



## Research paper

# Immunostimulation of bronchoalveolar lavage cells from recurrent airway obstruction-affected horses by different CpG-classes bound to gelatin nanoparticles

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## ABSTRACT

Recurrent airway obstruction (RAO) in horses has become a common problem in stabled horses in industrialized countries and deserves new therapeutic strategies. CpG-oligodeoxynucleotides (CpG-ODNs) were developed as effective immunostimulating agents to induce a Th2/Th1 shift. These agents showed a beneficial therapeutic effect in allergic diseases with predominant Th2 immunoresponse. CpG-ODN delivery by gelatin nanoparticles (GNPs) resulted in enhanced cellular uptake in murine and human *in vitro* studies and was a starting point for the present trial.

The aim of this study was to identify an optimal stimulating CpG motif in horses with regard to species specificity on equine bronchoalveolar lavage (BAL) cells, in terms of a possible specific immunomodulation effect (Th2/Th1 shift) by used CpG-ODN. Accordingly, GNPs were evaluated as a delivery system to improve CpG-ODN immunostimulation in equine BAL cells.

BAL fluid (BALF) was obtained from seven horses with moderate RAO and from four healthy horses and was subsequently incubated with five different CpG-ODN sequences (from A-, B- and C-class) and one ODN without any CpG motif. Release of three key cytokines (IL-4, IL-10 and IFN- $\gamma$ ) was quantified by ELISA to detect an allergy mediated Th2 immunoresponse (IL-4) as well as a proinflammatory Th1 response (IFN- $\gamma$ ). Due to its specific anti-inflammatory and anti-allergic effects, IL-10 was considered as a beneficial agent in pathophysiology of RAO.

Results showed a significant upregulation of IL-10 and IFN- $\gamma$  on the one hand and a downregulation of IL-4 on the other hand in RAO affected horses. Cell cultures from healthy horses had a significantly stronger response in cytokine release to all the applied stimuli in contrast to RAO derived cells. Comparing all five CpG sequences, A-class 2216 significantly showed the highest immunomodulatory effects on equine BALF cells and, hence, was chosen for follow-up preliminary clinical studies.

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**Abbreviations:** BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CpG-ODN, cytosine-phosphate-guanosine-oligodeoxynucleotides; CpG-GNP, GNP-bound CpG-ODN; GNP, gelatin nanoparticle; HPW, high purified water; RAO, recurrent airway obstruction; pDC, plasmoidal dendritic cell; TLR-9, toll-like receptor 9; Treg, T regulatory cells.

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## 1. Introduction

In the last decades, allergic airway diseases have dramatically increased in the northern hemisphere in industrialized countries in horses and humans (Kline, 2007; Braun-Fahrlander, 2009). Recurrent airway obstruction (RAO) has become one of the most common airway diseases in horses (Fey, 2006). Housing of horses in stables with permanent exposure to potentially allergenic organic and inorganic particles was reported to be a major trigger factor (Schmallenbach et al., 1998; Robinson, 2001; Millerick-May, 2009). Keeping horses on pasture leads to improvement of clinical signs, however, complete avoidance of allergens is not always possible (Robinson, 2001). The permanent inhalation of various antigens from mouldy hay, mite dust as well as endotoxin,  $\beta$ -glucan and other organic and inorganic particles causes airway neutrophilia and inflammation with a mixed Th1/Th2 immune response (Cordeau et al., 2004; Horohov et al., 2005). Although the clinical signs of RAO are well defined (Robinson, 2001), immunological mechanisms are controversially discussed till the present. However, a predominant Th2 allergic response was recently presumed (Horohov et al., 2009). In several studies, signs of predominant Th2 response such as high IL-4, IL-5 in bronchoalveolar lavage fluid (BALF) of RAO horses exposed to antigens were reported (Lavoie et al., 2001; Cordeau et al., 2004). Due to persistent chronic inflammatory reaction in small airways of the affected horses, Th1 participation was also confirmed (Ainsworth et al., 2003). Recently, proinflammatory and chemotactic IL-17 from Th17 cells has been demonstrated to be involved in the progression of RAO pathology (Debrue et al., 2005; Ainsworth, 2009).

As a potential therapeutic option for RAO (unmethylated CpG-ODN) have been explored (Fonseca and Kline, 2009). CpG-ODNs were introduced as effective immune stimulating agents to cause a Th2/Th1 immune shift (Kline, 2007). This shift further promotes an immunoglobulin isotype switch from IgE to IgG2 (Bohle, 2002). The prevalence of IgE is a matter of ongoing debate (Halliwell et al., 1993; Marti, 2009). In addition, a shift from pro-allergy mediating IL-4, IL-5 and IL-13 Th2 cytokines towards pro-inflammatory IFN- $\gamma$  and IL-12 Th1 cytokines was observed. Anti-inflammatory and antiallergic properties of Th2 cytokine IL-10 turned out to be of interest. Particularly IL-10 producing T regulatory cells (Tregs), and its balance towards Th2 cells, seemed to play an important role in immune homeostasis in the lung and thus could be used for therapy of RAO (Akdis et al., 2004; Hawrylowicz, 2009). Consequently, besides a conventional symptomatic therapy, the possibility to modulate cytokine level in order to avoid development of an allergic hypersensitivity may be a better therapeutic option.

