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Recombinant *Culicoides obsoletus* complex allergens stimulate antigen-specific T cells of insect bite hypersensitive Shetland ponies *in vitro*

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Background – Ponies may suffer from Insect bite hypersensitivity (IBH), an allergic IgE-mediated pruritic skin disorder, induced by allergens from biting midges of the *Culicoides* spp.

Hypothesis/Objectives – To determine whether recombinant *Culicoides obsoletus* allergens are able to activate T cells of ponies exposed to *C. obsoletus* and whether these allergen-specific responses differ between IBH-affected and healthy ponies.

Animals – Ten IBH-affected Shetland ponies and 10 age-matched healthy controls taken from the same stables, to ensure similar exposure to midges.

Method – Peripheral blood mononuclear cells (PBMC) were cultured with two different pools of recombinant *C. obsoletus* complex allergens to expand the allergen-specific T cells. These PBMC cultures were subsequently co-cultured with mature dendritic cells (DCs) loaded with the same antigens. Induction of Th1, Th2 and regulatory T (Treg) cells in these DC/PBMC co-cultures was assessed by analysis of IFN-γ, IL-4, IL-10 and FoxP3 expression levels using quantitative RT-PCR and phenotyping by flow cytometry.

Results – Recombinant *C. obsoletus* allergens increased IFN-γ mRNA expression levels, percentages of IFN-γ expressing (Th1) cells and $CD25^{high}FoxP3^{+}IL\text{-}10^{+}$ Tregs compared to unstimulated DC/PBMC co-cultures. Stimulation of IL-4 expressing Th2 cells by the recombinant allergens was far less pronounced. The DC/PBMC co-cultures did not reveal significant differences between healthy and IBH-affected ponies for any of the analysed parameters, except for higher IL-4 mRNA levels in IBH affected ponies after stimulation with one of the two allergen pools.

Conclusion and clinical importance – The recombinant *C. obsoletus* complex allergens can stimulate antigen-specific Th1 and IL10 producing Treg cells and are therefore promising candidates for the immunotherapy of IBH.

Introduction

Equine insect bite hypersensitivity (IBH) is the most common skin allergy in horses and ponies. Clinically it is characterized by intense itch and irritation caused by hypersensitivity reactions mainly to components of the saliva of midges of the genus *Culicoides*.[1–3] Welfare of horses suffering from IBH is seriously affected by this allergic reaction and by self-mutilation in an attempt to reduce the itch.

Various studies have shown that IBH is an IgE-mediated allergy that resembles type I allergies in humans.[4,5] In addition, there is evidence for the involvement of Th1-, Th2- and regulatory T (Treg)-type cytokines in IBH in Icelandic horses.[6–10] It has been shown that peripheral blood mononuclear cells (PBMC), stimulated with *Culicoides* whole body extract (WBE), from IBH-affected horses, produced higher levels of IL-4 and lower levels of IFN-γ than in healthy horses, suggesting a bias towards a Th2 immune responsiveness.[6] PBMC from affected horses also produced less IL-10 and TGFβ1, indicating that IBH is associated with a reduced regulatory immune response.[7] Furthermore,

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upon stimulation of PBMC with *Culicoides* WBE the expression of FoxP3 by $CD4^+CD25^+$ cells was significantly higher in healthy than in IBH-affected horses. Moreover, in skin biopsies of healthy Icelandic horses, significantly higher levels of mRNA for FoxP3 were found compared to the skin of IBH-affected horses.[10] In contrast, another study showed no significant differences in Foxp3 mRNA expression levels between skin biopsies of IBH-affected and healthy Shetland ponies.[11]

Current preventive and treatment options, including insect blankets, insect repellents, corticosteroids and antihistamines, fail to target the mechanisms underlying the allergy and often have limited efficacy and may have several adverse effects. Therefore, there is a strong need for allergen-specific immunotherapy for horses suffering from IBH. Two open immunotherapy trials using WBE of *Culicoides* spp. have reported controversial results.[12,13] The only double blind study did not show a beneficial effect in horses with IBH after 1 year of immunotherapy with a commercially available extract from *Culicoides nubeculosus*.[14]

Using WBE or recombinant allergens from *Culicoides* species that actually feed on horses (in particular those from the *Culicoides obsoletus* complex) is important in diagnostic assays for IBH.[15] The use of WBE of other *Culicoides* species (*C. nubeculosus* and *C. sonorensis*), which contrary to *C. obsoletus* can be cultured in the laboratory but are non-native for Dutch horses, dramatically decreased the sensitivity of these tests. It is therefore most likely that for optimal efficacy immunotherapy should be conducted with allergens from the *Culicoides* species by which the horses are mostly bitten.

Seven allergens from *C. obsoletus* complex midges (Cul o 1–Cul o 7) were recently identified and expressed in *Escherichia coli*.[16] They were all recognized by IgE present in plasma of horses with IBH and were able to induce an immediate type hypersensitivity reaction in skin of IBH-affected horses. Hence, these *C. obsoletus* recombinant allergens may be interesting candidates for immunotherapy. Whereas previous studies[17] focused on recognition of these recombinant allergens by IgE, their relevance for cellular immunity is still unknown. Moreover, studies aiming at the analysis of T-cell responsiveness in IBH have only used WBEs of *C. nubeculosus* and were conducted in Icelandic horses.[6,18]

In the present study we used a dendritic cell (DC)/PBMC co-culture system to determine whether the recombinant *C. obsoletus* allergen combinations P1 (Cul o 1, Cul o 2, Cul o 5) and P2 (Cul o 3, Cul o 5, Cul o 7)[17] were able to stimulate T cells of Shetland ponies and whether allergen-specific responses differed between IBH-affected and healthy ponies. We show that recombinant *C. obsoletus* allergens are capable of stimulating allergen-specific Th1 and Treg cells in DC/PBMC co-cultures of both healthy and IBH-affected ponies exposed to midges of the *C. obsoletus* complex. These cultures did not reveal significant differences between healthy and IBH-affected ponies, except for higher IL-4 mRNA levels in IBH-affected ponies after stimulation with allergen combination P1.

Material and methods

Animals

Twenty privately-owned Shetland ponies were included in this study. IBH-affected Shetland ponies ($n = 10$, age range 2–21 years) were selected based on clinical signs in the present IBH season and history, namely recurrent, pruritic skin lesions located at the mane and the base of the tail. Healthy (control) Shetland ponies ($n = 10$, age range 3–17 years) had no clinical signs or history of IBH and were age-matched as closely as possible to the IBH-affected ponies from the same stables. None of the ponies received corticosteroids or any other immunosuppressive or itch-reducing treatment. All animal experiments were approved by the Animal Ethics Committee of Utrecht University.

Production of recombinant allergens

The *C. obsoletus* complex allergens Cul o 1, Cul o 2, Cul o 3, Cul o 5 and Cul o 7 (Genbank accession numbers KC339671, KC339672, KC339673, KC339675 and KC339677) were produced as previously described.[16]

We used pools, P1 (Cul o 1, Cul o 2 and Cul o 5) and P2 (Cul o 3, Cul o 7 and Cul o 5), of recombinant allergens instead of single allergens, in order to increase the sensitivity of the assay by stimulating a greater variety of allergen-specific T cells. These allergen pools were based on a previous study that showed that Cul o 5, which is present in both pools, is the strongest of the seven recombinant allergens.[1] Cul o 5 allergen was combined with promising other allergens which also show high IgE binding reactivity in ELISA and high skin reactivity in intradermal tests. These additional allergens were different between P1 and P2 to assess potential profound differences in T-cell stimulation by these additional allergens. We did not include Cul o 4 and Cul o 6, because these allergens reacted relatively weakly with IgE and were less able to distinguish between healthy and IBH-affected horses.

