Development of Immunotherapy for Insect Bite Hypersensitivity in Horses

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Thesis for the degree of Philosophiae Doctor

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Þróun ónæmismeðferðar gegn sumarexemi í hestum

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Ágrip

Sumarexem er ofnæmi af gerð I í hrossum með framleiðslu á IgE mótefnum sem leiða til losunar á bólguþáttum. Ofnæmið orsakast af biti smámýs af ættkvísl *Culicoides*. Sumarexem er ítrekuð húðbólga og einkennist af kláða, hárlausum svæðum, sáramyndun og jafnvel sýkingum í sárunum. Útfluttir íslenskir hestar fá sumarexem í um 50% tilfella, séu þeir óvarðir á flugusvæðum, en önnur hestakyn og íslenskir hestar fæddir á flugusvæðum fá sumarexem í tíðninni 5-10%. Ofnæmisvakar úr þremur smámýs tegundum, *C. sonorensis*, *C. nubeculosus* og *C. obsoletus* hafa verið skilgreindir, tjáðir í *E.coli* og nokkrir í skordýrafrumum og byggi. Markmiðið með verkefninu var að þróa ónæmismeðferð gegn sumarexemi; bólusetningu með hreinsuðum ofnæmisvökum í ónæmisglæðum og meðhöndlun um munnslímhúð með erfðabreyttu byggi sem tjáir ofnæmisvaka.

Við þróun á ofnæmisvakasértækri ónæmismeðferð voru mismunandi sprautunarstaðir og ónæmisglæðar prófaðir. Heilbrigðir íslenskir hestar voru sprautaðir í húð og í eitla með litlu magni af ofnæmisvökum með eða án Th1 stýrandi glæðis. Bólusetning í eitla og í húð með ónæmisglæði örvaði myndun á sértækum IgG1 og IgG4/7 mótefnum. Bólusetning í eitla með glæði gaf ívíð sterkara svar og mótefnin sem mynduðust hindruðu að hluta til bindingu IgE við ofnæmisvaka. Í næstu tilraun voru íslenskir hestar sprautaðir í eitla með ofnæmisvökum í Alum eða blöndu af Alum og Monophosphoryl lipid A. Bólu-setningin örvaði sterka IgG1 og IgG4/7 mótefnasvörun sem hindruðu bindingu IgE við ofnæmisvaka. Munur á ónæmissvörun hópanna var mælanlegur í boðefnasvari í kjölfar örvunar á hvítfrumum en ekki í mótefnasvari. Marktækt meiri IFNγ og IL-10 framleiðsla var hjá Alum/MPLA hópnum samanborið við óbólusetta viðmiðunar hesta, þessi munur sást ekki hjá Alum hópnum. Þetta bendir til að Alum/MPLA blandan hvetji Th1/Tstjórnfrumu miðað svar.

Þróuð voru byggmél og byggblanda til að meðhöndla hross um munnslímhúð. Heilbrigðir íslenskir hestar voru meðhöndlaðir með byggi sem

tjáir ofnæmisvakann Cul n 2. Í kjölfar meðhöndlunar mældust sérvirk IgG1 og IgG4/7 mótefni í sermi og munnvatni. Þau gátu að hluta til hindrað bindingu IgE við Cul n 2 en einnig við Cul o 2 sem er sambærilegt prótein úr *C. obsoletus*.

Mikilvægt er að hafa endurraðaða ofnæmisvaka sem auðvelt er að hreinsa, hafa svipaða þriðja stigs byggingu og eftirbreytni og náttúrulegu próteinin þegar árangur ónæmismeðferðar er metinn og klínísk greining sjúkdómsins staðfest. Byggfræ eru vel til þess fallin að framleiða prótein og fyrir langvarandi geymslu proteina á stöðugu formi. Tveir ofnæmisvakar framleiddir í byggi voru jafn vel fallnir til notkunar í ELISA mótefnaprófi og samsvarandi vakar framleiddir í *E.coli* og skordýrafrumum.

Niðurstöðurnar sýna að bólusetning í eitla með hreinum ofnæmisvökum í blöndu af Alum/MPLA er vænleg leið í þróun á fyrirbyggjandi meðferð gegn sumarexemi og mögulega líka afnæmingu. Enn fremur fengust vísbendingar um að meðhöndlun um slímhúð munns með byggi sem tjáir ofnæmisvaka gæti verið góður valkostur. Þannig væri komist hjá vandasamri sprautun og kostnaðarsamri próteinhreinsun.

Lykilorð: Sumarexem, hestar, ónæmismeðferð, ofnæmisvakar, bygg.

Abstract

Insect bite hypersensitivity (IBH) is a type I allergy of horses with production of IgE and release of inflammatory mediators. It is caused by bites of midges of the genus *Culicoides*. The disease is a recurrent dermatitis characterized by pruritic skin and hair loss, which can result in secondary infections. All breeds of horses can be affected, but horses born in Iceland and exported are more frequently affected than Icelandic horses born abroad. Allergens have been identified at the molecular level from three *Culicoides* spp., *C. sonorensis*, *C. nubeculosus* and *C. obsoletus*. They have all been produced in *E. coli* and some in insect cells and barley. The aim of the study was to develop immunotherapy for IBH using vaccination with purified allergens in adjuvants and a method to treat horses via the oral mucosa with transgenic barley expressing allergens.

Different injection routes and adjuvants were tested for development of prophylactic immunotherapy. Healthy Icelandic horses were vaccinated intradermally or intralymphatically with a small amount of purified allergens with or without a Th1 adjuvant. The intradermal and the intralymphatic vaccinations with adjuvant resulted in significant generation of allergen specific IgG1 and IgG4/7, the highest response being observed following intralymphatic vaccination. Furthermore, the antibodies produced after intralymphatic vaccination with the allergens in an adjuvant were able to partly inhibit binding of IgE to the allergens, an important mechanism of allergen immunotherapy. The intralymphatic route was employed to compare the immune response induced after vaccination with purified allergens in Alum alone or in a mixture of Alum and Monophosphoryl lipid A (MPLA). The vaccinated horses mounted a strong IgG1 and IgG4/7 response and the antibodies had the capacity to block the binding of IgE to the allergens. The adjuvant groups only differed with regard to the cytokine response but not in the antibody response. Compared to unvaccinated control horses, the IFNγ and IL-10 induced following *in vitro* restimulation of peripheral blood

mononuclear cells (PBMCs) with an allergen was significantly higher in the Alum/MPLA but not in the Alum group. This indicated an increased Th1/Treg response after vaccination with Alum/MPLA.

A spiral bit and a barley flour mixture were developed to treat horses via the mucosa of the mouth. Healthy Icelandic horses were treated with barley expressing the Cul n 2 allergen. The horses receiving the Cul n 2 barley mounted a specific IgG1 and IgG4/7 response in the sera and saliva. The antibodies induced were able to partly inhibit binding of IgE to Cul n 2 as well as to Cul o 2, the corresponding allergen from *C. obsoletus*.

For evaluation of the benefit of immunotherapy as well as to confirm the diagnosis of IBH it is important to have recombinant allergens with similar three-dimensional structure and post-translational modification to the native proteins which can be easily purified. Barley grains are an excellent source of proteins and ideal for stable storage. Two allergens produced in barley showed similar performance in ELISA to that of the corresponding proteins expressed in *E. coli* and insect cells.

In conclusion, intralymphatic vaccination with purified allergens in the adjuvant mixture Alum/MPLA is a promising approach for development of a prophylactic and probably also therapeutic immunotherapy against IBH. Furthermore, a first study has demonstrated that oral immunotherapy with barley expressing allergens could be an attractive alternative to conventional allergen immunotherapy, avoiding the expensive purification of recombinant allergens and the need of injection.

Keywords: Insect bite hypersensitivity, horse, immunotherapy, allergens, barley.

Acknowledgements

The project was predominantly carried out at the Institute for Experimental Pathology, University of Iceland, Keldur, from 2012 to 2017 but also at Prof. Eliane Marti's lab at the University of Berne in Switzerland and at ORF Genetics in Iceland. I worked at Prof. Eliane Marti's lab for seven weeks in 2012 and 2014 and three weeks in 2015. I am grateful to those institutes for allowing me to do my PhD project at their facilities.

First of all I would like to thank my supervisors, Sigurbjörg Þorsteinsdóttir and Vilhjálmur Svansson, for their endless encouragement, guidance and motivation throughout this project. I am, as well, grateful for their friendship and kindness over the years.

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Contents

List of abbreviations

Alum	Aluminium hydroxide
AP	Alkaline phosphatase
APCs	Antigen presenting cells
ASIT	Allergen specific immunotherapy
AUC	Area under the curve
Cul n	*Culicoides nubeculosis*
Cul o	*Culicoides obsoletus*
Cul s	*Culicoides sonorensis*
Con A	Concanavalin A
DAMPs	Damage-association molecular patterns
DCs	Dendritic cells
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FcεRI	Fc epsilon receptor I
FoxP3	Forkhead box P3
HRP	Horseradish peroxidase
HSA	Human serum albumin
i.d.	Intradermal
i.l.	Intralymphatic
IBH	Insect bite hypersensitivity
IFNγ	Interferon gamma
IL-	Interleukin
LPS	Lipopolysaccharide

MeDALL	Mechanisms of the Development of Allergy
MPLA	Monophosphoryl lipid A
OD	Optical density
PAMPs	Pathogen-association molecular patterns
PBL	Peripheral blood leukocyte
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PRRs	Pathogen-recognition receptors
PVDF	Polyvinylidene difluoride
r-	Recombinant
RAO	Recurrent airway obstruction
ROC	Receiver operating characteristic
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SCIT	Subcutaneous immunotherapy
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGE	Salivary gland extract
SLIT	Sublingual immunotherapy
sLT	Sulfidoleukotriene release
TGFβ	Transforming growth factor
Th	T helper cell
TLRs	Toll like receptors
T_{reg}	T regulatory cell
WB	Western blot

List of figures

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: A pilot study.** Jonsdottir, S., Hamza, E., Janda, J., Rhyner, C., Meinke, A., Marti, E., Svansson, V., & Torsteinsdottir, S., *Vet Immunol Immunopathol*, 2015, 166(1-2), 8-21.

II. **A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A.** Jonsdottir, S., Svansson, V., Stefansdottir, S. B., Schupbach, G., Rhyner, C., Marti, E., & Torsteinsdottir, S., *Vet Immunol Immunopathol*, 2016, 172, 14-20.

III. **Oral administration of transgenic barley expressing a *Culicoides* allergen induces specific antibody response.** Jonsdottir, S., Svansson, V., Stefansdottir, S. B., Mantyla, E., Marti, E., & Torsteinsdottir, S. *Equine Vet J*, 2016.

Declaration of contribution

Paper I: This paper describes vaccination of horses with purified allergens with and without IC31® adjuvant. I took part in all steps of the experiment; the vaccination, analysis of the samples, data analysis and writing the paper. I participated in the sample collections, immunoblot analysis and *in vitro* stimulation done at Keldur. I participated in the RT-PCR and antibody ELISA carried out at Prof. Eliane Marti's lab at the University of Berne, Switzerland. I collected the data, participated in the data analysis and in the writing of the paper. I took care of the submission and the revision process.

Paper II: This paper describes vaccination of horses with purified allergens in Alum or in a mixture of Alum/MPLA. I took part in all steps of the experiment, planning and design as well as the vaccination and analysis of the samples to the data analysis and writing the paper. I participated in the sample collections and *in vitro* stimulation done at Keldur. I participated in the expression of the allergens in insect cells done at Keldur, it was also part of Sara Björk Stefánsdóttir's master's project. I participated in the RT-PCR and the antibody ELISA carried out at Prof. Eliane Marti's lab. I collected the data, participated in the data analysis and the writing of the paper. I took care of the submission and the revision process.

Paper III: This paper describes the development of a method to treat horses via the oral mucosa with transgenic barley expressing an allergen. The expression of the allergen in barley was performed at ORF Genetics and all work concerning the barley itself. I took part in all steps of the experiment, planning and design as well as the oral treatment and analysis of the sample to the data analysis and writing the paper. I participated in the development of the barley mixture, the treatments and sampling performed at Keldur. I

participated in the antibody ELISA performed at Prof. Eliane Marti's lab. I collected the data, participated in the data analysis and the writing of the paper. I took care of the submission and the revision process.

Unpublished data I: An attempt was made to induce allergy in horses against one allergen. I took part in all steps of the experiment. I participated in the sample collections and *in vitro* stimulation done at Keldur. The expression of the allergens in insect cells was done by Sara Björk Stefánsdóttir, a master student at our lab at Keldur. The antibody ELISA was carried out at Prof. Eliane Marti's and the measurements of cytokines in cell supernatant was performed at Prof. Bettina Wagner's lab at Cornell University, USA. I collected the data and participated in the data analysis.

Unpublished data II: Two allergens were expressed in three different expression systems and their performance in ELISA compared. This work was carried out at Keldur, at Prof. Eliane Marti's lab and at ORF Genetics. The expression of the allergens in insect cells was done by Sara Björk Stefánsdóttir and Sæmundur Bjarni Kristínarson, master's students at our lab at Keldur. The expression in barley was done at ORF Genetics and the expression in *E. coli* at Prof. Eliane Marti's lab. The ELISA's were carried at Prof. Eliane Marti's lab. I took part in planning, designing and collection of the data and did a major part of the data analysis.

