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Effect of synthetic agonists of toll-like receptor 9 on canine lymphocyte proliferation and cytokine production in vitro

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Abstract

Synthetic agonists of TLR9 containing novel DNA structures and R′pG (wherein R = 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine) motifs, referred to as immune modulatory oligonucleotides (IMOs), have been shown to stimulate T_H-1-type-immune responses and potently reverse allergen-induced T_H-2 responses to T_H-1 responses in vitro and in vivo in mice. In order to investigate the immunomodulatory potential of IMOs in dogs, canine peripheral blood mononuclear cells (PBMC) from healthy dogs were stimulated with three different IMOs and a control IMO, alone or in combination with concanavalin A (ConA). Lipopolysaccharide (LPS) was used as a positive control for B lymphocyte activation. Carboxyfluorescein diacetate succinimidyl ester and phenotype staining was used to tag proliferating T and B lymphocytes ($CD5^+$ and $CD21^+$) by flow cytometry. Real-time PCR and ELISA were processed to assay cytokine production of IFN-γ, IL-10, TGF-β, IL-6 and IL-10. Like LPS, IMOs alone induced neither proliferation of $CD5^+$ T cells nor $CD21^+$ B cells, but both LPS and IMO had the capacity to co-stimulate ConA and induced proliferation of B cells. In combination with ConA, one of the IMOs (IMO1) also induced proliferation of T cells. IMO1 also significantly enhanced the expression of IFN-γ on the mRNA and protein level in canine PBMC, whereas expression of IL-10, TGF-β and IL-4 mRNAs was not induced by any of the IMOs. These results indicate that in canine PBMC from healthy dogs, IMO1 was able to induce a T_H-1 immune response including T- and B-cell proliferation.

Keywords: TLR9 agonists; Immunomodulatory oligonucleotides; Flow cytometry; Canine lymphocyte proliferation; Cytokines; Toll-like receptor 9

1. Introduction

The immune system of vertebrates recognizes unmethylated cytidine–phosphate–guanosine (CpG) dinucleotides, which are present in bacterial DNA, as a danger signal (Krieg, 2001). CpG dinucleotides trigger a protective immune response that improves the ability of the host to eliminate the pathogen (Wagner, 1999). This

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; CpG, cytidine-phosphate-guanosine; IMO, immunomodulatory oligonucleotide; ODN, oligodeoxynucleotide; PE, phycoerythrin; RT, room temperature; TLR9, toll-like receptor 9; Treg, T regulatory cells.

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recognition is mediated by a molecular pattern recognition receptor called toll-like receptor 9 (TLR9) (Hemmi et al., 2000; Latz et al., 2004). As TLR9 agonists, CpG oligonucleotides initiate signaling pathways that lead to activation of several transcription factors including nuclear factor-κB and activator protein-1 (Stacey et al., 1996; Yi and Krieg, 1998). The ensuing biological effect includes activation of B cells, T cells, professional antigen-presenting cells (APCs), increased expression of MHC class II antigens, increased synthesis of RNA, DNA, cytokines (IL-6, IL-12, IFN-γ) and chemokines (Klinman et al., 1996; Pasare and Medzhitov, 2003a; Klinman, 2004; Li et al., 2005; Krieg, 2006). Due to this immunological activation, CpG oligodeoxynucleotides (CpG ODN) have a broad therapeutic potential in cancer treatment, infectious disease and allergy (Walker and Zuany-Amorim, 2001; Zuany-Amorim et al., 2002; Klinman, 2004; Krieg, 2006; Romagne, 2007). CpG ODN were tested for their use as allergen-adjuvants in specific immunotherapy and were shown to suppress established IgE-titers in mice (Johansen et al., 2005). They were also shown to prevent inflammatory disease manifestations in mice with established allergic disease, due to a potent T helper 1 (T_H-1) immune response (Kline et al., 1998; Jain et al., 2002).

Recent studies have described synthetic agonists of TLR9 containing novel DNA structure and synthetic immune stimulatory motifs (Kandimalla et al., 2001, 2003a,b, 2005), referred to as immune modulatory oligonucleotides (IMOs). A novel immune stimulatory motif R′pG (wherein R′ = 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine) has been shown to induce immune responses in a wide range of mammalian species. The R′pG motifs potently reverse allergen-induced T_H-2 responses to T_H-1 responses by inhibiting IL-5 secretion and augmenting secretion of IL-12 and IFN-γ in mice, and act as a T_H-1-type adjuvant with antigens and vaccines in mice (Kandimalla et al., 2003a,b; Li et al., 2005). Therefore, synthetic TLR9 agonists containing R′pG motifs could be useful to elicit T_H-1 type-immune responses as immunomodulatory therapy for allergic diseases, viral infections or neoplasia in dogs.

The aim of this study was to investigate the immunomodulatory potential of these synthetic agonists of TLR9 with R′pG motifs in canine cell-based studies. In particular, various synthetic agonists of TLR9 (IMO1–3 and control IMO4) were examined for their ability to induce proliferation of peripheral blood lymphocytes in peripheral blood mononuclear cells (PBMC) obtained from healthy dogs. We also investigated their ability to induce the expression of selected cytokines representing different T cell responses: IFN-γ as a T_H-1 cytokine, IL-4 as a T_H-2 cytokine, IL-10 and TGF-β as T regulatory cell (Treg) cytokines.

