned by the DCSCA were used. The healthy dog group consisted of two Beagles, and three Greyhounds (three females, two males; 3–6 years old, median 6 years) and the AD group consisted of one Beagle, three Beagle crossbreeds and one Labrador retriever (two females, three males; 4–9 years old, median 8 years).

Table 1

Different classes of CpG ODN used in the study.

Oligodeoxynucleotide	Reference number	Sequence 5'–3'
Class A	D19	GGTGCAT CG ATGCAGGGGG
Class B	1018	TGACTGTGAAC CGTTCG AGATGA
Class C	C274	TCCGTCGAACGTTCCG AGATGAT
Control	1040	TGACTGTGAACCTTAGAGATGA

CpG dinucleotides are shown in bold underlined text.

Control ODN; synthetic oligodeoxynucleotide without specific CpG motifs.

The experimental procedures in the present study fulfilled the requirements of the Utrecht University Animal Ethics Committee, under Dutch legislation (DEC number 2008.II.07067 and 2012.II.06.0892008).

Isolation and stimulation of PBMC

Twenty millilitres of blood were collected by jugular venepuncture into heparin anticoagulant tubes. PBMC were isolated by density gradient centrifugation. Heparinised blood, diluted 1:1 with RPMI-1640 (Gibco) was overlaid at a 2:1 volume ratio onto 10 mL Histopaque-1077 (Sigma–Aldrich) and centrifuged at 805 g for 30 min. After aspiration of cells at the interface, these were washed, counted and resuspended in culture medium, consisting of RPMI-1640 supplemented with 5% fetal calf serum (FCS; Gibco), 1% penicillin/streptomycin (Sigma–Aldrich) and 50 μ M of 2-mercaptoethanol (Sigma–Aldrich). Cells (2×10^6 cells/mL) were stimulated with 10 μ g/mL CpG ODN class A, B, C or control ODN (Dynavax Technologies; Table 1), or left unstimulated as a negative control, for 6 h or 18 h at 37 °C and 5% CO₂ in 96-well round bottom plates (Corning). Supernatants from unstimulated PBMC and those stimulated with either CpG class C ODN or control ODN, were collected after 18 h of culture and stored at –20 °C until further analysis.

Fluorescence activated cell sorting (FACS)

After culture for 18 h, cells were washed and resuspended in Dulbecco's phosphate buffered saline (DPBS; Lonza), supplemented with 2% FCS and 0.1% sodium azide (facsbuffer). Cells (1×10^6) were incubated with anti-CD11c (clone CA11.6A1, IgG1, AbD Serotec) for 30 min at 4 °C, washed and incubated with APC-conjugated goat anti-mouse IgG (clone Poly4053, IgG, Biolegend) as a secondary antibody for 30 min at 4 °C. Subsequently, after washing, non-specific binding was blocked with 10 μ L mouse serum for 10 min on ice, followed by incubation with FITC-labelled anti-CD3 (clone CA17.2A12, IgG1, AbD Serotec) and RPE-labelled anti-CD21 (clone CA2.1D6, IgG1, AbD Serotec) for 30 min at 4 °C. Single positive CD3, CD21 and CD11c cells were sorted on a Cytosort FACS (Becton Dickinson Influx) and collected in tubes containing facsbuffer. Thereafter, cells were pelleted, resuspended in 600 μ L Buffer RLT (Qiagen), supplemented with 2-mercaptoethanol according to the manufacturers protocol, and stored at –80 °C until RNA isolation.

Real-time quantitative PCR

RNA was extracted within 2 weeks of storage, using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions, incorporating an on-column DNase digestion step (Qiagen RNase-free DNase kit). RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Isogen Lifescience). The I-script cDNA synthesis kit (BioRad) was used, following the manufacturer's instructions. Real-time quantitative RT-PCR was performed to assess mRNA expression of canine IL-4, IL-6, IL-10, IL-12p40, IL-13, IL-18, IFN- α , IFN- γ , TGF- β , tumour necrosis factor (TNF)- α , FoxP3, thymus and activation-regulated chemokine (TARC), TLR9 and two reference genes (ribosomal proteins S5 and S19; RPS-5 and RPS-19, respectively) according to the method described by Brinkhof et al. (2006).