1 Introduction

1.1 The immune system

The immune system is a network of cells, organs and molecules that protects the host against pathogens and disease. It can be divided into innate and adaptive systems. The innate system offers the first defense upon encounter with a pathogen and the response is rapid. While the response of the adaptive immune system takes days to develop, it can eliminate pathogens more efficiently because of specific antigen receptors on the surface of lymphocytes (Murphy & Weaver, 2017b). The immune system reacts upon a danger signal caused by pathogens or damaged cells recognized by macrophages, dendritic cells or other sensor cells of the innate system. The cells express receptors, known as the pathogen-recognition receptors (PRRs), binding to simple molecules and regular patterns unique for pathogens, the pathogen-associated molecular patterns (PAMPs) (Murphy & Weaver, 2017b). The immune system can also respond to self-derived host molecules that indicate cellular infection, damage, stress or transformation termed damage-associated molecular patterns (DAMPs) (Murphy & Weaver, 2017c). When the antigen presenting cells (APCs) recognize PAMPs or DAMPs they are activated and can respond directly with effector activity or produce inflammatory mediators to recruit other cell types, such as innate NK cells or innate lymphoid cells (ILCs) (Murphy & Weaver, 2017b). In accordance with their cytokine patterns they can be divided into three groups, ILC1s, ILC2s and ILC3s, mirroring T helper (Th) 1, Th2 and Th17 lymphocytes. The ILCs do not have T- or B-cell receptors (reviewed in Morita et al., 2016). The APCs take up an antigen, process it and display it to naïve $CD4^+$ T helper cells that differentiate into any of the T cell subset, Th1, Th2, Th9, Th17, Th22, T regulatory (T_{reg}) or T follicular helper cells (Tfh). T cell differentiation is dependent on the source of the antigen (intracellular pathogen, extracellular pathogen, harmless protein, etc.) and the cytokines secreted by the APC or by other cells, such as ILCs. The cytokines that are essential for the differentiation and the cytokines produced by different T cell subsets are shown in figure 1.

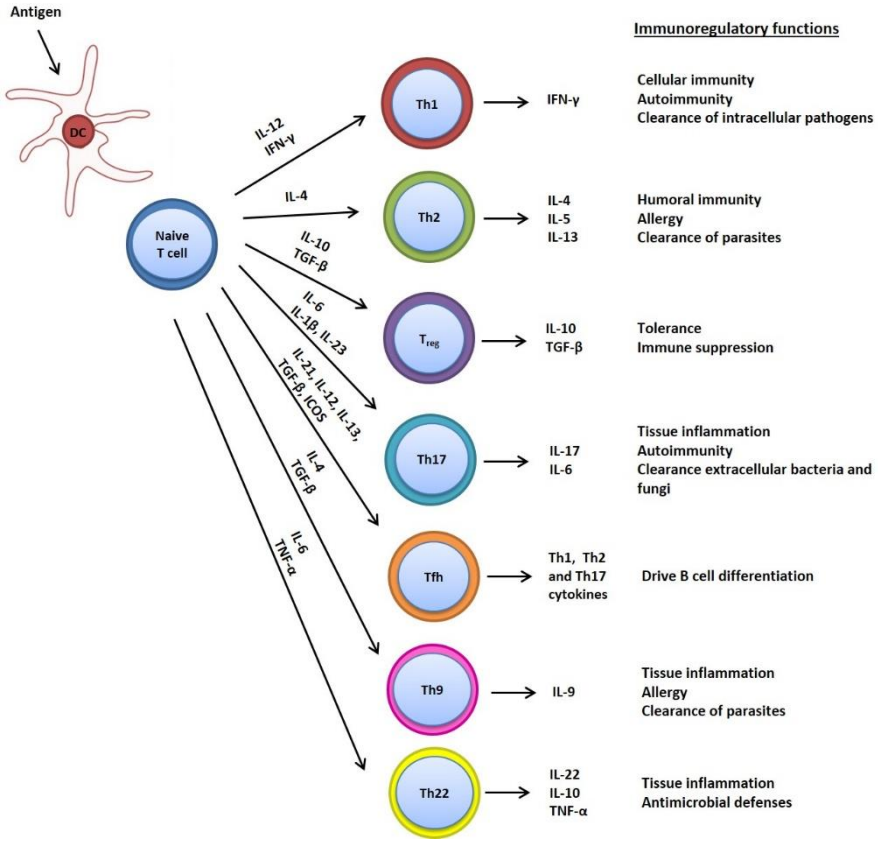

Figure 1: Differentiation of naïve CD4$^+$ T cells.
Naïve CD4$^+$ T cells differentiate into T cell subsets; Th1, Th2, T_{reg}, Th17, Tfh, Th9 and Th22 based on the antigen presented to them and the cytokines produced by the antigen presenting cells and by other immune cells in the environment. The figure was modified by Sara Björk Stefánsdóttir in accordance with Crotty (2014); Soyer et al. (2013).

1.2 Allergy

Allergy (type I hypersensitivity) is a type 2 immune response with production of IgE, activation of mast cells and basophils and release of inflammatory mediators. It is caused by harmless proteins from the environment that lack PAMPs, termed allergens (Murphy & Weaver, 2017a). What makes a protein an allergen is still not clear. It cannot be predicted with a simple rule and probably does not just depend on the features of the protein itself (reviewed in Scheurer et al., 2015).

Allergy involves two steps, first the sensitization and then an IgE mediated allergic response leading to a release of inflammatory mediators (Murphy & Weaver, 2017a). The symptoms and severity of allergic diseases differ and are dependent on the allergens and their entry. Seasonal rhinoconjunctivitis is caused by pollen or dust-mite feces in contact with the conjunctiva of the eye or the nasal mucosa, resulting in edema of these tissues and sneezing. A food allergy, on the other hand, caused for example by peanuts, fish and milk, can lead to vomiting, diarrhea and itching. Systemic anaphylaxis is the most severe allergic reaction. It can be caused by drugs, venoms or food entering the body intravenously, leading to increased vascular permeability, circulatory collapse and death (Murphy & Weaver, 2017a).

The type 2 immune response begins with inflammation and epithelial damage leading to a release of pre-made IL-25 and IL-33. The cytokines act as alarms, activating group 2 innate lymphoid cells (ILC2s) in the affected tissue. They secrete the Th2 cytokines, IL-4, IL-5, IL-9 and IL-13 into the tissue, triggering mucus production by the goblet cells, epithelial repair and arrival of immune cells (reviewed in Redpath et al., 2015). Upon first encounter of an allergen the DC instructs the naïve T helper cell to express IL-4 and IL-13, the first signal that a B-cell should switch to IgE production. The second signal is a co-stimulatory interaction between CD40 ligand on the T-cell surface and the CD40 on the surface of the B-cell. The specific IgE binds to the high affinity Fc epsilon RI (FcεRI) on mast cells and basophils. This process is called sensitization. Upon re-exposure the allergen binds to IgE bound to mast cell and basophil, causing cross-linking and activation. Activated mast cells and basophils release inflammatory mediators like histamine that cause an immediate increase in local blood flow and vessel permeability, leading to edema and local inflammation. The inflammatory mediators are also responsible for itching and sneezing (Figure 2). After activation of mast cells there is a recruitment of eosinophils and basophils at the site of the allergic reaction. The eosinophils are activated through cell surface receptors by the binding of cytokines, such as IL-5. Activated eosinophils can, firstly, release highly toxic granule proteins which can cause

tissue damage. Secondly, they can release prostaglandins, leukotrienes and cytokines that recruit and activate more eosinophils (Murphy & Weaver, 2017a).

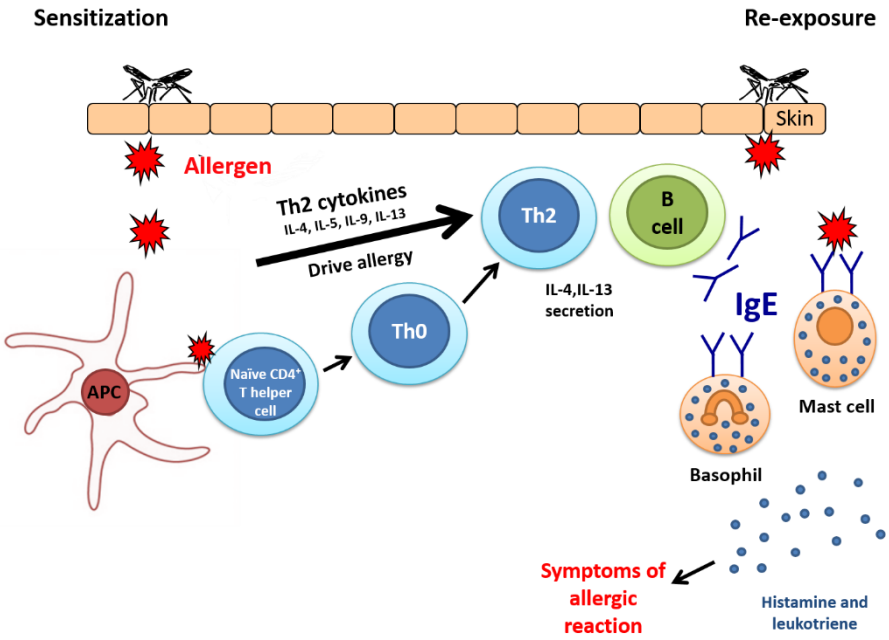

Figure 2: Schematic overview of type I hypersensitivity.
Sensitization occurs when the antigen presenting cell takes up the allergen, processes it and shows it to a naïve $CD4^+$ helper T cell that due to Th2 cytokines differentiates into a Th2 cell. The Th2 cell produces IL-4 and IL-13 instructing the B-cell to switch to IgE production together with the co-stimulatory interaction CD40 ligand and CD40. The specific IgE binds to FcεRI on mast cells and basophils. Upon **re-exposure** the allergen binds to mast-cell-bound IgE, causing cross-linking and activation. Activated mast cells release inflammatory mediators, such as histamine and leukotriene causing the symptoms of allergic reaction. Figure: Sara Björk Stefánsdóttir, modified in accordance with Allergy and Allergic diseases in *Janeway's immunobiology* 9th edition.

When sensitized individuals are re-exposed the allergic reaction starts immediately, but late-phase reaction can occur depending on the dose of the allergen. The late-phase reaction can occur 3-9 h after provocation and can persist for 24 h or longer. It is caused by continued synthesis and release of inflammatory mediators from mast cells and infiltrating eosinophils,

monocytes, basophils and lymphocytes. In healthy individuals antigens without danger signal are taken up by APC and shown to a naïve $CD4^+$ T helper cell that differentiates to T a regulatory cell (T_{reg}) due to IL-10 and TGFβ secreted by APC. The T_{reg} suppresses the T cell response and leads to tolerance to the antigen (Murphy & Weaver, 2017a).

A protein is a major allergen if it has more than 50% IgE reactivity. Allergens are named after the first three letters of the genus, then the first letter of the species and an Arabic number, e.g. the first allergen of *Aedes aegypti* is Aed a 1 (Arlian, 2002).

1.2.1 Insect allergy

Allergies to biting and stinging insects are known both in humans and animals (Arlian, 2002; Mueller et al., 2016). Common insects that bite are bedbugs, fleas, flies, mosquitoes and ticks and those that sting are ants, bees and wasps (Lee et al., 2016). Reactions to insect stings and bites are usually mild and local, but systemic reaction leading to anaphylaxis can occur (Peng et al., 2004).

The allergens of mosquitos originate in the salivary glands and they have been compared from different species. Peng *et al.* analyzed saliva or salivary gland extract from 10 species by immunoblot with pooled sera from human allergic patients for detection of IgG and IgE. These 10 species had 3 to 16 salivary allergens with molecular mass from 16 to 95 kDa, both species-shared and species-specific (Peng et al., 1998). The saliva of the female *Aedes aegypti* mosquito contains at least 55 protein transcripts and 10 of them have been identified as allergens (Peng et al., 2016; Peng et al., 1998), Aed a 1 – Aed a 8 and Aed a 10 – Aed a 11 (Table 1). Four of the 10 allergens have been expressed in insect cells, apyrase (Aed a 1, 68 kDa), D7 protein (Aed a 2, 37 kDa), unidentified salivary protein (Aed a 3, 30 kDa) and α-glucosidase (Aed a 4, 67 kDa) (Peng et al., 2016; Peng & Simons, 2007). Some allergens are found in more than one mosquito and a cross-reactivity between them has been shown. Aed a 1-specific polyclonal antibodies bound to a 68 kDa protein in 3 out of 4 species tested. Aed a 2 specific polyclonal antibodies bound to a 37 kDa protein in five *Aedes* species and in mosquitos

of two other genera *Ochlerotatus triseriatus* and *Culex quinquefasciatus* (Peng et al., 2001; Peng et al., 2006). The reaction to rAed a 1, 2 and 3 in a skin prick test differed. Reaction to rAed a 1 was 29-43%, to Aed a 3 32%, while only 10 % reacted to rAed a 2. None of the individual's negative in skin prick test responded to the three allergens. This shows the importance of using more than one allergen in an allergy test (reviewed in Peng & Simons, 2007).