Three distinctive CpG classes (A-, B- and C-class) with varying immunologic effects were previously investigated (Krieg, 2002). The key pattern-recognition receptor for these “danger signals” is the Toll-Like receptor 9 (TLR-9) which is located in endosomes (Krieg, 2002). Recently, equine TLR-9 was detected in monocytes, airway epithelial cells, capillary endothelium in the lungs and pulmonary intravascular macrophages (Schneberger et al., 2009). Fur-

thermore, species-specific immune stimulation depending on CpG motifs has been determined (Hartmann and Krieg, 2000; Rankin et al., 2001).

The beneficial use of delivery systems like lipid nanoparticles or gelatin nanoparticles (GNPs) to protect ODNs from degradation by nucleases and to enhance cellular uptake was recently demonstrated (Wilson et al., 2009; Zwioerek et al., 2008). GNPs from gelatin type A, obtained from porcine skin, were found to be immunologically inert (Zwioerek et al., 2008). The use of GNPs showed amplified CpG-ODN-related TLR-9 activation (Zwioerek et al., 2008).

Consequently, the aim of this study was to identify the optimal CpG motif to stimulate equine BAL cells *in vitro* for the first time and to detect specific immunomodulating effects (Th2/Th1 shift) on the cells derived from RAO and healthy horses treated with GNP-delivered CpG-ODN.

## 2. Materials and methods

### 2.1. Horses and physical lung examination

Based on published schemes (Gerber et al., 2004) horses were evaluated for clinical parameters (nasal discharge, coughing, breathing rate and thoracic percussion), blood gas chemistry, exercise examination, endoscopic exploration, cytology of tracheobronchial secretion (TBS) and bronchoalveolar lavage fluids (BALF). Patients were grouped into four categories of healthy horses and horses with mild, moderate and severe RAO. In the present study eleven horses (eight trotter, one pura raza espanol, one warmblood and one knobstrupper) within an age range of 6–22 years and a weight range of 470–560 kg were scored. Two groups were established, whereby the first group consisted of seven horses with mild to moderate RAO (mean age = 14.6 years) and the second group included four healthy horses (mean age = 12.3 years).

### 2.2. Oligodeoxynucleotides

To evaluate the optimal stimulating CpG motif in cultured equine BAL cells, three different CpG-ODN classes, with previously employed motifs in horses were compared. Five different CpG-ODNs and one ODN without a CpG motif were selected (Biomers GmbH, Ulm, Germany). Each CpG-ODN class was represented by two different sequences, except the A-class where only one sequence was available. All CpG-ODN classes were single stranded ODNs with a length of 20–30 bases. ODN 2041 (5'-CTG GTC TTT CTG GTT TTT TTC TGG-3') was used as a CpG-free sequence. The A class differs in backbone structure from the other classes. It consists of a backbone chimera of phosphorothioate\* (PS) and phosphodiester (PD) modified deoxyribose: CpG-ODN A 2216 (5'-G\*G\*G GGA CGA TCG TCG\*G\*G\*G\*G-3'). Two different B-classes were compared: CpG-ODN B 2142 (5'-TCG CGT GCG TTT TGT CGT TTT GAC GTT-3') and CpG-ODN B 2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3'). The C-class was represented by CpG-ODN C 2395: (5'-TCG TCG TTT TCG GCG CGC GCC G-3') and CpG-ODN C M362: (5'-TCG TCG TCG TTC GAA CGA CGT TGA T-3').

### 2.3. Preparation of ODN loaded GNP

The GNPs were prepared, according to the standard protocol of Coester et al. (2000), using porcine-derived gelatin type A Bloom 175, glutaraldehyde 25% solution, cholamine and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). All starting materials were purchased from Sigma (Taufkirchen, Germany). Cationization of the GNP dispersion (2 mg/ml) was performed with equimolar amounts of cholamine and EDC catalyst. The reaction mixture was incubated for 30 min, at room temperature with constant stirring at 500 rpm. The solution pH was adjusted to 4.7. For purification, particles were centrifuged at  $18,000 \times g$  for three times as well as washed and subsequently filtered through a membrane with 1.2  $\mu\text{m}$  pore size (Pall Life Sciences, Ann Arbor, USA). The ODNs were then bound as prepared onto positively charged GNPs by electrostatic interaction as described earlier (Zwiorek et al., 2008). Particle size and surface charge (Zeta potential) were quantified in 10 mM NaCl by a Zetasizer ZS Nano (Malvern Instruments, Malvern, UK). Loading of ODNs onto GNPs surface was conducted by adding 5% (w/w) of ODNs to GNP dispersions for best colloidal stability (Zwiorek et al., 2008).

A working concentration of 2.5 mg/ml for GNPs and 1.0 mg/ml for ODNs was used. In brief, 76.5  $\mu\text{l}$  of GNP stock solution was diluted by 230  $\mu\text{l}$  highly purified water (HPW) and mixed with 43.8  $\mu\text{l}$  of CpG-ODN stock solution by gentle stirring to obtain a final ODN concentration of 0.125 mg/ml. As references, 0.125 mg/ml CpG-ODN solution of each class (306.5 ml HPW, 43.8  $\mu\text{l}$  CpG-ODN stock solution) and 2.5 mg/ml GNP dispersion (274  $\mu\text{l}$  HPW, 76.5  $\mu\text{l}$  GNP stock solution) were prepared. All the samples were incubated for 90 min at room temperature with constant stirring at 300 rpm in a Thermomixer (Eppendorf, Hamburg, Germany). Thereafter, samples were ready for use. The samples were stored at 4°C and used within 48 h.

