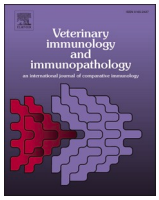
Veterinary Immunology and Immunopathology 243 (2022) 110351

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Research paper

An allergen-fused dendritic cell-binding peptide enhances *in vitro* proliferation of equine T-cells and cytokine production

Anja Ziegler [a,*], Judith Olzhausen [b], Eman Hamza [a,d], Ana Stojiljkovic [c], Michael H. Stoffel [c], Mattia Garbani [b], Claudio Rhyner [b], Eliane Marti [a]

[a] *Division of Neurological Sciences, Vetsuisse Faculty, University of Bern, Länggassstrasse 124, CH-3001 Bern, Switzerland*
[b] *Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Herman-Burchardstrasse 9, CH-7265 Davos Wolfgang, Switzerland*
[c] *Division of Veterinary Anatomy, Vetsuisse Faculty, University of Bern, Länggassstrasse 120, CH-3001 Bern, Switzerland*
[d] *Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Egypt*

ARTICLE INFO

Keywords:
Dendritic cell targeting
Horse
T-cell activation
Immunotherapy

ABSTRACT

Allergen-specific immunotherapy (AIT) constitutes the only curative approach for allergy treatment. There is need for improvement of AIT in veterinary medicine, such as in horses suffering from insect bite hypersensitivity, an IgE-mediated dermatitis to *Culicoides*. Dendritic cell (DC)-targeting represents an efficient method to increase antigen immunogenicity. It is studied primarily for its use in improvement of cancer therapy and vaccines, but may also be useful for improving AIT efficacy. Immunomodulators, like the Toll-like receptor 4 (TLR-4) agonist monophosphoryl lipid-A (MPLA) has been shown to enhance the IL-10 response in horses, while CpG-rich oligonucleotides (CpG-ODN), acting as TLR-9 agonists, have been shown to induce Th1 or regulatory responses in horses with equine asthma. Our aim was to evaluate *in vitro* effects of antigen-targeting to equine DC with an antigen-fused peptide known to target human and mouse DC and investigate whether addition of MPLA or CpG-ODN would further improve the induced immune response with regard to finding optimal conditions for equine AIT. For this purpose, DC-binding peptides were fused to the model antigen ovalbumin (OVA) and to the recombinant *Culicoides* allergen Cul o3. Effects of DC-binding peptides on cellular antigen uptake and induction of T cell proliferation were assessed. Polarity of the immune response was analysed by quantifying IFN-γ, IL-4, IL-10, IL-17 and IFN-α in supernatants of antigen-stimulated peripheral blood mononuclear cells (PBMC) in presence or absence of adjuvants. Fusion of DC-binding peptides to OVA significantly enhanced antigen-uptake by equine DC. DC primed with DC-binding peptides coupled to OVA or Cul o3 induced a significantly higher T-cell proliferation compared to the corresponding control antigens. PBMC stimulation with DC-binding peptides coupled to Cul o3 elicited a significant increase in the pro-inflammatory cytokines IFN-γ, IL-4, IL-17, as well as the anti-inflammatory IL-10, but not of IFN-α. Adjuvant addition further enhanced the effect of the DC-binding peptides by significantly increasing the production of IFN-γ, IL-4, IL-10 and IFN-α (CpG-ODN) and IL-10 (MPLA), while simultaneously suppressing IFN-γ, IL-4 and IL-17 production (MPLA). Targeting equine DC with allergens fused to DC-binding peptides enhances antigen-uptake and T-cell activation and may be useful in increasing the equine immune response against recombinant antigens. Combination of DC-binding peptide protein fusions with adjuvants is necessary to appropriately skew the resulting immune response, depending on intended use. Combination with MPLA is a promising option for improvement of AIT efficacy in horses, while combination with CpG-ODN increases the effector immune response to recombinant antigens.

Abbreviations: AIT, allergen-specific immunotherapy; BA, *Bacillus anthracis*; CD, cluster of differentiation; *Cul o*, *Culicoides obsoletus*; DC, dendritic cell; DCpep, DC-binding peptide FYPSYHSTPQRP; *E. coli*, *Escherichia coli*; GM-CSF, granulocyte-macrophage colony stimulating factor; IBH, insect bite hypersensitivity; MoDC, monocyte-derived dendritic cell; MPLA, monophosphoryl Lipid-A; ODN, oligodeoxynucleotide; TLR, toll-like receptor.

* Corresponding author at: Division of Experimental Clinical Research, Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Länggassstrasse 124, CH-3001 Bern, Switzerland.
E-mail address: anja.ziegler@vetsuisse.unibe.ch (A. Ziegler).

https://doi.org/10.1016/j.vetimm.2021.110351
Received 2 August 2021; Received in revised form 26 October 2021; Accepted 5 November 2021
Available online 9 November 2021

1. Introduction

Allergen specific immunotherapy (AIT) is considered the only curative treatment for allergies (Akdis et al., 2011). While AIT is used routinely for human hypersensitivity disorders such as allergic rhinitis (Roberts et al., 2018) or insect venom allergy (Dhami et al., 2017), it often leads to only partial improvement of clinical signs and severe side-effects can occur (Bukantz and Lockey, 2004; DaVeiga et al., 2011). Studies on AIT in horses suffering from insect bite hypersensitivity (IBH), an IgE-mediated type I hypersensitivity induced by bites of insects of the genus *Culicoides* (Schaffartzik et al., 2012) conclude that to date, clinical effects of AIT are questionable at best (Barbet et al., 1990; Ginel et al., 2014; Marteles et al., 2019). Therefore, there is still need for improvement of AIT in both humans (Jutel and Akdis, 2014; Sandrini et al., 2015) and in domestic animals (Mueller et al., 2018).