Table 1: The yellow fever mosquito (*Aedes aegypti*) allergens.

Apyrase	Aed a 1
Salivary D7 protein	Aed a 2
Undefined 30 kDa salivary protein	Aed a 3
α-glucosidae	Aed a 4
Sarcoplasmic Ca+ binding protein	Aed a 5
Porin 3	Aed a 6
Undefined protein	Aed a 7
Heat Shock cognate protein	Aed a 8
Tropomyosin	Aed a 10
Lysosomal aspartic protease	Aed a 11

Based on http://www.allergen.org/

The four allergens expressed in insect cells have been used in ELISA for detection of allergen specific IgE. In that study 29% of mosquito bite test positive patients had Aed a 1 specific IgE, 11% were positive to Aed a 2, and 46% to both Aed a 3 and Aed a 4 (Peng et al., 2016; Peng & Simons, 2007). The recombinant proteins bind IgE, induce histamine release when used to stimulate basophils, and induce skin reactions.

Systemic allergic reaction following Hymenoptera sting affects 0.3% - 3.5% adult population (Bilo & Bonifazi, 2008). The Hymenoptera order consists of apids; honey bees and bumblebees, vespids; hornets, wasps and yellow jacket and formicids; fire ants and harvest ants. The venom of these stinging insects contains proteins that can cause IgE-mediated reactions (Arlian, 2002). The venom of honey bee and yellow jacket are the ones most studied (Perez-Riverol et al., 2015). Honey bee (*Apis mellifera*) venom

contains 12 identified allergens (Table 2) and the four major allergens are phospholipase A2 (Api m 1, 16 kDa), hyaluronidase (Api m 2, 39 kDa), acid phosphatase (Api m 3, 43 kDa) and melittin (Api m 4, 3 kDa) (Perez-Riverol et al., 2015).

Table 2: The honey bee (*Apis mellifera*) allergens.

Phospholipase A2	Api m 1
Hyaluronidase	Api m 2
Acid phosphatase	Api m 3
Melittin	Api m 4
Dipeptidylpeptidase IV	Api m 5
CUB serine protease	Api m 7
Carboxylesterase	Api m 8
Serine carboxypeptidase	Api m 9
Icarapin variant 2	Api m 10
Marjor royal jelly protein	Api m 11
Vitellogenin	Api m 12

Based on http://www.allergen.org/

The yellow jacket (*Vespula vulgaris*) venom contains 5 allergens (Table 3). The three major allergens are Phospholipase A1 (Ves v 1, 34 kDa), hyaluronidase (Ves v 2, 38 kDa) and antigen 5 (Ves v 5, 23 kDa).

Table 3: The yellow jacket (*Vespula vulgaris*) allergens.

Phospholipase A1B	Ves v 1
Hyaluronidase	Ves v 2
Dipeptidylpeptidase IV	Ves v 3
Antigen 5	Ves v 5
Vitellogenin	Ves v 6

Based on http://www.allergen.org/

As the hyaluronidase is an allergen in both honey bees (Api m 2) and yellow jackets (Ves v 2), with over 50% amino acid identity, patients can be

sensitized to both bees and vespids, although it is not common (reviewed in Arlian, 2002). The most common allergens among the Hymenoptera order are phospholipase, hyaluronidase and antigen 5 (Spillner et al., 2014). The use of venom extract for diagnosis does not always reflect absolute sensitization (Ebo et al., 2014) as it can lead to double positive results, up to 59% of Hymenoptera allergic patients show positive response to honey bee venom and yellow jacket venom (Perez-Riverol et al., 2015). Double positive results can be true double sensitization to both venoms or may be based on IgE cross-reactivity (Spillner et al., 2014). The IgE cross-reactivity can be due to common epitopes of the homologous allergens in both venoms, or the IgE antibodies could be directed against related N-linked carbohydrate determinants (Ebo et al., 2014; Spillner et al., 2014). Recombinant non-glycosylated *E. coli* expressed Api m 1, Api m 2 and Ves v 5 are commercially available. With those non-glycosylated allergens it was possible to distinguish between true double positive sensitization and cross-reactivity in 29 patients using IgE ELISA (Mittermann et al., 2010).

1.3 The immune system of the horse

The immune response of horses is mediated by the same cells, molecules and mechanisms as in other mammals (Felippe, 2016). The immunoglobulin isotypes of the horse are IgM, IgD, IgG, IgE and IgA. The basic function of IgM is activation of complement; IgE binds to both high and low affinity IgE receptors and is increased in parasite infection and allergy. The IgA has neutralizing activity and is involved in mucosal immunity but the function of IgD is unknown (Perkins & Wagner, 2015). IgG is the major immunoglobulin in horse sera and colostrum and neutralizes many horse pathogens (Goehring et al., 2010; Sheoran et al., 1998). The immunoglobulins of the horse have been studied for a long time, as hyperimmune equine antisera are used in therapy and prophylaxis of human and animal diseases. First it was thought that the horse had three IgG subclasses, IgGa, IgGb and IgGc. Later T-protein was identified after vaccination with diptheria or tetanus toxoid, it was believed to be a homologue of IgA. But later with structural and antigenic studies it was recognized as IgG and termed IgG(T). The IgG

subclasses were identified based on antigenic differences and mobility in an immunoelectrophoretic analysis (reviewed in Lunn et al., 1995). Monoclonal antibodies were raised against them (Sheoran et al., 1998). Sequencing revealed that the horse has seven genes encoding gamma heavy chains, and the IgG subclasses have now been termed IgG1-IgG7 (Wagner et al., 2004). The old subclasses have been linked to the new ones as follows: IgGa is IgG1, IgGb is IgG4 and IgG7, IgGc is IgG6 and IgG(T) is IgG3 and IgG5 (Wagner, 2006). IgG4/7 is the most abundant IgG subclass in sera and colostrum (Sheoran et al., 2000). The seven IgG subclasses were expressed in CHO cells (Lewis et al., 2008) and monoclonal antibodies raised against them (Keggan et al., 2013). The antibodies can be used for isotype quantification and the antibody response of healthy and diseased horses can be compared (Keggan et al., 2013). The IgG subclasses have different functions and it has been shown that IgG3, IgG1, IgG4/7 can all bind complement, while IgG2, IgG5 and IgG6 cannot. In the same study IgG1, IgG3, IgG4/7 and IgG5 all evoked a strong respiratory burst from equine peripheral blood leukocytes (PBL), indicating that these five subclasses interact with Fc gamma receptor (FcγR) of the cells (Lewis et al., 2008). In microbial and viral infections IgG4/7, IgG1 and IgG3 are the important subclasses for protective immunity (Goehring et al., 2010; Lopez et al., 2002; Soboll Hussey et al., 2011; Svansson et al., 2009), while IgG(T) has been linked to sensitization (Wagner et al., 2006). The preferred subclasses in a protective vaccine are IgG1, IgG3 and IgG4/7 (Lewis et al., 2008).

Foals receive their immunoglobulins, cytokines and maternal immune cells through the colostrum not via the placenta. The immune response of adult horses differs from young foals as young foals respond rather weakly. The equine fetus produces IgM and IgG1; however the endogenous IgG4/7 production is first detected 16-20 weeks after birth and IgE production only 6-12 months after birth. The neonatal foals therefore need maternal support during the first weeks of life. Studies on vaccination performance in young foals have shown that they respond poorly to known vaccines (reviewed in Perkins & Wagner, 2015).

The most common allergic diseases of horses are insect bite hypersensitivity (IBH), recurrent airway obstruction (RAO) and urticaria. Section 1.4 gives a detailed description of IBH. RAO, also known as the heaves, has been suggested to be a hypersensitivity reaction to inhaled molds and organic dust, but the involvement of IgE in the disease is controversial (Marti et al., 2008; Wagner, 2016). The symptoms of RAO are coughing and an increased effort in breathing due to cholinergic bronchospasm and airway hyperreactivity (Pirie, 2014). Urticaria or hives has been associated with insect bite, food allergens and environmental allergens, leading to typical lesions affecting the skin of the horse. The lesions can be of different shapes, round, linear strips or doughnut-like (Wagner, 2016).

1.4 Insect bite hypersensitivity

Insect bite hypersensitivity is a recurring seasonal allergy of type I in horses with production of IgE. It is known worldwide but under different names such as summer eczema, sweet itch, *Culicoides*-hypersensitivity and Queensland itch (Bröstrom et al., 1987; Riek, 1953). The first report of IBH goes back to 1840 in France (Bröstrom et al., 1987; Henry & Bory, 1937) and 1888 in Australia (Riek, 1953). It was not known until the 1980s that *Culicoides* spp. (midges) were responsible for IBH (Fadok & Greiner, 1990; Quinn et al., 1983). Prior to that, different speculations regarding the causative agent included, in addition to insect bites, climate conditions, sunlight, grass allergy, nutritional deficiencies, bacteria, fungi and microfilariae (Bröstrom et al., 1987).

1.4.1 Epidemiology

IBH is well known in *Culicoides* endemic areas and all breeds of horses can be affected. The prevalence varies worldwide between 3 and 60% (reviewed in Schaffartzik et al., 2012). In Great Britain the prevalence is only 3%, whereas in Shire horses in Germany it is 37.7% and 60% in Queensland Australia (reviewed in Schaffartzik et al., 2012). Iceland is one of the few places in the world where *Culicoides* spp. are not endemic, so Iceland is IBH-free. However, Icelandic horses exported to Europe show a high prevalence

of IBH as more than 50% develop the disease two years post export if they are located in *Culicoides* infested areas and not protected from the bites (Bjornsdottir et al., 2006). However, the overall frequency in horses born in Iceland and exported is 26.2-34.5% (Bjornsdottir et al., 2006; Bröstrom et al., 1987). On the other hand, only 6.7-8.2% of Icelandic horses born in a *Culiciodes* area develop the allergy (Bröstrom et al., 1987; Halldordsottir & Larsen, 1991; Schaffartzik et al., 2012). The prevalence is influenced by the environment with the exposure to *Culicoides* as the main factor. Everything affecting the midges will have a direct influence on IBH prevalence and climate factors such as wind and moisture being the most important (Schaffartzik et al., 2012). The age of exposure to *Culicoides* seems to be crucial for the prevalence of IBH. Icelandic-born horses that were exported at 7-10 months of age developed the IBH at the same frequency as European-born Icelandic horses (Sommer-Locher et al., 2012). Genetic factors do influence the prevalence of IBH. However, this cannot be studied in horses born in Iceland and exported to a *Culicoides* rich environment due to the strong effect of first *Culicoides* exposure at adult age (Bjornsdottir et al., 2006). In a study on 1200 German-born Icelandic horses, the overall prevalence of IBH was 4.6%. Three groups of offspring were compared in terms of IBH prevalence. The offspring from two affected parents developed IBH in 12.2% cases, 6.5% from one affected parent and 2.9% if both parents were healthy (Marti et al., 2008).

1.4.2 Clinical signs

IBH is characterized by severe itching leading to loss of hair and formation of lesions, sometimes resulting in secondary infections (Bröstrom et al., 1987). If the eczema becomes chronic it can lead to fibrosis, thickening of the skin and scaling (Riek, 1953; Schaffartzik et al., 2012). The horses develop the lesions at the feeding site of the midges, often on the mane, neck and tail, that can result in a complete loss of the mane and part of the tail. Others show symptoms along the ventral midline, the face, ear, chest and withers

(Figure 3) (Anderson et al., 1996; Baker & Quinn, 1978; Schaffartzik et al., 2012; Wagner, 2016). Horses can have one lesion, few or many, depending on the severity of the disease (Wagner, 2016).

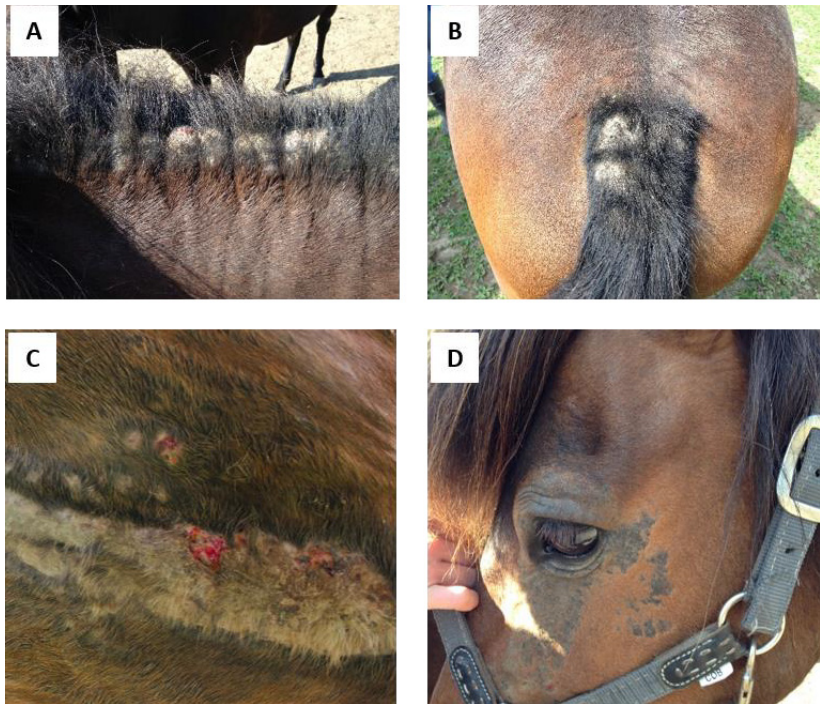

Figure 3: Clinical signs of insect bite hypersensitivity.
A) Mane**, B)** Tale**, C)** Belly**, D)** Face**.** The figures were kindly provided by Dr. Bettina Wagner.

