

***In vitro* effects of CpG oligodeoxynucleotides delivered by gelatin nanoparticles on canine peripheral blood mononuclear cells of atopic and healthy dogs – a pilot study**

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Background – Cytosine-phosphate-guanine (CpG) oligodeoxynucleotides offer a novel promising immunotherapeutic approach for atopic dermatitis (AD) both in humans and animals. Gelatin nanoparticles (GNP) enhance and prolong CpG-associated immunomodulatory effects and minimize adverse effects both *in vitro* and *in vivo*. Information about the effects of this combination in dogs is lacking.

Hypothesis/Objectives – The aim of this study was to evaluate immunological effects of CpG coupled to GNP on canine peripheral blood mononuclear cells (PBMCs) *in vitro*.

Animals – Eight dogs with AD, diagnosed by standard criteria and with a concurrent immediate hypersensitivity to house dust mites were included. Control samples were taken from eight healthy, age-matched control dogs without history or evidence of cutaneous or systemic illness.

Methods – Peripheral blood mononuclear cells of healthy and allergic dogs were incubated with CpG-GNP and the uptake of CpG-GNP was demonstrated using confocal laser scanning microscopy. Cell culture supernatant concentrations of interferon gamma (IFN- γ), interleukin (IL)-4, IL-6 and IL-10 were measured by Canine Cytokine Milliplex.

Results – No significant changes in IFN- γ and IL-4 were found when comparing PBMCs incubated with CpG and CpG-GNP with the negative controls in atopic and healthy dogs. Interleukin-6 was not detected in any of the groups. However, a statistically significant increase in IL-10 concentration was found after 24 h stimulation with CpG-GNP compared with CpG alone both in atopic and healthy dogs.

Conclusions and clinical importance – As IL-10 is considered an immunosuppressive cytokine playing a key role in peripheral tolerance; the reported CpG-GNP formulation could be a new approach in allergy treatment.

Introduction

Canine atopic dermatitis (AD) is a common skin disease in small animal practice with characteristic clinical features. It is associated in most but not all cases with the formation of immunoglobulin E (IgE) against environmental allergens¹ and is very similar to the human equivalent and thus considered a relevant model for human AD.² The pathophysiology involves the interplay of immunological,

genetic, skin barrier and environmental factors.³ In addition, cutaneous infections can influence the course of the disease.⁴ Immunological studies showed substantial evidence that T lymphocytes play a critical role in canine AD and that an imbalance in the T-cell populations characterizes different stages of the disease. The acute and chronic phase are characterized by high mRNA Th2 [interleukin (IL)-4] and Th1 responses [interferon (IFN)- γ], respectively.³ However, demonstration of biologically active cytokines is still lacking. In humans, an inhibited expression of IL-10 has been shown.⁵

Allergen-specific immunotherapy is a recommended therapeutic option for canine AD and was reported to be effective in controlling clinical signs in approximately 60% of treated dogs.^{6,7} Further, it is recommended to control infections if present and to restore skin barrier function.⁷ Glucocorticoids or calcineurin inhibitors are needed in some patients, and antihistamines and essential fatty acids may be also beneficial.⁷ However, cases of refractory canine AD are an unfortunate fact in small animal

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practice. Thus, research on new immunomodulatory therapies and studies investigating the aetiology and pathogenesis of this disease seem obligatory.

Novel immunomodulatory strategies for AD include cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODN), which signal through an endosomal membrane-based type of pattern recognition receptor (PRR), the Toll-like receptor 9 (TLR9).^{8,9} Toll-like receptor nine was identified in dendritic cells (DC), B cells and other human immune cell types.^{10–12} In dogs, there is only evidence that TLR9 mRNA is expressed in peripheral blood mononuclear cells (PBMC)¹³ and one report recognized its particular presence in macrophages.¹⁴ Cytosine-phosphate-guanine ODN has been shown to influence several signalling pathways in a variety of immune cells, leading to cytokine production in many mammalian species.^{13,15,16} It appears that the specific purines and pyrimidines surrounding the CpG motif, the phosphorothioated backbone, as well as the spacings between CpG motifs may influence both the level and the type of immune stimulation.^{17,18} The CpG motifs improve the antigen presenting function of DC, monocytes and macrophages, induce the proliferation of B lymphocytes, stimulate the immunoprotective activity of natural killer cells and recruit T cells to the site of ODN administration.^{19–21}

Recent studies have shown that the immune system responds to CpG motifs by inducing potent Th₁-like immune responses which can be harnessed for immune therapy of cancer, allergy, infectious diseases,²² autoimmune diseases and sterile inflammation.⁸ Cytosine-phosphate-guanines may also be used as adjuvant for vaccines.²³ It has been shown that CpGs are powerful stimulators of canine immune cells *in vitro*^{24,25} and *in vivo*.²⁶ Elevated IL-10 mRNA transcription after *in vitro* stimulation of canine PBMCs with ODN rich in CpG²⁷ and elevated IL-10 protein levels after stimulation with synthetic agonists of TLR9²⁸ are reported. *In vivo*, a shift towards a Th₁- and T-regulatory immune response in allergy treatment has been documented with CpGs in cats²⁹ and with CpG-allergen conjugates in humans³⁰, similar to what is seen during allergen-specific immunotherapy in canine atopic dermatitis.^{31,32} In humans, clinical efficacy of CpG-allergen conjugates³³ and CpG-virus-like-particle formulations administered together with allergens³⁴ has been demonstrated.