In recent years, allergy research has focused on the identification and production of highly purified recombinant allergens (Valenta et al., 2016; Pomés et al., 2018), also for IBH (Schaffartzik et al., 2010, 2011; van der Meide et al., 2013; Peeters et al., 2013; Novotny et al., 2021). This opens new possibilities to further improve AIT, as the recombinant allergens can easily be modified to make them more suitable for vaccination or immunotherapy.

Dendritic cells (DC) have unique abilities to initiate and modulate antigen-specific immune reactions. Targeting antigens directly to DC has been widely used to improve vaccines against tumors (Unger et al., 2014; Garu et al., 2016) and against infectious diseases (Curiel et al., 2004; McNulty et al., 2013; Sehgal et al., 2014) and could thus be an interesting approach to improve AIT efficacy by enhancing allergen uptake and presentation. DC-targeting entails selective antigen delivery to DC by using chimeric proteins composed of an antigen linked to a ligand that is recognized by DC (Alvarez et al., 2013; Kastenmüller et al., 2014) such as antibodies that target DC surface structures (Bonifaz et al., 2002) or a DC-binding peptide (Curiel et al., 2004). Curiel et al. (2004) showed that a peptide of 12 amino acids, named DCpep, was able to target human and mouse DCs and to enhance their ability to activate autologous T-cells. Improved protective immunity mediated by an oral vaccine against *Bacillus anthracis* (BA) using *Lactobacillus acidophilus* expressing BA protective antigen fused to DCpep was demonstrated (Mohamadzadeh et al., 2009). Furthermore, Owen et al. (2013) suggested in a pilot study that DCpep is also recognised by horse DCs amongst other species.

Vaccine adjuvants are important tools to enhance the efficacy of AIT by polarizing the immune response away from the detrimental T helper (Th) 2 response towards a protective regulatory immune reaction. In recent years, adjuvants acting as TLR agonists which constitute danger signals and consequently lead to an enhanced cross-presentation by DC and inflammation, have been investigated in numerous pre-clinical and clinical studies (reviewed in Jensen-Jarolim et al., 2020). The non-toxic LPS derivative Monophosphoryl lipid A (MPLA) acts as an agonist of Toll-like receptor (TLR) 4. It has been developed as an adjuvant for human AIT (Rosewich et al., 2013; Jensen-Jarolim et al., 2020), as well as for anti-tumour vaccines (Cluff, 2010). In veterinary medicine, MPLA was identified as a promising vaccine adjuvant candidate for equine AIT *in vitro* (Ziegler et al., 2017), as well as *in vivo* (Jonsdottir et al., 2016). CpG-rich oligonucleotides (CpG-ODN) act as TLR-9 agonists and have shown promising clinical effects in studies investigating immunotherapy for horses suffering from Recurrent Airway Obstruction (Klier et al., 2012, 2015, 2019), but also in humans (Senti et al., 2009).

The rationale of the present investigation is that employing the DC-targeting approach will open up new possibilities for enhancing and modulating the immune response to recombinant antigens in an equine model system. This might be of use for the development of novel vaccines or cancer therapeutics, but also for the development of an effective AIT for horses with IBH.

Therefore, our aims were to evaluate effects of the DC-binding peptide DCpep-antigen fusions on the ability of equine monocyte-derived DC (MoDC) (Hammond et al., 1999) to stimulate T-cells and to modulate the cytokine response of equine peripheral blood mononuclear cells (PBMC) in the presence or absence of TLR-agonists acting as adjuvants. The cytokines IFN-γ, IL-4, IL-10 and IL-17, representing key cytokines for Th1, Th2, regulatory T-cell and Th17 responses, respectively, were measured to investigate the nature of the resulting antigen-specific T-cell response. Experiments were first performed with ovalbumin (OVA) as a model antigen to assess recognition of DCpep by equine DC. Thereafter, we used recombinant Cul o3, an antigen-5-like protein from *Culicoides obsoletus*, which is a major allergen for equine IBH (Ziegler et al., 2018). Finally, we investigated whether addition of MPLA or CpG-ODN to PBMC stimulated with Cul o3 coupled to the DC-binding peptide would further modulate the induced immune response with regard to finding optimal conditions for equine AIT.

2. Materials and methods

2.1. Cloning, production and purification of proteins

N-terminal Hexahistidine-tagged constructs derived from ovalbumin (OVA) or from Cul o3 (accession number KC339673.1) were cloned in *Escherichia coli* (*E. coli*) by means of the expression vector pET17b (Merck Millipore, Billerica, MA, USA). Either DCpep (FYPSYHSTPQRP) or its GSSG-spaced tetramer DCpep4 were cloned C-terminally of the allergen module. DCpep4 was investigated, because of its higher avidity observed in previous studies (Garbani, unpublished data).

Recombinant proteins were produced in *E. coli* as described previously (Schaffartzik et al., 2011), with the following modifications: After binding to a HisTrap FF crude column (GE Healthcare, Glattbrugg, Switzerland), proteins were washed with 20 mM Tris-HCl pH8 / 6 M urea / 0.5 M NaCl / 5 mM Imidazole / 0.1 % Triton X-100 / 0.05 % Triton X-114 for endotoxin removal. Imidazole-eluted proteins were diluted to 150 μg/mL, dialyzed against 5 mM carbonate-bicarbonate buffer / 5 mM NaCl with a 6−8 kDa MWCO membrane (Spectrum Labs, Roncho Dominguez, CA, USA), concentrated ten times by centrifugation using amicon filter (amicon ultra-2; Merck Millipore), supplemented with 10 % glycerol at end concentration and stored at −80 °C. Molecular size and purity of all protein products were assessed on the 2200 TapeStation (Agilent technologies, Santa Clara, CA, USA) (Additional File 1).