1.4.3 Pathogenesis

IBH has been described in several studies as a type I allergy (Fadok & Greiner, 1990; Marti et al., 1999) and the involvement of IgE was confirmed by a Prausnitz-Küstner experiment (Wagner et al., 2006). IgE from affected horses was transferred into the skin of clinically healthy horses, which then responded to *Culicoides* extract in an intradermal test (Wagner et al., 2006). Not only IgE but also IgG1 and IgG(T) specific for *Culicoides* salivary gland proteins are at significantly higher levels in sera of IBH-affected horses as compared to healthy controls (Hellberg et al., 2006). The sensitization of mast cells can be shown by intradermal tests (Figure 4). However the

interpretation can be difficult (Lebis et al., 2002) and it has been demonstrated that mast cells from healthy horses can be sensitized with IgE specific for *Culicoides* allergens (Wagner et al., 2009). Type IV allergy has also been suggested as following an intradermal test a delayed response has been observed (Baker & Quinn, 1978; Fadok & Greiner, 1990).

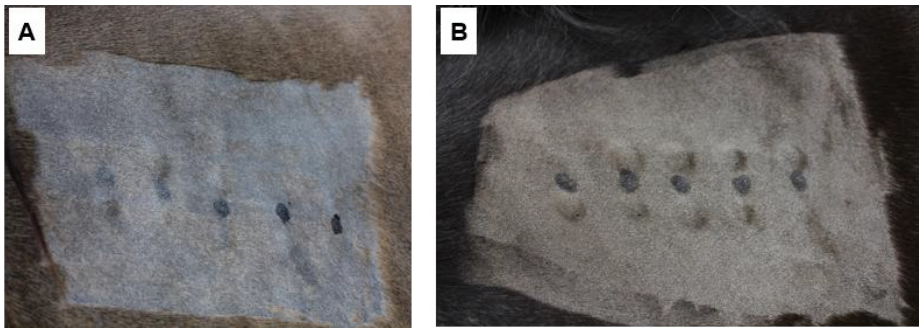

Figure 4: Intradermal test.
Intradermal test with *Culicoides* whole body extract and *Culicoides* recombinant allergens. **A)** Clinically healthy horse **B)** IBH-affected horse. The figures were kindly provided by Dr. Bettina Wagner.

Histopathological examinations of the lesions have revealed mixed perivascular to diffuse cellular infiltrate consisting of mononuclear cells and in acute lesions eosinophils (Fadok & Greiner, 1990; McKelvie et al., 1999). With immunohistochemical staining of the skin an increase in total T cell number in lesions compared to non-lesions was seen in the dermis from IBH affected horses. The lesions also contained a higher number of $CD4^+$ cells than the skin of healthy controls, but the number of $FoxP3^+$ cells, i.e. regulatory T cells was not significantly different between the groups (Heimann et al., 2011). At the mRNA level IL-13 was elevated in the skin of IBH affected horses as compared to healthy controls, but that was not the case for IL-4 and IFNγ. The mRNA level of IL-10 and $FoxP3^+$ was reduced in lesions compared to both non-lesion and healthy skin. The mRNA expression suggests that there is an imbalance between Th2 and T_{reg} cells (Heimann et al., 2011).

The Icelandic horse has been used to investigate the T cell response in the pathogenesis of IBH; horses born in Iceland and exported as adults (1st generation) are compared to Icelandic horses born in *Culicoides* infested areas (2nd generation). IBH is associated with changes in IL-4 producing T-cells. Polyclonal (mitogen) and allergen-specific stimulation of PBMCs from 1st and 2nd generation IBH-affected horses resulted in significantly higher levels of IL-4 than stimulation of PBMCs from healthy horses. Furthermore PBMCs from 2nd generation healthy horses produced more of the regulatory cytokine, IL-10.

(Hamza et al., 2007). Further studies of the 1st and 2nd generations showed that a supernatant of stimulated PBMCs from 2nd generation healthy horses could reduce the number of IL-4 producing T cells and IL-4 production when added to PBMCs from 1st generation IBH affected horses. The suppression of IL-4 production of PBMCs from 2nd generation healthy horses was shown to be a combined effect of IL-10 and TGFβ. This suggested that the 1st generation IBH horses have an impaired T regulatory response to *Culicoides* allergens (Hamza et al., 2008).

Stimulation of $CD4^+CD25^+$ regulatory T cells from 2nd generation healthy horses with *Culicoides* allergens induced more FoxP3 compared to 1st generation IBH affected horses. If IL-4 was added, FoxP3 induction was inhibited, suggesting that the decreased expression of FoxP3 in IHB horses could be a consequence of the increased IL-4 production by PBMCs of IBH horses. A subsequent study has demonstrated an impaired allergen-specific suppressive ability of T regulatory cells in IBH affected horses (Hamza et al., 2011; Hamza et al., 2012).

Horses in Iceland have a higher burden of endoparasites, higher faecal helminth egg counts, higher tapeworm specific IgG3/5 and higher total IgE than horses living in Switzerland. Following polyclonal and parasite antigen stimulation of PBMCs horses in Iceland displayed few IL-4 producing T-cells and showed high production of IL-10 and TGF-β1. When IL-10 and TGFβ specific antibodies were added to the stimulation the number of IL-4 producing T-cells was increased. The data indicates a strong T cell regulation of heavy parasite infections in contrast to what is seen in IBH (Hamza et al., 2010).

1.4.4 Diagnosis

IBH is mostly diagnosed using clinical signs and the history of recurrent symptoms. Commercial serological tests using whole body *Culicoides* extract have a low sensitivity and specificity (Frey et al., 2008). Functional tests measuring histamine (Wagner et al., 2008) or sulfidoleukotriene release (Baselgia et al., 2006) using whole body *Culicoides* extract give a somewhat better performance. IgE serology test with a pool of recombinant allergens originating from the most abundant *Culicoides* spp. in the area of the horses might be worth trying (van der Meide et al., 2012; van der Meide et al., 2014).

Currently, there is no efficacious treatment available for IBH and *Culicoides* avoidance is the best option to keep the symptoms mild or moderate. The midges are most active during the twilight and then it is important to house the horses and use blankets (Figure 5).

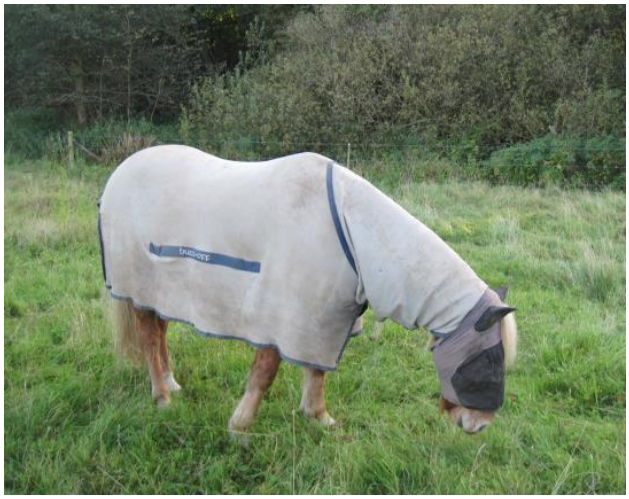

Figure 5: A blanket for protection against *Culicoides* bites.
The figure was kindly provided by Þórunn Guðmundsdóttir.

In severe cases steroids and anti-histamine are applied. Attempts have been made to inject horses with *Culicoides* whole body extract with contradictory results (Anderson et al., 1996; Barbet et al., 1990). In a double-blind, randomized placebo-controlled study where IBH affected horses received *Culicoides* extract or placebo no significant difference between the groups was detected (Ginel et al., 2014).

1.4.5 *Culicoides*

Culicoides are known as midges, no-see-ums and gnats. They belong to the order Diptera and are one of the smallest haematophagous flies, only 1 to 3 mm in size (Figure 6). Over 1400 species of *Culicoides* have been reported and they exist in almost all parts of the world except for the polar regions, New Zealand, the southern part of South America, the Hawaiian Islands and, until recently, Iceland. They need moist habitats such as pools, ponds or swamps. About 96% of them are blood sucking and attack animals, including humans (Mellor et al., 2000) and some *Culicoides* spp. have host preferences (Ninio et al., 2011). The bites cause discomfort and irritations and can cause allergic dermatitis such as IBH. The life cycle includes egg, four larval stages, pupa and adult, and they are short lived (Mellor et al., 2000; Mordue & Mordue, 2003). It is only the female that needs blood for the development of the eggs (Blackwell, 2001).

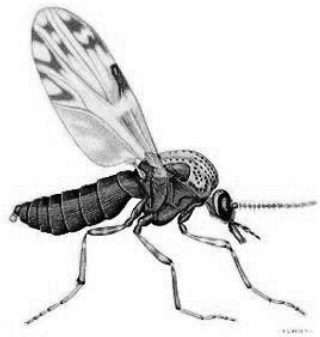

Figure 6: A midge (*Culicoides* spp.).

The midges are most active during the twilight but temperature and wind do affect their activity. Some *Culicoides* species are vectors transmitting pathogens such as African horse sickness virus (AHSV), infecting horses, and bluetongue virus (BTV), infecting all ruminants (Carpenter et al., 2013; Mellor et al., 2000). *Culicoides* species have different geographical distributions (Mellor et al., 2000). In Switzerland, for example *C. obsoletus* complex is the most common species (Cagienard et al., 2006; Casati et al., 2009) where IBH is a problem, but an outbreak of bluetongue virus has not

yet occurred as the *C. imicola* is not endemic (Cagienard et al., 2006; Casati et al., 2009).

Iceland is one of the few places in the world where *Culicoides* are not endemic and the only fly biting mammals is the black fly (*Simulium vittatum*) (Johannsson, 1988). However, in the summer of 2015 there was a small outbreak in the southwest of Iceland where *Culicoides (Wirthomyia) riouxi* attacked humans, causing great discomfort and itching. This was the first record of *Culicoides* in Iceland (Auður L. Arnþórsdóttir personal communication). This occurred again the next summer in the same but enlarged area close to Reykjavik. Little information is available on *Culicoides (Wirthomyia) riouxi* and there are no reports on this species biting horses.

1.4.6 *Culicoides* allergens

The allergens causing IBH are found in the salivary glands of the midges. Allergens have been identified from three different species, *C. sonorensis* (Cul s), *C. nubeculosus* (Cul n) and *C. oboletus* (Cul o) and expressed in *E. coli* and some in insect cells (Table 4) (Langner et al., 2009; Peeters et al., 2013; Schaffartzik et al., 2011; van der Meide et al., 2013).

Table 4: Isolated *Culicoides* allergens.
Amino acid identity (%) between homologous allergens of *C. nubeculosus* and *C. obsoletus.*

	C. nubeculosus	Amino acid identity	*C. obsoletus*
Antigen 5	Cul n 1[a]	70%	Cul o 3[b]
Hyaluronidase	Cul n 2[a]	75%	Cul o 2[b]
Cysteine peptidase	Cul n 3[a]	—	—
Secreted salivary protein	Cul n 4[a]	33%	Cul o 7[b]
Secreted salivary protein	Cul n 5[a]	—	—
Secreted salivary protein	Cul n 6[a]	—	—
Unknown salivary protein	Cul n 7[a]	41%	Cul o 5[b]
Maltase	Cul n 8[a]	78%	Cul o 1[b]
D7 related protein	Cul n 9[a]	40%	Cul o 6[b]
Secreted salivary protein	Cul n 10[a]	—	—
Trypsin	Cul n 11[a]	51%	Cul o 4[b]
Kunitz protease inhibitor	—	—	Cul o 1[c]
D7 related protein	—	—	Cul o 2[c]

[a] Schaffartzik et al., 2010 & 2011
[b] van der Meide et al., 2013
[c] Peeters et al., 2013

The importance of each allergen is evaluated by the binding of serum IgE from IBH affected horses as compared to healthy controls. The sensitization of basophils and mast cells to the allergens is measured by a cellular degranulation test and intradermal test. The maltase from *C. sonorensis* (rCul s 1) is the only expressed allergen from *C. sonorensis*. It was the first allergen to be expressed and it was done in insect cells. Seven out of the 8 IBH affected horses had specific IgE in sera and responded to Cul s 1 in an intradermal test. In a histamine release test all eight horses responded (Langner et al., 2009). Eleven Cul n allergens have been expressed in *E. coli*, rCul n 1 – 11; six of them are known proteins, rCul n 1 (antigen 5 like protein), rCul n 2 (hyaluronidase), rCul n 3 (putative cysteine endopeptidase), rCul n 8 (maltase), rCul n 9 (D7 related protein) and rCul n 11 (trypsin) (Table 4). Sera from 46 IBH affected horses were used to analyze the frequency of IgE binding. It ranged from 13 – 57%, with the highest percentage against rCul n 2. Eight of the eleven allergens tested in the intradermal test were able

to induce a type I skin reaction (Schaffartzik et al., 2010; Schaffartzik et al., 2011). There are seven isolated allergens from *C. obsoletus*; rCul o 1 (maltase), rCul o 2 (hyaluronidase), rCul o 3 (Antigen 5 like protein), rCul o 4 (trypsin), rCul o 6 (D7 related protein) (Table 4). They have all been expressed in *E. coli* and the maltase (rCul o 1) also in insect cells. Sera from 203 horses, 103 IBH affected and 100 healthy controls, was used to analyze IgE reactivity against rCul o 2 - 7. The frequency of IgE positive sera from IBH affected horses was 38-67% in contrast to 2-6% from the healthy controls. The highest frequency was observed for rCul o 5, an unknown salivary protein. Of the 103 IBH affected horses tested 95 horses or 92% reacted to at least one of the seven allergens. Fourteen horses were tested with an intradermal test, seven IBH-affected and 7 healthy controls. The highest serum IgE reactivity of IBH-affected horses was against rCul o 4 and rCul o 5 (van der Meide et al., 2013). In another study on allergens from *C. obsoletus* two additional allergens were described, also named rCul o 1 and rCul o 2, Kunitz protease inhibitor (rCul o 1) and D7-related protein (rCul o 2). IgE from 45% and 40% IBH affected horses bound to these two allergens (Peeters et al., 2013).