Production of recombinant horse cytokines GM-CSF and IL-4

The sequences of horse GM-CSF and horse IL-4 (Genbank, accession number NM_001082519.1 and NM_001081882.1) encoding the mature form (without signal sequence) of the proteins were ordered as synthetic genes (GeneArt, Life Technologies; Bleiswijk, the Netherlands) codon-optimized for expression in *E. coli* and with a *Bam*HI and *Hin*dIII restriction site at the 5′and 3′-ends, respectively. The products obtained were cut with *Bam*HI and *Hin*dIII, and cloned downstream of the hexahistidine encoding tag into the corresponding restriction sites of a pET15b derived vector, pET15bNew (Novagen; Billerica, MA, USA). The isolated pET15bNew plasmids encoding IL-4 or GM-CSF were used to transform *E. coli* BL21 CodonPlus (DE3) (Agilent Technologies; Amstelveen, the Netherlands). Expression, purification and refolding of the insoluble (inclusion bodies) horse GM-CSF was performed as described for recombinant carp chemokines.[19] The horse IL-4, produced using the same approach as described for GM-CSF, was present in the soluble protein fraction of the bacterial lysate, which was kept on ice. After addition of 0.4 mol/L NaCl, 25 mmol/L imidazole and 1% Triton × 114 soluble protein fraction was purified by incubation with chelating sepharose fast flow (Amersham-Biosciences GE Healthcare; Eindhoven, the Netherlands) charged with Ni^{2+} according to the manufacturer's instructions. The $Ni2^+$ beads were subsequently washed with 40 column volumes of ice-cold Phosphate Buffered Saline (PBS), containing an additional 0.4 mol/L NaCl, 25 mmol/L imidazole and 1% Triton × 114 to remove contaminating proteins and Lipopolysaccharide (LPS). The Ni^{2+} beads were then washed with five volumes of 40% isopropanol in PBS to remove the Triton- × 114 and subsequently washed with 10 column volumes of PBS containing an additional 0.4 mol/L NaCl. The his6-tagged, recombinant horse IL-4 was finally eluted with the same buffer containing 250 mmol/L imidazole. Protein-containing fractions were pooled and dialyzed extensively against PBS and, after filter sterilization (0.2 μm), stored at −20°C.

Production and FITC labelling of the monoclonal anti-equine CD4

Hybridoma cells producing monoclonal mouse antibody against equine CD4 (clone CVS4) (a generous gift of David P. Lunn from Colorado State University, USA) were cultured in Opti-MEM with 10% Fetal Calf Serum (FCS) (Gibco, Life Technologies, Bleiswijk, The Netherlands) at 37°C and 5% CO_2. After 3 weeks, cells were transferred into medium without FCS to simplify the antibody purification by a Gammabind Plus Sepharose column (Amersham Biosciences, GE Healthcare). After purification, the antibodies were labelled with FITC, using a FluoroTag conjugation kit (Sigma-Aldrich; Zwijndrecht, the Netherlands) according to the manufacturer's specifications.

PBMC collection

At the end of the IBH season, heparin blood (140 mL) was collected from the jugular vein of each pony with Intraflon two catheters (16G Ø1.6 mm; Vycon, Ecouen, France) under local anaesthesia with 2% lidocaine (B. Braun; Melsungen, Germany). The blood was mixed with 140 mL PBS, containing 2% horse serum. Of this mixture 35 mL was carefully pipetted into a SepMate™-50 (STEMCELL technologies; Grenoble, France) tube with Histopaque-1077 (Sigma-Aldrich) that was centrifuged at RT and 1,300 ***g*** for 20 min. The PBMC fraction harvested from the interphase was then washed twice with PBS containing 2% horse serum (Gibco, Life Technologies) and left on ice until further use.

Monocyte-derived DC

After PBMC isolation, 75% of the cells were labelled with a cross-reactive mouse anti-human CD14 (a monocyte marker, cross-reactive to horse according to the manufacturer) (biG 10, Biometec; Greifswald, Germany) diluted 1:100 and incubated at RT for 15 min. Cells were then washed twice with Macs buffer (PBS with 2% horse serum and 2 mmol/L EDTA; Gibco, Life Technologies) incubated with anti-mouse IgG microbeads (Miltenyi Biotec; Leiden, the Netherlands) and isolated via LS columns (Miltenyi Biotec) according to the manufacturer's specifications. The monocyte-depleted cell fraction was added to the 25% remaining PBMC to be used for antigen-specific T-cell generation.

In order to produce DC the CD14-positive cells (monocytes) were transferred into complete medium [RPMI 1640 (Gibco, Life Technologies), 10% horse serum, 1% Glutamax (Gibco, Life Technologies), 1% Pen/Strep (Gibco, Life Technologies) and 30 μmol/L β-mercaptoethanol (Sigma-Aldrich)] containing horse IL-4 (50 ng/mL) and horse GM-CSF (50 ng/mL) to induce monocyte differentiation into DC, and plated into a 24-well plate (1×10^6 cells/well), and incubated at 37°C and 5% CO_2 for 2 days.

After 2 days, 0.5 mL medium was carefully removed and replaced by 0.5 mL fresh complete medium with IL-4 and GM-CSF, both at 50 ng/mL. After three more days, cells were washed with complete medium and transferred to 96 flat-bottom well plates (0.5×10^6 cells/well). These immature DC were matured with 1 μg/mL LPS together with different stimulants: medium only (mock), 2.5 μg/mL Pokeweed mitogen (PWM) (Sigma-Aldrich), recombinant antigen pool P1 (1 μg/mL Cul o 1, 1 μg/mL Cul o 2, 1 μg/mL Cul o5) or pool P2 (1 μg/mL Cul o 3, 1 μg/mL Cul o 5, 1 μg/mL Cul o 7) and incubated for 24 h at 37°C and 5% CO_2.

After 24 h, 50 μL of each culture was used to measure DC maturation by fluorescence-activated cell sorting (FACS) analysis. For this purpose the DC cells were stained with mouse anti-human CD206[20] conjugated to phycoerythrin (PE) (a DC marker) (3.29B1.10, Beckman Coulter; Woerden, the Netherlands) and mouse anti-human CD86[21] conjugated to allophycocyanin (APC) (a DC maturation marker) (IT2.2, Biolegend, ITK Diagnostics; Uithoorn, the Netherlands), which were both cross-reactive with horse, and their respective isotype controls mouse IgG1 PE (MCA928PE, AbD Serotec; Puchheim, Germany) and mouse IgG2b APC (MPC-11; Biolegend ITK Diagnostics). Cells were washed and flow cytometry was performed with a FACS canto II (BD Biosciences; Erembodegem, Belgium) and results analysed with FlowJo software (Tree Star; Ashland, OR, USA).