Oligonucleotide primers were obtained from Eurogentec, based on published sequences (Spee et al., 2005; Brinkhof et al., 2006; Veenhof et al., 2010, 2011) or custom-designed, based on the dog genome assembly sequences available in Ensembl¹ or NCBI² using Primer-3 software³ (Table 2). A relative quantification method was used to analyse the fold changes in gene expression (Pfaffl, 2001). Results were normalised against the two reference genes for each sample. Determination of the expression ratio for each cytokine, chemokine and TLR9 was based on comparison of stimulated vs. unstimulated cells for each dog and leukocyte subset.

ELISA for IL-10 and IFN- γ

Measurement of IFN- γ and IL-10 in supernatants was performed using commercially available ELISAs (R&D Systems), quantified against standard curves of

¹ See: http://www.ensembl.org/Canis_familiaris/Info/Index.

² See: <http://www.ncbi.nlm.nih.gov/genome?term=canis%20lupus%20familiaris>.

³ See: http://biotools.umassmed.edu/bioapps/primer3_www.cgi.

Table 2

Details of primers used in the study.

Gene	Primer sequence 5'–3'	Primer sequence 5'–3'	T _m (°C)
IL-4 ^a	F: ccaaagaacacaagcgataagga	R: gtttccatgctgctgaggtt	61.0
IL-6 ^a	F: gagccaccaggaacgaaagaga	R: ccgggtagggaaagcagtagc	65.0
IL-10 ^a	F: cccgggctgagaaccacgac	R: aaatgcgctctcactgctccac	63.0
IL-12p40 ^a	F: ggacgtttcacatgctggt	R: ccactctgacctctctgct	59.0
IL-13 ^a	F: gagagctgggtcaacatca	R: tgcagtcggagacattga	59.0
IL-18 ^a	F: gaggatatccccgattctga	R: tccggaggactcatttctg	61.0
TNF- α ^a	F: cccgggctccagaaggtg	R: gcagcaggcagaagagtgtggtg	64.0
TGF- β ^b	F: caaggatctgggctggaagtga	R: ccaggaccttgctgtactgcgtgt	65.0
IFN- α ^c	F: tgggacagatgaggagactctc	R: gaagaccttctgggtcatcacg	55.2
IFN- γ ^a	F: agcgcaaggcgataaatg	R: gcggcctcgaacagatt	55.8
FoxP3 ^a	F: caaatgggtctgcaagtgg	R: gtgctctgcccttctcatct	59.0
TARC ^a	F: ggagccattctctatcagcag	R: ggctcggaacagatggacttg	64.5
TLR9 ^d	F: tcgctcagcaagacc	R: agtccactgatgcg	60.0
RPS-5 ^e	F: tcactggatgagaaccctt	R: cctgattcacacggcgtag	62.5
RPS-19 ^e	F: ccttctctcaaaaagtctggg	R: gttctcatctgtagggagcaag	61.0

T_m, melting temperature; F, forward/sense; R, reverse/antisense.^a Veenhof et al. (2010, 2011).^b Spee et al. (2005).^c Accession number: AB125936 for canine IFN- α .^d Accession number: AB104899.3 for canine TLR9.^e Brinkhof et al. (2006).

recombinant canine IFN- γ and IL-10, respectively. All supernatant samples were analyzed in triplicate.

Statistical analysis

To test for normal distribution, descriptive statistics and the Kolmogorov Smirnov or Shapiro–Wilk test were performed on data sets. To determine whether there was a significant difference between CpG ODN and control ODN for stimulation of selected gene expression or cytokine secretion in PBMC or PBMC subsets within dog groups, a paired Student's *t* test was performed on normally distributed data. The non-parametric Wilcoxon signed-rank paired *t* test was used for data not normally distributed. Analysis was performed using SPSS version 16 software with *P* < 0.05 considered statistically significant.

Results

Cytokine expression by healthy canine PBMC stimulated with different CpG ODN

Quantitative RT-PCR was performed to measure mRNA expression of the cytokines IL-4, IL-6, IL-10, IL-12p40, IL-13, TNF- α , TGF- β and IFN- γ in CpG ODN-stimulated PBMC of three healthy dogs. Whilst there appeared to be a slight degree of stimulatory capacity by class C ODN with regards to IL-6, IL-12p40 and IFN- γ , this was not statistically significant (Fig. 1a).