### 2.4. Preparation of cells and cell culture

BALF (50 ml sterile, warm, isotonic NaCl solution per 100 kg) was taken from every horse and centrifuged immediately by  $1200 \times g$  for 6 min. Supernatant was stored at  $-80^\circ\text{C}$  to conserve actual cytokine concentration. Total cell count was determined by manual counting using trypanblue with a Neubauer counting chamber (Laboroptik GmbH, Friedrichsdorf, Germany).  $2 \times 10^5$  cells (mixture of neutrophil granulocytes, alveolar macrophages and lymphocytes) in 230  $\mu\text{l}$  RPMI medium (Biochrom AG, Berlin, Germany) per well in a 96 well plate (Techno Plastic Products, Trasadingen, Switzerland) were incubated for 24 h at  $37^\circ\text{C}$  in 5% (v/v)  $\text{CO}_2$  atmosphere. The RPMI cell culture medium was supplemented with 10% FCS, 67.8  $\mu\text{g}/\text{ml}$  penicillin and 113  $\mu\text{g}/\text{ml}$  streptomycin.

### 2.5. Immunostimulation by ODN and cytokine quantification (ELISA)

Five different CpG-ODN sequences of three different classes and one ODN lacking a CpG motif, as described

above, were incubated with the equine BAL cells in triplicate. In detail, 0.275 mg GNPs loaded with 13.5  $\mu\text{g}$  (5%, w/w) ODN or 13.5  $\mu\text{g}$  of soluble ODN were added per well to compare the effects of unbound ODNs and ODNs bound to GNPs. To estimate the immunostimulating response of ODNs in cell culture, supernatant was taken after 24 h of incubation and analyzed by equine ELISAs (R&D Systems, Minneapolis, USA). Three key-cytokines namely IL-4, IL-10 and IFN- $\gamma$  were evaluated. The ELISAs were performed according to the manufacturer's protocol. Limits of detection of the applied ELISA assays were 15.6–2000, 156.25–20,000 and 31.2–4000 pg/ml, respectively. Given values were corrected by subtraction of untreated BAL cells, which served as a negative control.

### 2.6. Cell viability by MTT-assay

MTT assays were performed to evaluate cell viability following incubation with CpG-ODN and GNP-bound CpG-ODN. After removal of supernatant cell pellets were immediately resuspended in 300  $\mu\text{l}$  MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) working solution consisting of 82% PBS, 9% FCS and 9% of a 5 mg/ml MTT stock solution. Then 0.135 mg MTT reagent was added per  $2 \times 10^5$  cells. After 2 h of incubation at  $37^\circ\text{C}$  and in 5% (v/v)  $\text{CO}_2$  the culture plates were centrifuged at  $1200 \times g$  for 6 min. The supernatant was discarded and the remaining pellets were resuspended in 200  $\mu\text{l}$  DMSO to solubilize violet formazan crystals. The absorbance of each well was measured at 530 nm using wallac Victor<sup>2</sup> 1420 multilabel counter (Perkin Elmer, Fremont, CA, USA). Untreated BAL cells served as a reference for 100% viability. All measured experiments were conducted in triplicate.

### 2.7. Quantification of BAL cell subpopulations

To evaluate the percentile fraction of BAL cell subpopulations (macrophages, lymphocytes and neutrophil granulocytes) from RAO-affected and healthy horses, in each case 1 ml BAL was centrifuged two times at  $1200 \times g$  for 6 min to obtain cell pellets which were transferred to cytospot glass slides. Subsequently, they were stained using diff-quick® staining set (Medion diagnostics, Dürdingen, Switzerland) and 300 cells respectively were counted manual under microscope. The percentile fraction of macrophages, lymphocytes and neutrophil granulocytes was identified; mean values and S.D. out of at least three counts, respectively, were determined.

### 2.8. Statistical analysis

Data from cytokine quantification by ELISAs and MTT assays were analyzed using the Prism 5 software (Graphpad software Inc., La Jolla, USA). An unpaired Student *t*-test was employed to estimate significant differences between two independent groups such as stimulation of cells gained from RAO-affected versus healthy horses.  $P < 0.05$  was considered as statistical significant (displayed as \*,  $P < 0.01$  as \*\*,  $P < 0.001$  as \*\*\*) data from experiments and the data is reported as mean  $\pm$  S.D.

### 3. Results

#### 3.1. Quality control and formulation study of GNP-bound CpG-ODN

Particle sizes and size distribution determined before and after loading exhibited a mean particle diameter of 250 nm and homogenous size distributions with polydispersity index values throughout below 0.15. The loading efficiency of CpG-ODN was at least 98% (w/w).

After 48 h GNP-bound CpG-ODN formed visible aggregations and cytokine stimulation was reduced significantly. Therefore, all GNP formulations were freshly prepared and used immediately for the experiments.