Adverse effects following CpG ODN treatment are possible; however, there is no evidence that modest doses are directly toxic or cause autoimmune disease in healthy animals.^{35,36}

Colloidal particulate delivery systems exhibit the same size range as microbes and are therefore preferentially phagocytosed by DCs.³⁷ Consequently, they have shown efficacy in delivery of subunit vaccines based on proteins, peptides and DNA.³⁸ It was previously shown that gelatin nanoparticles (GNP) are effectively taken up by TLR9 possessing cells.³⁹ The GNP carrier system for ODN in this study prevents enzymatic degradation, transports CpG to the desired site of action and is biodegradable and biocompatible.⁴⁰ Further, a nebulized GNP-based CpG formulation triggered expression of IL-10 and showed partially positive clinical effects in immunotherapy of allergic horses.⁴¹

Our study aims were to demonstrate cellular uptake of CpG-GNP by canine PBMCs and to evaluate the direct influence on cytokine release by PBMCs of atopic and healthy dogs. Indirect influence of CpG and CpG-GNP on PBMCs by means of stimulated macrophages was also investigated. From information gained in this study a new therapeutic strategy in canine AD might be obtained.

Materials and methods

Animals

The procedure was approved by the local animal welfare authorities (District Government of Upper Bavaria, Field of interest 54, 80534 Munich, Germany).

Eight dogs with AD [six females and two males with an age range of 14 months to 4.8 years (median 4.6 years) and a median duration of disease of 4.6 years], diagnosed by standard criteria⁴², ruling out differential diagnoses and with a concurrent immediate hypersensitivity to house dust mites documented by intradermal testing or a positive serum test for allergen-specific IgE were included. The group consisted of two mixed breed dogs and one of each of the following breeds: golden retriever, Labrador retriever, shi tzu, bichon frise, bull terrier and Weimeraner. The atopic dogs did not show any signs of skin infection and were not treated with antibiotics.

Control samples were taken from eight healthy (non-atopic) control dogs (four females and four males with an age of 2.3–8.3 years, median 6.1 years); six of these were mixed breeds, one was a German shorthair pointer and one a Vizsla. All healthy dogs had no history or evidence of cutaneous or systemic illness. All dogs from both groups did not receive any form of immunosuppressive agents for at least 6 weeks before blood collection.

CpG-ODN material

Oligodeoxynucleotides with the sequence GGTGCATCGATG-CAGGGGGG²⁴ were used with a full phosphorothioate backbone (Biomers, Ulm, Germany). For demonstration of intracellular localization by confocal laser scanning microscopy (CLSM), a batch of CpG was labelled with fluorescent dye Atto 540 (Biomers).

Preparation of GNP

The unloaded GNP were prepared by a two-step desolvation method as previously described.⁴³ Briefly, a 5% (w/w) solution of porcine gelatin was subjected to controlled desolvation by acetone leading to separation of the high- and low-molecular-weight fractions of the gelatin. Thereafter, the high-molecular-weight fraction was labelled by covalent attachment of Alexa 633 dye (Invitrogen, Darmstadt, Germany) and subsequently subjected to a second desolvation step by acetone, whereby spherical colloid nanoparticles formed spontaneously. Cationization was carried out by attaching permanent positive charges from quaternary cholamines to the surface of readily prepared spheres and processing at a pH of 4.5 and room temperature (RT) for 30 min.⁴⁴ Using this method the zeta potential of the GNP changed from negative to positive. The concentration of GNPs was determined gravimetrically with a UMX2 micro-balance (Mettler Toledo, Giessen, Germany) by a standard protocol.⁴⁴ Intensity-weighted particle sizes and zeta potential were obtained by positron correlation spectroscopy (PCS) using a Zetasizer ZS Nano (Malvern Instruments, Herrenberg, Germany). To exclude the impact of viscosity on PCS particle size results, the viscosity of particle samples was determined with automated microviscometer (Anton Paar, Ostfildern-Scharnhausen, Germany). For Zeta potential measurement before and after loading, GNP stock dispersions were diluted in 10 mM NaCl at a 1:10 ratio (v/v) and measured in folded capillary cells (Malvern Instruments).

ODN loading onto cationized GNP

Five milligrams of aseptically prepared nanoparticles were incubated with 250 µg of ODN in a total volume of 3339 µL of phosphate buf-

ferred saline (PBS) at pH 7.4 (5% w/w) for 2 h at 22°C and under gentle shaking using a thermomixer (Eppendorf, Hamburg, Germany). For quality control, the loading efficiency was checked photometrically at a wavelength of 260 nm (Thermo Fisher Scientific, Schwerte, Germany) in the samples' supernatant after ultracentrifugation and accepted if the loading was at least 97% (w/w).

