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RESEARCH PAPER

A Nebulized Gelatin Nanoparticle-Based CpG Formulation is Effective in Immunotherapy of Allergic Horses

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ABSTRACT

Purpose In the recent years, nanotechnology has boosted the development of potential drug delivery systems and material engineering on nanoscale basis in order to increase drug specificity and reduce side effects. A potential delivery system for immunostimulating agents such as Cytosine-Phosphate-Guanine-Oligodeoxynucleotides (CpG-ODN) needs to be developed to maximize the efficacy of immunotherapy against hypersensitivity. In this study, an aerosol formulation of biodegradable, biocompatible and nontoxic gelatin nanoparticle-bound CpG-ODN 2216 was used to treat equine recurrent airway obstruction in a clinical study.

Methods Bronchoalveolar lavage fluid was obtained from healthy and allergic horses to quantify Th1/Th2 cytokine levels before and after inhalation regimen. Full clinical examinations were performed to evaluate the therapeutic potential of this nebulized gelatin nanoparticle-based CpG formulation.

Results Most remarkable was that regulatory anti-inflammatory and anti-allergic cytokine IL-10 expression was significantly triggered by five consecutive inhalations. Thorough assessment of clinical parameters following nanoparticle treatment indicated a partial remission of the allergic condition.

Conclusion Thus this study, for the first time, showed effectiveness of colloidal nanocarrier-mediated immunotherapy in food-producing animals with potential future applicability to other species including humans.

KEY WORDS CpG-ODN · gelatin nanoparticles · hypersensitivity · immunotherapy · inhalation

ABBREVIATIONS

BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CpG-ODN	cytosin-phosphate-guanosin-oligodeoxynucleotides
GNP	gelatine nanoparticle
HPW	highly purified water
RAO	recurrent airway obstruction
TLR-9	toll-like receptor 9
Treg	T regulatory cells

INTRODUCTION

The rationale of this study was to evaluate a new potentially curative therapeutic approach to treat an allergic disease in horses based on nanocarrier-mediated immunotherapy. A prominent self-assembling nanocarrier system which has been commercialized is chemotherapeutic albumin-bound paclitaxel Abraxane® (1). However, this formulation is composed of a nano-aggregate (2). Still no solid nanoparticle delivery system

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is commercially available and, up to today, and only few are in the stage of clinical trials (3). Particularly in drug delivery of immunotherapeutic agents, nanoparticles seem appealing as wider use of immunotherapy is urgently needed to treat or prevent increasing prevalence and severity of allergic airway diseases both in humans and in animals, such as horses (4). Such immunotherapy might be implemented by Cytosine-Phosphate-Guanine-Oligodeoxynucleotides (CpG-ODN) (5).

Like human asthma, recurrent airway obstruction (RAO) in horses is considered as a common multifactor allergic airway hypersensitivity reaction elicited by environmental exposure to potential allergens (6) and heritable components (7). A promising immunotherapeutic strategy against allergic conditions has already entered human clinical phase IIa studies involving CpG-ODN (8) as synthetic analog of natural microbial CpG-DNA (9). This Toll-like receptor 9 (TLR9) agonist demonstrated efficiency in allergic diseases due to its immunomodulating potential to cause a Th2/Th1 shift (4,5,9). This shift was associated with a downregulation of proallergic Th2 cytokines (IL-4) and an upregulation of antiallergic Th1 cytokines (IFN γ) (10).

Electrostatically GNP-bound CpG-ODNs or even RNA-oligonucleotides were proven to be protected against early degradation by nucleases via steric shielding (11–13). Furthermore, cellular uptake of CpG-ODN into target cells was enhanced by nanoparticulate delivery (11). Moreover, nebulized GNP-bound CpG-ODN was found to remain stable and effective in an *in vitro* aerosolization study. Previously, out of 6 prospective ODNs, the authors found A-class to be most potent in eliciting antiallergic cytokine releases *in vitro* from bronchoalveolar lavage fluid (BALF) cells (14) Therefore, CpG-ODN 2216 was chosen for the present clinical trial on local targeting of the lung. Furthermore, this specific CpG-ODN GNP formulation was found to be stable in physiological conditions and thus, found eligible for the present *in vivo* study.