#### 3.2. Cytokine release in cell culture upon stimulation by CpG-ODN/GNP-bound CpG-ODN

##### 3.2.1. IL-10 release

In order to compare the effect of GNP-bound CpG-ODN versus soluble CpG-ODN on cytokine release from BAL cells, six sequences of ODNs (to include five CpG-ODNs) were tested. Throughout, IL-10 showed the highest release in absolute concentration values of the three quantified cytokines. As a general observation, cells from healthy horses secreted significantly ( $P=0.0047$ ) higher amounts of IL-10 than those of RAO-affected horses. In Fig. 1a IL-10 stimulation of soluble CpG-ODN is shown. Out of the six different ODNs, CpG 2216 (A-class) showed the highest IL-10 release for both examined groups. IL-10 release was 400 pg/ml in cells from healthy horses and 220 pg/ml in those from RAO horses. The B and C class triggered only low levels of IL-10 release in RAO-derived cells, with concentrations of about 125 pg/ml. C-classes appeared to result in higher stimulation (350 pg/ml) than B-class (200 pg/ml) in cell culture from healthy horses. The employed B-classes (ODN 2142 and ODN 2006) and C-classes (ODN M362 and ODN 2395) showed almost identical release behavior in cell culture from RAO horses. In Fig. 1b IL-10 stimulation by GNP-bound CpG-ODNs is shown. Most noticeable was a significant higher release of IL-10 of the cells from healthy horses ( $P=0.0051$ ) compared to RAO-affected horses. Furthermore the GNP-bound CpG-ODN 2216 (A-class) induced the highest IL-10 concentration (540 pg/ml) which surpassed the release value by soluble CpG-ODNs as shown in Fig. 1a. On the contrary the stimulation by other particle-bound CpG-ODNs (B- and C-class) was lower than those by soluble CpG-ODN.

##### 3.2.2. IL-4 release

In contrast to IL-10, IL-4 release upon stimulation by six soluble ODNs did not differ ( $P=0.614$ ) between RAO and healthy horses derived cells (Fig. 2a). Both absolute values were low and inter and intra variations were high. However, as shown in Fig. 2b, difference of high significance ( $P=0.001$ ) was found when comparing GNP-bound CpG-ODN stimulated cell cultured from RAO and healthy horses for IL-4 release. The latter resulted in negative values (Fig. 2b) while those of RAO-affected horses were positive and not distinguishable from those provoked by soluble CpG-ODN ( $P=0.9469$ ). Furthermore GNP-bound CpG-ODN

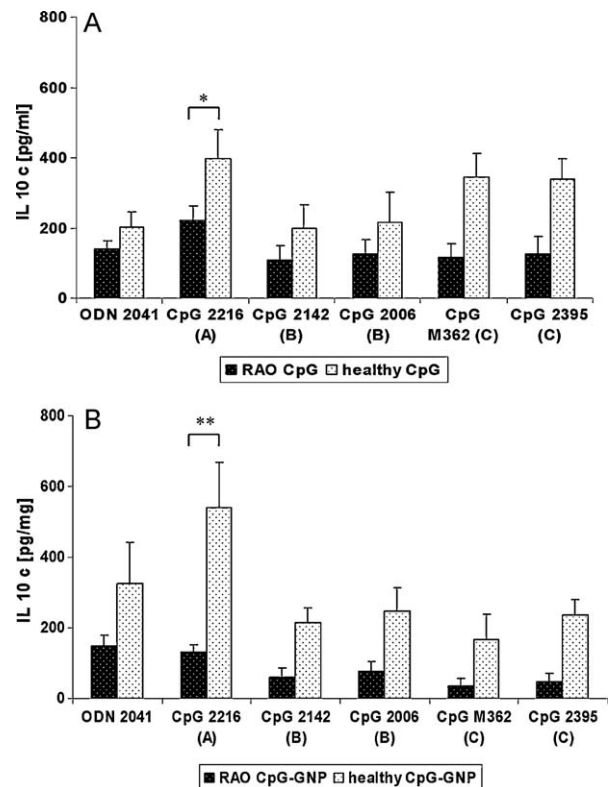


**Fig. 1.** IL-10 expression from BALF cells from healthy and RAO-affected horses with different CpG classes. BAL cells derived from RAO-affected horses (individuals  $n=7$ , measuring points  $n=3$ ) (black bars) or healthy horses (individuals  $n=5$ , measuring points  $n=3$ ) (white bars) were incubated with  $13.5 \mu\text{g}$  of soluble CpG-ODN (A) or GNP-bound CpG-ODN (CpG-GNP) (B). Cells from healthy horses secreted significantly higher amounts of IL-10 than those of RAO-affected horses ( $P=0.0047$ ). CpG 2216 A triggered the highest IL-10 release for both cells groups. Depicted values are group means  $\pm$  S.D. and corrected by subtraction of individual negative controls (untreated BAL cells).

lowered IL-4 release of cells of healthy horses considerably more than soluble CpG-ODN ( $P=0.0018$ ) (Fig. 2a).

##### 3.2.3. IFN- $\gamma$ release

In Fig. 3 IFN- $\gamma$  release from RAO and healthy horses derived cell cultures is displayed after stimulation by soluble CpG-ODNs. On average, no significant difference could be observed between the mean values of cells from RAO-affected and healthy horses ( $P=0.3514$ ). However, the six employed ODNs revealed distinctive effects. As seen for IL-10, the highest release was induced by CpG 2216 A-class and accounted for  $94 \pm 10$  pg/ml in RAO and  $135 \pm 19$  pg/ml in healthy horses derived cell cultures. All other ODNs induced lower amounts between 15 and 50 pg/ml (Fig. 3a). A tendency towards higher release by healthy horse derived cells could be assumed but was not statistically significant (Fig. 3a). In contrast, GNP-bound ODNs led to a clear discrimination between RAO and healthy derived cells in terms of IFN- $\gamma$  in Fig. 3b ( $P=0.008$ ). Here, CpG 2216 A-class stimulated the highest IFN- $\gamma$  secretion as well, accounting for  $76 \pm 14$  pg/ml (healthy) and  $25 \pm 10$  pg/ml (RAO). In this individual example (CpG 2216),