2. Materials and methods

2.1. Synthetic agonists of TLR9

Synthetic agonists of TLR9 containing R′pG motifs were synthesized as described elsewhere (Kandimalla et al., 2003a,b) by Idera Pharmaceuticals (Cambridge, MA, USA). The three TLR9 agonists containing R′pG motifs and control IMO used in the study were referred to as IMO1 (5′-TCAGT**R′**GTTAG-X-GATTG**R′**TGA-CT-5′), IMO2 (5′-T**R′**GAA**R′**GTTCT-X-TCTTG-**R′**AAG**R′**T-5′) and IMO3 (5′-T**R′**GTA**R′**GTACT-X-TCATG**R′**ATG**R′**T-5′) and control IMO4 (5′-ACA-CACCAACT-X-TCAACCACACA-5′) (wherein **R′** and X stand for 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine and glycerol, respectively) in the text. All four compounds contained phosphorthioate backbone and the purity was greater than 92% full-length product with the rest being 1, or 2 nucleotides shorter as determined by anion-exchange high performance liquid chromatography, capillary gel electrophoresis, and/or denaturing polyacryl amide gel electrophoresis (PAGE). All compounds were characterized by matrix-assisted laser desorption/ionization-time-of-flight mass for their sequence integrity. All four compounds contained < 0.05 EU of endotoxin as determined by limulus amebocyte lysate assay.

2.2. Animals

Six healthy beagle dogs, with no previous history of disease from Novartis Centre de Recherche Santé Animale, St. Aubin, Switzerland were used as blood donors. Four males (4–7 years old) and two females (6 and 7 years old) were kept in their usual housing conditions. All procedures applied for this study were approved by the local animal welfare authorities.

2.3. Blood sampling

Fifty milliliters of blood was collected from each dog by veinpuncture of the jugular vein using tubes containing EDTA.

2.4. Isolation of PBMC

PBMC were prepared by density gradient centrifugation of EDTA–blood samples obtained from the

above-mentioned dogs. Therefore, undiluted EDTA blood was layered 1:2 onto Histopaque®-1077 (Sigma–Aldrich, Buchs, Switzerland). After centrifugation (1100 × *g* for 30 min at room temperature (RT)), the cells from the interface were aspirated by pipette and washed three times by suspension in Ca^{2+}–Mg^{2+}-free PBS and centrifugation and adjusted to 1×10^6 living cells/ml in PBS.

2.5. Stimulation of PBMC

Stimulation of PBMC was performed twice (1 month apart) in all six dogs with the carboxyfluorescein diacetate succinimidyl ester (CFSE) division technique. As the fluorescence intensity of CFSE-stained cells halves with each cell division, cells that have divided are easily identified and enumerated by flow cytometry. CFSE staining was performed by incubation of 1×10^7 PBMC/ml with a final concentration of 0.5 μM CFSE (Vybrant® CFDA SE Cell Tracer Kit, Invitrogen, OR, USA) at 37 °C for 10 min. The cells were washed three times with RPMI-1640 medium supplemented with 10% FCS, penicillin–streptomycin, L-glutamine and sodium bicarbonate, hereafter designated as complete medium. 1×10^6 cells/ml were cultured in 24-well flat bottom plates in a total volume of 1 ml of complete medium with the following final concentrations of stimulants: concanavalin A (ConA) 1 μg/ml, ConA 1 μg/ml + lipopolysaccharide (LPS) 10 μg/ml, IMO 10 μg/ml and IMO 10 μg/ml + ConA 1 μg/ml. Concentrations of stimulants were determined based on titrations in preliminary experiments (data not shown). Unstimulated cells in complete medium were used as negative control. The cells were incubated for 3 and 4 days at 37 °C in a humidified atmosphere with 5% CO_2. At days 0 and 3, 100 IU/ml recombinant human IL-2 (Peprotech, London, UK) was added to all cultures.

2.5.1. Identification of canine B and T lymphocytes

The following monoclonal antibodies were used for indirect staining: CD5 (clone YKIX322.3; IgG2a; Serotec, Oxford, UK) as a T cell marker and CD21 (clone CA2.1D6; IgG1; Serotec) as a B cell marker. R-phycoerythrin (R-PE) conjugated $F(ab')_2$ fragment donkey anti-rat IgG was used as secondary antibody for CD5 and R-phycoerythrin (R-PE) conjugated $F(ab')_2$ fragment goat anti-mouse IgG was used as secondary antibody for CD21. These antibodies used were obtained from Jackson ImmunoResearch Europe Ltd., Suffolk, UK. Directly conjugated rat IgG2a antibody (PE) and mouse IgG1 antibody (PE) were used as isotype controls (Southern Biotech, Birmingham, AL). Additionally, samples including only secondary antibodies were used as controls for non-specific staining.

PBMC cultures were harvested on days 3 and 4 for phenotyping. A cell suspension of 50 μl containing 1×10^6 cells was incubated on ice with the primary antibody for 15 min (CD5 1:300, CD21 1:640), washed once with PBS, resuspended in 50 μl PBS followed by incubation on ice with the secondary antibody for 15 min (anti-rat IgG 1:100, anti-mouse IgG 1:100). After another washing, cells were resuspended in PBS and immediately analyzed by flow cytometry.

2.5.2. Flow cytometric analysis

Data were acquired on a BD™ LSRII flow cytometer (BD, San Jose, CA, USA), and were processed using FlowJo software (Tree Star, Ashland, OR, USA).

Propidium iodide was used to identify dead and damaged cells in order to exclude these from analysis. Appropriate single stained controls (single-CFSE, single-PE, single-propidium iodide, native cells) were used in all experiments to set the electronic compensation on the flow cytometer to eliminate spectral overlaps between fluorochromes.

In the FlowJo software, a gate was set around the lymphocyte population by means of their characteristic location on forward, side scatter dot plots. A minimum of 30,000 events within the gate was collected for each antibody. Defined by a CFSE versus PE dot plot, $CD5^+$ and $CD21^+$ lymphocytes were gated. Cells that did not proliferate (CFSE high) were determined by gating on unstimulated cells. Cells with decreased CFSE fluorescence intensity (CFSE low) were considered to have proliferated and were gated as $CD5^+$ CFSE low and $CD21^+$ CFSE low. The percentage of CFSE low $CD5^+$ or $CD21^+$ cells was used to compare the ability of the different stimulants to induce proliferation in the different population of PBMC.