Endotoxin contamination was assessed using a qualitative *in vitro* end-point endotoxin assay (ToxinSensor™, GenScript, Piscataway, NJ, USA) and LPS levels in the working protein dilutions were shown to be below 3 I.U./ mg protein.

All chemicals were obtained from Sigma-Aldrich (St. Louis MO, USA), unless stated otherwise.

2.2. Fluorophore labeling of proteins

Fluorophore labeling was performed using Dylight 488 Amine-Reactive Dye (Thermo Scientific, Waltham, MA, USA) and quantified according to the manufacturers' instructions by measuring the absorption at 493 nm using a NanoDrop1000 Spectrophotometer (Thermo Scientific).

2.3. Horses and blood samples

Blood samples were collected from the jugular vein of eighteen horses (12 geldings, 6 mares; mean age 14 years (range 4–26)) belonging to various breeds (Icelandic, Freiberger, Warmblood, Arabian) using Sodium-Heparin containing vacutainers (Vacuette®; Greiner, St. Gallen, Switzerland). The experiments with MoDC employing the model antigen OVA were performed using blood from five to twelve healthy horses. For all experiments with Cul o3 and for cytokine production by PBMC with OVA, six horses with a history of clinical IBH over several years and sensitization to *Culicoides* allergen extract confirmed by a Cellular

Antigen Stimulation Test (CAST, Bühlmann Laboratories, Schönenbuch, Switzerland) and six healthy control horses were used. The control horses had no clinical signs and history of skin diseases and lived in the same environment as the allergic horses. A detailed overview on which horses were used for the respective experiments can be found in the supplementary material (Additional File 2). The study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (No. BE 51/13). Informed consent was obtained from the horse owners for including the animals in the present study.

2.4. In vitro generation of equine MoDC

PBMC were isolated by two subsequent Ficoll density gradient centrifugations, using Biocoll ρ = 1.090 g/mL and 1.077 g/mL (both Biochrom GmbH, Berlin, Germany) as described in (Mauel et al., 2006; Ziegler et al., 2016). Monocytes were isolated by magnetic separation (MACS technology, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) according to standard protocols by the manufacturer, using a monoclonal anti-equine CD14 antibody (clone 105) (Kabithe et al., 2010) and secondary goat anti-mouse-coated micro beads. Cells were then separated on a LS column (Miltenyi Biotec) and differentiation into MoDC was induced by culturing $CD14^+$ monocytes for three days in the presence of recombinant equine IL-4 and GM-CSF as described by Moyo et al. (2013).

2.5. Antigen-uptake experiment

Immature MoDC were re-suspended at 200′000 cells per 150 μL medium and incubated for 90 min at 37 °C with 20 μg/mL fluorescently labelled DCpep-OVA or OVA, corresponding to 500 nM. In order to account for the variable degree of labelling and the resulting differences in fluorescence intensity, the amount of DCpep-OVA or OVA was corrected according to the absorption at 493 nm. The antigen-primed MoDC were washed and stained with the surface marker antibody anti-human CD206 conjugated to PE (Beckman Coulter, Nyon, Switzerland), known to cross-react with equine CD206 (Moyo et al., 2013). After antigen uptake and surface marker staining as described above, MoDC from five horses were transferred to a 96-well flat-bottom plate (Greiner bio-one, Frickenhausen, Germany) at 25,000 cells per well and fixed with 2 % paraformaldehyde before using DAPI (Sigma-Aldrich) to stain the nuclei. Cells were then visualized in the INCell Analyzer2000 system (GE Healthcare). Twenty-five frames per well were randomly selected and imaged with a Nikon 60 × 0.70NA objective. The following filter sets were used: 350_50x & 455_50 m (DAPI), 490_20x & 525_36 m (FITC) and 579_34x & 624_40 m (Texas Red). For optimal focusing of the images, we took advantage of the laser autofocus option provided by the system. For each antigen condition, six replicates were used, yielding 150 frames per condition. High content analysis was performed using the INCell Investigator1.6.2 software (GE Healthcare). The object segmentation function was used to segregate nuclei, cells and antigen particles, respectively. Briefly, nuclear segmentation relied on the whole nuclear signal with a minimum target area of 30 μm^2. Intensity segmentation was used for identifying single MoDC based on the cell surface marker CD206 signal. Antigen particles were segregated by vesicle segmentation (granule size range = 0.1−1 μm). Only antigen particle signal overlapping 100 % with the cell surface signal were considered for analysis as intracellular antigen particles. To determine the amount of antigen taken up per cell, we compared the area ratios between DCpep-OVA and OVA alone. The area ratio was defined for every single cell as the ratio between the total area covered by the intracellular antigen particles and the total cell area. Additionally, the proportion of MoDC with signals of intracellular antigen particles was determined.