Antigen-specific T-cell generation and stimulation with antigen-loaded DC

PBMC, including the $CD14^+$ depleted cells (see above), were re-suspended in complete medium (RPMI 1640 (Gibco, Life Technologies), 10% horse serum, 1% Glutamax (Gibco, Life Technologies), 1% Pen Strep (Gibco, Life Technologies) and 30 μmol/L β-mercaptoethanol (Sigma-Aldrich) and plated in a 24-well plate (1×10^6 cells/well). Before incubation with antigens, PBMC were allowed to recover for at least 1 h at 37°C and 5% CO_2. Cells were then stimulated with recombinant antigen Pool-1 (P1 = 1 μg/mL Cul o 1, 1 μg/mL Cul o 2, 1 μg/mL Cul o 5) and Pool-2 (P2 = 1 μg/mL Cul o 3, 1 μg/mL Cul o 5, 1 μg/mL Cul o 7), 2.5 μg/mL PWM (positive control), or medium only (mock).

After 2 days, 0.5 mL medium was carefully removed and replaced by 0.5 mL fresh complete medium with 2 ng/mL recombinant equine IL-2 (Kingfisher Biotech; Saint Paul, MN, USA). After three more days PBMC were harvested and washed with complete medium. These cultures were then plated into a 96-well v-bottom plate (0.5×10^6 cells/well) for antigen-specific T-cell stimulation. Matured antigen-loaded (PWM, P1, P2 or mock) DC were washed with complete medium and added (0.05×10^6 cells/well) to their corresponding antigen stimulated PBMC in a ratio of 1:10 (DC:PBMC). After one hour of recovery at 37°C and 5% CO_2, additional antigen (PWM, P1, P2 or mock) and 2 μL of monensin (Sigma-Aldrich), to block secretion of produced cytokines, were added and cultures were incubated at 37°C and 5% CO_2 for 6 h. Subsequently, cells were harvested and washed two times with PBS buffer and left on ice. Cell suspensions were sampled for FACS analysis and the remaining cells were re-suspended in 0.5 mL Trizol (Invitrogen, Life Technologies; Bleiswijk, the Netherlands) and frozen at −20°C for mRNA extraction.

Flow cytometry

Stimulated-cell suspensions were simultaneously stained for either CD3 (T-cell receptor marker), CD4, CD25 (alpha receptor of IL2), FoxP3 (Treg marker) and IL-10 or for CD3, CD4, IFN-γ and IL-4. Briefly, all cells were stained with Fluor506, a fixable viability dye (e-Biosciences; Vienna, Austria), according to the manufacturer's specifications. Subsequently, surface and intercellular staining was performed for 20 min at 4°C.

In order to identify the $CD3^+CD4^+CD25^{high}FoxP3^+IL\text{-}10^+$ cells (corresponding to IL10 expressing Tregs), labelling was first performed with cross-reactive goat anti-human CD25[22] (AF-223-NA, R&D systems; Abingdon, UK) followed by its secondary antibody donkey anti-goat IgG PE (F0107; R&D systems,) together with the FITC-labelled monoclonal mouse antibody specific for equine CD4, as detailed above. After CD4 staining, cells were fixed and permeabilized using a FoxP3 staining kit (e-Biosciences) according to the manufacturer's specifications. After fixation, the cells were further stained with rat anti-human CD3 Pacific Blue (cross-reactivity with horse indicated by the manufacturer) (CD3-12, AbD Serotec; Puchheim, Germany), rat anti-mouse FoxP3[9] Alexa 647 (FJK-16s; e-Biosciences) and mouse anti-bovine IL-10 biotin[6] (CC320, MCA2111B; AbD Serotec) followed by PerCP-labelled streptavidin (e-Biosciences). All antibodies were cross-reactive for horse. Rat IgG1Alexa 647 isotype control (MCA1123A647; AbD Serotec) was used for FoxP3 staining and for the nonlabelled CD25 and IL-10 antibodies only the secondary antibody was added to determine unspecific binding.

In order to identify the $CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}4^+$ cells, labelling was first performed with mouse anti-equine CD4 and cells were subsequently fixed. After fixation and permeabilization, rat anti-human CD3 Pacific Blue (CD3-12, AbD Serotec) and secondary labelling was performed for CD4 with anti-mouse PE (e-Biosciences) and intercellular staining with cross-reactive mouse anti-bovine IFN-γ FITC (CC302, MCA1783F; AbD Serotec), and mouse anti-equine IL-4 Alexa 647 (12H8, Wagner Lab; Cornell University, Ithaca, NY, USA). Mouse IgG1 FITC and mouse IgG1 Alexa 647 isotype controls (GM4992 and MG121; Thermo Fisher Scientific, Life Technologies, Bleiswijk, The Netherlands) were used to determine nonspecific staining. Cells were washed and flow cytometry was performed with a FACS canto II. Data were analysed using FlowJo software. For the

$CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}4^+$ fraction only FACS measurements with more than 200 events in the final gate were used for analysis; however, for the $CD3^+CD4^+CD25^{high}FoxP3^+IL\text{-}10^+$ staining a lower value of 100 events was accepted to prevent many samples being lost for analysis.

Qpcr

Messenger RNA extraction from the frozen Trizol samples was performed as previously described.[11] In short, 0.1 mL 100% chloroform was added to 0.5 mL of the Trizol samples and incubated at RT for 3 min. After centrifugation (15 min at 16,000 ***g*** at 4°C) the water-phase was used to extract mRNA using an RNeasy kit (Qiagen; Venlo, the Netherlands) according to the manufacturer's specifications. The mRNA concentrations were measured with a Nanodrop ND-1000 (Thermo Scientific; Etten-Leur, the Netherlands). All mRNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories; Veenendaal, the Netherlands) according to the manufacturer's instructions. QPCR was performed for the following genes: *18s*, *CD3ζ*, IFN-γ, IL-4, IL-10 and FoxP3, as described previously.[11] Relative expression of all cytokines were normalized against *18s* using the Pfaffl method[23] and a fixed point from the standard curve was used as calibrator. Subsequently mRNA expression levels were assessed relative to the corresponding *CD3ζ* expression, to relate the cytokine expression to the T cells in the DC/PBMC co-cultures.

Statistical methods

Statistical analyses were carried out using GraphPad Prism 4.00 (Graphpad Software, La Jolla, CA, USA). As our data were not normally distributed, the Mann–Whitney U-test was used to compare values from IBH-affected ponies with those from control ponies. For a number of ponies insufficient cells were left for analysis of the cultures without PWM or recombinant allergen (mock control). To maintain enough power for the statistical analysis, the values of control and IBH-affected horses were combined for these mock cultures provided that the maximum and the minimum value of the control mock group resided between the mean $\pm$ 2 $\times$ SD of the IBH-affected mock group. These combined mock groups were compared with the stimulated cultures of IBH-affected and control ponies using the Mann–Whitney U-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$). Results were considered significant at $P \leq 0.05$.

Results

Increased IFN-γ and IL-4 mRNA expression upon T-cell stimulation

In order to increase the likelihood of detecting allergen-specific T-cell responses, cells were first expanded by culturing PBMC for 5 days in the presence of the recombinant allergen pools and IL2. These T-cell enriched PBMC cultures were then co-cultured with antigen-loaded DC. The DC were matured by LPS to increase antigen presentation to the antigen-specific T cells and thereby maximize cytokine expression by these T cells.