2.6. Cytokine assay

2.6.1. Stimulation of PBMC

PBMC (1×10^6 cells/ml) were seeded in 48-well plates in a total volume of 1 ml of complete medium together with 1 μg/ml LPS or 0.5 μg/ml ConA, both used as positive controls. IMOs were tested with three final concentrations: 1 μg/ml, 5 μg/ml or 10 μg/ml. Medium alone was used as negative control. Cells were incubated for 16 h, 24 h, 48 h and 72 h at 37 °C and 5% CO_2. At each time point cell culture supernatant was collected, centrifuged and stored at −20 °C for ELISA. Cells were collected at each time point and total RNA

was isolated as described in Section 2.6.3 and stored at −20 °C for real-time PCR (RT-PCR).

2.6.2. Sandwich ELISA test to measure cytokine proteins

Secretion of IFN-γ, IL-10 and IL-6 was measured after 16 h, 24 h, 48 h, and 72 h of stimulation by sandwich ELISA (canine IFN-γ duoSet ELISA development kit DY781; canine IL-6 duoSet ELISA development kit DY1609; canine IL-10 antibodies AF735, BAF735, MAB7351, 735CL010/CF all from R&D systems, Abingdon, UK). ELISA plates were incubated with antibodies (anti-IFN-γ 1 μg/ml, anti-IL-6 2 μg/ml and anti-IL-10 1 μg/ml) over night at RT and then blocked with PBS containing 1% BSA at RT for 1 h. Cell culture supernatants and cytokine standards were diluted with PBS/1% BSA (IFN-γ 2000 pg/ml, IL-6 4000 pg/ml, IL-10 8000 pg/ml) and added. Plates were covered with a new adhesive sheet and incubated at 37 °C for 2 h. After washing, plates were incubated with biotinylated antibodies diluted with PBS/1% BSA (anti-IFN-γ 100 ng/ml, anti-IL-6 200 ng/ml, anti-IL-10 100 ng/ml) at room temperature for 2 h. Plates were washed and then further incubated at RT for 40 min in the dark after addition of streptavidin-conjugated antibodies diluted 1:200. After washing, plates were developed with tetramethylbenzidine (R&D systems, Abingdon, UK) for 30 min at RT in the dark. Reaction was stopped by adding stop solution ($2NH_2SO_4$) and the color change was measured immediately on a SpectraMax® Plus^{384} (Molecular Devices, Basel, Switzerland) at 490 nm.

2.6.3. Real-time PCR to measure mRNA expression

The mRNA expression of IFN-γ, IL-4, IL-10 and TGF-β was measured after 16 h, 24 h, 48 h, and 72 h of stimulation with real-time PCR. After RNA isolation using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland), RNA was cleaned with Turbo DNA-free kit (Ambion, Rotkreuz, Switzerland) and reverse transcription was performed using High Capacity cDNA Archive Kit (Applied Biosystems, Rotkreuz, Switzerland). The following primer pairs were used: QuantiTect® Primer Assays for IFN-γ (QT00897036), IL-4 (QT00896931), IL-10 (QT01119804) and TGF-β (QT00898219) all from Qiagen, Hombrechtikon, Switzerland. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH, QT00915355, Quiagen, Hombrechtikon, Switzerland) was used as endogenous control. RT-PCR was carried out using the Power SYBR® Green PCR Master Mix (Applied Biosystems) on a RT-PCR machine (Mx3005P™QPCR System, Stratagene, Amsterdam, Netherlands). Real-time PCR was performed according to the manufacturer's instructions. The amounts of mRNAs of cytokines including IFN-γ, IL-4, IL-10 and TGF-β, were measured in comparison to that of GAPDH in each sample. A relative quantification method was used that quantifies differences in expression levels by comparing the cycle threshold (CT) values of target samples with a calibrator (non-stimulated sample). The CT value was defined as the number of PCR cycles at which a significant increase in reporter fluorescence was first detected, and was used for quantification of the mRNA expression of cytokines. The CT value of the calibrator (non-stimulated/medium) was subtracted from that of the target cytokine to calculate the ΔCT value. The CT value of the calibrator and the target samples were normalized to an endogenous control (GADPH). All samples were examined in duplicate and the mean value of the ΔCT was calculated for each sample for further statistical analysis.

2.7. Statistical analysis

Data obtained were analyzed using the statistical software package NCSS 2007 (www.ncss.com) and SAS® version 8.2 (www.sas.com). Descriptive statistics and box plots from data acquired from flow cytometry were used to describe the proliferation of all stimulants. In order to compare ConA and ConA-costimulations in detail (a) the relation (ratio) of each ConA co-stimulation to ConA (serving as a baseline) was calculated per time point and (b) for each sample the mean of the two (time points) ratios was derived. Some ratios were not normally distributed within groups. A non-parametric one-sample test (Wilcoxon Signed-Rank Test) was used – for each ConA co-stimulation group – to test the hypothesis that this ratio was significantly different from 1 (one).

A repeated measurements analysis of variance (ANOVA) with corrected multiple comparison (Dunnett method) was used to assess differences in cytokine expression between the study groups. As the group/time interaction was not significant, it was removed from comparison.

Differences were considered significant when $p < 0.05$.

3. Results

3.1. Flow cytometry—lymphocyte proliferation

Preliminary experiments showed that in contrast to other species LPS alone did not induce proliferation of canine B cells (Fig. 1). However, co-stimulation of