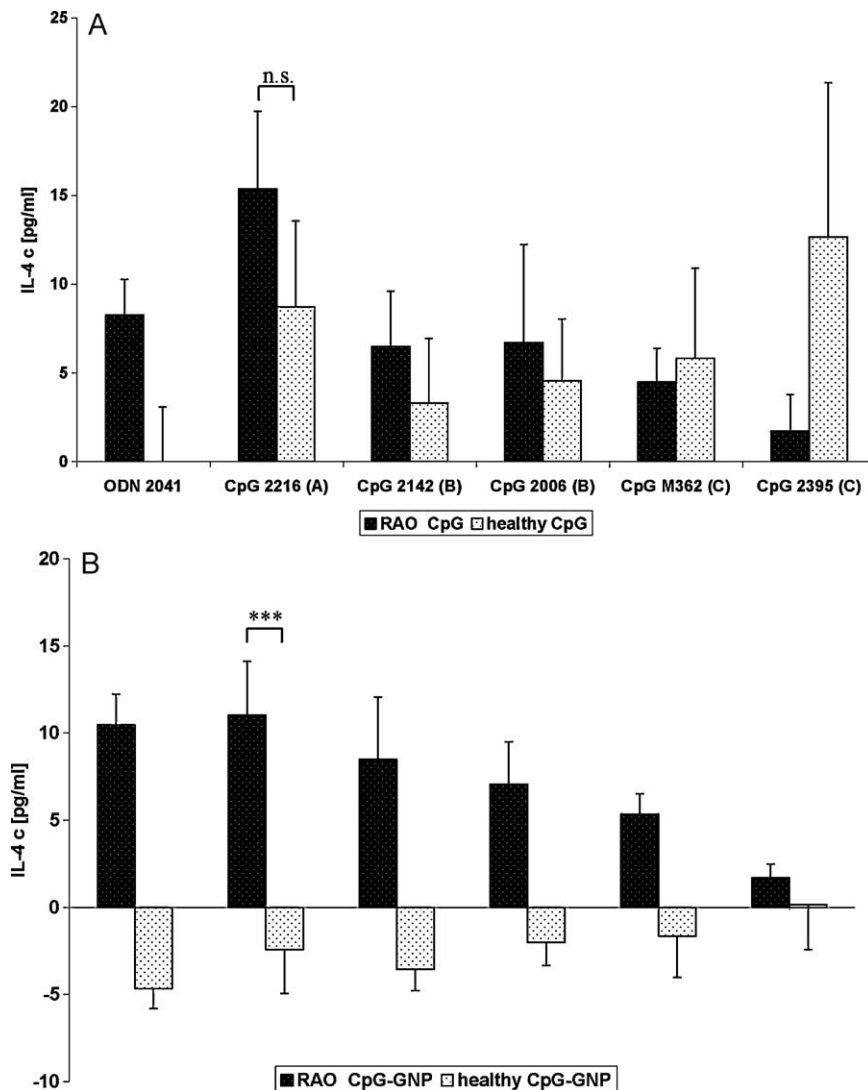


**Fig. 2.** IL-4 expression from BALF cells from healthy and RAO-affected horses with different CpG classes. BAL cells derived from RAO-affected horses (individuals  $n = 7$ , measuring points  $n = 3$ ) (black bars) or health horses (individuals  $n = 5$ , measuring points  $n = 3$ ) (white bars) were incubated with  $13.5 \mu\text{g}$  of soluble CpG-ODN (A) or GNP-bound CpG-ODN (CpG-GNP) (B). Cells from healthy horses secreted significantly lower amounts of IL-4 than those of RAO-affected horses ( $P = 0.001$ ) when treated with GNP-bound CpG-ODN (CpG-GNP) (B). Incubation with soluble CpG-ODN did not result in such significantly distinguishable release (A). Depicted values are group means  $\pm$  S.D. and corrected by subtraction of individual negative controls (untreated BAL cells) which particularly resulted in negative values. The original IL-4 ELISA results were positive before correction.

a marginally statistically not significant difference was found ( $P = 0.05$ ). Also, no or slight release from RAO cell cultures was found (Fig. 3b), while no statistical significance occurred between the healthy derived cell cultures treated by CpG-ODN in comparison with GNP-bound CpG-ODN ( $P = 0.45$ ).

### 3.3. MTT assay

BAL cells from RAO-affected and healthy horses were used to investigate *in vitro* cell viability after 24 h of incubation with regard to detectable differences after exposition to soluble CpG-ODNs compared to GNP-bound CpG-ODNs. Four mean values were averaged out of the individual viability results of the six employed ODNs: cells from

RAO horses treated with CpG-ODN, cells from RAO horses treated with GNP-bound CpG-ODN, cells from healthy horses treated with CpG-ODN and cells from healthy horses treated with GNP-bound CpG-ODN (Fig. 4). Untreated BAL cells were considered as 100% viability reference. In the healthy group, no significant differences were detectable in viability between soluble CpG-ODN and GNP-bound CpG-ODN administration. Moreover, no significant differences were seen between the six ODNs examined individually. Of all evaluated means of the four above mentioned groups, lowest viability was observed in cells from RAO-affected horses treated with soluble CpG-ODN ( $69.7 \pm 6.6\%$ ). In comparison, cells from RAO-affected horses treated with GNP-bound CpG-ODN showed the highest viability, on average  $104.3 \pm 6.4\%$ , which was significantly higher. As an