Maturation of the cultured DC 1 day after addition of antigen and LPS as determined by FACS analysis was approximately 50% as indicated by CD86 expression on the DC (see Figure S1 in Supporting information).

In order to determine the effect of recombinant allergen-specific stimulation of T cells, mRNA expression of *CD3ζ*, IFN-γ, IL-4, IL-10 and FoxP3 was measured with RT-QPCR and normalized against *18s* rRNA levels. The normalized values of IFN-γ, IL-4, IL-10 and FoxP3 were divided by the normalized *CD3ζ* (see Figure S2 in Supporting information) values to correct for the number of T cells in the DC/PBMC co-cultures.

Expression of IFN-γ mRNA in T cells from control ponies was significantly higher after stimulation of the DC/PBMC co-cultures with (respectively) P1 ($P = 0.0326$), P2 ($P = 0.0242$) and PWM ($P = 0.0003$) compared to the unstimulated (mock) cultures. Similarly, T cells from IBH-affected ponies expressed significantly more IFN-γ mRNA after P1 ($P = 0.0040$), P2 ($P = 0.0213$) and PWM ($P = 0.0001$) stimulation (Figure 1a) than the mock controls.

The expression of IL-4 mRNA was significantly upregulated in T cells from control ponies stimulated with PWM ($P = 0.0003$) compared to the mock culture. T cells stimulated with P2 ($P = 0.004$) and PWM ($P = 0.0103$) from IBH-affected ponies expressed significantly more IL-4 mRNA compared to the mock. In addition, we found significantly more IL-4 mRNA expression in T cells stimulated with P1 antigens from IBH-affected ponies compared to control ponies ($P = 0.0152$) (Figure 1b), whereas this was not the case for P2 and PWM stimulation.

IL-10 mRNA was only significantly upregulated between mock and PWM ($P = 0.0345$)-stimulated T cells from IBH-affected ponies (Figure 1c).

There were no significant changes in FoxP3 mRNA expression between T cells exposed to the different stimuli compared to the mock culture (Figure 1d).

None of the mRNA levels showed significant differences between IBH-affected and control ponies, except for the abovementioned IL-4 mRNA levels following cell culture with P1, which were higher for IBH-affected than for healthy horses (Figure 2).

Increased percentage of IFN-γ $^+$ T cells from IBH-affected and control ponies after stimulation with allergens

Flow cytometry was used to determine the percentages of CD4 -positive cells expressing IFN-γ or IL-4 in the DC/PBMC co-cultures (Figure 2). The percentage of IFN-γ expressing T cells was significantly upregulated in DC/PBMC co-cultures from control ponies stimulated with P1 ($P < 0.0001$), P2 ($P < 0.0001$) and PWM ($P < 0.0001$) compared to mock stimulation. T cells from IBH-affected ponies also expressed significantly more IFN-γ when stimulated with PWM ($P < 0.0001$), P1 ($P < 0.0001$) and P2 ($P < 0.0001$) compared to the mock (Figure 2a). There were no significant changes in percentage IL-4 expressing T cells between mock-stimulated T cells and the different stimuli or between T cells from IBH-affected and control ponies (Figure 2b). Representative FACS plots for all different stimuli are shown for one IBH-affected and one control pony in Figure 2c.

Increased percentage of $CD25^{high}FoxP3^+$ IL-10-producing T cells after antigenic stimulation

$CD25^{high}$ T cells within the CD4-positive population (see above) were gated and the percentages of cells positive for FoxP3 and IL-10 within this population determined. The CD25, FoxP3 and IL-10 isotype controls showed no staining (Figure 3b).

We found a significant increase of $CD25^{high}$FoxP3-positive cells in T cells from control ponies stimulated with P1 ($P = 0.0006$), P2 ($P = 0.0367$) and PWM ($P = 0.0010$)