Blood sampling and PBMC isolation

Twenty millilitres of blood were collected from the jugular vein using ethylenediaminetetraacetic acid (EDTA)-containing tubes on day 0 and day 10. Peripheral blood mononuclear cells were isolated by density gradient centrifugation as previously reported.⁴⁵ Briefly, 15 mL centrifugation tubes were filled with 7 mL Ficoll separation gradient (Biochrom, Berlin, Germany) with a density of 1.077 g/mL and covered with 7 mL blood previously diluted 1:1 with PBS. After centrifugation without final braking (40 min, 1200 *g*, RT), the concentrated PBMC were removed by pipetting. The cells were washed using PBS at RT and resuspended thereafter in standard cell culture medium consisting of 10 mL RPMI-1640 cell culture medium (Biochrom) with 10% bovine calf serum (Biochrom) and 1% penicillin-streptomycin (Biochrom). Peripheral blood mononuclear cells viability and number were determined by Trypan blue staining (Biochrom) and cell counting in a Neubauer chamber (Biochrom). In all experiments, 98–100% of cells were viable. The isolated cells were stored at 4°C and used within 1 h.

CpG-GNP uptake by PBMCs of healthy dogs

A total of 25×10^5 PBMCs were transferred to each chamber of a μ -slide eight-well microscopy chamber slides (Ibidi, Planegg/Martinsried, Germany), covered with 300 μ L standard cell culture medium and incubated.⁴⁶ The cultured PBMCs were then incubated with fluorescence labelled CpG-GNP for 2 h. The supernatant was then carefully removed and the cells washed using 300 μ L PBS. After washing, the PBMC membranes were labelled with 100 μ L Concanavalin A (ConA)/Alexa 488 (Invitrogen) solution for 1 min and washed three times with PBS. Nuclei of the PBMC were stained with 50 μ L Hoechst 33342 dye (Invitrogen) for 5 min. After the final PBS washing, cells were fixed with 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany). Chamber slides were finally prepared with a cover medium consisting of 10% Mowiol 488 and 2.5% diazabicyclooctan (Sigma-Aldrich, Taufkirchen, Munich, Germany), and visualized using a Zeiss 510 LSM NLO confocal laser scanning microscope (Carl Zeiss Microscope Systems, Munich, Germany).

Cultivation and stimulation of PBMC

A total of 2×10^6 PBMCs were cultured in 24-well Nunc flat bottom culture plates (Thermo Fisher Scientific) in a total volume of 1 mL of standard cell culture medium at 5% CO₂ and 37°C, which was exchanged every 2 days. Over the next 7–14 days non-adherent cells were removed with the medium every other day and fresh standard cell culture medium was added. During cultivation by the aforementioned protocol the adherent cells represented macrophages.⁴⁶ The following substrates were added to the wells containing macrophages: 1.5 μ g of the positive control ConA (Biochrom), 75 μ g CpG and 1500 μ g of CpG-GNP containing 75 μ g CpG. Unstimulated cells in cell culture medium only and cells incubated with GNP alone served as negative controls. The macrophages were then incubated for 2 h at 5% CO₂ and 37°C. The supernatant was removed thereafter and the cells were washed twice with PBS at RT. To prove an indirect effect of CpG and CpG-GNP on PBMC through stimulated macrophages, 2×10^6 PBMCs from the same dogs were added to the wells containing macrophages and incubated for 24 h at 5% CO₂ and 37°C, and the cell culture supernatants were consequently collected and stored at –80°C.

The direct effects of CpG and CpG-GNP on PBMC were explored by stimulating 2×10^6 PBMCs from the same dogs without the intermediate presence of macrophages, using the stimulants and negative controls as specified above. The PBMC culture supernatants were collected after incubation for 24 h and then stored at –80°C until use.

Quantification of cytokines

Cell culture supernatant concentrations of IFN- γ , IL-4, IL-6 and IL-10 were measured by Canine Cytokine Milliplex (Merck Millipore, Darmstadt, Germany) according to manufacturer instructions. The cytokines were quantified by a Bio-Plex 200 reader (Bio-Rad, Munich, Germany) equipped with the Luminex xMAP technology detection system. Calculation of the result was performed with the Bio-Plex Manager Software 4.1.1. (Bio-Rad). The sensitivity limit was 0.2 pg/mL and an eight-point standard curve from 0.2 to 3.200 pg/mL was used.

Statistical analysis

To compare the cytokine production in the various groups a repeated measures ANOVA (or with data not distributed normally a Friedman test) with Dunn's post test was used. For comparison of the data between groups an ANOVA (or a Kruskal–Wallis test with data not normally distributed) with Dunn's post test was used. The level of significance was set to $P < 0.05$. The statistical analyses were performed with Prism 5.0 d (Graphpad Software, San Diego, USA).

Results

Formulation quality control

The GNP featured a hydrodynamic diameter of 247 nm (± 1.13 nm) before and 243.1 nm (± 2.51 nm) after loading. The zeta potential measured in 10 mM NaCl remained constant at +18.0 mV and at a conductivity of 1.18 mS/cm. The surface loading of CpG remained at 98% (m/m) or higher 2 h after complexation.

Uptake of CpG-GNP by macrophages of healthy dogs

The cellular uptake of the CpG-loaded GNP formulations in PBMCs was confirmed by CLSM and is shown in Figure 1. The CpG and GNP appeared as fluorescent yellow and red spots, respectively, or as a mixture thereof within the cells taking up CpG-GNP, which were characterized by a blue-stained nucleus and a green cell membrane.