2.6. [^{3}H] thymidine incorporation assay to determine T-cell proliferation

Antigen uptake was performed as described above using 20 μg/mL of OVA, DCpep-OVA, Cul o3 or DCpep4-Cul o3 respectively, as well as the recall antigen tetanus toxoid (Schweizerisches Serum und Impfinstitut Bern, Switzerland) and medium only, as controls. After antigen uptake, MoDC were washed and cultured in a 96-well round bottom tissue culture plate (Sarstedt, Nümbrecht, Germany) at 20′000 MoDC per well in quadruplicates. Freshly isolated $CD5^+$ T-lymphocytes were enriched by positive selection using MACS technology as described above and according to standard protocols by the manufacturer, employing an anti-equine CD5 mAb (clone CVS5, Abd Serotec, Kidlington, UK). 100’000 autologous $CD5^+$ T-lymphocytes were added to the MoDC that had been matured as described (Moyo et al., 2013), and co-cultured for five days at 37 °C/ 5% CO_2. Five μCi/ well [^{3}H] thymidine (Perkin Elmer, Waltham, MA, USA) was added for the last 18 h of culture. DNA was then harvested onto a glass fiber filter plate and thymidine incorporation was measured on a scintillation counter (Inotech, LabLogic Systems Inc. Brandon, FL, USA).

2.7. Cytokine production by PBMC

To determine the effect of DCpep-coupled antigen in the presence or absence of immunomodulatory adjuvants on cytokine production, 500’000 PBMC were cultured in 200 μl RPMI complete medium in a 96-well round bottom tissue culture plate (Sarstedt) for 48 h in the presence of 5 μg/mL of the respective antigen Cul o3 or DCpep4-Cul o3 as well as 5 μg/mL each of the immunomodulators C-class CpG-ODN (D-SL03, Invivogen) or MPLA (from *Salmonella minnesota* R595, InvivoGen). Cells incubated with complete medium alone served as negative control. After incubation, cell culture supernatants were harvested and stored at −80 °C until used. IFN-α, IFN-γ, IL-4, IL-10 and IL-17 and were determined at the Department of Population Medicine and Diagnostic Sciences, Cornell University with a bead-based multiplex assay (Horse Cytokine 5-plex Assay) (Wagner and Freer, 2009). Results were reported as either pg/mL (IFN-α, IL-4, IL-10) or U/mL (IFN-γ, IL-17).

2.8. Statistical analyses

Statistical analyses were carried out using the software program NCSS11 (NCSS, Kaysville, UT, USA). Descriptive statistics showed that the data were not normally distributed. Therefore, a non-parametric paired-sample Wilcoxon (signed rank) test was used to compare the antigen conditions DCpep-OVA with OVA and DCpep4-Cul o3 with Cul o3, respectively (Figs. 1–5), as well as to compare the effects of DCpep4-Cul o3 with DCpep4-Cul o3 combined with either MPLA or CpG-ODN (Fig. 6/ Additional File 4). Overall, p-values ≤ 0.05 were considered significant.

3. Results

3.1. Significantly higher uptake of DCpep-OVA than OVA by equine MoDC

Automated image acquisition and analysis of immature MoDC primed with fluorophore labelled antigen by fluorescence microscopy revealed intracellular localisation of fluorescent antigen particles (Fig. 1a). A significantly higher percentage of MoDC had taken up DCpep-OVA than OVA (median 70.3 % *vs*. 33.2 %, $p < 0.001$, Fig. 1b). Fig. 1c shows that significantly more DCpep-OVA was taken up per cell than OVA (median area ratio 8.7 % *vs*. 1.8 % respectively, $p < 0.001$).

3.2. DCpep-OVA enhances T-cell proliferation and cytokine production by PBMC

In line with the results of the antigen-uptake experiments, DCpep-OVA-primed MoDC induced a significantly higher T-cell proliferation than OVA-primed MoDC as measured by [^{3}H] thymidine incorporation assay (median = 9073 *vs*. 5492 counts per minute (cpm), $p < 0.05$,