Consequently, the chosen administration via inhalation should ensure minimal systemic side effects equivalently to known inhalative administration of corticosteroids in and circumvent known bioavailability issues regarding nanoparticulate systemic administration (12).

MATERIALS AND METHODS

Nanoparticle Production and Loading

GNPs were manufactured according to an established protocol (15) 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). The cationization of GNPs and electrostatic binding of CpG-ODN onto the particle surface has been described previously (11). Particle size and surface charge (Zeta potential) were quantified in 10 mM NaCl before and after loading by a Zetasizer ZS Nano (Malvern Instruments, Malvern, UK), and likewise before and after nebulization. The target surface loading was set to 5% (weight of CpG-ODN per weight of GNPs), which was previously shown to result in colloidal stability of the formulation (11). Consequently, the Zeta potential was measured as an indicator of colloidal stability of the prepared GNP-bound CpG-ODN dispersions. The percentile loading efficiency of CpG-ODN, refers to the initial mass of CpG-ODN, onto the GNP surface was checked photometrically and then calculated as follows, wherein OD stands for optical density:

$$\text{DNA loading} = \left(1 - \left(\frac{\text{OD of GNP} - \text{DNA supernatant} - \text{OD of DNA control supernatant}}{\text{OD of DNA control supernatant}}\right)\right) \times 100(\%)$$

Oligodeoxynucleotide

CpG-ODN A-class was synthesized by Biomers GmbH (Ulm, Germany), consisting of a single-stranded ODN with a length of 20 bases and the subsequent sequence: 5′-G*G*G GGA CGA TCG TCG* G*G*G *G*G-3′ with its specific chimera backbone structure consisting of phosphorothioate* (PS) and phosphodiester (PD) modified deoxyribose.

CpG/GNP Formulation

A working concentration of 3 mg/ml for GNPs and 1.0 mg/ml for CpG-ODNs was adjusted. For *in vitro* cell cultures, 278 μg of GNPs loaded with 14 μg of CpG-ODNs were applied per well which corresponded to 0.87 mg/ml GNP and 0.044 mg/ml of CpG-ODN. As references, CpG-ODN solutions of each group with a concentration of 0.044 mg/ml and GNP dispersion concentrated 0.87 mg/ml were added per well. For inhalation studies, 3.75 mg GNPs loaded with 187.5 μg CpG-ODN in a total volume of 2.5 ml highly purified water (HPW) were used per inhalation treatment. All GNP formulations were prepared freshly in aseptic conditions and used within two hours for the experiments.

Nebulization

For inhalation studies, an Equine Haler™ spacer (Equine HealthCare Aps, Hoersholm, Denmark, see item "A" in Fig. 1) and an AeroNeb Go™ vibrating mash (VM) nebulizer (Aerogen, Galway, Ireland, see item "B" in Fig. 1) were combined by a 90° glass connector with ground joints (see item "C" in Fig. 1) that suitably matched the aerosol generator part's outlet diameter and the spacer's inlet and allowed for constant aerosol output to be inhaled by the recipient within 5 to 10 min. The negative control (placebo) contained only an aqueous (HPW) GNP dispersion (1.5 mg/ml) while the medication (verum) consisted of the above described complex of GNPs (1.5 mg/ml) and CpG-ODN 2216 (0.075 mg/ml). Healthy as well as RAO-affected horses were inhaled three times with two-day intervals between individual administrations followed by a control BAL. In RAO-affected horses, two additional subsequent inhalations and one final BALF examination for disease development monitoring were added. Clinical symptom examinations, blood gas analysis, endoscopic exploration and cytology of tracheobronchial secret (TBS) were performed at the beginning, after three and –in RAO-affected individuals- after five inhalations.

