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

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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.

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-κB (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 (Wernette 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	TGACTGTGAA**CG**TT**CG**AGATGA
Class C	C274	T**CG**T**CG**AACGTT**CG**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 overlayered 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 μM of 2-mercaptoethanol (Sigma–Aldrich). Cells (2×10^6 cells/mL) were stimulated with 10 μg/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% CO_2 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 μL 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 Cytopeia FACS (Becton Dickinson Influx) and collected in tubes containing facsbuffer. Thereafter, cells were pelleted, resuspended in 600 μL 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 Ensembl[1] or NCBI[2] using Primer-3 software[3] (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.