Prior to the isolation of the *Culicoides* allergens four allergens (Smi v 1 – Sim v 4) had been identified and expressed in *E. coli* from *S. vittatum* some of them proteins corresponding to the *Culicoides* allergens (Schaffartzik et al., 2009).

1.5 Allergen-specific immunotherapy

Allergen-specific immunotherapy (ASIT) was first reported by Noon in 1911 where hay fever patients were injected subcutaneously (s.c.) with pollen extract, resulting in decreased sensitivity to the pollen (Noon, 1911). To date, ASIT is the only curative treatment against allergic diseases. Benefit of successful ASIT for patients is reduced symptom duration and severity upon allergen exposure, decrease in antiallergic drugs use and improvement in overall quality of life (Cuppari et al., 2014). Important factors affecting the performance and safety of ASIT are the route of administration, adjuvants and allergens (Chesne et al., 2016).

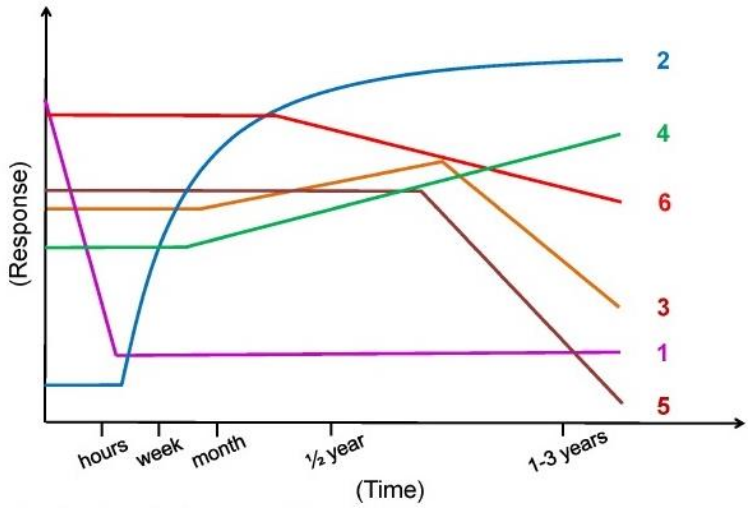

Figure 7: Immune regulation during the time course of allergen-SIT.
Specific immune responses are observed during the course of allergen-SIT. 1. An early desensitization effect including decrease in mast cell and basophil degranulation soon after the first administration of allergens. 2. Generation of allergen-specific T_{reg} cells and suppression of effector cells. 3. An early increase and a late decrease in specific IgE levels. 4. A relatively early increase in specific IgG4. 5. A late decrease in type I skin test reactivity. 6. A decrease in tissue mast cell and eosinophil numbers and a release of their mediators after a few months. The figure and figure text are reprinted from Fujita et al. (2012) with permission from Biomed Central.

The *in vivo* mechanisms of ASIT have not yet been completely defined; however, the induction of peripheral T cell tolerance to allergens is the central event in successful ASIT (Akkoc et al., 2011). Within the first week of ASIT in humans there is a decrease in mast cells and basophils degranulation and it remains low during the treatment. The generation of allergen-specific T regulatory cells and the suppression of effector cells begins early in the ASIT and six months post the first injection the T_{reg} response has almost reach its peak. The specific IgE level in sera remains the same for the first month of treatment; it then slightly increases with a substantial decrease later on in the treatment. There is also an increase in specific IgG4 after the first month of ASIT and late decrease in type I skin test reactivity. The number of tissue mast cells and eosinophils, as well as the release if their mediators decreases after few months of ASIT (Figure 7) (reviewed in Fujita et al., 2012).

Dendritic cells (DCs) at the site of administration induce regulatory T cells and type 1 regulatory T cells (Tr1). These allergen-specific T_{regs} produce the anti-inflammatory cytokines, IL-10 and TGFβ. The inflammatory cytokines suppress IgE production by B cells and IL-10 induces production of blocking-IgG4 and IgA. The IgG4 is thought to bind to the allergens before they reach surface-bound IgE on mast cells and basophils. The effector cells in allergy, mast cells, basophils and eosinophils are suppressed directly by the IL-10 and TGFβ. The Tregs also influence the generation of DCs, leading to further development of IL-10 producing DCs. The T regulatory cells also suppress Th2 cells and therefore they cannot produce Th2 cytokines, IL-4, IL-5, IL-9 and IL-13, which are essential for the differentiation, survival and activity of mast cells, basophils, eosinophils and mucus producing cells (Figure 8) (reviewed in Akkoc et al., 2011).

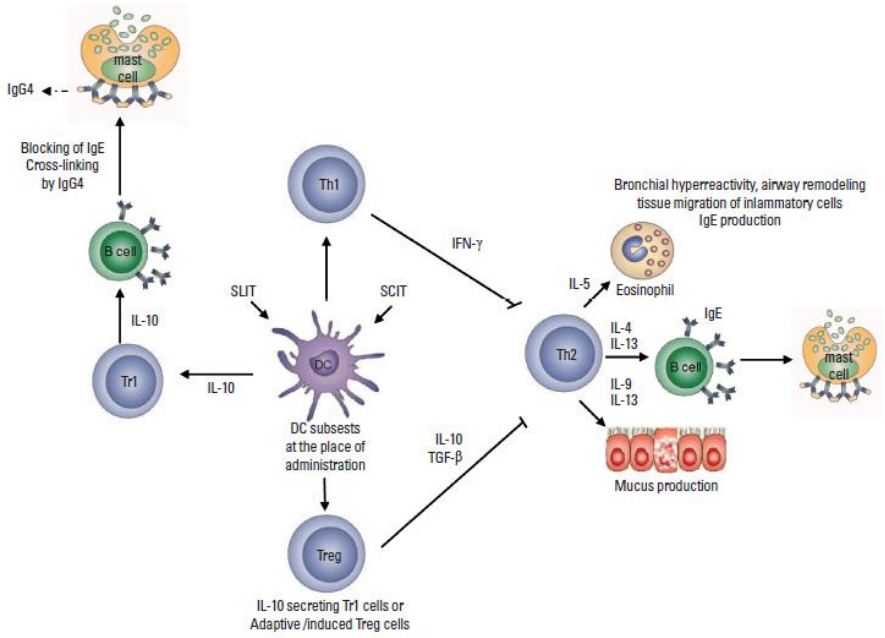

Figure 8: Mechanism of ASIT.
Both subcutaneous and sublingual SITs first affect the regional antigen-presenting cell, namely the local dendritic cell subset in the place of administration and draining lymph nodes. Although *in vivo* mechanisms are not clearly known, these dendritic cells induce Treg (CD4+CD25+FoxP3+) cells and Tr1 cells (IL-10+). Treg cells and regulatory cytokines (such as interleukin-10 (IL-10) and transforming growth factor-β, TGFβ) may contribute to the control of allergen-induced immune responses in several different ways. Treg cells utilize multiple suppressor factors to regulate the immune response.IL-10 and TGF-β suppress IgE production and IL-10 induces inflammatory immunoglobulin isotype, IgG4. These two cytokines directly suppress allergic inflammation induced by effector cells such as mast cells, basophils and eosinophils. TReg cells influence the generation of dendritic cells and promote the development of IL-10-producing dendritic cells. In addition, Treg cells inhibit Th2 cells, which can no longer provide cytokines such as IL-3, IL-4, IL-5, and IL-9. These cytokines are required for the differentiation, survival and activity of mast cells, basophils, eosinophils and mucus producing cells, as well as for the tissue homing of Th2 cells. SIT, specific immunotherapy; SLIT, sublingual immunotherapy; SCIT, subcutaneous immunotherapy; Treg, T regulatory cells. The figure and figure text is reprinted from Akkoc et al. (2011) with permission from the Allergy, Asthma & Immunology Research journal.

1.5.1 The route of administration

Different routes for administration are used for allergen immunotherapy. Subcutaneous immunotherapy (SCIT) is the most common and has been applied for over a hundred years. The drawback is the long treatment period, 3-5 years with up to 56 injections under the supervision of a medical professional. Although this route has its limitations it still is the golden standard (reviewed in Cox & Calderon, 2010). One alternative is noninvasive sublingual immunotherapy (SLIT) where patients apply the allergen extract under the tongue and allow it to dissolve (Canonica et al., 2009). The mouth is considered an immune privileged site as the oral mucosa remains non-inflamed even though it is consistently exposed to antigens from food and microbes (Novak et al., 2008).

The drawbacks of SCIT and SLIT are the long duration with the risk of side effects, and less than 5% of allergic patients that would benefit from it undergo the therapy (Chesne et al., 2016; von Moos et al., 2011). The new routes of administration should preferably overcome these disadvantages. The routes that are currently being investigated are intralymphatic, oral, epicutaneous, intradermal and intranasal, and some of them look promising (Cuppari et al., 2014; Hochfelder & Ponda, 2013). The traditional subcutaneous injection was compared to intralymphatic injection in a randomized control trial for grass pollen immunotherapy. The patients in the intralymphatic group were injected 3 times with 1000 standardized quality units (SQ-U) in Alum in week 0, 4 and 8, whereas, the patients in the s.c. group were injected 54 times over 3 years with approximately 4.000.000 SQ-U in Alum. Intralymphatic injection caused fewer adverse events and the recovery was faster than in the s.c. group. The recovery in both groups 3 years post the first injection was comparable and did not differ significantly (Senti et al., 2008).

1.5.2 Adjuvants

Adjuvants are compounds that modify and enhance the immune response to antigens (reviewed in Chesne et al., 2016). They can be broadly classified into three groups: delivery systems, immunomodulatory molecules and

combinations of them. The delivery systems are non-immune stimulatory components that provide more effective antigen presentation. Immunomodulatory molecules activate the innate immune system directly. The third group of adjuvants is the combination of delivery system and immunomodulatory molecules (Reed et al., 2013). Glenny *et al.* demonstrated for the first time the adjuvant effect of aluminum (Alum) in 1926. Alum, aluminum hydroxide or aluminum phosphate, a mineral salt that still is the predominant adjuvant used in human vaccines (Petrovsky & Aguilar, 2004). Even though Alum has been in use for almost 90 years the mechanism of how it works is not completely understood (Lambrecht et al., 2009). Alum is classified as a delivery system as it was thought to function by forming a depot for antigen and by promoting antigen uptake by APC. Now the function is believed to be through inflammasome and to trigger a necrotic cell death with release of uric acid, which is a known DAMP (Coffman et al., 2010; Lambrecht et al., 2009). Virosomes and emulsions are also examples of delivery systems (Reed et al., 2013). Adjuvants that are immunomodulatory molecules work through the PRRs on APC, e.g. monophosphoryl lipid A (MPLA) that binds TLR-4. It is a detoxified derivative of lipopolysaccharide (LPS) isolated from *Salmonella minnesota* (Didierlaurent et al., 2009). Another PAMP adjuvant is CpG oligonucleotides that activate the sensor cells of the innate immune system through TLR-9 (Coffman et al., 2010). The third group of adjuvants is the combination of delivery systems and immunomodulatory molecules. The adjuvant system 04 (AS04) where aluminum hydroxide and MPLA are mixed together is used in human papilloma virus vaccine against cervical cancer (Garçon et al., 2011). Another adjuvant mixture is IC31®, consisting of artificial antimicrobial peptide KLK acting as a delivery system and the TLR-9 ligand oligodeoxynucleotide ODN1a (Szabo et al., 2013). IC31® is in clinical trials for a protein subunit vaccine against tuberculosis (Mearns et al., 2017).

Adjuvants are included in ASIT to reduce the allergen dose and the frequency of the injections and thereby to increase safety (Chesne et al., 2016) and alum is the most commonly used (Exley, 2014). SLIT is usually

performed without an adjuvant; it requires a 50-100 times higher dose of allergens compared to SCIT. Therefore a considerable effort is put in testing adjuvants to enhance SLIT and several candidates are in clinical trials (Moingeon et al., 2017).