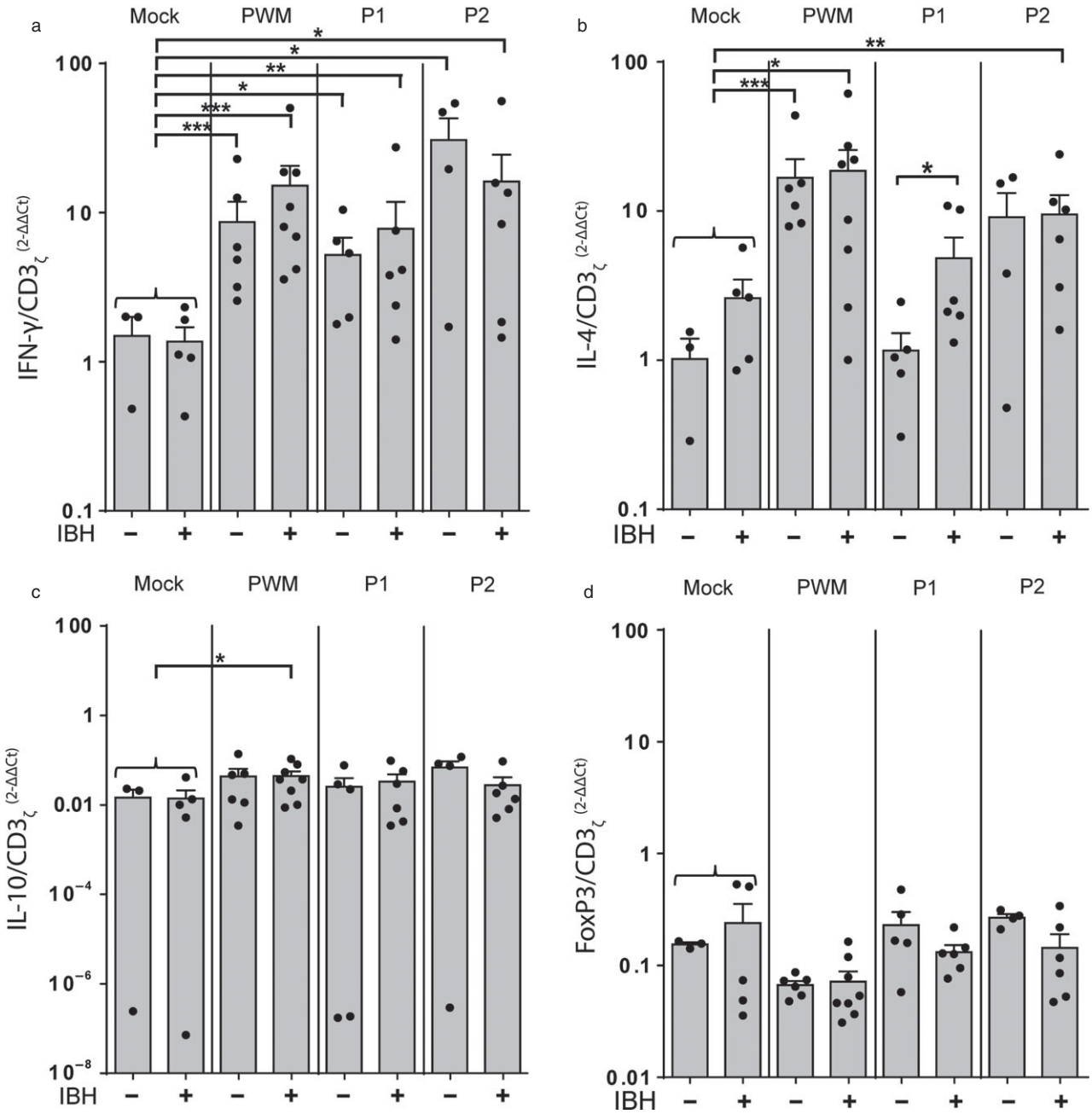

Figure 1. Expression of IFN-γ, IL-4, IL-10 and FoxP3 mRNA in dendritic cell/peripheral blood mononuclear cell (DC/PBMC) co-cultures stimulated with *Culicoides obsoletus*-specific antigens. DC/PBMC co-cultures derived from insect bit hypersensitivity (IBH)-affected and control ponies were stimulated with pools of recombinant *C. obsoletus* antigen [P1 = Cul o 1, Cul o 2, Cul o 5; P2 = Cul o 3, Cul o 5, Cul o 7), Pokeweed Mitogen (PWM) = (positive control)] or left unstimulated (mock) for 6 h in the presence of monensin. The mRNA expression of IFN-γ, IL-4, IL-10 and FoxP3 was determined by qRT-PCR and normalized against the housekeeping gene *18s* using the Pfaffl method[23] and subsequently divided by the *CD3ζ*/*18s* value of the same sample. The dots represent individual ponies and the bars represent the means of all samples in the groups. Significant differences are indicated as followed $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. (a) Expression of IFN-γ/CD3ζ (Th1 cytokine). (b) Expression of IL-4/CD3ζ (Th2 cytokine). (c) Expression of IL-10/CD3ζ (immune regulatory cytokine). (d) Expression of FoxP3/CD3ζ (Treg cell marker).

compared to unstimulated T cells (Figure 3a). The IBH-affected ponies showed significant differences between unstimulated T cells and P1 ($P = 0.0051$) and PWM ($P = 0.0189$) stimulation. Additionally, all $CD3^+CD4^+$ $CD25^{high}FoxP3^+$ cells expressed IL-10 (Figure 3b).

Discussion

Insect bite hypersensitivity is an IgE mediated pruritic skin disorder in horses and ponies caused by the bites of *Culicoides* midges. Seven allergens from *C. obsoletus* complex midges (Cul o 1–Cul o 7) have been produced as recombinant allergens in *E. coli* and shown IgE reactivity in horses with IBH.[16] In the present study we have shown in DC/PBMC co-cultures that recombinant allergen combinations P1 (Cul o 1, Cul o 2 and Cul o 5) and P2 (Cul o 3, Cul o 5 and Cul o 7) are capable of stimulating antigen-specific T cells of both healthy and IBH-affected Shetland ponies exposed to midges of the *C. obsoletus* complex.

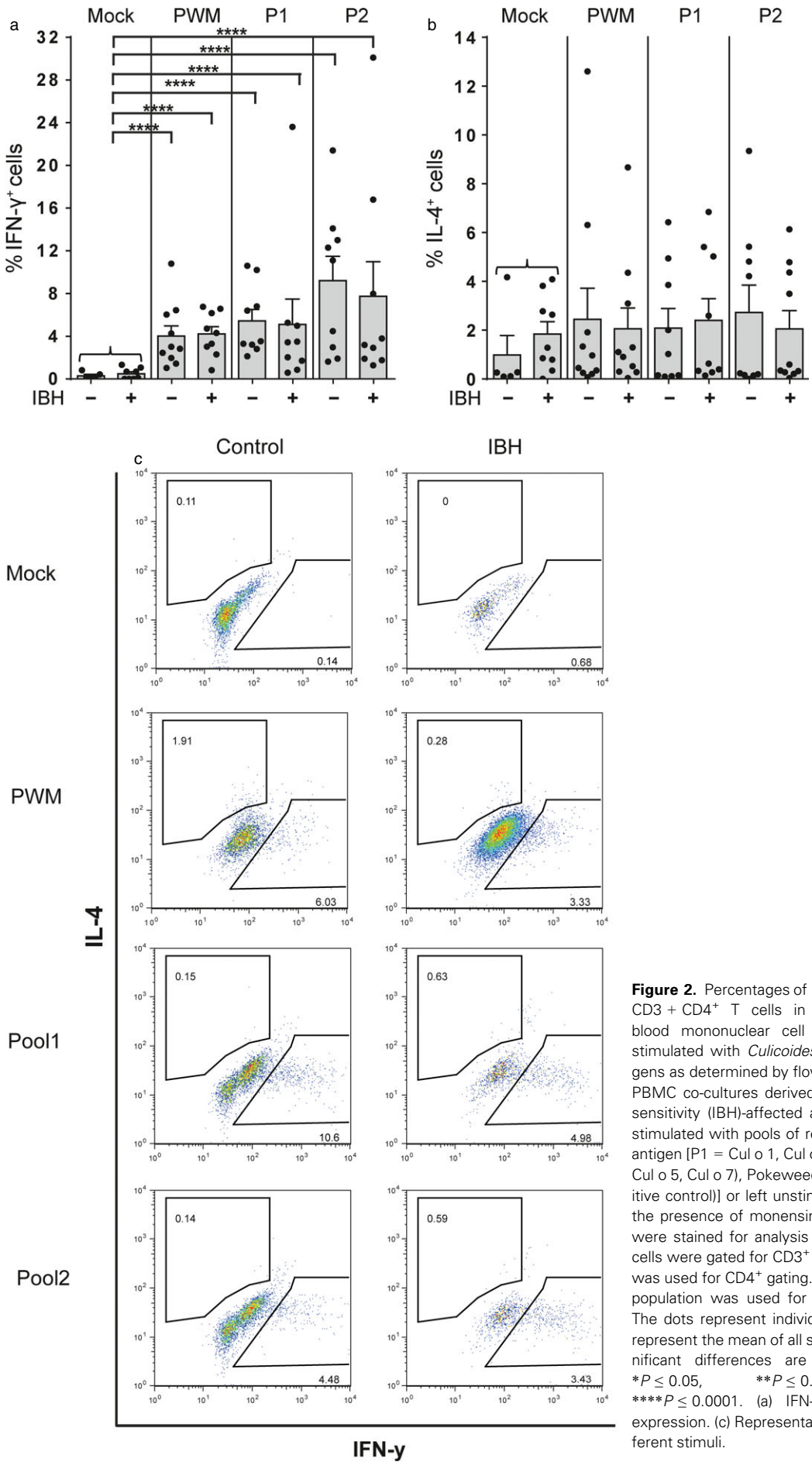

Figure 2. Percentages of IFN-γ and IL-4 expressing CD3 + CD4$^+$ T cells in dendritic cell/peripheral blood mononuclear cell (DC/PBMC) co-cultures stimulated with *Culicoides obsoletus*-specific antigens as determined by flow cytometry (FACS). DC/PBMC co-cultures derived from insect bite hypersensitivity (IBH)-affected and control ponies were stimulated with pools of recombinant *C. obsoletus* antigen [P1 = Cul o 1, Cul o 2, Cul o 5; P2 = Cul o 3, Cul o 5, Cul o 7), Pokeweed Mitogen (PWM) = (positive control)] or left unstimulated (mock) for 6 h in the presence of monensin. Subsequently, all cells were stained for analysis by flow cytometry. Live cells were gated for CD3$^+$ and the CD3$^+$ population was used for CD4$^+$ gating. Subsequently, the CD4$^+$ population was used for IFN-γ and IL-4 analysis. The dots represent individual ponies and the bars represent the mean of all samples in the group. Significant differences are indicated as followed $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. (a) IFN-γ expression. (b) IL-4 expression. (c) Representative FACS plots for all different stimuli.