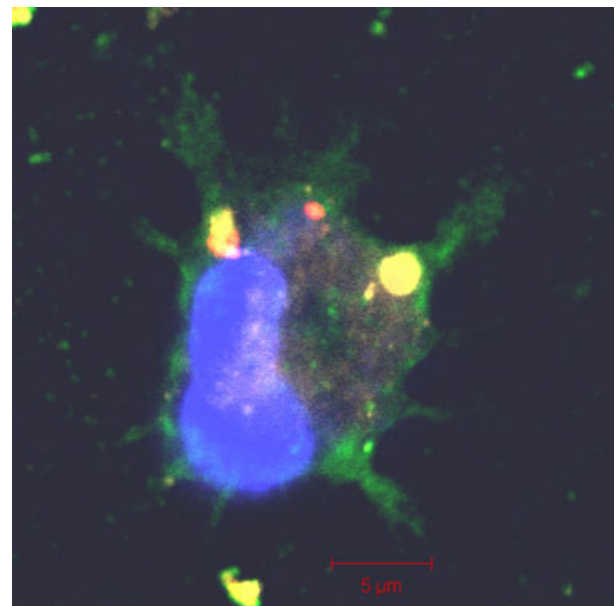


Figure 1. Cellular uptake of cytosine-phosphate-guanine (CpG; yellow) and gelatin nanoparticles (GNP; red) formulations by macrophages of healthy dogs. The nucleus of the macrophages is stained blue and the cell membrane is stained green.

The yellow of the CpG was brighter than the red of the GNP in some incidences owing to the more precise wavelength excitation provided by the CLSM device's lasers. Moreover, visual inspection from various perspectives of three-dimensional reconstructions of the recombined optical cuts prepared by the CLSM software showed that the yellow and red signals were covered by the green signal (i.e. located within the cells that had taken up the dye).

Quantification of cytokines

There were no significant differences in IFN- γ (Figure 2) and IL-4 (Figure 3) concentrations between the positive and negative control and the CpG and the CpG-GNP formulations tested in both the directly and indirectly stimulated PBMC group. Interferon- γ was released by PBMCs

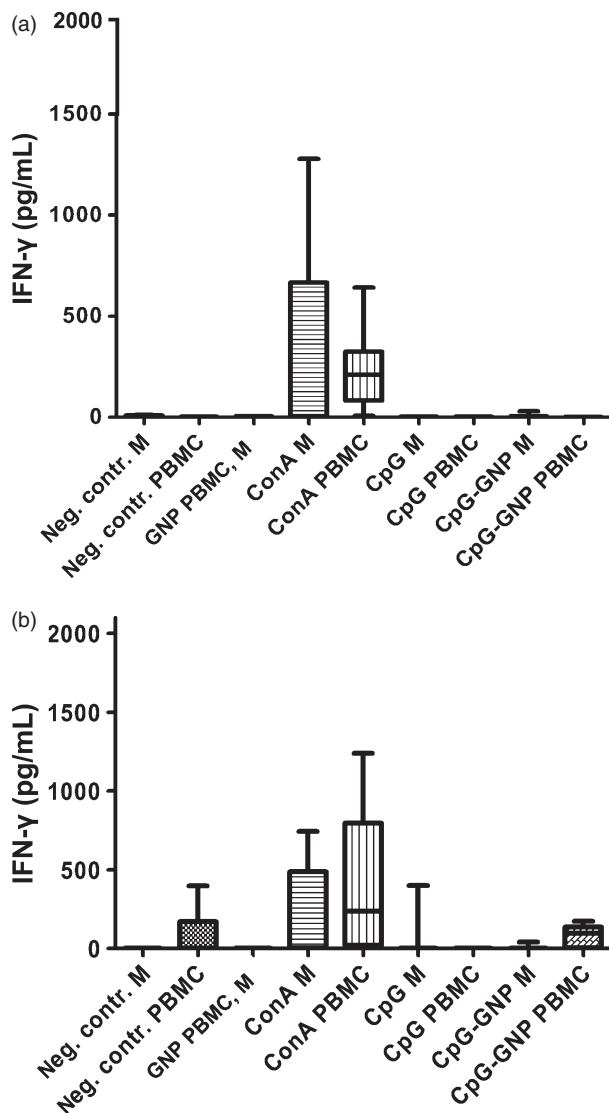


Figure 2. (a) Interferon-gamma (IFN- γ) concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells of dogs with atopic dermatitis. (b) Interferon gamma (IFN- γ) concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells (M) of healthy dogs. ConA, Concanavalin; neg. contr., negative control; PBMC, directly stimulated peripheral mononuclear cells; M, indirectly stimulated peripheral mononuclear cells.