Clinical Examination and Lung Scoring

A lung scoring system based on (16) was developed comprising clinical parameters (nasal discharge, breathing rate), blood gas chemistry, endoscopic exploration, cytology of TBS and of BALF. Hence, 12 horses of a mean weight of 477.7 kg and aged 12.0 years on average were scored. The applied scoring system allowed grouping the patients into four categories (healthy, mild, moderate and severe RAO). For the clinical trial, three groups of horses were established; the first group (n=4) consisting of healthy horses (mean age of 8.8 years) for the placebo negative control, the second group (n=4) consisting of healthy horses for compatibility study (mean age of 10.4 years) and the third group (n=4) consisting of moderate RAO-affected horses for therapeutic efficiency verification (mean age of 16.8 years). The important arterial blood gas parameter "partial pressure of oxygen" (PaO_2) was measured by a Radiometer Copenhagen NPT 7 series (Radiometer GmbH, Willich-Schiefbahn, Germany). Physiological values for PaO_2 were set to 100 mmHg (±5 mmHg). Moreover, percentages of neutrophile granulocytes from a total cell count out of the TBS cytology were calculated after staining using the Diff-Quick® staining set (Medion diagnostics, Düdingen, Switzerland). The physiological range of the breathing rate was defined as 8 to 16 breaths per minute while higher values were considered as pathological.

In addition, to provide for a preliminary safety estimation of the present therapy, potential local or systemic adverse effects were monitored: endoscopically visible local hyperaemia, follicular hyperplasia, clinical detectable signs like increased nasal discharge, coughing, bronchospasm, increased breathing rate and worsening of arterial blood gas values. Further, to evaluate systemic side effects, leucocytes, differential blood cell count, fibrinogen plasma concentration and inner body temperature were performed as commonly known in the art.

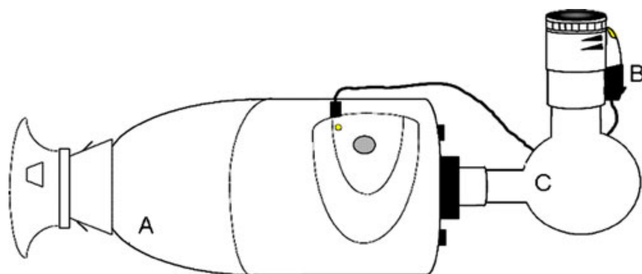

Fig. 1 Plan of the inhalation device consisting of an Equine Haler™ spacer (A) and an AeroNeb Go™ vibrating mash (VM) nebulizer (B) with respective control unit, combined by a 90° glass connector with ground joints (C).

Cell Culture

BALF was taken from each horse before and after inhalation regimen (50 ml/100 kg body weight sterile, warm, isotonic NaCl solution). The thus obtained cell suspension was centrifuged by 1200 g for 6 min. Supernatants were stored immediately at −80°C for cytokine conservation. Total cell count was determined and 2×10^5 cells were seeded per well. Incubation with the relevant six ODNs was set to 24 h. Supernatants were analyzed for IL-4, IL-10 and IFN-γ by equine duo set ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, USA).

The study was approved by the regional legal agency for animal experiments (Nr. 55.2-1-54-2531-31-10).

Statistical Analysis

Comparisons between groups (normally distributed) were carried out using the unpaired Student's *t*-test. N is shown in parenthesis for each calculation in figure captions. All statistical analysis was performed using Prism Graph (Version 5.0, GraphPad software Inc., La Jolla, USA).

RESULTS

Quality Control of GNP-Bound CpG-ODN Formulation

Particle sizes and size distribution of GNPs determined before and after loading with CpG-ODNs and nebulization

by the VM-device exhibited a mean particle diameter of about 250 nm on average and homogenous size distributions with polydispersity index values throughout below 0.15. The loading efficiency of CpG-ODN onto the GNP surface was at least 98% (weight of surface-bound CpG-ODN / weight of employed CpG-ODN). The Zeta potential of the loaded cationized GNPs was at least 20 mV in 19 mM NaCl. HPW dispersions remained visibly stable and could be nebulized without causing any obstruction to the VM-device.