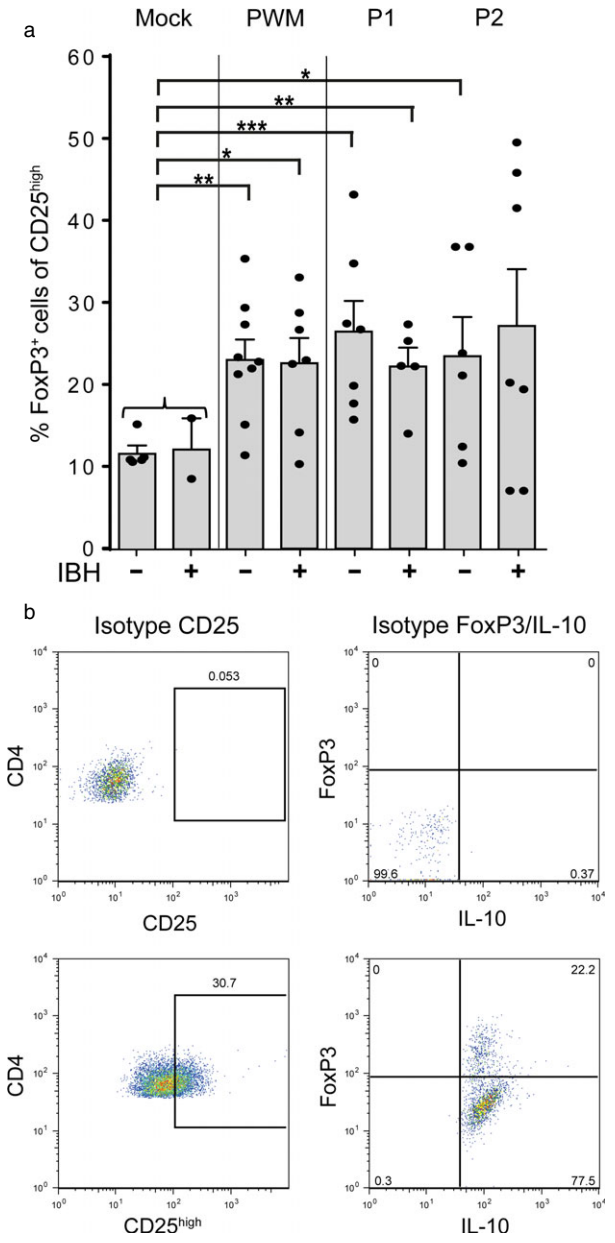

Figure 3. Percentage of FoxP3 and IL-10-expressing $CD3^+CD4^+$ $CD25^{high}$ cells in Dendritic Cell/Peripheral Blood Mononuclear Cell (DC/PBMC) co-cultures stimulated with *Culicoides obsoletus*-specific antigens as determined by flow-cytometry. DC/PBMC co-cultures derived from insect bite hypersensitivity (IBH) and control ponies were stimulated with pools of recombinant *C. obsoletus* antigen [P1 = Cul o 1, Cul o 2, Cul o 5; P2 = Cul o 3, Cul o 5, Cul o 7), Pokeweed Mitogen (PWM) = (positive control)] or left unstimulated (mock) for 6 h in the presence of monensin. Subsequently, all cells were stained for analysis by flow cytometry (FASC). Live cells were gated for $CD3^+$ and the $CD3^+$ population was used for $CD4^+$ gating. Subsequently de $CD4^+$ population was used for $CD25^{high}$ gating. The CD25high gate was used for FoxP3 and L-10 analysis. The dots represent individual ponies and the bars represent the mean of all samples in the group. Significant differences are indicated as followed $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. (a) Expression FoxP3, a Treg marker. (b) FASC plots for isotype controls from CD25, FoxP3 and IL-10.

In order to examine the presence of antigen-specific T cells and a possible difference in Th1/Th2 balance between IBH-affected and control ponies, IFN-γ and IL-4 expression levels in allergen stimulated and unstimulated (mock) cultures were compared. QPCR showed that IFN-γ mRNA was significantly upregulated in DC/PBMC co-cultures from both control and IBH-affected ponies stimulated with the recombinant allergen pools P1 and P2, compared to unstimulated cultures. In addition, flow cytometry showed a significant increase in the percentages of IFN-γ expressing T cells in the cultures after stimulation with P1 and P2. These data show for the first time antigen-specific *in vitro* stimulation of $CD4^+$ T-cells by recombinant *C. obsoletus* complex allergens in T-cell cultures from ponies exposed to these midges. Antigenic stimulation of IL-4-producing Th2 cells by the recombinant allergens was less pronounced. Still, antigenic stimulation with allergen P2 significantly upregulated IL-4 mRNA expression in cells from IBH-affected ponies compared to unstimulated cultures and P1 induced a significant higher IL-4 mRNA expression in cultures from IBH-affected than in cultures from control ponies. This indicates antigenic stimulation with both allergen pools and a tendency for a stronger Th2 response in IBH-affected than control ponies. Flow cytometry analysis did not show a significant increase in the percentage of IL-4 expressing cells in any of the stimulated cultures, including the PWM control. The cell cultures of some individual horses did seem to react to the allergen pools by a considerable increase in their percentage of IL-4-producing T cells, but for more than half of the ponies this was not the case.

Apart from a higher IL-4 mRNA expression in cultures of IBH-affected ponies stimulated with allergen P1, antigenic stimulation in the DC/PBMC co-cultures did not reveal a clear difference in Th1/Th2 response between IBH and healthy horses. One study observed a more pronounced Th2 bias in IBH-affected horses.[6] They obtained increased IL-4 mRNA levels and a higher percentage of IL-4-positive T cells in PBMC cultures of IBH-affected compared to healthy Icelandic horses after polyclonal (concanavalin A – conA) and antigenic (WBE of *C. nubeculosus*) stimulation. They also observed lower IFN-γ mRNA expression and a lower percentage of IFN-γ-positive T cells after conA stimulation of PBMCs from IBH-affected compared to those from healthy horses. The differences between the results of that study[6] and our observed bias towards a Th1 and Treg response might be the result of the different horse populations (Shetland ponies or Icelandic horses) or the different culture conditions used in both studies. On the one hand, the much longer *in vitro* culture time that we used and the stimulation with LPS-maturated DCs may have resulted in selective expansion and activation of T cells resulting in less clear representation of the *in vivo* Th1/Th2 balance than the overnight PBMC stimulation used in the other study.[6] On the other hand, the DC/PBMC co-cultures described here seem to be a sensitive way to detect antigen-specific T cells, as IFN-γ mRNA levels and the percentage IFN-γ-positive T-cells after stimulation with the recombinant allergens were comparable to those obtained with mitogenic (PWM) stimulation. Hamza *et al.*[6] could not detect IFN-γ expression after antigenic stimulation of PBMCs with *C. nubeculosus* WBE.