Cytokine expression by PBMC of AD-affected dogs stimulated with CpG ODN class C

Since CpG class C ODN has been described to possess qualities of CpG class A and B, being a strong inducer of IFN- α and capable of stimulating B-cells (Marshall et al., 2003, 2005), and as the only CpG-containing ODN suggestive of having stimulatory capacity in the healthy dogs (Fig. 1a), CpG class C ODN was chosen for evaluation of immunostimulatory effects on PBMC from dogs affected with AD. In addition to the cytokines IL-4, IL-6, IL-10, IL-12p40, IL-13, TNF- α , TGF- β and IFN- γ , real-time quantitative PCR was also used to measure IL-18, FoxP3 and TARC mRNA expression. Significantly greater expression of IL-6 (*P* = 0.001), IL-12p40 (*P* = 0.005) and IFN- γ (*P* = 0.021) was found after stimulation with CpG class C compared to control ODN (Fig. 1b). No differences in expression were observed for the other genes evaluated.

Cytokine and TLR9 expression of PBMC subsets of healthy dogs and dogs affected with AD after stimulation with CpG ODN class C

PBMC from five healthy and five AD-affected dogs were stimulated with CpG ODN class C and control ODN for 18 h. TLR9 mRNA

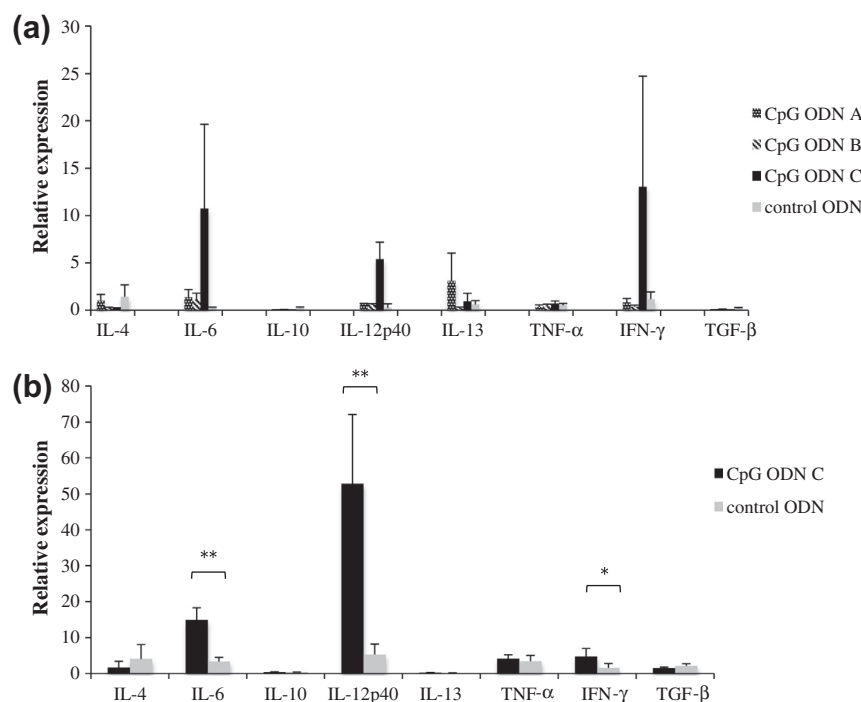


Fig. 1. Relative cytokine mRNA expression in PBMC derived from (a) three healthy control dogs and (b) 10 dogs affected with AD. Cells were stimulated for 6 h with 10 μ g/mL of three different CpG ODN classes (A, B, C) or a control ODN for the control dogs and with CpG ODN class C or a control ODN for the AD dogs. Relative expression (fold change) was determined by calculating a ratio, comparing stimulated vs. unstimulated (culture medium only) cells for each dog, normalised against the two housekeeping genes. Each bar represents the group mean + SEM; **P* < 0.05; ***P* < 0.01.

Table 3

Cytokine expression within PBMC subsets of healthy control and AD-affected dogs stimulated with CpG class C and control ODN for 18 h.