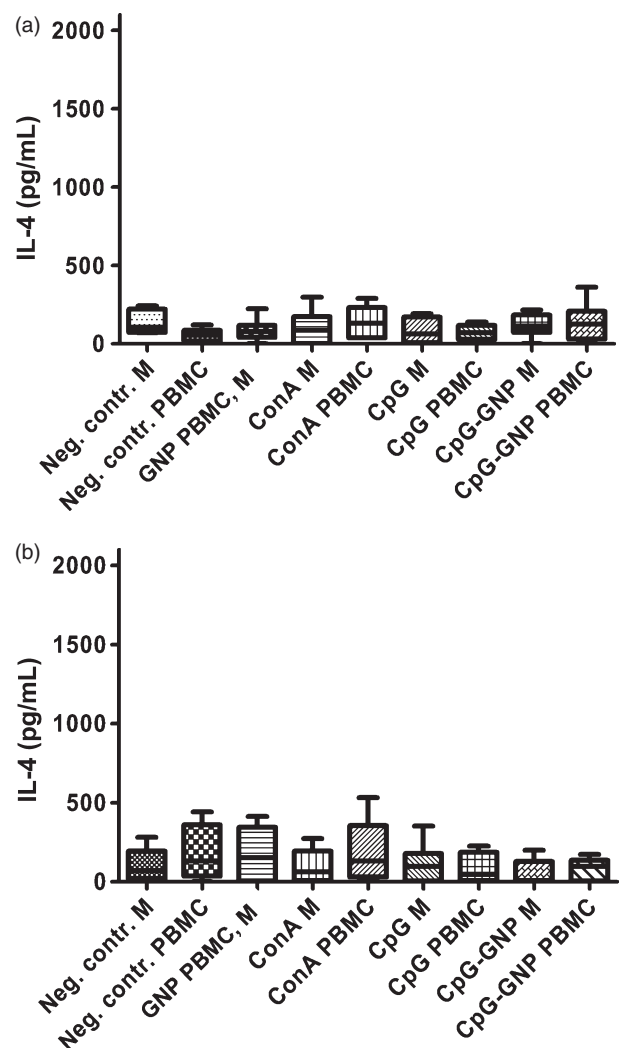


Figure 3. (a) Interleukin-4 (IL-4) concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells of dogs with atopic dermatitis. (b) Interleukin-4 (IL-4) concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells of healthy dogs. ConA, Concanavalin; neg. contr., negative control; PBMC, directly stimulated peripheral mononuclear cells; M, indirectly stimulated peripheral mononuclear cells.

only after direct and indirect stimulation with the positive control ConA in atopic and healthy dogs, but there was no statistically significant release of IFN- γ mediated by CpG alone or CpG-GNP.

Indirect stimulation of PBMCs by macrophages, which were stimulated by ConA, GNP alone, CpG alone and CpG-GNP did not yield any significant changes in the *in vitro* concentration of IL-10 (Graph 3a and 3b). In contrast, direct stimulation of PBMCs by CpG-GNP resulted in significantly higher concentrations of IL-10 when compared with negative controls and with CpG alone in healthy and atopic dogs (Figure 4). In addition, in atopic dogs ConA significantly increased concentrations of IL-10 in comparison with the negative control and with CpG alone, but not compared with CpG-GNP (Figure 4a). For healthy dogs, release of IL-10 upon stimulation with ConA did not significantly differ from CpG alone or CpG-GNP (Figure 4b). Gelatin nanoparticles did not induce any cytokine release compared with negative controls. As data for cytokine

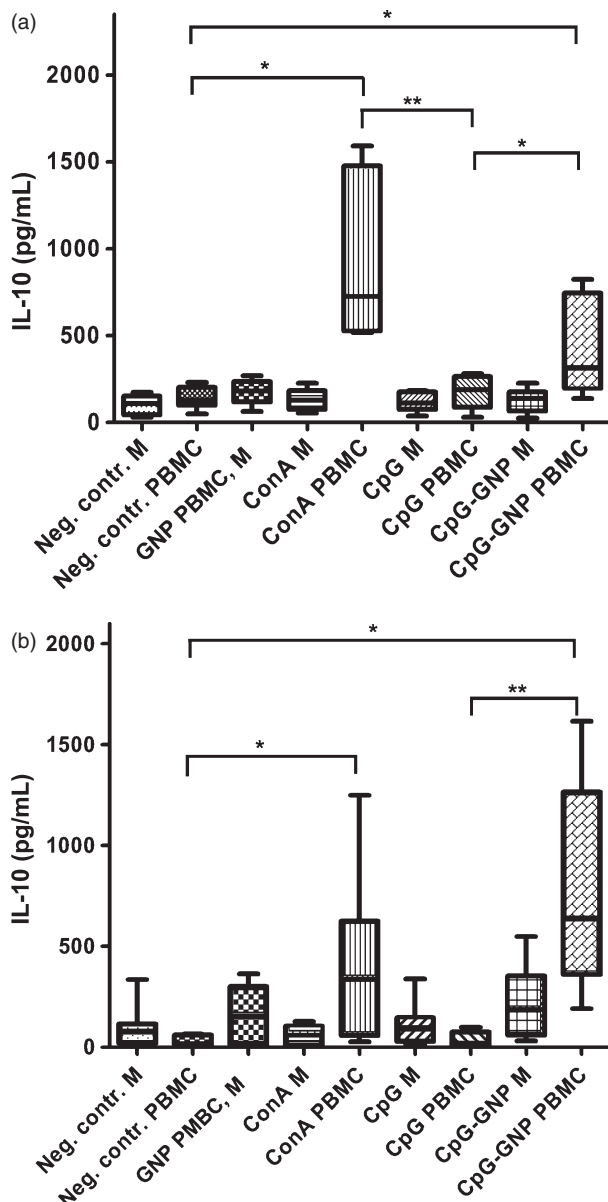


Figure 4. (a) Interleukin-10 (IL-10) concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells of dogs with atopic dermatitis. (b) Interleukin-10 concentration in supernatants of directly and indirectly stimulated peripheral blood mononuclear cells of healthy dogs. ConA, Concanavalin; neg. contr. negative control; PBMC, directly stimulated peripheral mononuclear cells; M, indirectly stimulated peripheral mononuclear cells; * $P < 0.05$; ** $P < 0.01$

release by GNP did not differ in direct (PBMC) and indirect (M) stimulation, this data was combined in one column in Figures 2–4.