In addition, to the Th1\Th2 balance, Treg cells have been suggested to play a major role in the control of

allergies in humans[24] and horses.[18] For this reason we examined FoxP3 and IL-10 mRNA expression in the DC/PBMC co-cultures by QPCR and the percentage of $CD3^+CD4^+CD25^{high}$ expressing FoxP3 and IL-10 cells by flow cytometry. The mRNA levels for IL-10 and FoxP3 were much lower than those for IFN-γ and IL-4 and no significant differences in FoxP3 and IL-10 mRNA expression were observed between unstimulated and P1, P2 or PWM stimulated DC/PBMC co-cultures (Figure 1). However, in contrast to these QPCR data performed on the total DC/PBMC co-cultures, flow cytometry analysis of the $CD3^+CD4^+CD25^{high}$ subpopulation in these cultures, did reveal an increase of FoxP3- and IL-10-producing cells after stimulation with PWM and with the recombinant allergen pools P1 and P2. This indicates that P1 and P2 are able to stimulate the development of antigen-specific Tregs. The overall FoxP3 and IL-10 data suggest that P1 and P2 can activate Treg cells to produce the immune suppressive cytokine IL-10 *in vitro*.

In conclusion, pools of the recombinant *C. obsoletus* complex allergens, that were previously shown to bind to IgE of IBH-affected horses, can stimulate allergen-specific Th1 and IL10-producing Treg cells in DC/PBMC co-cultures of *C. obsoletus* complex exposed ponies. This is promising as several studies indicate that healthy horses have higher levels of Th1 and Treg cells and produce higher levels of IL10 upon antigenic stimulation than IBH-affected horses.[6,7,9,25] Moreover, successful antigen-specific immunotherapy of humans with insect venom allergies is associated with a Th1 skewing of the immune response and increased IL-10 production.[26–29] Therefore, these recombinant *C. obsoletus* complex allergens are promising candidates for specific immunotherapy of IBH-affected horses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. DC maturation. PBMC were isolated from blood of IBH and control ponies. After CD14+ monocyte isolation, cells were incubated with IL-4 and GM-CSF for 5 days. At Day 5 these innate DC were matured with LPS alone or LPS together with different stimulants: PWM (pokeweed mitogen), P1 (Cul o 1, Cul o 2, Cul o 5) or P2 (Cul o 3, Cul o 5, Cul o 7). Maturation was assessed by flow cytometry after staining cells for CD86 (mature DC marker) and CD206 (immature DC marker) and their isotype controls.

Figure S2. Expression of *CD3ζ* mRNA, a T-cell receptor marker in antigen-stimulated DC/PBMC co-cultures. Antigen-specific T cells derived from IBH-affected and control ponies were stimulated with mature unloaded (Mock) or antigen-loaded, monocyte-derived DC [P1 = Cul o 1, Cul o 2, Cul o 5; P2 = Cul o 3, Cul o 5, Cul o 7; or PWM = (positive control)] for 6 h in the presence of monensin. The mRNA expression of *CD3ζ* was determined by qRT-PCR and normalized against the housekeeping gene *18s* using the Pfaffl method[23]. The dots represent individual ponies and the bars represent the mean of all samples in the group.

Résumé

Contexte – Les poneys peuvent être atteint d'hypersensibilité aux piqures d'insectes (IBH), une dermatose prurigineuse allergique médiée par les IgE, induite par les allergènes de morsures de Culicoides spp.

Hypothèses/Objectifs – Déterminer si les allergènes recombinant de Culicoides obsoletus peuvent activer les cellules T de poneys exposés à *C. obsoletus* et si ces réponses spécifiques d'allergènes diffèrent entre les poneys atteints d'IBH et les poneys sains.

Sujets – Dix poneys Shetland atteint d'IBH et 10 témoins sains du même âge et de la même écurie, assurant une exposition similaire aux moucherons.

Méthode – Les cellules mononuclées de sang périphérique (PBMC) ont été mises en culture avec deux différents lots d'allergènes recombinants de *C. obsoletus* complex pour développer les cellules T spécifiques d'allergène. Ces cultures de PBMC ont été ensuite co-cultivées avec des cellules dendritiques matures (DCs) pourvues des mêmes antigènes. L'induction de Th1, Th2 et T régulatrices (Treg) dans ces co-cultures de DC/PBMC était évaluée par l'analyse des niveaux d'expression de IFN-c, IL-4, IL-10 et FoxP3 par RT-PCR quantitative et le phénotypage par cytométrie de flux.

Résultats – Les allergènes recombinant de *C. obsoletus* augmentaient l'expression d'ARNm d'IFN-c, les pourcentages de cellules (Th1) exprimant IFN-c et Tregs CD25highFoxP3+IL-10+ comparé aux co-cultures DC/PBMC non-stimulées. La stimulation des cellules Th2 exprimant IL-4 par les allergènes recombinant était nettement moins prononcée. Les co-cultures DC/PBMC ne révélaient pas de différence significatives entre les poneys sains et les poneys atteint d'IBH pour aucun des paramètres analysés à l'exception de niveaux d'ARNm d'IL-4 plus élevés chez les poneys atteints d'IBH après stimulation avec l'un des deux pools d'allergènes.

Conclusion et importance clinique – Les allergènes recombinant de *C. obsoletus* complex peuvent stimuler les Th1spécifiques d'antigène et les cellules Treg produisant IL10 et sont ainsi des candidats prometteurs pour l'immunothérapie de l'IBH.

Resumen

Introducción – los ponies pueden sufrir hipersensibilidad por picadura de insecto (IBH), una enfermedad pruriginosa alérgica de la piel mediada por IgE de inducida por alergenos de los insectos picadores del género *Culicoides*.

Hipótesis/Objetivos – determinar si alergenos recombinante *C. obsoletus* son capaces de activar los linfocitos T de ponies expuestas a *C. obsoletus*, y si estas respuestas específicas de alergeno difieren entre ponies afectados y ponies sanos.

Animales – 10 ponies de raza Shetland afectados con IBH, y 10 controles sanos con edades similares tomados de los mismos establos, para asegurar una exposición similar a los insectos.

Métodos – se cultivaron células mononucleares de sangre periférica (PBMC) con dos diferentes cócteles de alergenos recombinantes *C. obsoletus* para expandir los linfocitos T específicos de alergenos. Estos cultivos de PBMC fueron después co-cultivados con células dendríticas maduras (DC) cargadas con el mismo tipo de antígenos. Se evaluó la inducción de Th1, Th2, y linfocitos T reguladores (Treg) en los co-cultivos DC/PBMC analizando los niveles de expresión de IFN-c, IL-4, IL-10, FoxP3 mediante RT-PCR cuantitativa y fenotipado mediante citometría de flujo

Resultados – los alergenos recombinantes de *C. obsoletus* incrementaron la expresión de mRNA de IFN- los porcentajes de linfocitos T expresando IFN-c, y los linfocitos Treg positivos con a CD25, con expresión alta de FoxP3, y positivos IL-10 comparados con co-cultivos DC/PBMC no estimulados. La estimulación de linfocitos Th2 expresando IL-4 por los alergenos recombinantes fue mucho menos pronunciada. Los co-cultivos de DC/PBMC no revelaron diferencias significativas entre animales sanos y animales afectados por IBH en ninguno de los parámetros realizados, a excepción de mayores niveles de mRNA de IL-4 en los ponies afectados por IBH tras la estimulación con uno de los dos cócteles de antígeno.