PBMC subtype	Cytokine	Control dogs		AD dogs	
		Control ODN	CpG ODN C	Control ODN	CpG ODN C
CD3 ⁺	IFN- α	9.0 $\pm$ 8.4	11.1 $\pm$ 11.2	1.6 $\pm$ 1.8	20.2 $\pm$ 37.3
	IFN- γ	3.1 $\pm$ 2.1	87.2 $\pm$ 140.7*	2.8 $\pm$ 0.7	39.9 $\pm$ 53.8
	IL-10	1.4 $\pm$ 0.7	4.1 $\pm$ 2.7*	1.6 $\pm$ 0.2	2.0 $\pm$ 0.3
	IL-13	0.9 $\pm$ 0.3	3.3 $\pm$ 4.2*	2.2 $\pm$ 1.7	7.4 $\pm$ 9.8
	TNF- α	1.5 $\pm$ 0.4	2.8 $\pm$ 1.5	1.2 $\pm$ 0.2	2.4 $\pm$ 1.1
	TGF- β	1.5 $\pm$ 0.8	1.9 $\pm$ 1.1	0.8 $\pm$ 0.4	2.1 $\pm$ 1.2
	FoxP3	0.9 $\pm$ 0.2	1.0 $\pm$ 0.3	0.9 $\pm$ 0.1	1.1 $\pm$ 0.1
	TLR9	1.1 $\pm$ 0.4	1.3 $\pm$ 0.8	0.6 $\pm$ 0.3	2.1 $\pm$ 0.7*
CD21 ⁺	IFN- α	0.7 $\pm$ 0.5	72.7 $\pm$ 123.1*	2.2 $\pm$ 0.9	81.3 $\pm$ 99.8
	IL-10	1.0 $\pm$ 0.2	0.7 $\pm$ 0.4	1.2 $\pm$ 0.6	0.7 $\pm$ 0.1
	TNF- α	1.9 $\pm$ 1.1	1.7 $\pm$ 1.2	3.0 $\pm$ 3.4	1.7 $\pm$ 1.3
	TGF- β	1.4 $\pm$ 0.5	0.9 $\pm$ 0.6	1.5 $\pm$ 0.7	1.1 $\pm$ 0.5
	TLR9	1.3 $\pm$ 0.6	1.5 $\pm$ 1.4	0.6 $\pm$ 0.4	1.7 $\pm$ 3.0
CD11c ⁺	IFN- α	6.5 $\pm$ 5.6	257.3 $\pm$ 289.1	4.6 $\pm$ 5.2	44.9 $\pm$ 63.5
	IFN- γ	3.1 $\pm$ 2.7	3.9 $\pm$ 3.2	4.9 $\pm$ 3.6	8.5 $\pm$ 14.0
	IL-10	0.9 $\pm$ 1.2	0.4 $\pm$ 0.3	0.6 $\pm$ 0.6	1.0 $\pm$ 0.6
	IL-12p40	6.8 $\pm$ 10.8	15.4 $\pm$ 21.6	0.7 $\pm$ 0.7	1.9 $\pm$ 1.3
	TGF- β	0.9 $\pm$ 0.5	1.0 $\pm$ 0.6	0.5 $\pm$ 0.3	1.8 $\pm$ 0.2
	TLR9	1.0 $\pm$ 0.7	1.5 $\pm$ 1.3	0.7 $\pm$ 0.3	0.8 $\pm$ 0.7

Values shown are means $\pm$ SD.* Significant difference ($P < 0.05$) within each group comparing CpG ODN C and control ODN.

expression was then measured in the CD3⁺, CD21⁺ and CD11c⁺ cell populations. Compared to control ODN, stimulation with CpG ODN class C resulted in increased TLR9 expression ($P = 0.011$) within the CD3⁺ cell population of the AD-affected dogs (Table 3, Fig. 2), but no significant differences in TLR9 expression were observed for the other two cell subsets.

Cytokine and transcription factor expression, measured within the CD3⁺, CD21⁺ and CD11c⁺ subsets, are shown in Table 3. In the control group, significant differences between CpG ODN class C and control ODN stimulation were found for the cytokines, IL-10 ($P = 0.043$), IL-13 ($P = 0.043$) and IFN- γ ($P = 0.043$) within the CD3⁺ cell population. Within the CD21⁺ cell population from control dogs, expression of IFN- α showed a significant difference ($P = 0.043$) upon stimulation with CpG ODN class C compared to control ODN. Within the CD11c⁺ cell subset, no differences in expression of the measured cytokines were observed. No effect of CpG ODN class C on cytokine or FoxP3 mRNA expression by any of the subpopulations was seen in the AD-affected dogs.