The IL-6 concentration was below the detection threshold in all groups (atopic and healthy animals, direct and indirect stimulation, respectively) and, consequently, could not be evaluated.

Discussion

Previous proliferation assays showed that CpGs alone are taken up by canine immune cells.²⁵ In this study, uptake of CpG-GNP by canine immune cells was visualized

directly by CLSM. This demonstrated that uptake is a precondition for CpG and/or CpG-GNP-related immunological effects. Furthermore, the present findings for CpG-GNP uptake by canine PBMCs confirm previous uptake studies involving murine dendritic cells.³⁹

In vitro immunological effects of CpG-GNP or CpG alone on canine PBMCs were compared with controls in two different populations: healthy dogs and dogs with confirmed AD. A bead-based flow cytometric method was used for the measurement of cytokines.^{47,48} There was no significant increase in concentrations of any of the cytokines analysed after indirect stimulation of PBMCs through macrophages, which were initially stimulated with ConA, CpG-GNP, GNP alone or CpG alone. This result is in accord with those of House *et al.*¹⁴ who showed that TLR9 is not the primary PRR pathway in canine macrophages. The production of cytokines is mainly controlled by RNA and proteolytic processing.⁴⁹ Therefore, degradation of pre-existing cytokines in the 24 h incubation time is possible but rather unlikely as cytokine production was shown in directly stimulated PBMCs subjected to the same incubation conditions. Based on the demonstrated uptake of CpG and CpG-GNP after 2 h, it is unlikely that the stimulation time for macrophages as inducers for a PBMC response was too short. Similarly, a low viability of cells in the macrophage cultures seems improbable as, in atopic and healthy dogs, production of IFN- γ was detected after stimulation with ConA. This observation is in accord with the previous finding that macrophages require other partner cells for an effective response to CpG.¹⁰ A weak point in this study was that we did not use GpC ODN to show that this effect was induced specifically by the CpG sequence. As a previous study had already showed that the immunostimulatory effects of the CpG ODN sequence used here were superior to the GpCs we did not consider it to be necessary to include them.²⁴

There were no significant changes in IL-4 concentrations after direct and indirect PBMC stimulation with CpG-GNP or CpG compared with the negative controls. The first *in vivo* study in canine atopic patients using liposome-plasmid DNA complexes showed a significant decrease in serum IL-4 and pruritus scores.²⁶ Although IL-4 is a key mediator in the pathogenesis of AD it cannot always be detected in atopic individuals.⁵⁰

The most significant finding was a statistically significant increase in IL-10 concentrations in healthy and atopic dogs after direct PBMC stimulation with CpG-GNP compared with CpG alone and negative control. In addition to the Th2 imbalance, which seems to be predominant in the acute phase of AD, the expression of the anti-inflammatory cytokine IL-10 was shown to be suppressed in humans suffering atopic dermatitis.⁵ As this cytokine is known to suppress Th1 and Th2 cells, its induction via CpGs could be one possible therapeutic approach for AD. Furthermore, it was previously shown that clinical improvement after allergen-specific immunotherapy in atopic dogs was associated with elevated IL-10 concentrations in addition to decreased serum IgE.³¹

The higher IL-10 production after CpG-GNPs stimulation shows that GNPs can enhance the immunomodula-

tory effects of CpG in dogs, as it was shown previously in mice.³⁷ This provides preliminary evidence for the use of GNP-formulated CpG in canine species. Similar *in vivo* effects were recently reported in horses.⁴¹ However, stimulation with GNP alone did not result in any release of cytokine, which is in line with earlier findings from human and murine immune cells.³⁷

The source of IL-10 could be B cells, dendritic cells and/or Treg cells.⁵¹ In this study an ODN sequence with a fully phosphorothioated backbone was used and probably enhanced the CpG action on B cells, which, as a consequence, produced more IL-10.¹⁷ This backbone modification was selected to achieve increased resistance to endonucleases, increased half-life and thus greater immunomodulatory effects. No release of IL-6 was detected in this study. This is of particular importance as IL-6 was shown previously to have a suppressive effect on Treg cells.⁵² Owing to the fact that Treg cells are the primary mediators of peripheral tolerance, playing a pivotal role in modulating chronic inflammatory diseases⁵³, a downregulation by released IL-6 would have been detrimental to the desired development of immunotolerance in the treatment of dogs with AD.

In contrast to previous reports, IFN- γ was not detected after stimulation with CpG.^{22,24} This fact needs to be elucidated in further studies, but could result from inhibition of IFN- γ production by high IL-10 concentrations. Interleukin-10 reduces proliferation of DCs, blocks IL-12 secretion and thus suppresses IFN- γ indirectly.^{54,55}

The fact that IL-6 could not be detected in any of the groups could be because of the low sensitivity of the Milliplex methodology for this cytokine, as shown previously.⁵⁶ However, our results show that this test procedure seems to be promising and we recommend that this test should be re-evaluated in future studies in the canine species.