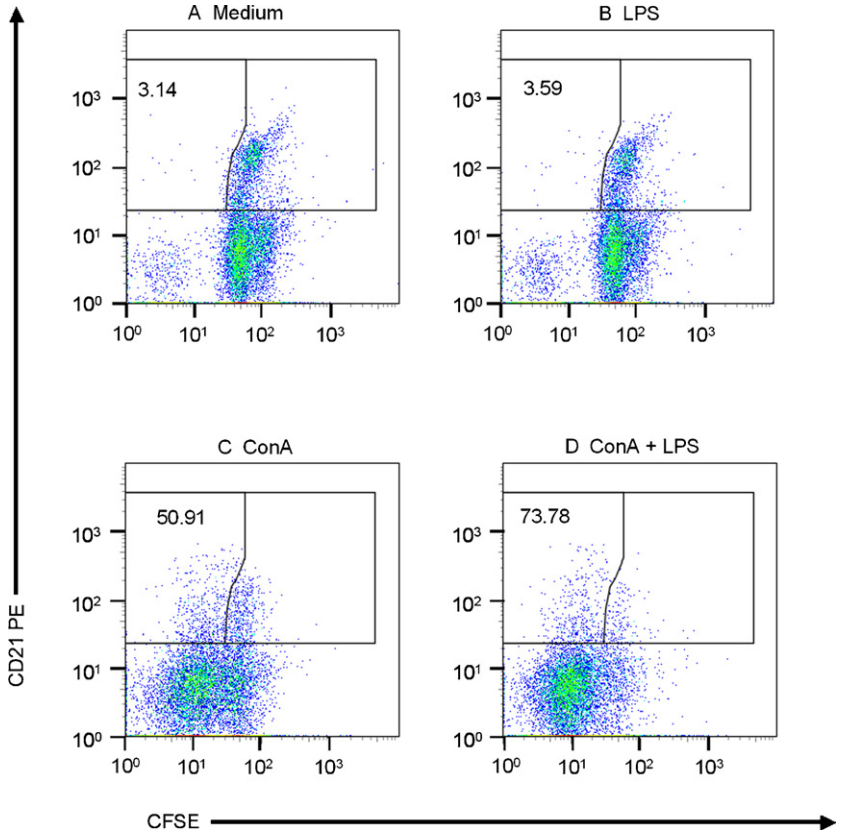

Fig. 1. Effect of ConA, LPS and ConA/LPS co-stimulation on canine B-lymphocyte proliferation. Canine PBMC from a healthy dog were stained with CFSE and cultured with different stimulants for 3 days. Lymphocytes were stained with anti-CD21 to detect canine B cells. Bivariate dot plots of canine lymphocytes from a control unstimulated cell culture (A), an LPS stimulated culture (B), a ConA stimulated culture (C), and a ConA and LPS stimulated culture (D) is shown. A polygonal gate was used to define the $CD21^+$ and $CD21^+$ CFSE low (left part of $CD21^+$ gate) cells. The percentages of $CD21^+$ CFSE low of total $CD21^+$ cells are given. The result is representative for data obtained with a total of four dogs.

ConA and LPS increased B cell proliferation compared to ConA monostimulation (Fig. 1). Therefore, the co-stimulation of ConA and LPS was integrated into our studies with IMOs as positive controls.

Fig. 2 shows a representative experiment for the effect of IMO1. While IMO1 had no effect on the proliferation of T and B lymphocytes alone, a co-stimulation with ConA resulted in the induction of proliferation. For the data shown in Fig. 3, the extent of proliferation of cells induced by three different IMOs and control IMO4 was measured twice in six dogs.

Either IMO1–3 or control IMO4 used in this study as monostimulants (B1–B4) had no effect on proliferation of T and B cells in PBMC cultures, whereas stimulation with ConA (C, D and E1–E4) always resulted in a strong increase in proliferation of lymphocytes. It appeared that like LPS some IMOs had co-stimulatory effects with ConA, in particular for the proliferation of $CD21^+$ cells. This was further investigated by determination of the ratio of the values obtained with ConA co-stimulation to ConA alone, including a statistical analysis (Fig. 4). Co-stimulation of ConA and LPS induced significantly more proliferation of $CD5^+$ T cells at days 3 and 4. Co-stimulation of ConA and IMO1 showed only significant effect on T cells at day 3 while IMO2, IMO3 and control IMO4 did not significantly change ConA-induced T-cell proliferation.

$CD21^+$ B cell proliferation was significantly increased with ConA and LPS or ConA and IMO1 co-stimulation at both days when compared to ConA monostimulation.

B cell proliferation with co-stimulation with ConA and IMO2, IMO3 and control IMO4 did show significant difference to ConA monostimulation at day 3 but not at day 4.

3.2. *ELISA—protein level of cytokines*

Table 1 shows an overview of the cytokine production of stimulated cells with IMO1–3 and