IFN- γ and IL-10 secretion after stimulation of PBMC of healthy and AD-affected dogs with CpG ODN class C

An increase in IFN- γ secretion was seen after stimulation with CpG ODN class C, compared to control ODN in both healthy and

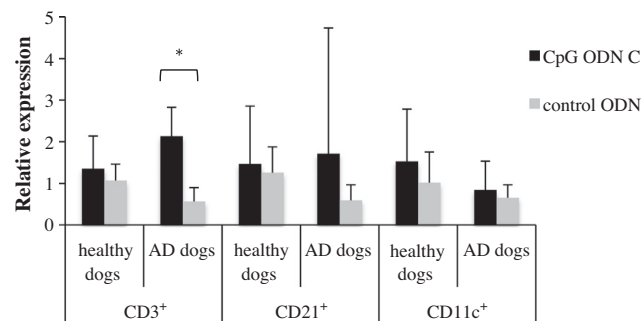


Fig. 2. Relative TLR9 mRNA expression in PBMC subsets, derived from five healthy control and five AD-affected dogs. Cells were stimulated for 18 h with CpG ODN class C or control ODN (both 10 μ g/mL). After FACS sorting, relative expression (fold change) was determined for the indicated cell subsets, comparing CpG ODN class C stimulated vs. unstimulated (culture medium only) cells for each dog. Each bar represents the group mean $\pm$ SD; * $P < 0.05$.

AD-affected dogs ($P = 0.006$ and $P = 0.006$, respectively; Fig. 3a). IL-10 secretion was not enhanced above background levels when cells from AD-affected or healthy dogs were stimulated with CpG ODN class C (Fig. 3b).

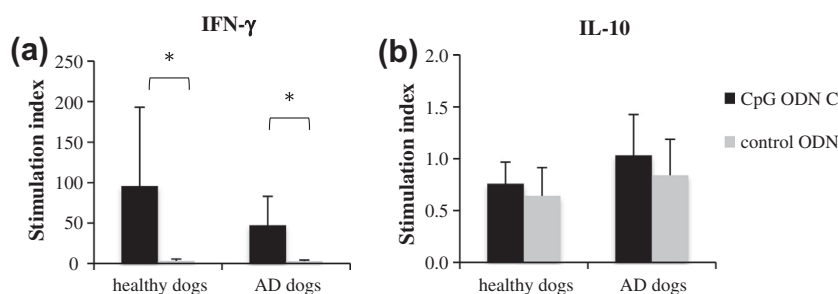


Fig. 3. (a) IFN- γ and (b) IL-10 secretion measured by ELISA within culture supernatants of PBMC of five healthy control and five AD-affected dogs after stimulation with CpG ODN class C or control ODN (both 10 μ g/mL) for 18 h. The stimulation index was calculated as the concentration of cytokine in stimulated cells compared to unstimulated cells. Each bar represents the group mean $\pm$ SD; * $P < 0.05$.

Discussion

Studies of the effects of CpG ODN on canine leukocytes are important, to establish the immunomodulatory potential of these compounds in dogs affected with AD. Our study showed low level reactivity of healthy canine PBMC to CpG ODN class C, but none to CpG ODN class A or B. Similarly, CpG ODN class B has been shown to have poor stimulatory capacity on canine PBMC proliferation *in vitro* (Wernette et al., 2002). Furthermore, CpG ODN class A has been shown to give inconsistent results as assessed by cytokine production by canine PBMC (Kurata et al., 2004; Rostaher Prelaud et al., 2013). In our study, CpG ODN class C induced significant expression of the Th1 cytokines IL-12p40 and IFN- γ , as well as the pro-inflammatory cytokine IL-6 in PBMC from AD-affected dogs. In the healthy dogs, the low sample number, together with the high degree of individual variability in the responses to stimulation, may have been factors leading to results being not significant within the healthy control group.