Conclusiones e importancia clínica – el complejo de alergenos recombinante de *C. obsoletus* puede estimular los linfocitos Th1 específicos de antígeno y linfocitos Treg productores de IL-10 y son por tanto candidatos prometedores para la inmunoterapia de IBH.

Zusammenfassung

Hintergrund – Ponys können an einer Insektenstichhypersenisibilität (IBH), einer allergischen IgE-vermittelten juckenden Hauterkrankung leiden, die von Allergenen der beißenden Mücken der Culicoides spp. verursacht wird.

Hypothese/Ziele – zu bestimmen, ob rekombinante Culicoides obsoletus Allergene imstande sind T Zellen von Ponys, die C. obsoletus ausgesetzt sind, zu aktivieren und herauszufinden, ob diese Allergen-spezifischen Antworten sich zwischen den IBH-betroffenen und gesunden Ponies unterscheiden.

Tiere – Es wurden zehn IBH-betroffene Shetland Ponies und 10 altersangepasste gesunde Kontrollponies aus denselben Stallungen verwendet, um eine ähnliche Exposition zu Mücken zu gewährleisten.

Methoden – Es wurden periphere mononukleäre Blut-Zellen (PBMC) mit zwei unterschiedlichen Pools an rekombinanten C. obsoletus Komplexallergenen kultiviert, um die Allergen-spezifischen T Zellen zu expandieren. Diese PBMC Kulturen wurden in der Folge gleichzeitig mit reifen dendritischen Zellen (DCs) kultiviert, die mit denselben Allergenen beladen waren. Eine Induktion der Th1, Th2 und regulatorischen T (Treg) Zellen in diesen DC/PBMC Co-Kulturen wurde mittels Analyse der Exprimierungslevels von IFN-c, IL-4, IL-10 und FoxP3 mittels quantitativer RT-PCR und Phänotypisierung mittels Flowzytometrie beurteilt.

Ergebnisse – Rekombinante *C. obsoletus* Allergene erhöhten die mRNA Exprimierungslevels von IFN-c, sowie prozentual die IFN-c exprimierenden (Th1) Zellen und CD25highFoxP3+IL-10+tregs im Vergleich zu unstimulierten DC/PBMC Co-Kulturen. Die Stimulierung von IL-4 exprimierenden Th2 Zellen durch rekombinante Allergene war wesentlich weniger betont. Die DC/PBMC Co-Kulturen zeigten für keine der analysierten Parameter, außer für hohe IL-4 mRNA Werte bei IBH betroffenen Ponys nach der Stimulierung mit einem der beiden Allergenpools signifikante Unterschiede zwischen gesunden und IBH-betroffenen Ponys.

Schlussfolgerungen und klinische Bedeutung – Die rekombinanten *C. obsoletus* Komplexallergene können Antigen-spezifische Th1 und IL-10 produzierende Treg Zellen stimulieren und sind daher vielversprechende Kandidaten für eine Immuntherapie bei IBH.

要約

背景 – ポニーはCulicoides spp.の小昆虫咬傷からのアレルギーを含む、アレルギー性IgE介在性そう痒性皮膚疾患である昆虫刺傷過敏症(IBH)に罹患するかもしれない。

仮説/目的 – 組み換えCulicoides obsoletusアレルゲンがC. obsoletusに暴露されたポニーのT細胞を活性化できるかどうかおよびこれらのアレルゲン特異的反応がIBHに罹患したポニーと健康なポニーの間で異なるかを判断すること。

供与動物 – 10頭のIBH罹患シェットランドポニーおよび同様の小昆虫への暴露が保証されている、同じ厩舎の年齢が合致した10頭の健康な対象動物。

方法 – 末梢血単核細胞(PBMC)をアレルゲン特異的T細胞を増殖させるために2種類の異なった組み換えC. obsoletus複合体アレルゲンのプールで培養した。これらのPBMC培養をその後、同じアレルゲンを付加した成熟樹状細胞(DCs)と共培養した。このDC/PBMC 共培養中のTh1、Th2ならびに制御性T (Treg)細胞誘導を定量的RT-PCRとフローサイトメトリーによる表現型検査を用いて、IFN-c、 IL-4、 IL-10およびFoxP3の発現レベルを解析することで評価した。

結果 – 組み換えC. obsoletusアレルゲンは共培養した刺激していないDC/PBMCと比較し、 IFN-c mRNA発現レベル、IFN-C発現(Th1)細胞およびCD25highFoxP3+IL-10+ Tregsを増加させた。組み換えアレルゲンによるIL-4発現Th2細胞の刺激は明らかでなかった。DC/PBMC共培養は2種類のアレルゲンプールの1種類を用いて刺激した後のIBH罹患ポニーにおけるIL-4mRNAレベルが高かったことを除き、解析したパラメーター全てで健康なポニーとIBH罹患ポニーの間で有意差は明らかでなかった。

結論および臨床的な重要性 – 組み換えC. obsoletus複合アレルゲンはアレルゲン特異的Th1およびIL10産生Treg細胞を刺激することができ、それゆえIBHの免疫療法に関する有望な候補となる。

摘要

背景 — 矮种马可能会患昆虫叮咬过敏症(IBH),由蠓(库蠓属)抗原诱发,是过敏性IgE介导的瘙痒性皮肤病。

假设/目的 — 确定接触过退化库蠓的矮种马,用重组退化库蠓过敏原是否可激活其T细胞,并且这些过敏原特异性反应在IBH患马和健康马间是否不同。

动物 — 10只患有IBH的设得兰矮种马,以及10只来自同一马厩年龄相仿的设得兰矮种马,作为健康对照组,以确保接触昆虫情况一致。

方法 — 用重组退化库蠓复合过敏原,扩增过敏原特异性T细胞,在两个不同池中培养外周血单核细胞(PBMC)。随后,用载有相同抗原的成熟树突细胞(DCs) 与PBMC共同培养。使用定量RT-PCR和流氏细胞术表型分析仪,通过分析IFN-c、IL-4、IL-10 和FoxP3的表达水平,评估DC/PBMC 混合培养物中,Th1、Th2 和 调节T (Treg) 细胞的诱导情况。

结果 – 与未受刺激的DC/PBMC混合培养物相比,重组退化库蠓过敏原增强了IFN-c mRNA的表达水平,调高了IFN-c表达(Th1) 细胞的百分比,以及CD25highFoxP3+IL-10+ Treg的百分比。重组过敏原对表达IL-4TH2细胞的刺激不明显。任何参数均未显示健康小马和IBH患马的DC/PBMC复合培养物有明显差异,除了IBH患马的两个过敏原池,其中一个刺激后IL-4mRNA水平较高。

总结和临床意义 – 重组退化库蠓复合过敏原,能够刺激过敏原特异Th1和产生IL10的调节T细胞,因此IBH免疫疗法有望作为候选疗法。