Immunological Control of Inhalation Therapy Efficiency *In Vitro*

To evaluate the efficiency of envisaged inhalation therapy, BALF was obtained before and after the regimen from RAO-affected and healthy horses. Data from healthy individuals served as physiological reference. BALF cells were stimulated *in vitro* by CpG-ODN 2216. Figure 2a shows *in vitro* IL-10 expression of cells derived from RAO-affected horses treated by CpG-ODN 2216 and GNP-bound CpG-ODN 2216 both before (*pre*) and after (*post*) inhalation. After inhalation treatment, a significantly ($P<0.0001$) higher IL-10 release (390 pg/ml) in BAL cell cultures stimulated by GNP-bound CpG-ODN 2216 was observed compared to the state before inhalation treatment (83 pg/ml) (Fig. 2a). A similar trend of IL-10 release was observed after stimulation (389 pg/ml) by soluble CpG-ODN 2216 (Fig. 2a) compared to the value of 139 pg/ml before inhalation regimen ($P=0.0002$). On the other hand, IL-4 *in vitro* expression was decreased significantly after inhalations of both soluble ($P=0.0298$) and GNP-bound ($P=0.0282$) CpG-ODN 2216 (Fig. 2b). In contrast, IFN-γ release *in vitro* was low and did not result in significant changes or a general and trend after treatment by GNP-bound CpG-ODN 2216 ($P=0.1414$) or by soluble CpG-ODN 2216 ($P=0.4870$) before *versus* after inhalation treatment of RAO-affected horses.

Immunological Control of Inhalation Therapy Efficiency *In Vivo*

Figure 3a clearly depicts the increase of IL-10 expression detected in BALF supernatant in RAO-affected horses. While three inhalations led to a significant 3.8-fold increase ($P=0.0473$) in IL-10 expression, a 6.9-fold increase was found after five inhalations (Fig. 3a). Therefore, the average IL-10 levels differ significantly ($P=0.034$) before starting and after finishing the full five inhalation regimen applied to RAO-affected horses. Healthy horses exhibited a 2.14 fold augmentation in IL-10 expression after pulmonal administration of GNP-bound CpG-ODN, confirming the principle of action (Fig. 3a). However, differences in expression levels before and after inhalation were marginally statistically significant ($P=0.089$). In contrast, no significant difference ($P=0.289$) was found when comparing healthy horses before and after three inhalative administrations of blank GNPs, which were given as placebos (Fig. 3a). *In vivo* secretion of IL-4 and IFN-γ was analyzed in BALF supernatants before and after inhalation regimens. IL-4 levels were below detection threshold *in vivo*. For IFN-γ, a significant impact of