At the protein level, stimulation of PBMC from both AD-affected and healthy dogs with CpG ODN class C led to increased secretion of IFN- γ , but not IL-10, which was in agreement with the quantitative RT-PCR results. A difference in kinetics might have resulted in a loss of significance of IFN- γ expression in the CD3⁺ subset of AD-affected compared to healthy dogs. We demonstrated that PBMC subsets of atopic and control dogs can respond differently to CpG class C stimulation. Expression of IL-10 mRNA was upregulated when CD3⁺ cells from healthy dogs were stimulated with CpG ODN class C (Table 3), but this was not reflected in the IL-10 ELISA (Fig. 3). Differences in culture time might have played a role in this discrepancy. Alternatively, IL-10 production by CD3⁺ T cells might have been too low for detection in the ELISA, when assessing the mixed PBMC population, or a more prolonged culture time with CpG ODN might be required for detection of IL-10 secretion. Interestingly, in a recent study, CpG ODN class A was found to be capable of increasing IL-10 secretion, but only when complexed with gelatine nanoparticles (Rostaher Prelaud et al., 2013). This suggests a potential requirement for a carrier to transport the CpG ODN to the site of detection (TLR9 is typically located in intra-cellular vesicles), as also seen by the higher efficacies acquired by VLP-conjugated CpG ODN in human clinical studies (Klimek et al., 2011; Beeh et al., 2013). Currently, it is not known whether similar potentiation of CpG ODN class C activity would enhance stimulation of IL-10 secretion by PBMC in AD-affected dogs.

In control dogs, CD21⁺ B cells were stimulated to produce more IFN- α , which is in agreement with the previously described B-cell stimulatory capacity of CpG class C in mice and primates (Marshall et al., 2003, 2005). Brinkmann et al. (1993) showed that, in humans, IFN- α is capable of increasing the frequency of IFN- γ -producing CD4⁺ T cells. Therefore, a canine B cell-mediated, IFN- α -induced response to CpG ODN could be one explanation for the significant increase in IFN- γ expression in the control group in this study, particularly since T cells have been shown not to respond directly to stimulation with CpG ODN (Hornung et al., 2002). In humans, IFN- α is also a potent inhibitor of existing Th2 responses by inducing degradation of the transcription factor GATA-3 (Huber et al., 2010). However, this effect was not observed in canine PBMC, since control CD3⁺ T cells produced significantly more IL-13 upon CpG ODN stimulation.

Taken together, the results of the present study suggest that canine T cells from healthy dogs respond to CpG ODN class C with a mixed Th1/Th2/Treg cytokine profile. Although we could not detect any responsiveness to CpG ODN class C in the CD11c⁺ population, containing dendritic cells and other antigen presenting cells (Ricklin Gutzwiller et al., 2010), it is not possible to distinguish between a direct effect of CpG ODN on T cells and indirect stimulation via other cells in the culture. In contrast, the effects of CpG ODN in

humans have been attributed predominantly to their stimulation of plasmacytoid dendritic cells (Kadowaki et al., 2001).

Previous research has shown expression of TLR9 in canine PBMC, spleen and lymph node cell populations (Hashimoto et al., 2005; Huber et al., 2011). In our study, TLR9 mRNA expression could be detected in all tested PBMC subsets, without any requirement for stimulation. An increase in TLR9 mRNA expression was seen after stimulation with CpG class C, specifically within the CD3⁺ cell population of atopic dogs. However, since no enhanced cytokine responses could be detected within this population, the biological significance of the increased TLR9 expression remains unclear.

Conclusions

Results of the present study indicate that PBMC from healthy dogs and those affected with AD are sensitive to stimulation with CpG class C ODN and respond by upregulating gene expression of a range of cytokines. In healthy dogs, stimulation of the B-cell population led to upregulation of expression of IFN- α , which might indirectly act to induce IFN- γ production by T cells in a paracrine manner. However, when T cells from healthy dogs were stimulated with CpG ODN class C, a mixed Th1/Th2/Treg cytokine response was observed. From this study, based on the induced Th2-cytokine response in healthy dogs and a lack of response in terms of IL-10 in AD-affected dogs, there is little evidence to support the use of CpG ODN class C in future therapeutic strategies for canine AD.

Conflict of interest statement

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was funded by the Utrecht University and Zoetis, United Kingdom. The authors thank Edith Hessel and Dynavax Technologies for their contribution to this study.

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