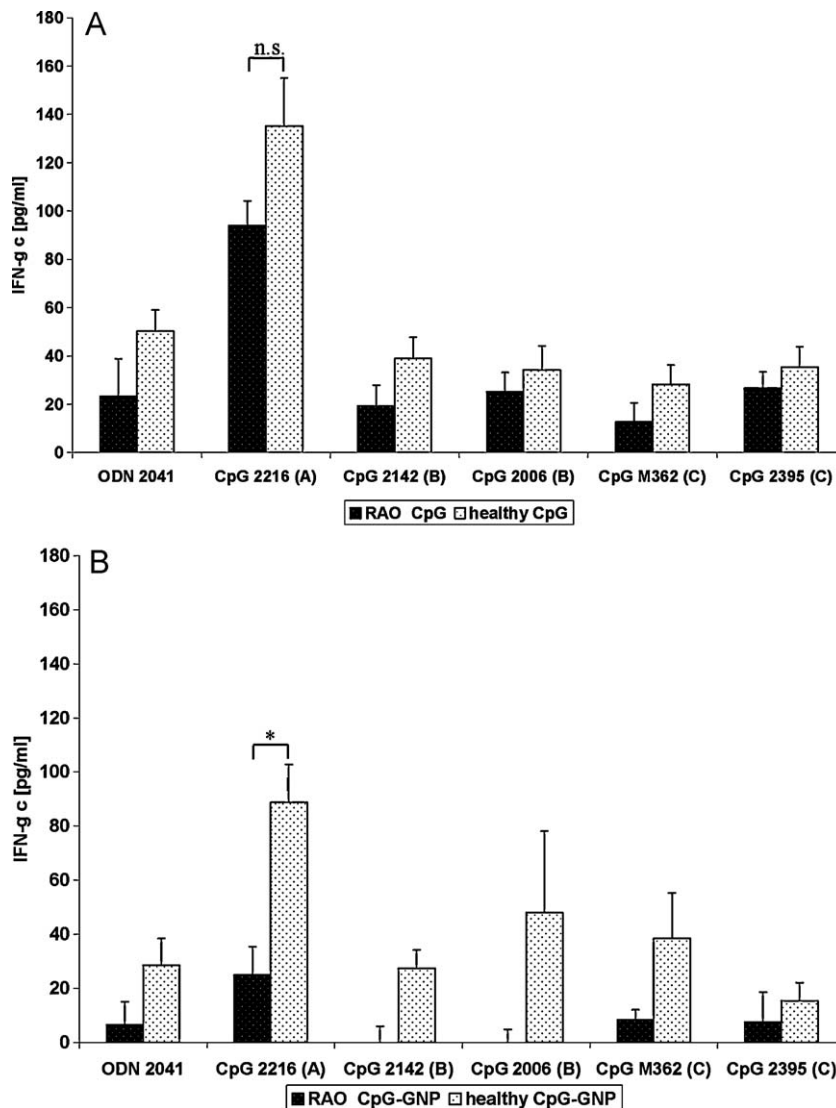


**Fig. 3.** IFN- $\gamma$  expression from BALF cells from healthy and RAO-affected horses with different CpG classes. BAL cells derived from RAO-affected horses (individuals  $n=7$ , measuring points  $n=3$ ) (black bars) or health horses (individuals  $n=5$ , measuring points  $n=3$ ) (white bars) were incubated with 13.5  $\mu\text{g}$  of soluble CpG-ODN (A) or GNP-bound CpG-ODN (CpG-GNP) (B). Cells from healthy horses secreted significantly higher amounts of IFN- $\gamma$  than those of RAO-affected horses ( $P=0.008$ ) when treated with GNP-bound CpG-ODN (CpG-GNP) (B). Incubation with soluble CpG-ODN did not result in such significantly distinguishable release (A). CpG-ODN 2216 A-class triggered overall highest IFN- $\gamma$  concentrations. Depicted values are group means  $\pm$  S.D. and corrected by subtraction of individual negative controls (untreated BAL cells).

example, difference between cells treated with CpG-ODN 2216 A-class and cells treated with GNP-bound CpG-ODN 2216 A-class was found to be significant ( $P=0.0405$ ) as indicated in Fig. 4. However, mean viability of cells from healthy horses exposed to soluble CpG-ODN and to GNP-bound CpG-ODN was  $94.2 \pm 4.0\%$  and  $91.7 \pm 8.0\%$ , respectively, which did not involve any significant difference ( $P=0.512$ ). Likewise, no significant difference was identified e.g. in the CpG-ODN 2216 A-class ( $P=0.5319$ ). Cells derived from RAO-affected and healthy horses did not differ in viability when treated with GNP bound CpG-ODN 2216 A-class ( $P=0.808$ ). Summarizing, BAL cells gained from RAO-affected horses showed significant higher cell viability in MTT assay when incubated with GNP-bound

CpG-ODN in comparison to soluble CpG-ODN. This difference was not seen in assays with cells gained from healthy horses.

### 3.4. Quantification of BAL cell subpopulation

In healthy horses, BALF cell population in healthy horses consisted of 50% ( $\pm 9.53$ ) macrophages, 42% ( $\pm 9.81$ ) lymphocytes and 7% ( $\pm 7.31$ ) neutrophils, respectively, without considering epithelial cells. In BALF of RAO-affected horses, 37% ( $\pm 8.72$ ), 50% ( $\pm 11.94$ ) and 12% ( $\pm 13.16$ ) were determined, respectively. For comparison, lymphocytes range 30–50%, neutrophils 0–8% and macrophages 45–70% in healthy horses' BALF (Bain, 1997).