1.5.3 Production of recombinant allergens

Standardized allergen extracts are used in ASIT, either native or modified (Jongejan & van Ree, 2014). Different approaches have been tried to lower the risk in ASIT by modifying the extracts, e.g. chemically, such as by denaturation with aldehydes with the aim of reducing the IgE reactivity. Another disadvantage of natural extracts is the difficulty in making standardized solutions as the amount, potencies and immunogenicity of individual allergens cannot be controlled (reviewed in Valenta et al., 2016).

Advances in molecular biology and genetic engineering have enabled the production of recombinant proteins. Eukaryotic and prokaryotic expression systems are used to obtain high yields of pure proteins. The common expression systems are bacteria, yeast, insect cells, mammalian cells and plants. *Escherichia coli* (*E. coli*) is the most widely used expression system for production of heterologous proteins (Terpe, 2006). *E. coli* has been used for production of therapeutic recombinant proteins since 1982 and is still in use (Wells & Robinson, 2017). The advantages of *E. coli* include the fact that the genome can easily be modified, the cultivation is easy and with a high product yield, and expression is inexpensive. The disadvantages of *E. coli* are the lack of post-translational modification like glycosylation and the proteins can be in insoluble inclusions and have to be denatured and then refolded. There is also a high risk of an endotoxin contamination (Demain & Vaishnav, 2009; Wells & Robinson, 2017).

Yeasts are single-cell eukaryotic organisms that are used in production of recombinant proteins as they are cost effective, their proteins go through post-translational modification and are easy to work with. The two most commonly used strains are *Saccharomyces cerevisiae* and *Pichia pastoris*. The advantages of yeasts over *E. coli* are the glycosylation, the ability to produce disulfide bonds and that the proteins can be secreted into the

medium. The drawback is that the glycosylation can be hypermannosylation and if the proteins are aimed at human use the post-translational modification is not humanized (Demain & Vaishnav, 2009; Wells & Robinson, 2017).

Insect cells have been used to produce recombinant proteins most commonly with the use of baculoviruses. Recombinant baculoviruses that contain the gene of interest are made and insect cells infected with the virus for production of the target protein. The proteins go through post-translational modification without complication; they can be N- and O-glycosylated, phosphorylated and the cleavage of signal peptide occurs. Proteins are properly folded and it is easy to scale up the production. The disadvantages of insect cells as an expression system are that some proteins can become aggregated intracellularly, making purification difficult, a low level of expression, incorrect glycosylation as compared to mammalian cells, and the overall process is expensive (Demain & Vaishnav, 2009).

Plants are eukaryotes capable of producing complex proteins which go through post-translational modifications, such as glycosylation. Plant glycans contain xylose, not seen in mammalian or insect cells (Brooks, 2006). The gene of interest is either inserted into a plant virus or bacteria used to infect the plant or directly into the DNA of the plant. The use of plants as a production system for recombinant proteins has some advantages over the other expression systems discussed. The production cost is low and the scale up is easy as plants can be grown on an agricultural scale requiring only water, sun and minerals. The production is safe, endotoxin-free and free of mammalian pathogens (Demain & Vaishnav, 2009; Magnusdottir et al., 2013). The protein expression can be directed into one specific tissue of the plant, as has been done in barley, where the recombinant protein is only expressed in the seeds, the natural storage environment for long-term conservation (Magnusdottir et al., 2013).

Different recombinant allergens or hypoallergenic allergens, e.g. recombinant allergen fragment, mutated allergens, mosaic hybrids and denatured proteins, have been studied. The aim is to reduce IgE reactivity, retain T cell epitopes, reduce allergenicity and induce the production of blocking IgG antibodies (Linhart & Valenta, 2012).

2 Aims

Insect bite hypersensitivity (IBH) is a type I allergy of horses caused by bites of insects of the genus *Culicoides*. All breeds of horses can be affected but the allergy affects up to half of all Icelandic-born horses that are exported to a *Culicoides* rich environment. The allergens are the *Culicoides* salivary gland proteins and they have been expressed in *E. coli* and purified. No treatment is currently available. An imbalance between Th1, Th2, and T regulatory cells is important for the pathogenesis of the disease, opening the possibility to develop an allergen specific immunotherapy by inducing Th1 and T regulatory cells.

The aim of the project was to develop an immunotherapy against IBH.

The specific aims of the project were to:

- Compare intradermal and intralymphatic injections and test different Th1 focusing adjuvants for immunotherapy.
- Produce Cul n 2, Cul n 3 and Cul n 4 in barley for oral treatment and for application in immune assays.
- Develop a method to treat horses via the oral mucosa, with barley expressing a *Culicoides* allergen.
- Compare Cul n 3 and Cul n 4 produced in *E. coli*, insect cells and barley grains in immune assays.

3 Materials and methods

This chapter summarizes the main materials and methods used in the project, as more detailed description can be found in the papers. The experiments not included in the papers are described in more detail.

3.1 Allergens

Two allergens from *C. nubeculosus*, Cul n 3 and Cul n 4 were used in a comparison study (Unpublished data II). The *Cul n 3* gene (GenBank: HM145951.1) is 1173 bp with calculated size 45 kDa coding for a putative cysteine endopeptidase. The *Cul n 4* gene (GenBank: HM145952.1) is 459 bp with calculated size 17 kDa coding for secreted salivary protein. These two allergens expressed in three different systems were compared in *in vitro* tests.

3.1.1 Production in *E. coli* (Paper I - III)

At the beginning of the project the allergens had been expressed in *E. coli* and were kindly provided by Dr. Eliane Marti (Schaffartzik et al., 2011). The allergens were amplified from λZAP II cDNA library that was made from salivary glands of *Culicoides nubeculosus*. All allergens were purified under denaturing conditions with the 6xHis tag and either dissolved in PBS or dH_2O. The *E. coli* expressed allergens were used both for vaccination and detection of specific antibody response after vaccination.

3.1.2 Production in insect cells (Paper II)

Cul n 3 and Cul n 4 allergens were expressed in insect cells with the Bac- to Bac Baculovirus expression system (Invitrogen). The genes were amplified with gene specific primers. The Cul n 3 was cloned into pFastBac-HBM-TOPO vector and the Cul n 4 into pFastBac-1 vector. After isolation of rBacmid DNA, containing the gene of interest, Sf-9 insect cells were transfected with the rBacmid to generate r-Baculovirus. The viruses were amplified in Sf-9 cells in SF-900 medium with 1% FBS. The r-proteins were

produced in High five insect cells in an SF-900 medium without FBS. The proteins were purified under native conditions and dialyzed against 2xPBS before used.

3.1.3 Production in barley seeds (Paper III)

Cul n 2, Cul n 3 and Cul n 4 from *C. nubeculosus* were expressed in barley in collaboration with ORF Genetics (Magnusdottir et al., 2013). Cul n 3 and Cul n 4 were purified with the 6xHis tag and dissolved in 2xPBS. The Cul n 2 was expressed as a truncated form without the HQ-tag.

3.2 Experimental horses (Papers I - III)

Horses used for vaccination, oral treatment and allergy development were healthy Icelandic horses located in Iceland. The experiments were accepted by the National Animal Research Committee of Iceland (no. 0113-16, no. 2013-03-02 and no. 2014-03-01).

For the analysis of the performance of the rCul n 3 and rCul n 4 in IgE ELSA sera from 168 adult horses (97 IBH affected and 71 non-affected control horses) living in Switzerland were used (Unpublished data II). These were 70 Icelandic-born Icelandic horses (47 with IBH and 23 H) that had been living in a *Culicoides* infested area in Switzerland for at least 4 summers. Additionally, sera from 47 European-born Icelandic horses (24 with IBH and 23 H) and from 51 horses (26 with IBH and 25 H) belonging to various breeds (Warmbloods [n=17], Franches-Montagnes [n=22], Ponies [n=6], Tinker [n=1], Arabian [n=1] and Andalusian [n=1]), were tested. Diagnosis of IBH was based on clinical history and typical clinical signs, recurring every summer with a period of remission in winter. The horses assigned to the healthy control group had no history of skin problems and no preventive measures had been taken in the summer, such as blankets.

3.3 Immunological assays (Papers I - III)

3.3.1 Antibody ELISA

Allergen specific antibodies were detected with ELISA in the serum and saliva of horses. The plates were coated with 0.2 µg of each recombinant allergen. Unspecific binding was blocked with blocking buffer and the samples were added at different concentrations depending on the antibody being tested. Primary antibody specific for IgG1, IgG1/3, IgG4/7, IgG5, IgG6, IgG(T), IgE or IgA, was then added and incubated. Secondary antibody, AP- or HRP-labelled anti-mouse was added prior to the substrate. The absorbance was measured at 405 nm or 450 nm. *Papers I-III*.

3.3.2 Competitive inhibition ELISA

Competitive inhibition ELISA was used to analyze whether the antibodies generated by the immunotherapy could inhibit the binding of IgE (from IBH affected horse) to an allergen used in the immunotherapy. The ELISA was carried out as the IgE *antibody ELISA* except sera from treated horses were added, both before and after treatment in different dilutions, prior to sera from an IBH affected horse (diluted 1:5). Then the procedure was as for the *antibody ELISA*. The percent inhibition was calculated using the following formula.

$$\%\ \text{inhibition} = 100 - \left(\frac{OD\ of\ IBH\ serum\ after\ inhibition\ with\ serum\ from\ treated\ horse}{OD\ of\ IBH\ serum} \times 100\right).$$

Papers I - III.

3.3.3 Immunoblot analysis

The immunoblot analysis was used to detect allergen specific antibodies, total IgG or IgG1, in serum. Purified allergens (1-4 µg) or *C. nubeculosus* salivary gland extract (SGE) were separated by SDS-PAGE under denaturing conditions and the proteins transferred to a PVDF membrane. Before incubation with the sera, the unspecific binding was blocked with 2% tween 20 and the membrane cut in strips. The sera were diluted 1:2000 when tested

on the r-allergens and 1:30 when tested on the SGE and the strips incubated overnight at 4°C. The secondary antibody AP-labelled anti-horse was applied and the strips incubated for 1 h at RT before the bound antibodies were detected with BCIP/NBT staining. *Paper I.*

3.3.4 Stimulation of PBMCs *in vitro*

Stimulation of PBMCs with allergens was carried out for cytokine measurement. The protocol was developed throughout the project. In general, isolated PBMCs were stimulated with 1.25-10 µg of r-allergens, for 24 h, 48 h or 96 h. Cells were harvested for RT-PCR analysis of the cytokine mRNA and the supernatant used for measuring the proteins with bead-based multiplex assay. The development led to a current set-up; 200 µL of 3 x 10^6 cells/mL incubated in a 96 well flat bottom plate for 24 and 48 h with the concentration 1.5-3 µg/mL of the r-allergens; cells were also incubated with mitogen as the positive control and in medium alone as the negative control. Cell supernatant was harvested and the cytokines measured with the bead-based multiplex assay (Wagner & Freer, 2009). *Papers I-II.*

3.3.5 Sulfidoleukotriene release test

Sulfidoleukotriene release (sLT) test was used to analyze whether vaccinated horses were sensitized following vaccination. Leukocytes were incubated at 37°C with *C. nubeculosus* extract for 40 min and the supernatant harvested. The sLT was determined by using CAST 2000-ELISA. *Paper I.*

3.3.6 Skin test

Skin tests were used to analyze whether vaccinated horses were sensitized following vaccination. The neck of the horse was shaved and 1 and 0.1 µg/mL of the allergens injected i.d. and immediate early response was measured 30 min post injection. *Papers I and II.*

3.4 Vaccinations and oral treatment (Papers I, II and III)

3.4.1 Intradermal and intralymphatic vaccination with r-allergens with or without IC31® (Paper I)

Twelve healthy Icelandic horses were divided into four groups. All horses received four *E. coli* expressed allergens, rCul n 1, rCul n 2, rCul n 5 and rCul n 9, three times at four and five week intervals, 10 µg of each allergen per injection (Figure 9). Six horses were vaccinated i.d., three with the allergens alone and three with the allergens in 250µL IC31® adjuvant. Six horses were vaccinated i.l., three with allergens alone and three with the allergens in IC31® adjuvant.

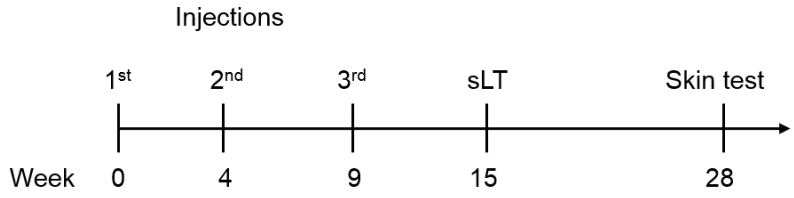

Figure 9: Time points of the injections, sulfidoleukotriene release test (sLT) and the skin test.

Blood was collected before vaccination, two weeks after each vaccination and at weeks 19 and 30. Allergen specific antibody response was tested in sera by ELISA and WB. PBMCs were isolated for *in vitro* stimulation with the allergens (*E. coli* expressed) for detection of cytokine response. Six weeks after the last vaccination leukocytes were incubated with *C. nubeculosus* extract to determine the sulfidoleukotriene release. Six months after the last vaccination a skin test was performed with the allergens and the immediate early response measured.