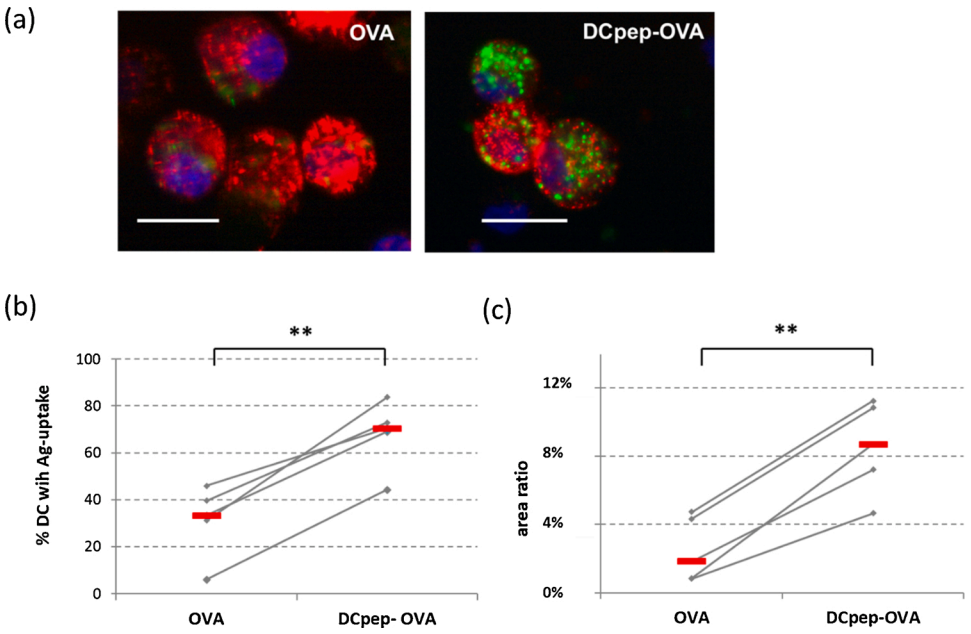


Fig. 1. Uptake of OVA and DCpep-OVA by MoDC, respectively, as visualized by fluorescence microscopy (IN Cell Analyzer 2000) (a). Nuclear staining with DAPI (blue) and surface staining with anti–CD206 (red) were used for cell delineation. Intracellular antigen is visible as green fluorescent particles. 150 fields of vision per condition were randomly chosen, and images are representative of experiments done with five horses. White scale bars represent 20 μm. Percentage of MoDC with intracellular antigen (b), as well as antigen uptake per cell (c), depicted as the area ratio (= total area covered by intracellular antigen particles per total cell area) were compared between OVA and DCpep-OVA. Individual lines represent separate horses, with the red bars representing the medians. Non-parametric, paired sample Wilcoxon (signed rank) test was used to compare between OVA and DCpep-OVA, as indicated by asterisks: ** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

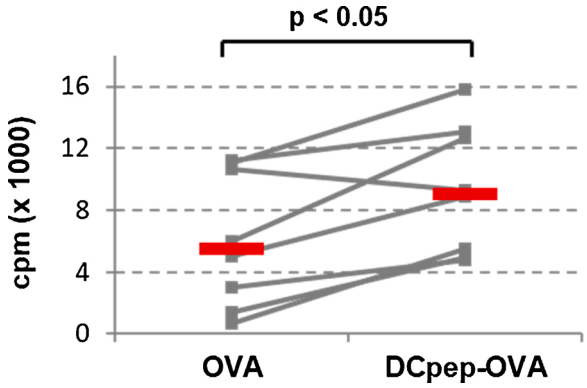


Fig. 2. Induction of T-cell proliferation by MoDC primed with DCpep-OVA and OVA, respectively, as measured by [3 H] thymidine incorporation assay. Results are displayed as counts per minute (cpm) x 1000 with cpm of the non-antigen primed control subtracted. Each symbol indicates a separate horse (n = 8), with bars indicating the median value. Non-parametric, paired sample Wilcoxon (signed rank) test was used to determine significant differences between the antigen conditions.

Fig. 2). As expected, the positive control tetanus toxoid, considered a recall antigen as horses get routinely vaccinated against tetanus, induced the highest proliferation (Additional File 3).

Furthermore, compared to OVA, DCpep-OVA significantly enhanced the production of IL-4 (median DCpep-OVA 1191 pg/mL; median OVA 793 pg/mL, $p < 0.01$), IL-10 (1354 pg/mL; 217 pg/mL, $p < 0.001$) and IL-17 (988 U/mL; 363 U/mL, $p < 0.01$) by PBMC (Fig. 3). Conversely, no significant difference was detected for IFN-γ (395 U/mL and 263 U/mL, respectively).

3.3. Effect of DCpep4 coupled to Cul o3

After having shown that DCpep fused to the prototype antigen OVA was able to enhance antigen uptake and immune activation, we investigated the effect of a DC-targeting peptide fused to the recombinant *Culicoides* allergen Cul o3. The GSSG-spaced tetramer DCpep4 was investigated, because of the higher avidity observed in previous studies (Garbani, unpublished data). Co-culture of MoDC primed with Cul o3 or DCpep4-Cul o3 with autologous $CD5^+$ T-cells resulted in a marked increase in the proliferation of T-cells with DCpep4-Cul o3 compared to Cul o3, in all horses (median = 2101 *vs.* 855cpm, $p < 0.01$, Fig. 4).

Next, cytokine production by PBMC stimulated with Cul o3 or DCpep4-Cul o3 was investigated. Consistent with the DCpep-OVA results, the PBMC cultured in the presence of DCpep4-Cul o3 resulted in a significantly higher production of IFN-γ, IL-4, IL-10 and IL-17 compared to Cul o3 (Fig. 5). No IFN-α was induced (Additional File 4).

3.4. Immunomodulators further enhance cytokine production by DCpep4-Cul o3 stimulated PBMC

Addition of C-class CpG-ODN to PBMC stimulated with DCpep4-Cul o3 was shown to further increase significantly the production of IFN-γ, IL-4, IL-10 and IL-17, as well as inducing a marked production of IFN-α (Fig. 6/ Additional File 2). Similarly, addition of MPLA to PBMC stimulated with DCpep4-Cul o3 resulted in a further significant increase in IL-10 (Fig. 6/ Additional File 2). Interestingly, MPLA was found to significantly suppress production of IFN-γ, IL-4, and IL-17 elicited by DCpep4-Cul o3 (Fig. 6/ Additional File 4).

4. Discussion

AIT is widely accepted as the gold standard therapy for hypersensitivity disorders. Despite the documented beneficial effects of AIT, improvement it still needed as complete and sustained clinical remission after cessation of AIT is relatively rare. Amongst other approaches, current efforts to improve AIT focus on the use of highly pure recombinant proteins instead of extracts (Valenta et al., 2016). However, while these allergens often possess a reduced IgE-reactivity (Karamloo et al., 2005; Holzhauser et al., 2020) and are thus safer for use in AIT, they may also exhibit a reduced immunogenicity and therefore lower efficacy (Würtzen et al., 2007).