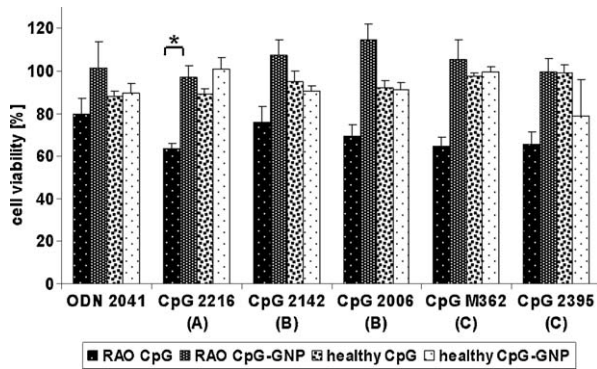


**Fig. 4.** MTT-assay of BALF cell viability. Four groups mean values  $\pm$  S.D. of cell viability obtained by MTT assay are depicted from left to right: cells derived from RAO-affected horses treated with soluble CpG-ODN or with GNP-bound CpG-ODN (CpG-GNP) followed by cells derived from healthy horses treated with soluble CpG-ODN or with GNP-bound CpG-ODN (CpG-GNP). A statistically significant difference ( $P < 0.0001$ ) occurred when comparing soluble CpG-ODN versus GNP-bound CpG-ODN (CpG-GNP) impact on RAO derived cells. No difference was prevalent for healthy horses' derived-cells.

#### 4. Discussion

One major aim of this study was to identify optimal stimulating CpG-ODN motifs in equine BAL cells, concerning Th2/Th1 shift via TLR-9 activation. Optimal TLR-9 agonist sequences were reported to be 5'-GACGTT-3' in murine (Bauer et al., 2001) and 5'-GTCGTT-3' in human PBMCs (Hartmann and Krieg, 2000). The motif 5'-GTCGTT-3' was found to be beneficial for different animal species including the horse (Rankin et al., 2001). In contrast, CpG-ODN sequences containing this motif (2142 and 2006 B-class) were not most effective in stimulating equine BAL cells in the present study. This contrary could probably be due to different prevalent cell populations between PBMCs and BAL cultures. For horses, Ólafsdóttir et al. (2004) described an ideal sequence 5'-CTCGTT-3' which has not been confirmed by other groups yet. Due to deficiency of knowledge in the action of various CpG motifs on equine BAL cells, motifs with a record of any equine employment were chosen and their distinctive effects were compared. Previously CpG-ODN B 2142 and CpG-ODN C 2395, as well as ODN 2041, were used *in vitro* only in equine PBMCs cultures which is a known and well established model in contrast to BAL cells (Liu et al., 2008, 2009). However, as RAO is located in small airways the authors decided to harvest cells from this region to investigate effects on this specialized group of lung cells.

This is the first study which compared the effects of all three CpG-ODN classes *in vitro* on equine BAL cells. Furthermore, two different motifs per class except for the A class were investigated to gain more insight into motif depending CpG-ODN immunostimulation on equine BAL cells.

With regard to the results, the optimal stimulating sequence among five explored CpG-ODNs in equine BAL cells was the A-class 2216. The detectable IL-10 upregulation was higher than expected as CpG-ODNs were previously known for potent IFN- $\gamma$  release in general and CpG-ODN A-class for IFN- $\alpha$  release in particular (Krieg,

2002). Characteristics of the A-class which could influence the efficacy are its exceptional backbone structure (backbone chimera), palindrome motif and poly guanine tail which all contributes to a special structure-effect interaction (Krieg, 2006). The actual IL-10 cytokine release profile was related to heterogeneous cell composition in BAL (macrophages, neutrophil granulocytes and lymphocytes). TLR-9 receptors were identified as the primary target of CpG-ODNs (Krieg, 2002). Via the TLR-9 pathway CpG-ODN 2216 (A-class) induced human pDCs which activated CD4<sup>+</sup> CD 25<sup>-</sup> towards IL-10 producing CD4<sup>+</sup> CD 25<sup>+</sup> by direct cell contact (Moseman et al., 2004).

All six ODNs treated BAL cells from healthy horses showed higher releases of IL-10 and IFN- $\gamma$  as compared to cells from RAO-affected horses. This was observed regardless of the formulations (CpG-ODN, GNP-bound CpG-ODN). The most likely explanation is a difference in cell types. As RAO is expected to be associated with Th2 upregulation (Horohov et al., 2009), less stimulation or possibly a lack of Th1 and Treg effects is likely to happen. On the contrary, healthy individuals have normal regulatory T cell capability which is demonstrated in this study by high levels of IL-10 in stimulated cell cultures of healthy horses.

All three cytokines detected in BAL cell cultures obtained from healthy horses could serve as important parameter for non allergic lungs under homeostatic immunologic conditions without excessive Th2 response. Therefore, detected cytokine concentrations in healthy individuals could be regarded as reference for a CpG-ODN based immunotherapy of RAO-affected individuals.

With regard to the observation that no significant difference of IL-10 release within B- and C-class by cells from RAO-affected horse was detectable, we could hypothesize that the class is more determining than the individual CpG-ODN sequence.