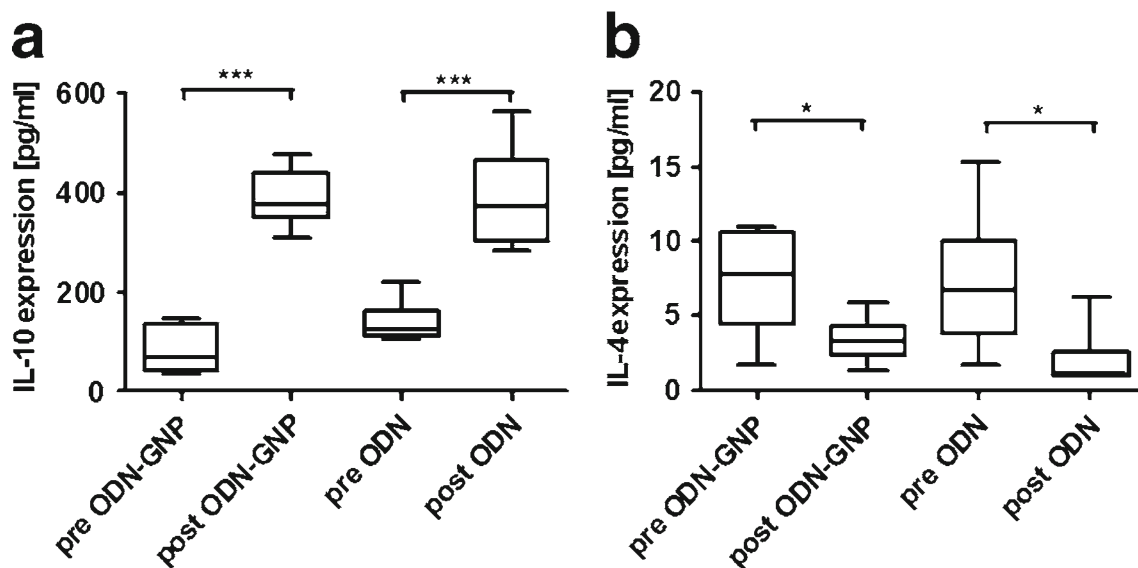

Fig. 2 *In vitro* stimulation of key cytokines by ODN-GNP from BALF cells gained from RAO-affected horses before (pre) and after (post) *in vivo* inhalation therapy, respectively. (**a**) IL-10 release from cell cultures stimulated by soluble CpG-ODN 2216 and GNP-bound CpG-ODN 2216 is increased by inhalation therapy of GNP-bound ODN and subsequent *in vitro* stimulation (in triplicate) by GNP-bound CpG-ODN 2216 or by soluble CpG-ODN 2216 on average. (**b**) IL-4 release is accordingly decreased.

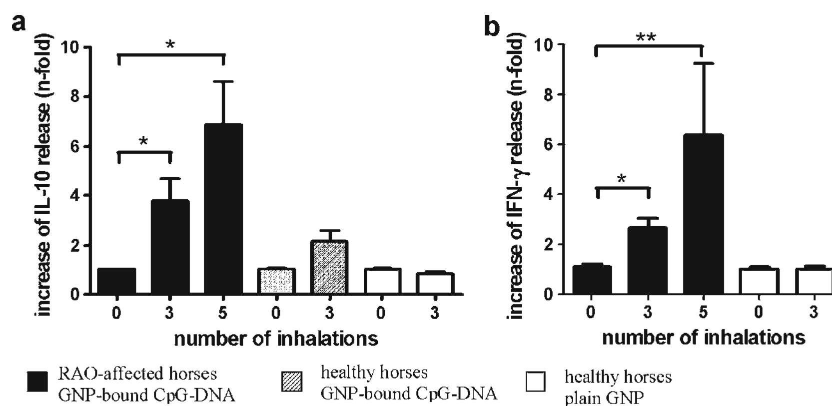

Fig. 3 Distinctive effect of GNP-bound CpG-ODN *versus* plain GNPs on cytokine release *in vivo* before and after inhalation therapy. (**a**) IL-10 release is expressed as relative n-fold increase based on 1 as initial value before inhalation. IL-10 release in *RAO-affected horses* (n=4) before and after three and five inhalation of GNP-bound CpG-ODN (black bars), from *healthy horses* (n=4) before and after three inhalations of GNP-bound CpG-ODN (grey bars), and from *healthy horses* (n=4) before and after three inhalations of *plain GNP* (white bars). (**b**) IFN-γ release in RAO-affected horses before and after three and five inhalation of GNP-bound CpG-ODN (black bars) and from healthy horses before and after three inhalations of plain GNP (white bars).

GNP-bound CpG-ODN was observed. Figure 3b reveals a constant increase after three and five consecutive inhalations compared to IFN-γ levels in BAL supernatants before the regimen (P=0.0034). Placebo administration did not result in altered cytokine expression (P=0.8322) (Fig. 3b) while IFN-γ data could not be obtained from healthy individuals treated with GNP-bound CpG-ODN.

Control of Inhalation Therapy Efficiency by Assessment of Clinical Symptoms

Generally, monitoring of the above identified adverse effects revealed no occurrence of the same. Consequently, a preliminary estimation could be drawn that the present therapy seems to be safe in horses.