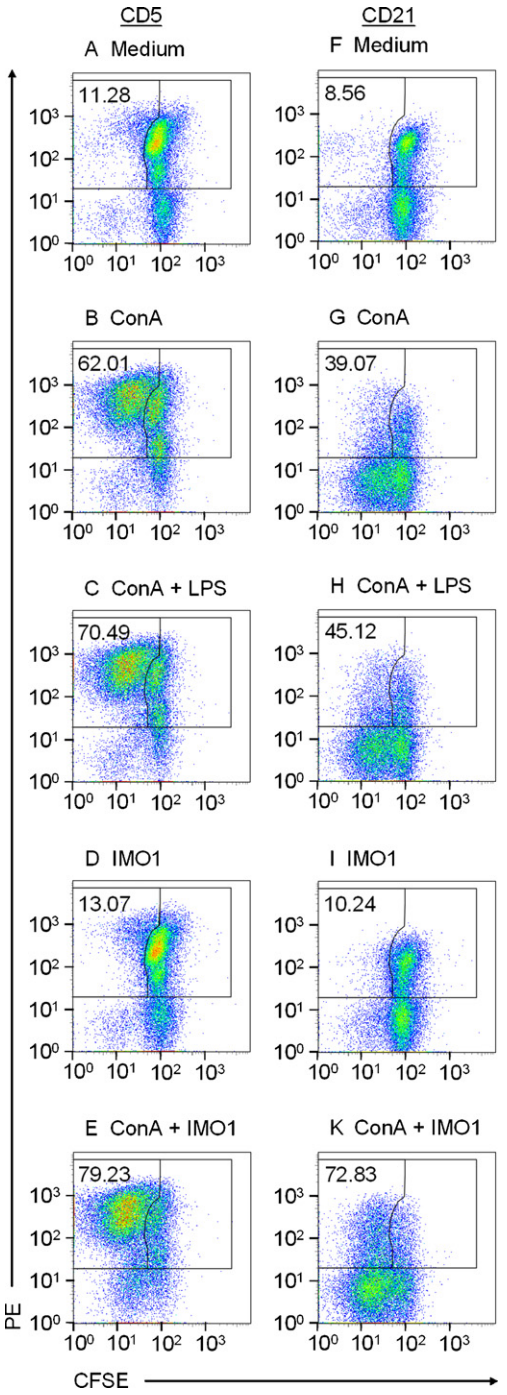

Fig. 2. Effects of ConA, LPS and IMOs on canine T- and B-lymphocyte proliferation. Canine PBMC were labeled with CFSE and cultured with different stimulants for 3 days and stained for CD5 (A–E) and CD21 (F–K). Vertical axis shows R-phycoerythrin (PE) staining for $CD5^+$ T cells and $CD21^+$ B cells, horizontal axis shows CFSE staining. A polygonal gate was used to define the populations with high and low CFSE intensity expressing CD5, and CD21, respectively. Due to limited space dot plots of unstimulated cells (A and F), cells cultured with ConA (B and G), ConA + LPS (C and H), IMO1 (D and I) and ConA + IMO1 (E and K) of a representative experiment out of 24 are shown.

control IMO4 at three different concentrations and their comparison to unstimulated cells (ratio to untreated).

IFN-γ protein: We obtained an average amount of 182 pg/ml IFN-γ with the LPS stimulation and 434 pg/ml IFN-γ with the ConA stimulation after 24 h. IMO1–3 and control IMO4 used in this study induced IFN-γ protein production. Compared to medium nearly all inter-quartiles of the data from IMO1–3 and control IMO4 were above the unstimulated cells (medium line 1) (Fig. 5A–C). Medians of IFN-γ production with 1 μg/ml of all IMOs were between 1.5-fold and 3-fold (Fig. 5A), whereas medians with 5 μg/ml and 10 μg/ml were between 3-fold and 6-fold (Fig. 6B and C). The highest upper quartile has shown almost a 15-fold increase with 10 μg/ml of IMO1 at 16 h after stimulation (Fig. 5C). This IMO also showed an upper extreme of an almost 30-fold increase (Fig. 5C).

Statistical analysis demonstrated a significant increase of IFN-γ protein production by IMO1–3 and control IMO4 at all concentrations (p-value) (Table 1).

IL-6: in comparison with untreated cells, the amount of IL-6 induced by IMOs was maximally 1.4-fold above the negative control. This slight increase induced by IMO1–3 (5 and 10 μg/ml) was statistically significant with the tests used (Table 1).

IL-10: although the ratio to untreated cells was minimally increased, all IMOs at all concentrations showed statistically significant increase except for control IMO4 at 1 μg/ml (Table 1).

3.3. RT-PCR—mRNA level

IFN-γ: when compared to medium only medians of IMO1 and IMO2 showed at all time points an increased mRNA production at least 1.5-fold with 5 μg/ml and 10 μg/ml (Fig. 6B and C). IMO1 demonstrated a higher distribution of the upper quartile at 5 μg/ml and 10 μg/ml than IMO2 and 3 and control IMO4 (Fig. 6B and C). The highest upper quartile showed a 10-fold increase with 10 μg/ml of IMO1 at 72 h after stimulation (Fig. 6C).

However, the increased IFN-γ mRNA production was statistically significant only with IMO1 at 10 μg/ml ($p < 0.0117$).

IL-10 and TGF-β: When compared to untreated cells the amount of IL-10 and TGF-β mRNA induced by IMOs, control IMO, and positive controls was equal to or lower than that produced by cells in culture medium only (data not shown).

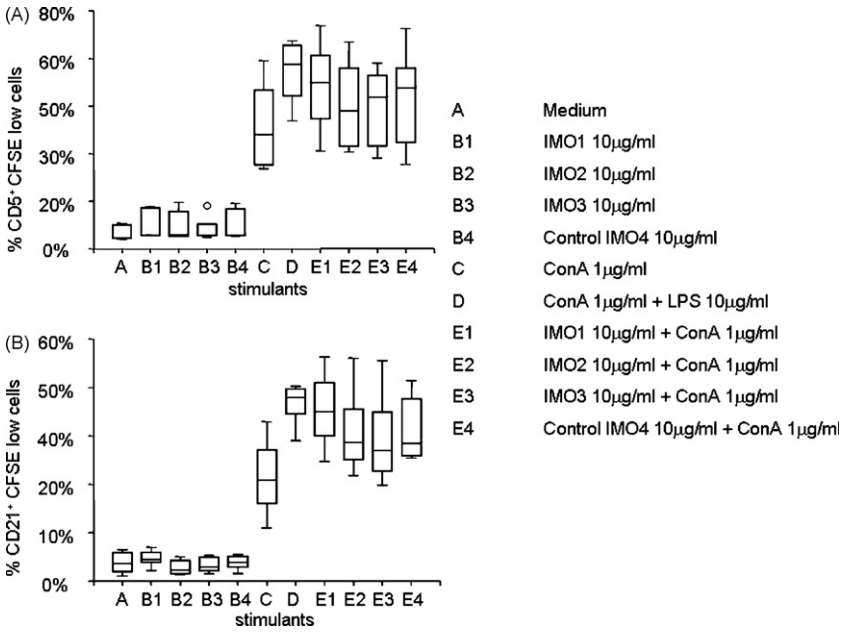

Fig. 3. Summary of effects of IMOs on canine T- and B-lymphocyte proliferation. Box plot data representing the proliferation of $CD5^+$ (A) and $CD21^+$ (B) CFSE low cells measured after 3 days and expressed as percent (%) of proliferated cells in media with different stimulants.

IL-4: There was no IL-4 mRNA production observed either with IMOs, LPS or with untreated cells. As expected, stimulation with 0.5 μg/ml of ConA, used as a positive control, induced 50-fold more IL-4 than the negative control ($p < 0.0001$) (data not shown).