3.4.2 Vaccination intralymphatically with r-allergens in Alum or in Alum/MPLA (Paper II)

Twelve healthy Icelandic horses were divided into two groups and vaccinated i.l. with four *E. coli* expressed allergens, rCul n 3, rCul n 4, rCul n 8 and rCul n 10, 3 times at four week intervals, 10 µg of each per injection (Figure 10).

Six horses received the allergens in aluminum hydroxide (alum, 500 µg) and six horses in a mixture of alum and monophosphoryl lipid A (MPLA, 50 µg).

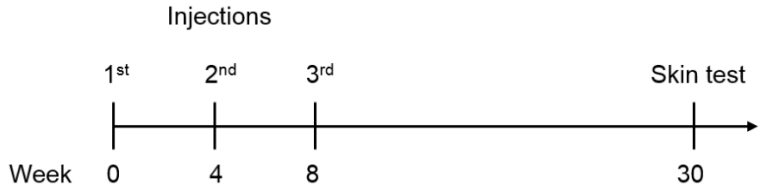

Figure 10: Time points of the injections and the skin test.

Blood was collected prior to vaccination, two weeks after each vaccination, at weeks 16, 24 and 32. The level of allergen specific antibodies was measured in sera by ELISA. PBMCs were isolated two weeks after the last vaccination (week 10) as well as from six healthy control horses. Isolated PBMCs were stimulated with 2 µg/mL of Bac-rCul n 3 for detection of cytokines. Six months (week 30) after the last vaccination an intradermal test was performed with the allergens and the immediate early response measured.

3.4.3 Oral treatment with transgenic barley (Paper III)

Bits to treat horses via the mucosa of the mouth were developed as well as the barley mixture. Seven healthy Icelandic horses were included in the study, four horses were treated with ground barley expressing rCul n 2 and three horses with a normal barley. The horses were treated six times, over a period of 18 weeks, receiving 400 g barley (≈40 mg rCul n 2). Eight months after the last treatment horses were boosted with an additional 100 g of barley (Figure 11).

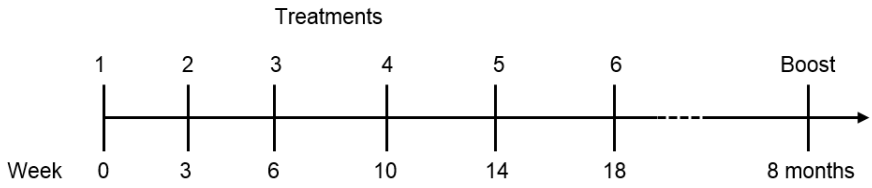

Figure 11: Time points of the treatments and boost.

Blood and saliva were collected before and two weeks after treatment and before and two weeks after the barley boost. Two weeks after the barley boost PBMCs were isolated for *in vitro* stimulation with *E. coli*-rCul n 2 and rCul o 2, expressed in *Pichia* (*Pichia*-rCul o 2), the corresponding protein from *C. obosletus*. Allergen specific response was measured by ELISA on *E. coli*-rCul n 2 and *Pichia*-rCul o 2 allergens.

3.4.4 An attempt to develop Cul n 4 allergy in horses (Unpublished data)

3.4.4.1 Animals

Five healthy Icelandic horses were included in the study. For *in vitro* stimulation of PBMCs five additional horses were used as untreated controls.

3.4.4.2 Injections and sampling

The horses were injected with Bac-rCul n 4 in Alum, 3 times with 10 µg s.c. at three week intervals and subsequently on weeks 13 and 16 with 50 µg i.d. A skin test was performed in week 20 and 11 months post the first injection the horses were boosted s.c. with 10 µg before *in vitro* stimulation was carried out on isolated PBMCs (Figure 12).

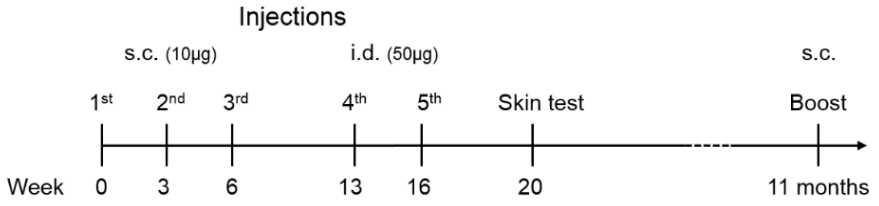

Figure 12: Time points of the injections, skin test and the boost.

Blood was collect in weeks 0, 3, 6, 8, 13, 15, 16, 18, 20 and 22 for isolation of sera and then two weeks after the boost for isolation of PBMCs. Sera were stored at -20°C until used in ELISA and PBMCs were used directly for stimulation.

3.4.4.3 ELISA

Sera taken in weeks 0, 3, 6, 8, 13, 15, 16, 18, 20, 22 were used to detect specific IgE, IgG1, IgG4/7, IgG5 and IgG(T). The ELISA plate was coated with Barley-rCul n 4 and the procedure was carried out as described in section *3.3.1 Antibody ELISA*.

3.4.4.4 Stimulation of PBMCs and determination of cytokines

For detection of cytokines the horses were boosted with one injection of Bac-rCul n 4 (10 µg). Two weeks after the boost the PBMCs were isolated from the five horses as well as from five untreated controls. The stimulation was carried out in 24 well plates, 2.5 x 10^6 cells/well. The cells were stimulated with 2 µg/mL and 4 µg/mL of Bac- and Barley-rCul n 4, mitogen or left unstimulated for 48 h. The supernatant was harvested and the cytokines measured with the bead-based multiplex assay (Wagner & Freer, 2009). The values are shown after medium correction.

3.4.4.5 Skin test

Four weeks after the last injection a skin test was performed, horses were injected in the neck on the opposite side of the i.d. injections with 0.1 and 1 µg of Bac-rCul n 4. Immediate early response was measured 30 min post injection.

3.5 Statistical analysis

Statistical analysis in the project was done with NCSS 8 (NCSS Statistical Software) or GraphPad Prism 6. The graphs were either plotted with Microsoft Excel (Microsoft office Professional Plus 2013) or GraphPad Prism 6. In all statistical tests a p-value ≤ 0.05 was considered significant. *Papers I – III.*

For the *unpublished data II* the following statistical analysis was carried out

To evaluate the performance of the IgE ELISA with rCul n 3 or rCul n 4 each produced in the three expression systems, non-parametric receiver operating characteristic (ROC) analysis was done and calculations of the area under the empirical curve (AUC) were performed using the statistical

software program NCSS 10 (NCAA Statistical Software). Furthermore, ROC curves were used to select the optimal cut-off points. For each allergen, the value giving the best accuracy, i.e. highest combined number of true positives (TP) and true negatives (TN), was chosen to determine the ELISA cut-off values. Correlations between IgE levels against the Cul n 3 or Cul n 4 allergens produced in the different expression systems were calculated with the Spearman-Rank correlation coefficient

A linear mixed effect model was used to further analyze the data. The data was log-transformed before statistical analysis and analyzed separately for rCul n 3 and rCul n 4. Two sample t-test was used to test for difference between healthy and IBH horses within each protein. A random effect ANOVA was used to test for difference between proteins for healthy and IBH horses separately with the protein as fixed effect and the individual horse as a random effect. Statistical analysis were performed in R and we fitted linear random effect models using the nlme-package (Linear and Nonlinear Mixed Effects Models. R package version 3.1-124).

We fitted 4 models for each rCul n 3 and rCul n 4 using maximum likelihood and compared them with likelihood ratio tests and the Akaike Information Criterion (AIC). The full model (FM) contained disease status (Healthy or IBH affected), protein and origin of birth as fixed factors and all two-way and three-way interactions between them. We included the individual horse as a random factor and had different variance for the IBH affected and healthy horses. The first reduced model (R1) had the same structure for the random effect but we excluded the three-way interaction between the fixed factors. The second reduced model (R2) had the same structure for fixed factors as FM but the structure of the random effect was simpler with the same variance for IBH and healthy horses. The final reduced model (R3) had the same fixed effect structure as the FM but no random effect. The multcomp-package (Hothorn et al., 2008) was used for postHOC-tests to analyze what parameters are different. Differences between healthy and IBH horses was tested within each protein for each group of origin and for differences between origin groups within each diseases status for each protein.

4 Results

4.1 Intradermal and intralymphatic vaccination with r-allergens with or without Th1 adjuvant (Paper I)

4.1.1 Total IgG response following 3rd injection

Total IgG response was analyzed with immunoblot two weeks post the 3rd injection. Sera from the twelve horses were tested on *E.coli*-rCul n 1, rCul n 2, rCul n 5 and rCul n 9 before treatment and after the last injection.

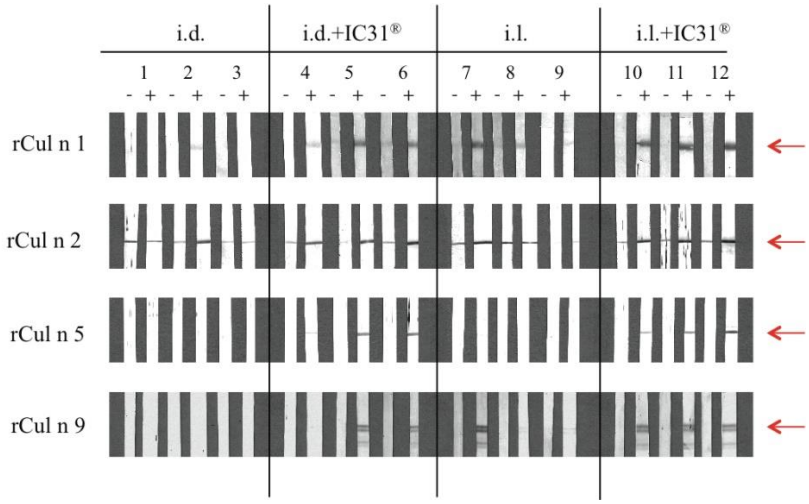

Figure 13: Total IgG response after the 3rd injection.
PVDF membrane strips with *E. coli*-rCul n 1, rCul n 2, rCul n 5 and rCul n 9 were incubated with sera (diluted 1:2000) taken before (-) and after the last injection (+) from the twelve vaccinated horses (1-12). Secondary antibody AP-labelled anti-horse (1:2000).

The horses vaccinated with the allergens in IC31® showed stronger IgG response than without adjuvant, and this was more distinct for the horses vaccinated i.l. (Figure 13). For rCul n 2 a low background was observed; however a stronger response was seen on the strips incubated with the post vaccination sera for the IC31® groups.

4.1.2 Allergen-specific IgG subclasses

To analyze the IgG subclass profile following the vaccination sera taken at weeks 0, 2, 7, 11, 19 and 30 were tested by ELISA on *E.coli*-rCul n 1, rCul n 2, rCul n 5 and rCul n 9 (Figure 1 in Paper I). The response against rCul n 2 is shown as representative of the four allergens (Figure 14).

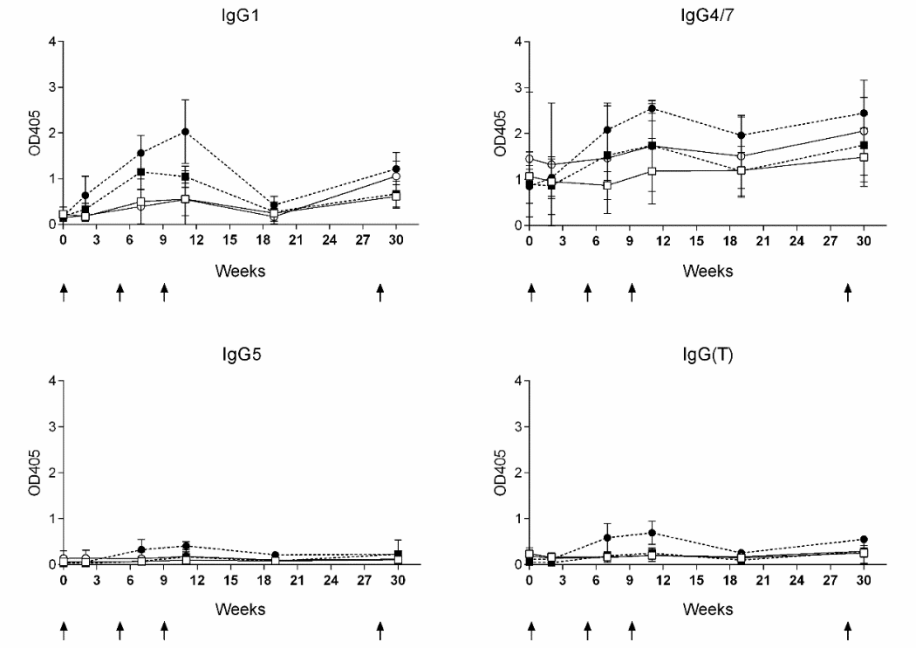

Figure 14: IgG subclass response against *E. coli*-rCul n 2.
Time course of Cul n 2 specific IgG response measured by ELISA, corrected OD values and standard deviation. Intradermal injection without adjuvant (—□—), intradermal injection with IC31® (- - ■ - -), intralymphatic injection without adjuvant (—○—) and intralymphatic injection with IC31® (- - ● - -). The first three arrows indicate vaccination time points and the last the skin test at week 28.