The breathing rate per minute was assessed to differ healthy from RAO-affected individuals. The latter exhibited a breathing rate of 19.6 (±1.47) breaths per minute (bpm) before treatment (Fig. 4a) which was significantly higher than the measured value of 13.6 (±0.98) bpm in healthy horses (P= 0.0094). The regimen (five doses) lowered the rate significantly down to 12.8 (±0.80) bpm (P=0.0036) (Fig. 3a).

Healthy horses had a PaO_2 of 94 mmHg (±.07) (Fig. 4b). In contrast, RAO-affected horses showed a PaO_2 of 86.75 mmHg (±2.29) (Fig. 4b). This mean value was significantly improved (P=0.0153) towards 95.6 mmHg (±1.69) by the full regimen of five inhalations (Fig. 4b). Thereafter, no statistically significant difference was observed compared with the healthy animals (P=0.5384).

The percentage of neutrophile granulocytes within the TBS of RAO-affected horses was high (70%±0.50) before treatment and differed significantly from values of healthy horses exhibiting 26% (± 6.0) (P=0.0004) (Fig. 4d). Treatment with GNP-bound CpG-ODN contributed to a significant decrease down to 50% (±2.04) by three inhalations (P< 0.0001) and down to 40% (±6.52) by five inhalations (P= 0.0048), respectively (Fig. 4d). After five applications of GNP-bound CpG-ODN no statistically significant difference could be observed compared to healthy horses (P=0.195). Furthermore, Fig. 4d shows that in healthy horses neither the GNP-bound CpG-ODN (P=0.3472) nor the placebo (P=0.8171) resulted in a significant change of neutrophile percentages, respectively.

DISCUSSION

From present *in vitro* data, a major impact of inhalation therapy on RAO-affected individuals can be observed, especially in IL-10 release. According to the cytokine release profile, the increase of IL-10 could be due to the fact that the inhalation therapy triggeres anti-allergic IL-10 producing cells in BAL. The in parallel observed decreased expression of IL-4 after inhalation regimen might indicate that the Th2-mediated proallergic immune response was reduced. Although Th1 upregulation (IFN-γ) was expected after treatment with CpG-ODN, surprisingly observed low IFN-γ levels *in vitro* could be considered beneficial in chronic inflammatory processes (17). In contrast to high IL-10 release values found *in vitro* from BALF cells after inhalation treatment, low amounts of both IFN-γ and IL-4 could be regarded as a negative feedback mechanism triggered by IL-10 (18,19).

Moreover, as a key cytokine to mediate tolerance, IL-10 was of primary interest in the evaluation of cytokine expression after administration of GNP-bound CpG-ODN *in vivo*. In addition, observed increased IFN-γ expression by GNP-bound CpG-ODN met the expectation of CpG-ODN-driven Th1 activation and might partly be explained by the previously discussed Th2/Th1 shift away from Th2