4. Discussion

The investigations reported here reveal the immunostimulatory effects of synthetic agonists of TLR9 containing R′pG motifs (IMO1–3) and control IMO4 on

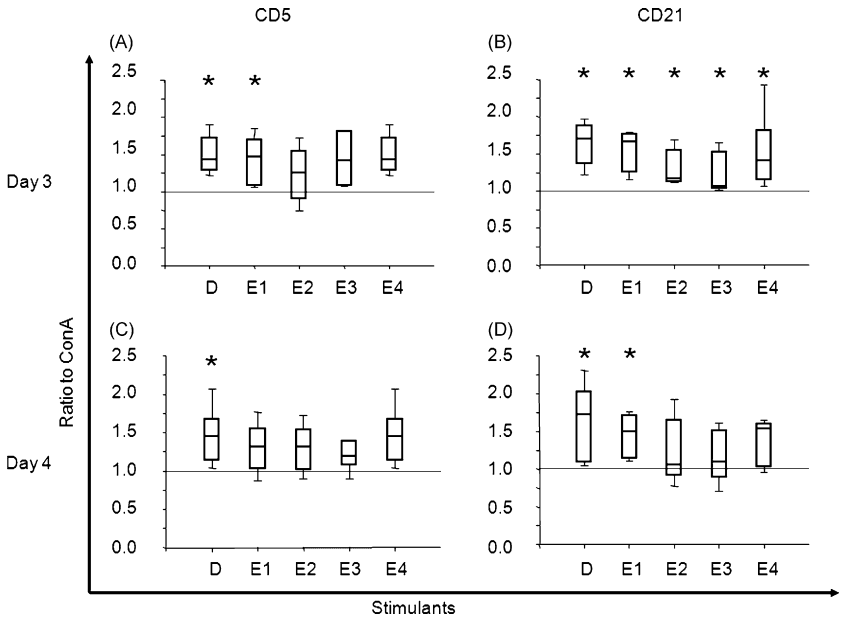

Fig. 4. Co-stimulatory effects of IMOs on canine T- and B-lymphocyte proliferation. Box plot data representing the ratio of ConA costimulated CFSE low cells to ConA monostimulation. D = ratio ConA + LPS to ConA; E1 = ratio ConA + IMO1 to ConA; E2 = ratio ConA + IMO2 to ConA; E3 = ratio ConA + IMO3 to ConA and E4 = ratio ConA + control IMO4 to ConA. The asterisk (*) indicates significant difference to ConA monostimulation (*Wilcoxon signed rank test; $p < 0.05$).

Table 1
ELISA: cytokine production of stimulated cells in comparison to medium-treated cells

Stimulant	Concentration	Ratio to untreated			*p*-Values (corrected)[a]		
		IL6	IL10	IFN-γ	IL6	IL10	IFN-γ
IMO 1	1	1.19	1.38	2.74	0.0547	**0.0001**	**<0.0001**
	5	1.29	1.39	4.33	**0.0005**	**<0.0001**	**<0.0001**
	10	1.40	1.44	4.48	**<0.0001**	**<0.0001**	**<0.0001**
IMO 2	1	1.09	1.31	2.02	0.7528	**0.0020**	**0.0031**
	5	1.25	1.36	3.39	**0.0050**	**0.0004**	**<0.0001**
	10	1.28	1.29	3.22	**0.0010**	**0.0044**	**<0.0001**
IMO 3	1	1.06	1.23	2.76	0.9924	**0.0393**	**<0.0001**
	5	1.21	1.38	3.95	**0.0227**	**0.0001**	**<0.0001**
	10	1.22	1.35	4.10	**0.0125**	**0.0005**	**<0.0001**
Control IMO 4	1	1.05	1.14	2.19	0.9965	0.4884	**0.0006**
	5	1.08	1.31	2.55	0.8573	**0.0020**	**<0.0001**
	10	1.12	1.26	2.95	0.4373	**0.0138**	**<0.0001**
LPS		1.57	1.50	2.12	**<0.0001**	**<0.0001**	**0.0012**
ConA		0.85	1.51	7.33	0.0838	**<0.0001**	**<0.0001**

Table shows the difference (ratio) compared to untreated cells (medium only) for IL-6, IL-10, and IFN-γ measurements using ELISA after stimulation of PBMC with three different concentrations (1 μg, 5 μg, and 10 μg/ml) of various IMOs. The last columns show the corrected *p*-values for the correspondent ratios. In bold, statistical significant data ($p < 0.05$). Note that all IMOs at all concentrations induce a statistically significant INF-γ production after stimulation to canine PBMC.

[a] *p*-Values corrected for multiple comparison.

canine PBMC in vitro. Co-stimulation of PBMC with ConA and IMOs resulted in proliferation of $CD5^+$ T cells (IMO1) and $CD21^+$ B cells (IMO1–3 and control IMO4). Furthermore we could show enhanced proliferation of canine lymphocytes stimulated with ConA and LPS co-stimulation. We were able to demonstrate that IMO1 induced increased expression of IFN-γ mRNA as well as the protein. All three IMOs and control IMO induced only minimal IL-10 and IL-6 production at the protein level. However, we did not observe induction of IL-4, IL-10 and TGF-β mRNA.

Our data demonstrate species differences in the response of T and B cells to LPS and ConA. Enhanced $CD21^+$ B cell proliferation occurred with the combination of ConA and LPS, when compared to ConA alone. While LPS is a well known mitogen for human and mouse B cells which does not require T-cell help, LPS monostimulation did not show any effect on proliferation of canine B cells. A lack of IL-2 cannot explain this observation since our cultures were supplemented with recombinant IL-2. Further investigations are required to determine whether LPS-driven canine B-cell proliferation requires other T-cell cytokines or even a cognate interaction of B cells with activated T cells.