The first aim of the present study was to evaluate *in vitro* whether antigen-uptake by equine DCs can be enhanced by use of a DC-binding peptide. Developing a system for efficient targeting of antigen to equine DCs has numerous potential clinical applications beyond AIT, such as anti-tumour vaccination or vaccination against infectious agents. In our study, we therefore first investigated the effect of fusion of DCpep to a prototype antigen (OVA) on its recognition and uptake by equine MoDC. We could demonstrate a significantly increased uptake of

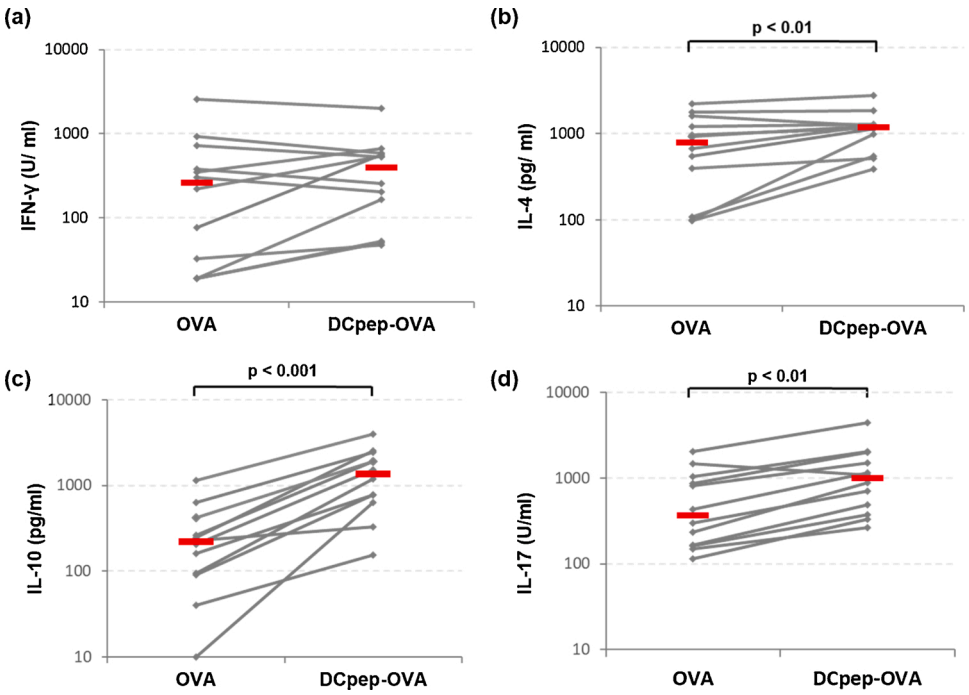


Fig. 3. Production of IFN-γ (a), IL-4 (b), IL-10 (c) and IL-17 (d) by PBMC stimulated with 5 μg/mL OVA or DCpep-OVA, respectively, as shown on a log scale. Cell culture supernatants were harvested and analyzed by a bead-based multiplex assay. N = 12 horses. Bars indicate the medians. Non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences between the antigen conditions.

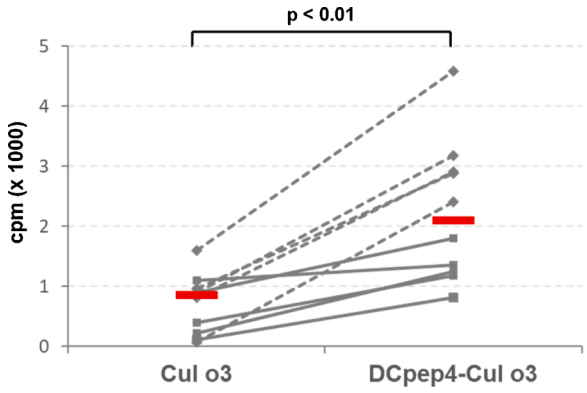


Fig. 4. T-cell proliferation induced by MoDC primed with DCpep4-Cul o3 or Cul o3, as measured by [3 H] thymidine incorporation assay. Results are displayed as cpm x 1000 with cpm of the non-antigen primed control subtracted. Each symbol indicates a separate horse with n = 5 IBH-affected horses (dotted lines) and n = 5 healthy horses (continuous lines). Black bars indicate the overall median values. Non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences.

DCpep-OVA compared to OVA. While the ligand for DCpep is unknown, Curiel et al. demonstrated that it binds to distinct, saturable DC surface epitopes (Curiel et al., 2004). Unfortunately, no follow-up studies have been published. We can, however, assume that the ligand must be a molecule which is well-conserved between species (Owen et al., 2013). We then investigated whether the increased uptake of DCpep-OVA by equine DC is functionally relevant. Indeed, induction of T-cell proliferation was significantly more effective using DCpep-OVA-primed MoDC compared to OVA alone.

Next, we aimed at evaluating the effect of DCpep when fused to a major allergen for equine IBH (Novotny et al., 2021) that had already been shown to induce cytokine production by PBMC *in vitro* (Meulenbroeks et al., 2015). Co-culture experiments of MoDC primed with Cul o3 or DCpep4-Cul o3 with autologous T-cells confirmed the findings with DCpep-OVA/ OVA. DCpep4-Cul o3 induced a significantly higher T-cell proliferation overall, when compared to Cul o3. Due to limited availability of DCpep4-Cul o3, antigen uptake experiments were not repeated. This constitutes a limitation of the study.

Surprisingly, no significant difference could be observed between IBH-affected and healthy horses in terms of T-cell proliferation or cytokine production when cells were stimulated with Cul o3 or DCpep4-Cul o3. The lack of difference between IBH-affected and healthy horses might be due to the heterogeneity within the IBH-horse group. Hamza et al. had demonstrated a heterogenous IL-4 response in IBH-affected horses following *Culicoides in vitro* re-stimulation, linked to the heterogenous suppressive capacity of the regulatory T-cells (Hamza et al., 2013). Alternatively, it might be attributed to the use of a single recombinant allergen instead of the many allergens and proteins present in *Culicoides* extract. Recently, component-resolved diagnostics have greatly enhanced our knowledge on the relevance of different recombinant *Culicoides* proteins as allergens for IBH.