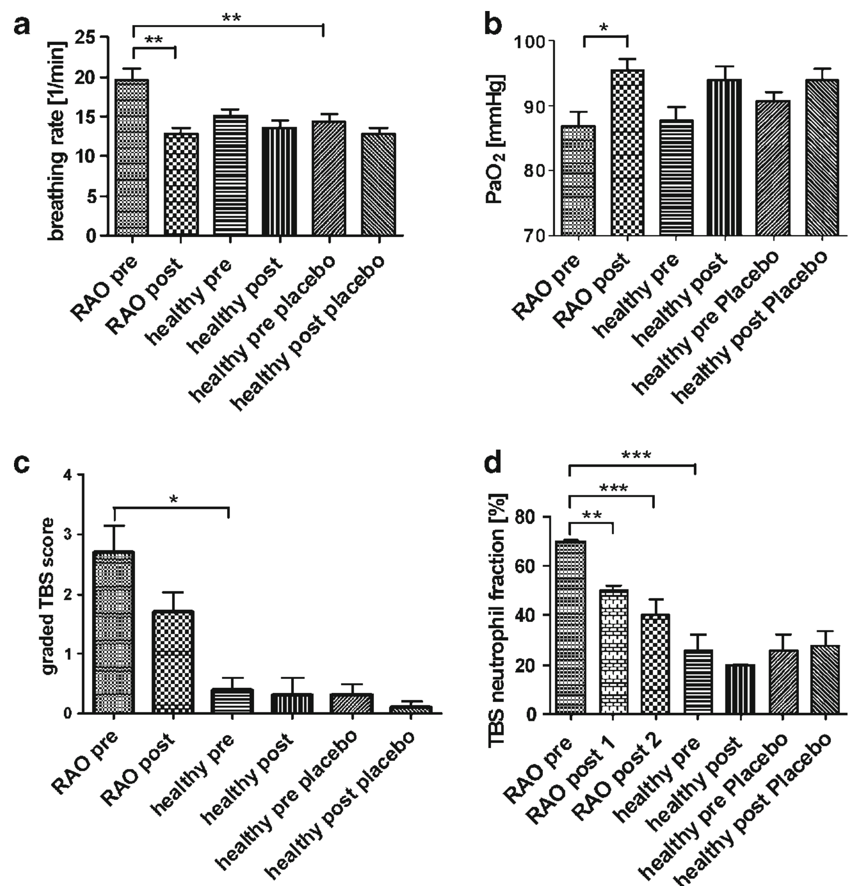

Fig. 4 Therapeutic effect of GNP-bound CpG-ODNs *versus* plain GNPs (negative control) on important clinical parameters. (**a**) Breathing rate before and after treatment by GNP-bound CpG-ODN in RAO-affected horses (n=4), before and after treatment by GNP-bound CpG-ODN in healthy horses (n=4) and before and after treatment by GNPs (placebo) in healthy horses (n=4, left to right). (**b**) Oxygen partial pressure in arterial blood before and after respective treatments. (**c**) Occurrence of tracheobronchial secretion before and after respective treatments. (**d**) Percentile of neutrophil granulocytes in the TBS before and after respective treatments.

associated proallergic pathways (20), as long as the excess IL-10 is not of Th2 offspring. Nevertheless, the parallel increase of IFN-γ in combination with suppressive cytokine IL-10 was earlier reported to be beneficial in challenging allergic conditions (21).

In conclusion, IL-10 could be induced by administration of CpG-GNP through inhalation in RAO-affected horses. Recent studies (14) led to the decision to employ the CpG-ODN 2216 (A-class) in the present study due to superior IL-10 inducing properties from equine BALF cells compared to representatives of the B- and C-class. We hypothesized that IL-10 could be beneficial in avoiding an equine allergic inflammatory immune response towards allergen contact and could therefore prevent tissue injury by neutrophile degranulation.

In humans, an induction of IL-10 by established anti-allergic medication such as glucocorticoids or allergen immunotherapy was attributed to the activation of Treg cells (22). Asthmatic human patients showed lower amounts of Tregs in BALF compared to healthy ones which corresponds to the lack of peripheral tolerance (23). Therefore, it was hypothesized that the increased IL-10 expression could be related to higher or nearly physiologic Treg cell numbers in the lower airways. Furthermore, it is known that CpG-ODN activates plasmoidal dendritic cells (24) which reside in the lower airways (25). In addition, it was shown earlier that CpG-ODN promoted an IL-10 release by dendritic cells which led to an increased IL-10 mediated Treg induction (26). Thus, it can be hypothesized from the present study that the GNP-bound CpG-ODN triggered release of pro-tolerance IL-10 and, therefore, possibly contributed to Treg cell-mediated peripheral tolerance. This would be beneficial as an innovative and alternative treatment of allergic airway diseases beyond horses (12,17,24).