Considering that ConA is described as a classical T-cell mitogen with no effect on B cells, it was also interesting to note that ConA had mitogenic activity for canine B cells. Nevertheless, such effects have also been described for mouse and human B cells and demonstrated to be mediated by T-cell ''help''(Brochier et al., 1976; Piguet et al., 1976)—also a likely explanation for our results.

An IMO containing R′pG motif in a different sequence context than the ones used in the present study induced potent proliferation of PBMC obtained from a number of vertebrate species including human, monkey, pig, horse, sheep, goat, rat and chicken (Kandimalla et al., 2003a,b). The present results of IMOs in canine immune cells indicate that the canine immune system may respond differently to TLR9 agonists compared with other vertebrate species. IMO1–3 (like LPS, an agonist for TLR4) tested as monostimulants in our study failed to cause significant canine lymphocyte proliferation in vitro suggesting that this species reacts differently to TLR ligands. Furthermore, control IMO4 was not stimulatory in human and mouse models (Putta et al., 2006). Our results demonstrate that control IMO4 despite lack of an immune stimulatory motif showed some level of immune stimulation comparable to IMO2 and 3. This could also indicate that IMO2 and 3 are not immunostimulatory in dogs.

Consistent with a previously reported study (Wernette et al., 2002), ConA monostimulation-induced proliferation of T and B cells of canine PBMC in contrast to TLR9 agonists like CpG or IMO monostimulation. A different IMO containing R′pG motif has

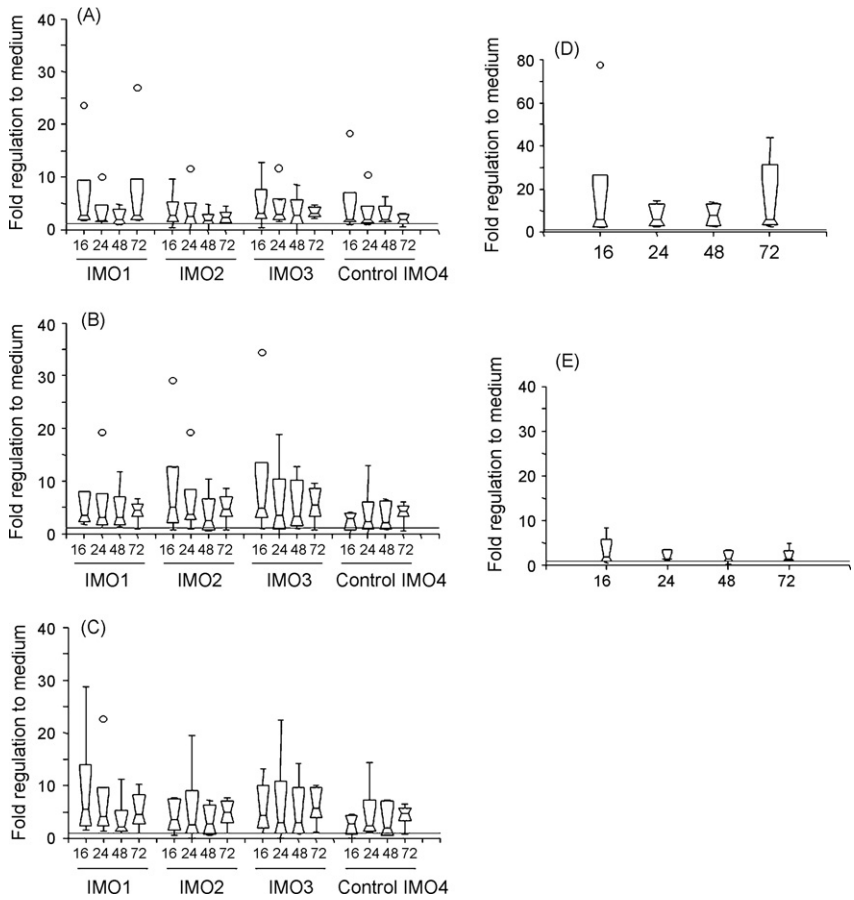

Fig. 5. Effects of ConA, LPS and IMOs on IFN-γ protein production. IFN-γ ELISA test from isolated canine PBMC ($n = 6$) stimulated in vitro with different concentration of various IMOs. Experiments were done in duplicates. Each IMO was tested at three different concentrations: (A) 1 μg/ml, (B) 5 μg/ml, and (C) 10 μg/ml. Protein production was measured 16 h, 24 h, 48 h, and 72 h after stimulation. ConA 0.5 μg/ml (D) and LPS 1 μg/ml (E) were used as positive controls. The horizontal line (value 1) represents the relative IFN-γ protein production of unstimulated cells (culture medium = baseline). Box plot data represent the calculated fold regulation compared to baseline. Box plots demonstrate the median value, the lower and upper quartile, the whiskers (lower and upper extremes) and the outlier dots of the data distribution for each IMO, concentration and time point.

been shown to induce immune responses in a TLR9-dependent fashion in a number of vertebrate species (Kandimalla et al., 2003a,b). It has thereby an activating effect on antigen presenting cells as well as a direct effect on B lymphocytes which also express TLR9. The mechanism of enhanced proliferation with co-stimulation of ConA and IMOs on T and B cells, will require further investigations including the characterization of TLR9 expression in the canine immune system. This is important considering that the cell populations that express TLR9 differ between species. For example, mice differ from primates and pigs in that they express TLR9 not only in plasmacytoid dendritic cells and B cells, but also in macrophages, monocytes, and myeloid dendritic cells (Guzylack-Piriou et al., 2004; Iwasaki and Medzhitov, 2004). In cats B cells, CD4+ and CD8+ T cells express TLR9 and their relative expression varies among different immune tissues (Ignacio et al., 2005). Consequently, dependent on the distribution of TLR9 in the canine immune system, direct target cells for IMO could be the B cells themselves and/or dendritic cells which could be activated by IMO to promote lymphocyte activation through production of cytokines.