IL-10 decreases IL-4, IL-5 and IFN- $\gamma$  (van Scott et al., 2000; Taylor et al., 2006) and could therefore be beneficial to interfere with chronic allergic and inflammatory pathophysiology of RAO. With regard to an expected upregulation of Th1 cytokine by CpG-ODN, IFN- $\gamma$  release was relatively low. A possible explanation could be the high amounts of IL-10 which could have inhibited IFN- $\gamma$  release.

The absolute values of IL-4 concentration in cell cultures were considerably low and below the detection limit of 15.6 pg/ml by the ELISA standard curve. However, raw data values were higher before being corrected by subtracting the untreated BAL cell result considered as a negative control. Therefore the observed trend that cells from healthy horses exhibited lower IL-4 release than cells from RAO-affected horses was considered convincing with regard to earlier findings (Cordeau et al., 2004; Horohov et al., 2005). This is in accordance with the presumption of Th2 downregulation, according to expected Th2/Th1 shift and probably also due to high IL-10 amounts with negative feedback on IL-4 release. These results support the idea of CpG-ODN prominent role in the downregulation of allergic Th2 cytokines (Bohle, 2002; Vollmer and Krieg, 2009). Furthermore, GNPs obviously discriminated CpG-ODN action towards IL-4 release in healthy horses' derived cells.

CpG-ODNs also induce B cells to produce IL-10, which acts in a counter regulatory fashion to limit the inflam-

matory response to CpG-ODN (Kline and Krieg, 2001). This would be beneficial in chronic inflammatory response like RAO. It is known that a pure Th1 response with IFN- $\gamma$  could contribute to exacerbation of allergic diseases (Umetsu and DeKruyff, 2006). IFN- $\gamma$  is also produced in lungs of patients with asthma and seems to contribute to severity of the allergic reaction (Umetsu and DeKruyff, 2006). Th1 cells on their own do not counterbalance Th2 cells but even could augment inflammatory reactions in lungs in an *in vivo* model (Umetsu and DeKruyff, 2006). On the contrary, IFN- $\gamma$  was found to contribute to inhibitory effects in asthma and allergy in combination with suppressive IL-10 from Tregs (Stock et al., 2004).

The MTT assay revealed that various formulations had distinctive impacts on viability of tested BAL cells. The most important observation was the significant difference between soluble CpG-ODN and GNP-bound CpG-ODN treated cells of RAO-affected horses. The GNP-formulated CpG-ODNs were less cytotoxic on average for the cell population present in the RAO BAL which is in accordance with earlier findings for other cells (Bourquin et al., 2008). Values above 100% could be interpreted as higher viability than untreated BAL cells because of GNP-bound CpG-ODN dependent stimulation which resulted in prolonged individual survival rates. Total cell numbers of RAO-affected horses were higher in comparison to healthy horses, however turn over rate was higher as well. In summary, GNPs were shown as an effective and biocompatible nucleotide delivery system in an equine *in vitro* study. Furthermore, GNPs are expected to protect short-lived active A-class from otherwise early degradation (Senti et al., 2009) in a currently conducted follow-up *in vivo* study.

Results of CpG-ODN treated cells derived from healthy horses were significantly reduced compared to the reference but still higher than values of cells derived from RAO-affected cells. However, both groups' viabilities remained far below those of all cells treated with a GNP-bound CpG-ODN formulation. This is evident from the IL-10 release profiles characterized by significantly higher release from healthy horses-derived cells than from cells from RAO-affected horses. On the contrary, higher viability of GNP-bound CpG-ODN treated cells did not correspond to higher IL-10 or IFN- $\gamma$  release. One explanation could be an overstraining phenomenon of the regulatory cytokine producing cells, which were supposed to be present in lower number in RAO BAL than in BAL of healthy horses. The difference in treatment response could be a proof of distinction of RAO and healthy cells on cytokine level *in vitro*. Due to the fact that cell isolation kits for equine BAL cells are not commercially available, it was not possible to stimulate separately isolated cell types for quantification of cytokines.

Although cell typing data covering macrophages, overall lymphocytes and neutrophils suggest a trend towards elevated lymphocyte and neutrophil percentiles in BALF obtained from RAO-affected horses, the differences are not significant compared to healthy horses. This might be due to considerable variations in low to moderate-level of disease. Thus, no significant correlation between cytokine release and individual cell population within BALF could be established. However, it should be taken into account that

the measured cytokines are produced by a variety of cells beyond types T- and B-lymphocytes (macrophages, mast cells, monocytes, natural killer cells and ceratinocytes). Moreover, the percentage amount of subtypes within the lymphocytes might have changed while the total amount remained constant.

The study offered comparable conditions as *in vivo* where a heterogenic composition of relevant cells is present. Therefore, intercellular interaction could probably be better reflected to finally predict *in vivo* therapeutic effects. Interestingly, previous human pediatric studies found that numbers of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells appeared in decreased amounts in children suffering from asthma (Hartl et al., 2007). Extrapolating this finding to the present equine scenario, a lowered number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in BALF of RAO-affected horses could have caused the observed depletion in IL-10 expression.

In conclusion, CpG-ODN 2216 A-class was identified as most efficient stimulating motif out of the six examined ODNs to trigger IL-10 and IFN- $\gamma$  release in equine BALF cells. As a potent delivery system GNPs, were partially able to enhance CpG-ODN impact (IL-4 and IL-10 in cells from healthy horses) and was found to be biocompatible. Consequently, administration of GNP-bound CpG-ODN by inhalation is currently under examination as an effective anti hypersensitivity therapy instead of conventional symptomatic treatment  $\beta_2$ -sympathomimetic agents, mucolytic agents and corticosteroids.

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