Recent observations show that around 50 % of IBH-affected horses are sensitized to Cul o3 and that there are in fact several recombinant *Culicoides* allergens with higher percentages of sensitized horses and which, therefore, are better suited for equine AIT using recombinant allergens (Novotny et al., 2021). Further experiments, ideally employing a combination of several "top" recombinant IBH-allergens targeted to DC, will be needed to find the ideal allergen composition for highest efficacy of AIT.

The concentration of all tested cytokines was significantly higher upon stimulation with DCpep-OVA or DCpep4-Culo3 compared to OVA and Cul o3, respectively. This indicates a general immune activation and suggests that DCpep may be able to enhance the immunogenicity of the allergens. Interestingly, the increase in cytokine production was more pronounced for DCpep4-Cul o3 compared to Cul o3 than for DCpep-OVA compared to OVA. Whether this reflects the previously observed higher avidity of the DCpep tetramer (Garbani, unpublished data) cannot be deduced from these experiments. This constitutes a limitation of our study and further experiments are needed to compare DCpep and DCpep4 fused to the same allergen.

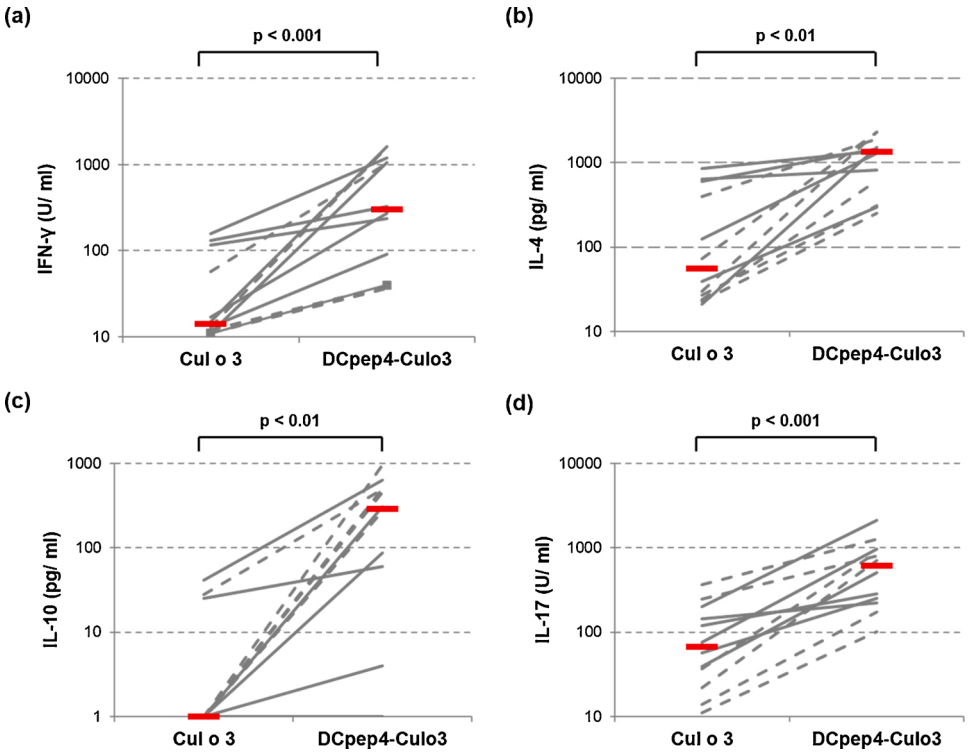


Fig. 5. Production of IFN-γ (a), IL-4 (b), IL-10 (c) and IL-17 (d) by PBMC stimulated with 5 μg/mL Cul o3 and DCpep4-Cul o3, respectively, as shown on a log scale. Cell culture supernatants were harvested and analyzed by a bead-based multiplex assay. Each symbol indicates a separate horse with n = 6 IBH-affected horses (dotted lines) and n = 6 healthy horses (continuous lines). Bars indicate the overall median values. Non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences between the antigen conditions.