The heterogeneous cell population in PBMC cultures makes it difficult to interpret the role of the different phenotype of immune cells. Indirect activity of present monocytes may influence the biological effects of lymphocytes. If single types of cell populations such as B or T cells were sorted from PBMC or if lymphocytes were purified from monocytes, a reduction of variability might be achieved and the individual response of

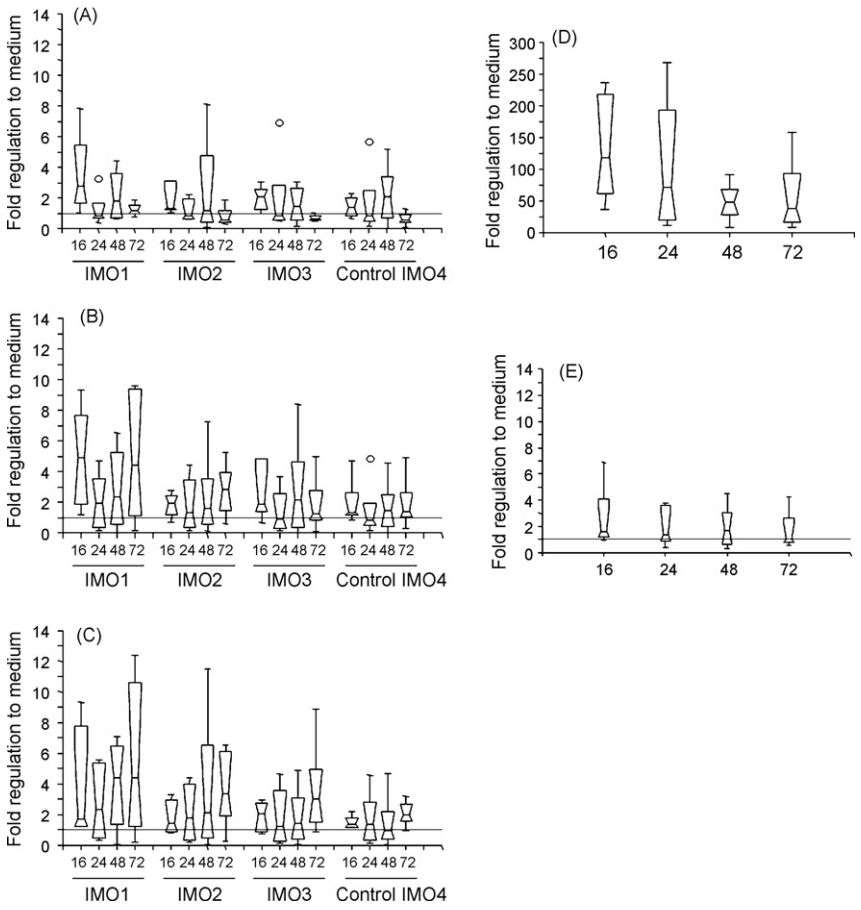

Fig. 6. Effects of ConA, LPS and IMOs on IFN-γ mRNA production. Steady-state mRNA levels determined by Q-PCR presented as relative change compared to GADPH. Experiments were done in duplicates. Data represents the relative fold regulation of the CT values for INF-γ mRNA. For each IMO, three different concentrations were tested: (A) 1 μg/ml, (B) 5 μg/ml, and (C) 10 μg/ml. mRNA production was measured 16 h, 24 h, 48 h, and 72 h after stimulation. ConA 0.5 μg/ml (D) and LPS 1 μg/ml (E) were used as positive controls. The horizontal line (value 1) represents the relative IFN-γ mRNA production of unstimulated cells (medium). Box plot data represent the calculated fold regulation compared to medium. Box plots demonstrate the median value, the lower and upper quartile, the whiskers (lower and upper extremes) and the outlier dots of the data distribution for each IMO, concentration and time point.

isolated cell populations to stimulation with IMOs would be defined more clearly. Furthermore, experiments with different combinations of cell population may provide an answer to the question of which interactions between monocytes, T and B cells and their cytokine pattern are necessary to trigger proliferation and result in a distinct cytokine pattern in vitro.

To investigate the immunomodulatory potentials of IMO1–3 and control IMO4, selected cytokines representing different T cell responses were tested: IFN-γ as a T_H-1 cytokine, IL-4 as a T_H-2 cytokine, IL-10 and TGF-β as Treg cytokines. IFN-γ is the signature cytokine of T_H-1 subsets and plays a major role in diverting T_H-2 responses to T_H-1 responses. In our study we were able to show that IMOs effectively induced IFN-γ production in dogs. The immunostimulatory activity of IMOs via their induction of T_H-1 cytokine IFN-γ could be advantageous for immunotherapy for allergic diseases, viral infections and neoplasia in dogs.

Since IL-4 is the signature cytokine of T_H-2 subsets, it is advantageous that none of the IMOs induced the expression of this cytokine in our study.

TLR9-ligand administration induces IFN-γ and IL-10 production from Tregs which induce and enhance indolamine 2,3 dioxygenase (IDO) expression (Hayashi and Raz, 2006). Treg cells have an immunosuppressive function and a cytokine pattern distinct from either T_H-1 or T_H-2 cells and play an important role in the healthy immune response to allergens (Akdis, 2006).

However, in our study we did not observe an increase in IL-10 and TGF-β mRNA and only a twofold increase

of IL-10 at the protein level, which was statistically significant. However, the biological relevance of that response remains unclear.

It was demonstrated that IL-6 secreted by dendritic cells following stimulation with CpG-ODN reversed the suppressive activity of Treg cells (Pasare and Medzhitov, 2003b). The fact that we found a minimal increase of IL-6 at the protein level may be advantageous as Treg cells play a major role in the healthy immune response.

In conclusion, our results demonstrate that synthetic agonists of TLR9 containing R′pG motifs can enhance T_H-1 responses and proliferation of canine PBMC obtained from healthy dogs in vitro and warrant further studies on their immunomodulatory effects in dogs suffering from allergic diseases, viral infections or neoplasia.

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