The concurrent use of CpG with a GNP carrier increased IL-10 production by PBMCs in both atopic and healthy dogs. Results from this study suggest that CPG and GNP could be an additional treatment option for canine AD. Further clinical studies testing the efficacy, safety and optimal dosing schedule are warranted.

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Résumé

Contexte – Les oligodéoxynucléotides cytosine-phosphate-guanine (CpG) offrent une nouvelle approche immunothérapeutique prometteuse de la dermatite atopique (AD) à la fois chez l'homme et chez l'animal. Les nanoparticules de gélatine (GNP) améliorent et prolongent les effets immunomodulateurs associés aux CpG et minimisent les effets secondaires *in vitro* et *in vivo*. On ne connaît aucune information sur les effets de cette combinaison chez le chien.

Hypothèses/Objectifs – Le but de cette étude était d'évaluer les effets immunologiques de CpG couplé à GNP sur les cellules mononucléées sanguines périphériques (PBMCs) *in vitro*.

Sujets – Huit chiens avec AD, diagnostiquée par les critères standards et avec une hypersensibilité immédiate concomitante aux acariens de poussière de maison ont été inclus. Des échantillons de contrôle ont été prélevés sur huit chiens sains du même âge sans antécédents ou de signes de maladie systémique ou cutanée.

Méthodes – Les cellules mononucléées sanguines périphériques des chiens sains et allergiques ont été incubées avec CpG-GNP et l'adjonction de CpG-GNP a été évaluée par microscopie laser confocale. Les concentrations de surnageant de culture cellulaire d'interféron gamma (IFN- γ), d'interleukine (IL)-4, IL-6 et IL-10 ont été mesurées par Canine Cytokine Milliplex.

Résultats – Aucune modification significative en IFN- γ et IL-4 n'a été trouvée en comparaison avec les PBMCs incubées avec CpG et CpG-GNP avec les contrôles négatifs chez les chiens atopiques et sains. L'interleukine-6 n'a été détectée pour aucun des groupes. Cependant, une augmentation statistiquement significative en concentration d'IL-10 a été trouvée après 24h de stimulation avec CpG-GNP comparé à CpG seul, à la fois chez les chiens atopiques et sains.

Conclusions et importance clinique – Comme l'IL-10 est considérée comme une cytokine immunosuppressive jouant un rôle clé dans la tolérance périphérique, la formulation rapportée CpG-GNP pourrait être une nouvelle approche du traitement de l'allergie.

Resumen

Introducción – los oligodeoxinucleótidos citosina-fosfato-guanina (CpG) ofrecen una nueva forma de tratamiento inmunoterapéutico frente a la dermatitis atópica (AD) en seres humanos y en animales. Las nanopartículas de gelatina (GNP) aumentan y prolongan los efectos inmunomodulatorios asociados a CpG y minimizan los efectos adversos tanto *in vitro* como *in vivo*. La información acerca de los efectos de esta combinación en perros no está disponible

Hipótesis/Objetivos – el propósito de este estudio fue evaluar los efectos inmunológicos de CpG combinado con GNP en células mononucleares de sangre periférica (PBMCs) *in vitro*.

Animales – se incluyeron en el estudio ocho perros con AD diagnosticada con criterios estándar y con hipersensibilidad inmediata concurrente a ácaros del polvo. Las muestras control se obtuvieron de ocho perros sanos de edad similar sin historia clínica ni evidencia de enfermedades cutáneas o sistémicas.

Métodos – células mononucleares de sangre periférica de perros sanos y alérgicos se incubaron con CpG-GNP y la internalización de CpG-GNP se demostró utilizando microscopia confocal de barrido de láser. Se evaluaron las concentraciones de interferón gamma (IFN- γ), interleukina 4 (IL-4), IL-6 y IL-10 mediante un Milliplex de citoquinas caninas.

Resultados – no se observaron diferencias significativas en las concentraciones de IFN- γ ni de IL-4 cuando se incubaron PBMCs con CpG y con CpG-GNP con los controles negativos de perros atópicos y sanos. No se detectó IL-6 en ninguno de los grupos. Sin embargo se observó un incremento estadísticamente significativo en las concentraciones de IL-10 tras 24 hr de estimulación con CpG-GNP comparado con CpG en solitario tanto en perros atópicos como en perros sanos.

Conclusiones e importancia clínica – ya que la IL-10 se considera una citoquina inmunosupresora que juega un papel importante en la inducción de tolerancia periférica, la formación de CpG-GNP puede ser un nuevo método para el tratamiento de la alergia.

Zusammenfassung

Hintergrund – Cytosin-Phosphat-Guanin (CpG) Oligodeoxynukleotide bieten sich als neue immuntherapeutische Herangehensweise für die atopische Dermatitis (AD) sowohl beim Menschen als auch beim Tier an. Gelatin Nanopartikel (GNP) verstärken und verlängern den CpG-assoziierten immunmodulatorischen Effekt und minimieren sowohl *in vitro* als auch *in vivo* Nebenwirkungen. Entsprechende Informationen über die Auswirkungen dieser Kombination fehlen bei Hunden.

Hypothese/Ziele – Das Ziel dieser Studie war es, die immunologischen Auswirkungen von CpG, gekoppelt an GNP, auf die peripheren Blutmononukleazellen (PBMCs) *in vitro* zu untersuchen.