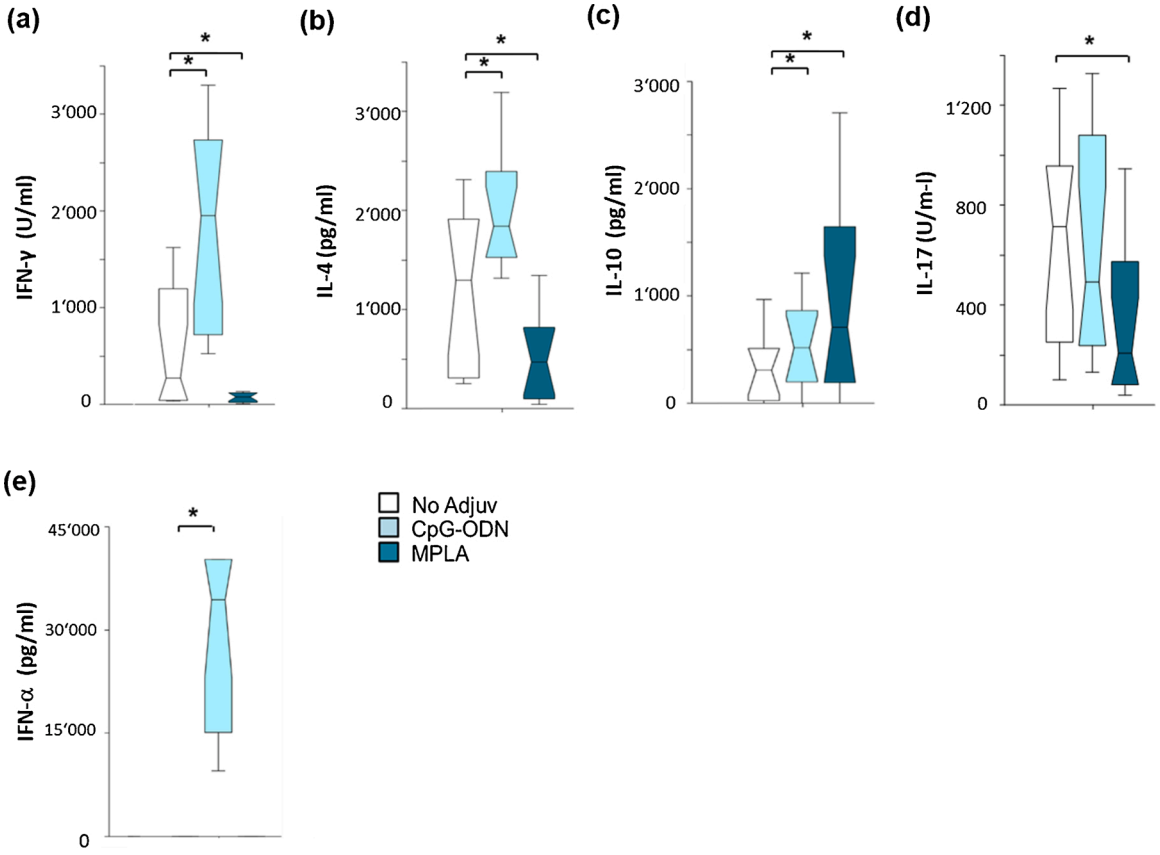


Fig. 6. Effects of immunomodulators on production of IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d) and IFN-α (e) by equine PBMC stimulated with 5 μg/mL DCpep4-Cul o3 only or combined with 5 μg/mL each of C-class CpG-ODN or MPLA. N = 12 horses. Cell culture supernatants were harvested and analyzed by a bead-based multiplex assay. Non-parametric paired sample Wilcoxon (signed rank) test was used to determine significant differences between the no-adjuvant condition (white boxplots) and CpG-ODN (light blue boxplots) or MPLA (dark blue boxplots), respectively, as indicated by asterisks: * $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

As modulation of cytokine environment towards a regulatory immune response is crucial in determining the success of therapeutic AIT vaccinations, selection of a suitable adjuvant is of great importance (Filì et al., 2014; Burakova et al., 2018). Our last objective was, therefore, to evaluate effects of DCpep in combination with prospective vaccine adjuvants for equine AIT with regard to their potential combined use. CpG-ODN, added in combination with DCpep4-Cul o3, elicited a marked further up-regulation of the effector cytokines IFN-γ and IL-4 as well as a weak, but significant upregulation of the regulatory cytokine IL-10. The strong induction of IL-4 together with the rather weak upregulation of IL-10 indicates that the C-class CpG-ODN D-SL03 used in this study is probably an unsuitable adjuvant for AIT, also in combination with allergens fused to DCpep4-Cul o3. However, the induced effector cytokine response may hold promise for its application in equine anti-infectious or anti-tumour vaccine formulations, especially as it was also found to be a strong inducer of IFN-α, confirming a previous study by our group (Ziegler et al., 2017). While our group had already shown MPLA to be an effective inducer of IL-10 *in vitro* by equine PBMC (Ziegler et al., 2017), a significant further upregulation of IL-10 was observed in PBMC stimulated with DCpep4-Cul o3 and MPLA, in comparison to PBMC stimulated with DCpep4-Cul o3 alone. While both compounds individually are able to induce a marked regulatory response by stimulating IL-10 production, additionally, a synergistic effect in further enhancing to regulatory nature of the immune response could be observed when combining the DCpep approach with MPLA as an adjuvant. With regard to using DCpep-antigen fusions in AIT vaccines, our study shows that MPLA significantly suppresses the unwanted induction of IL-4 production by DCpep4-Cul o3, confirming that adjuvants may often be more influential in skewing the cytokine response than the antigen itself. Therefore, selection of a suitable adjuvant formulation is crucial in counterbalancing the potent, but rather unspecific, T-cell activating capacity of the DC-binding peptide protein fusions to achieve an optimal vaccine formulation for a safe and efficacious equine AIT. Further studies are needed to this effect.

5. Conclusion

In conclusion, we have demonstrated that targeting equine DCs with allergens fused to DC-binding peptides enhances antigen-uptake and T-cell activation. This may be a useful approach to increase the immune response against recombinant antigens. Targeting DC with recombinant allergens fused to DC-binding peptides in combination with MPLA is a promising option for future improvement of AIT efficacy in horses, while combination with CpG-ODN appears to increase the effector immune response to recombinant antigens.

Ethics approval and consent to participate

The study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (No. BE 51/13). Informed consent was obtained from the horse owners for including the animals in the present study.

Funding

This work was supported by the Morris Animal Foundation grant D16EQ-039, by a grant of the Division for Neurological Sciences (formerly Department of Clinical Research VPH), Vetsuisse Faculty, University of Bern and by the Swiss National Science Foundation grant no. 310030-160196/1. The funding sources had no involvement in the study design, the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the article for publication.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We are grateful to Dr. Bettina Wagner at the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA for providing the anti-equine CD14 antibody.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110351.

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