Beside cytokine-based immunologic parameters, the clinical impact of the hereby proposed therapeutic regimen was assessed. As no local or systemic adverse effects such as endoscopically visible local hyperaemia, follicular hyperplasia, clinical detectable signs like increased nasal discharge, coughing, bronchospasm, increased breathing rate and worsening of arterial blood gas values, derivative leucocytes, differential blood count, fibrinogen plasma concentration and inner body temperature were observed after inhalation of GNP-bound CpG-ODN (CpG-GNP), good biocompatibility of the applied doses and regimen is preliminarily presumed. This is the first time an *in vivo* application of nanoparticle-bound immunostimulating DNA via inhalation in horses is reported.

Moreover, as the inhalation regimen lowered the breathing rate of RAO-affected horses significantly, it

can therefore be regarded as efficient. The determination of the most important blood gas oxygen (PaO_2) was used to evaluate the extent of gas exchange and the response to treatment (6). The magnitude of gas exchange abnormality correlates with the severity of bronchiolitis and other relevant clinical signs (6).

With regard to the clinical examination of the TBS, the percentage of neutrophile granulocytes in the TBS is a strong indicator for RAO (27). Moreover, it is regarded as one of the most decisive parameters to evaluate RAO. Consequently, an observed average decrease of 40% in neutrophils in TBS after five inhalations with GNP-bound CpG-ODN could be regarded as one of the most important clinical ameliorations after this immunotherapy. The percentage of neutrophiles within the TBS is directly related to the severity of the RAO condition. Therefore, it can be deducted that the severity of the pathogenesis was significantly reduced after five applications of GNP-bound CpG-ODN according to the present study.

The differences in treatment protocol for healthy (three days) and allergic horses (five days) were set due to a lack of expected improvement in clinical signs after three times of administration in healthy horses. Both groups were inhaled three times and clinical parameters were determined, whereby healthy horses showed no significant changes. In contrast RAO-affected horses showed significant improvement after three times of inhalation. The question was imminent what kind of ongoing improvement in quality and quantity of measured parameters could be seen by an augmentation of inhalations. Ethical appreciation of values regarding animal benefit vs. burden, available resources as well as practicability, the numbers of inhalations were consequently limited to three in healthy horses. Additionally, no further change was expected in healthy horses upon further treatment. In contrast, partly significant improvement of clinical parameters after two more inhalations in RAO-affected horses were expected and later observed, accordingly. Therefore, the inhalation protocol was fitted to that aspect. Moreover, in ongoing follow-up studies featuring significantly higher numbers of participating individuals, healthy horses were no longer included into the study design due to the expected non-significance of differences before and after the inhalation regimen regarding both clinical and immunological parameters.

Previously, Treg activation was related to reduction in activity and number of neutrophile granulocytes by promoting their rate of apoptosis (28). Human neutrophiles express all known TLRs except TLR3 (28). Moreover, TLRs were shown to possess a crucial impact on Treg stimulation and function (28,29). Interestingly, previous investigations confirmed that Treg cells inhibit neutrophiles other than by direct cell-cell contact mechanism (CTLA-4/B7-1 mechanism) and especially through IL-10 action (30). Furthermore, this mechanism was advantageously involved in the treatment of allergic diseases (17,22). Therefore, with regard to the present study it is concluded that the observed impressive IL-10 induction by the hereby proposed treatment can be directly related to the decreasing neutrophile percentage in TBS, and therefore directly contributes to the regimen's anti-RAO effectiveness.

CONCLUSION

This study represents the first-time use of nanoparticle-mediated immunomodulatory therapy in food-producing animals. The applied nanoparticulate CpG-ODN formulation appeared to be safe as no above specified side effects were observed, although this finding should be regarded as preliminary until histological confirmation is at hand from different studies. Moreover, it constitutes the first time inhalative employment of a colloidal nanoparticulate carrier system in a preliminary clinical study which implied both the proof of immunomodulatory concept on cytokine level and a significant reduction of clinical symptoms. Thus, the present formulation offers a curative therapy for an otherwise difficult to treat common allergic disease in horses and likely beyond. Consequently, enlarged follow-up studies are currently in progress in order to elucidate the full potential of this first applied inhalative nanoparticle-based immunotherapy.

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