Tiere – Acht Hunde mit AD, die anhand von Standardkriterien und mittels begleitender Allergie vom Soforttyp auf Hausstaubmilben diagnostiziert worden waren, wurden inkludiert. Kontrollproben wurden von acht gesunden, alters-angepassten Hunden ohne einer Anamnese oder Evidenz einer Haut – oder systemischen Erkrankung, genommen.

Methoden – Periphere Blutmononukleazellen von gesunden und allergischen Hunden wurden mit CpG-GNP inkubiert und die Aufnahme von CpG-GNP durch die Zellen mittels konfokaler Laser-Scanning-Mikroskopie demonstriert. Die Konzentrationen von Interferon Gamma (IFN- γ), Interleukin (IL)-4, IL-6 und IL-10 wurden mittels Canine Zytokine Milliplex im Überstand gemessen.

Ergebnisse – Es wurden keine signifikanten Veränderungen bei IFN- γ und IL-4 beim Vergleich der PBMCs, die mit CpG und CpG-GNP inkubiert worden waren, mit den Negativkontrollen von atopischen und gesunden Hunden, gefunden. Interleukin-6 wurde in keiner der Gruppen gefunden. Trotzdem wurde ein statistisch signifikanter Anstieg der IL-10 Konzentration nach 24 stündiger Stimulation mit CpG-GNP im Vergleich mit CpG alleine, sowohl bei atopischen wie auch bei gesunden Hunden, gefunden.

Schlussfolgerungen und klinische Bedeutung – Da IL-10 als immunsuppressives Zytokin betrachtet wird, das eine Schlüsselrolle in der peripheren Toleranz spielt, könnte die CpG-GNP Formulierung eine neue Herangehensweise bei der Behandlung von Allergien darstellen.

要約

背景 - シトシン-リン酸-グアニン (CpG) オリゴデオキシヌクレオチドはヒトと動物のアトピー性皮膚炎 (AD) に対する新規の有望な免疫療法となりうる。ゼラチンナノ粒子 (GNP) は *in vitro* および *in vivo* の両方において CpG 関連性免疫調整効果を増強かつ延長し、また有害事象を最小限にする。イヌにおけるこの組み合わせの効果に関する情報は少ない。

仮説/目的 - この研究の目的は *in vitro* でイヌ末梢血単核細胞 (PBMCs) における GNP と結合した CpG の免疫学的な効果を評価することである。

供与動物 - 標準的な基準で診断しハウスダストマイトへの再発性即時型過敏反応を示す8頭のADのイヌを組み入れた。コントロールは皮膚あるいは全身性の疾患の兆候や病歴のない、8頭の健康で年齢の合致したイヌから採取した。

方法 - 健康およびアレルギー犬の末梢血単核細胞を CpG-GNP とともに培養し、CpG-GNP の吸収は共焦点レーザー走査顕微鏡を用いて証明した。インターフェロンガンマ (IFN- γ)、インターロイキン (IL)-4、IL-6、IL-10 の細胞培養上清濃度を Canine Cytokine Milliplex を用いて測定した。

結果 - アトピー犬と健康犬において CpG および CpG-GNP とともに培養した PBMC と陰性コントロールを比較したとき、IFN- γ および IL-4 の有意な変化は認められなかった。インターロイキン-6 はいずれの群でも検出されなかった。しかしながら、アトピー犬および健康犬の両方において CpG-GNP で 24h 刺激した後の IL-10 濃度は CpG 単独刺激したものと比較し、統計学的に有意な増加が認められた。

結果および臨床的な重要性 - IL-10 は末梢での免疫寛容において鍵となる役割を果たす免疫抑制性サイトカインと考えられているため、報告された CpG-GNP の組み合わせはアレルギー治療において新しい提案となるかもしれない。

摘要

背景 - 胞嘧啶磷酸鸟嘌呤 (CpG) 寡脱氧核苷酸有望が异位性皮炎 (AD) 患者和患病动物提供一种新型免疫治疗方法。凝胶纳米粒子 (GNP) 可增强并延长 CpG 相关的免疫调节效应，且体内和体外不良反应最小。犬的这种组合功效的资料很少。

假设/目的 - 本研究旨在评估 CpG 与 GNP 联合对犬外周血液单核细胞 (PBMCs) 的体外免疫效应

动物 - 8 只 AD 患犬，通过标准条件诊断，以及并发户尘螨的速发型过敏反应。从 8 只年龄相仿的，没有病史、皮肤或全身疾病症状的健康犬上取得对照样本。

方法 - 于 CpG-GNP 中培养健康犬和过敏犬的外周血液单核细胞，使用共聚焦激光扫描显微术证实 CpG-GNP 摄取。通过多种犬细胞因子测量细胞培养上清液的干扰素 γ ，白介素 (IL)-4，IL-6 和 IL-10 浓度。

结果 - 异位性皮炎和健康犬 PBMCs 的 CpG 与 CpG-GNP 培养阴性对照相比，未发现 IFN- γ 和 IL-4 有显著变化。所有组均未检测到白介素-6。然而与单独使用 CpG 相比，CpG-GNP 刺激 24 小时后发现，异位性皮炎患犬和健康犬的 IL-10 的浓度统计学上显著增加。

结论和临床价值 - 认为 IL-10 是一种在外周耐受中起重要作用的免疫抑制细胞因子；CpG-GNP 合剂可能成为治疗过敏症的一种新方法。