Repeated measures ANOVA revealed that vaccination with 10 µg of each allergen resulted in a highly significant increase in antibody level (Table 1 in Paper I) although it differed between the IgG subclasses and the allergens. The IgG1 was significantly higher after the 1st vaccination compared to week 0 and the antibody level further increased with the 2nd and the 3rd vaccination. The IgG1 titer dropped with time (week 19) but was boosted with the skin test at week 28. For IgG4/7 the results were similar but not as clear because the

value of the sera taken before vaccination was high (mean OD value against rCul n 2 is 0.82). An increase of IgG4/7 was seen after the 2nd and the 3rd vaccination; the antibody level then decreased (week 19) but was boosted with the skin test. For IgG5 and IgG(T) the level was lower than for IgG1 and IgG4/7 but a significant increase was observed after the 2nd vaccination. The level then decreased and was boosted with the skin test (Figure 12). The use of IC31® resulted in a higher response and horses vaccinated intralymphatically showed the strongest IgG response even though it only differed significantly for IgG1 and IgG1/3. The difference between the injection sites did not reach significance (Table 1 in Paper I).

4.1.3 Antibody binding to salivary gland proteins

Binding of allergen specific IgG1 to the corresponding proteins in the salivary gland from *C. nubeculosus* was analyzed by immunoblot. Strips with salivary gland extract were incubated with sera taken before and after the 3rd vaccination. The IgG1 antibodies generated by the vaccination with recombinant allergens were able to bind to the corresponding proteins in the salivary glands extract from *C. nubeculosus* (Figure 3 paper I). The use of IC31® resulted in the strongest IgG1 response against all four allergens, although it was difficult to distinguish between Cul n 2 and Cul n 5 because of similar size (Figure 3 in Paper I).

4.1.4 Inhibitory capacity of the sera from i.l.+ IC31® horses

To investigate the inhibitory capacity of the antibodies generated with the vaccination, sera taken before (week 0) and after the 3rd vaccination (week 11) from the horses injected intralymphatically with IC31® were analyzed for IgE blocking capacity to the allergens. The sera were diluted 1:10, 1:20 and 1:40 before being applied to an allergen coated plate. After incubation serum from an IBH positive horse was added to the plate at a dilution of 1:5 and IgE detected.

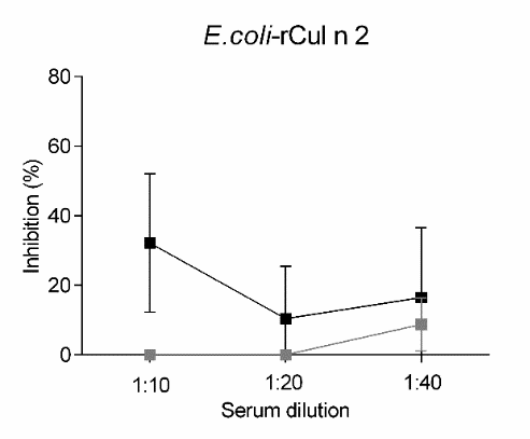

Figure 15: Inhibition of IgE binding to *E.coli*-rCul n 2 of sera.
Mean percent inhibition by pre- and post- vaccination sera from the three horses vaccinated intralymphatically with IC31®, diluted 1:10 – 1:40 and applied to an ELISA plate coated with *E.coli*-rCul n 2 prior to adding serum from an IBH positive horse at a dilution of 1:5. Pre-vaccination sera (■) and post-vaccination sera (■) with standard deviation.

The blocking capacity of the group differed between the allergens tested. The mean ranged from 54.4% - 18.4% at a dilution 1:10. Inhibitory capacity was dilution-dependent and resulted in a similar capacity for pre- and post-sera when diluted 1:40 (Figure 15 and figure 2 in Paper I).

4.1.5 Cytokines and FoxP3 mRNA expression

The relative IFNγ, IL-4, IL-10 and FoxP3 expression was estimated after *in vitro* stimulation of PBMCs with the *E. coli* expressed allergens before vaccination and after the 3rd vaccination. The values after the 3rd vaccination are shown in relation to the values obtained before vaccination (week 11/week 0). The data from the 4 r-allergens was pooled to enhance the statistical power of the groups (n=3) as no significant difference was obtained after stimulation with a single allergen (Figure 4 in Paper I). The IL-4 expression was significantly higher in the group vaccinated i.l. without adjuvant compared to i.d. with adjuvant. Both groups receiving the IC31® had a significantly higher IL-10 expression than those vaccinated i.d. without IC31®. There was no significant difference between the groups in the IFNγ and FoxP3 expression.

4.1.6 Allergen-specific IgE and sensitization tests

For analyzes of sensitization, allergen-specific IgE was measured at different time points. A sulfidoleukotriene release test and an intradermal test were performed. The IgE ELISA was tested on sera taken at weeks 0, 2, 7, 11, 19 and 30 on the allergens. No IgE was detected following the vaccination nor after the skin test (Figure 16).

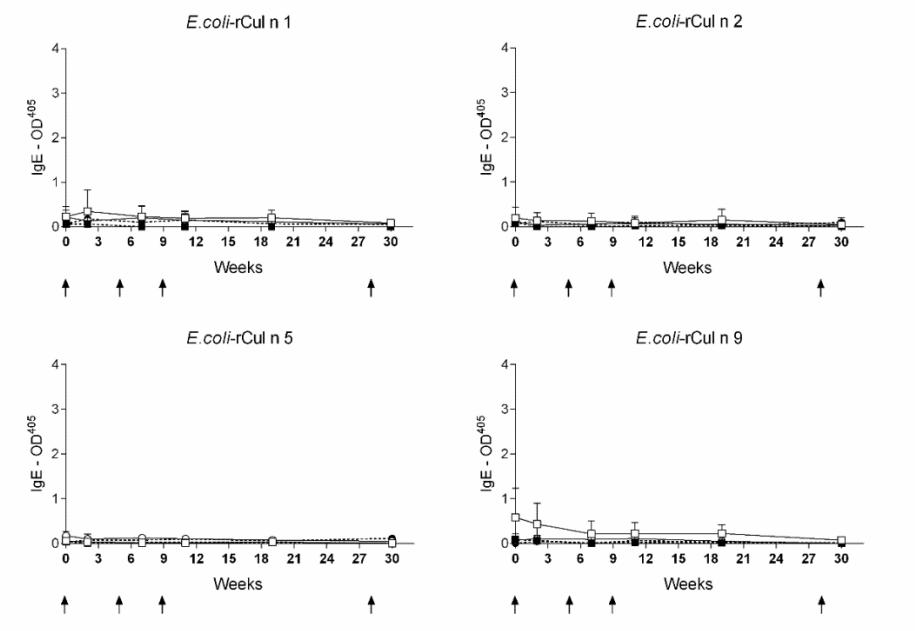

Figure 16: Time course of allergen-specific IgE response.
Corrected OD values and standard deviation. Intradermal injection without adjuvant (—□—), intradermal injection with IC31® (- - ■ - -), intralymphatic injection without adjuvant (—○—) and intralymphatic injection with IC31® (- - ● - -). The first three arrows indicate vaccination time points and the last the skin test at week 28.

Before and six weeks post vaccination PBL from the horses were stimulated with an extract of *C. nubeculosus* and ConA as a positive control. Sulfidoleukotriene was only detected after stimulation with ConA but not after stimulation with the extract (data not shown). At week 28 an intradermal test was done on the horses and additionally on three unvaccinated controls, on the opposite site of the vaccination on the neck of those horses intradermally

vaccinated. The horses were injected in the neck with 1 and 0.1 µg of the allergens and histamine as the positive control and PBS and dH_2O as the negative control. All horses responded strongly to the histamine but not to the allergens 30 min post injection (data not shown).

4.2 Vaccination intralymphatically with r-allergens in Alum or Alum/MPLA (Paper II)

Paper II is a short communication, therefore this section contains additional and more detailed results.

4.2.1 Allergen-specific antibody response

The allergen specific IgG and IgA antibody response was analyzed in sera from twelve vaccinated horses against *E. coli*-rCul n 3, rCul n 4, rCul n 8 and rCul n 10. In addition the response was tested against Cul n 3 and Culn 4 expressed in insect cells and compared to the *E. coli*-rCul n 3 and rCul n 4. The IgG subclass response was tested by ELISA at weeks 0, 2, 6, 10, 16, 24 and 32.

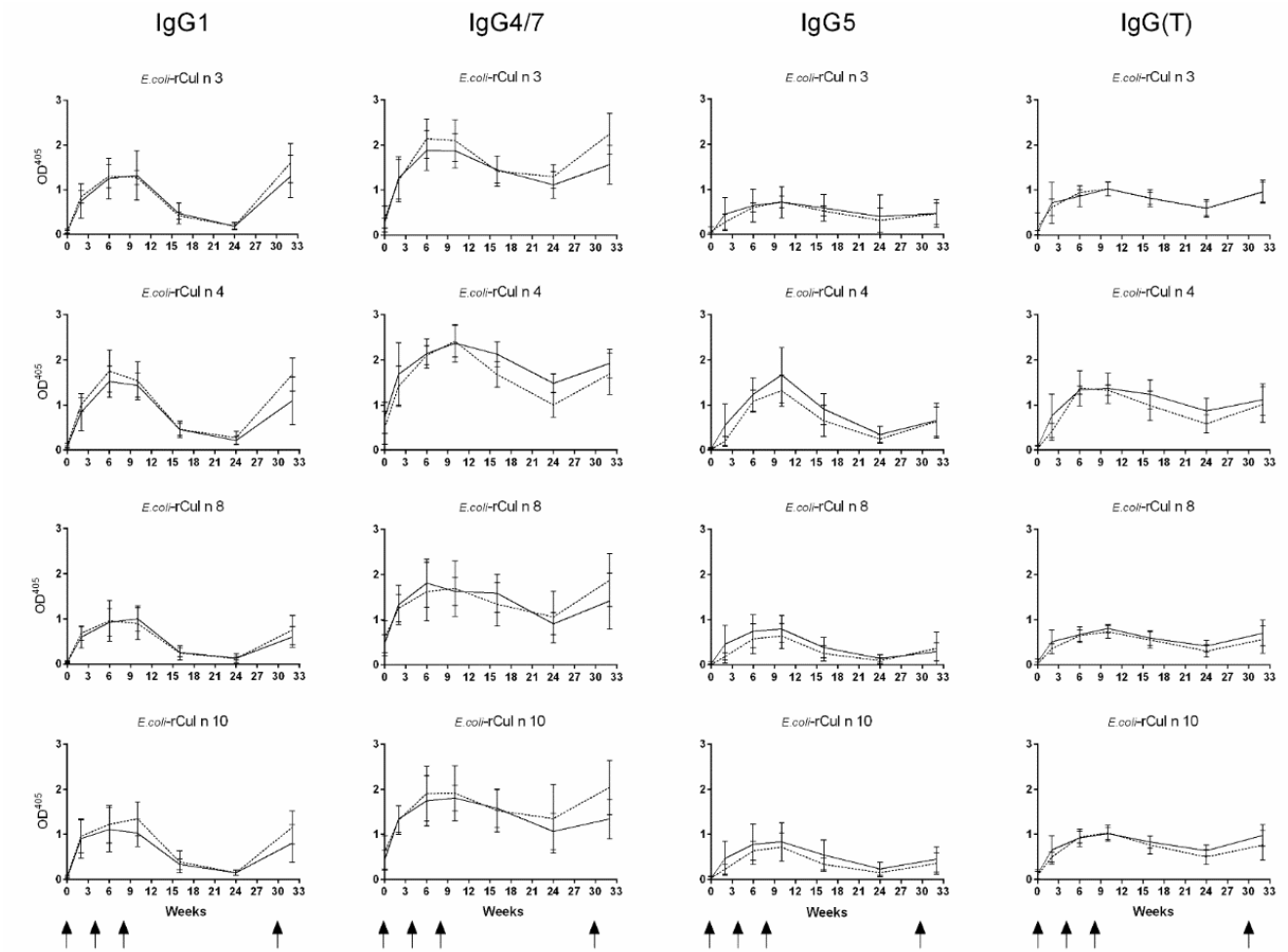

Figure 17: Time course of IgG subclass response against *E.coli* produced allergens measured by ELISA.
Corrected OD values and standard deviation for the two groups of horses at different time points, Alum (—) and Alum/MPLA (- - -) horses. The first three arrows indicate the vaccination time points and the last the intradermal test at week 30.

Significant IgG subclass response was generated for both groups with 10 µg of each of the four recombinant allergens two weeks after the first vaccination (Figure 17 and Table 1 in Paper II). The response further increased until week 6 and to a small extent until week 10 (2 weeks after the 3rd vaccination), then decreased again until week 24. The intradermal test performed at week 30 boosted the allergen specific antibody response. Horses vaccinated with allergens in Alum and in Alum/MPLA responded in a similar manner and a significant difference between the groups was only observed for IgG1 and IgG1/3 (Table 1 in Paper II). This was due to the fact that the mean IgG1 and IgG1/3 in the Alum/MPLA group was slightly but consistently higher than in the Alum group, resulting in a smaller increase after vaccination (Figure 17).

IgG subclass response was also tested on Cul n 3 and Cul n 4 expressed in insect cells at selected time points, weeks 0, 6, 10, 24 and 32. Expression in insect cells should result in a protein that is closer to the native allergen.

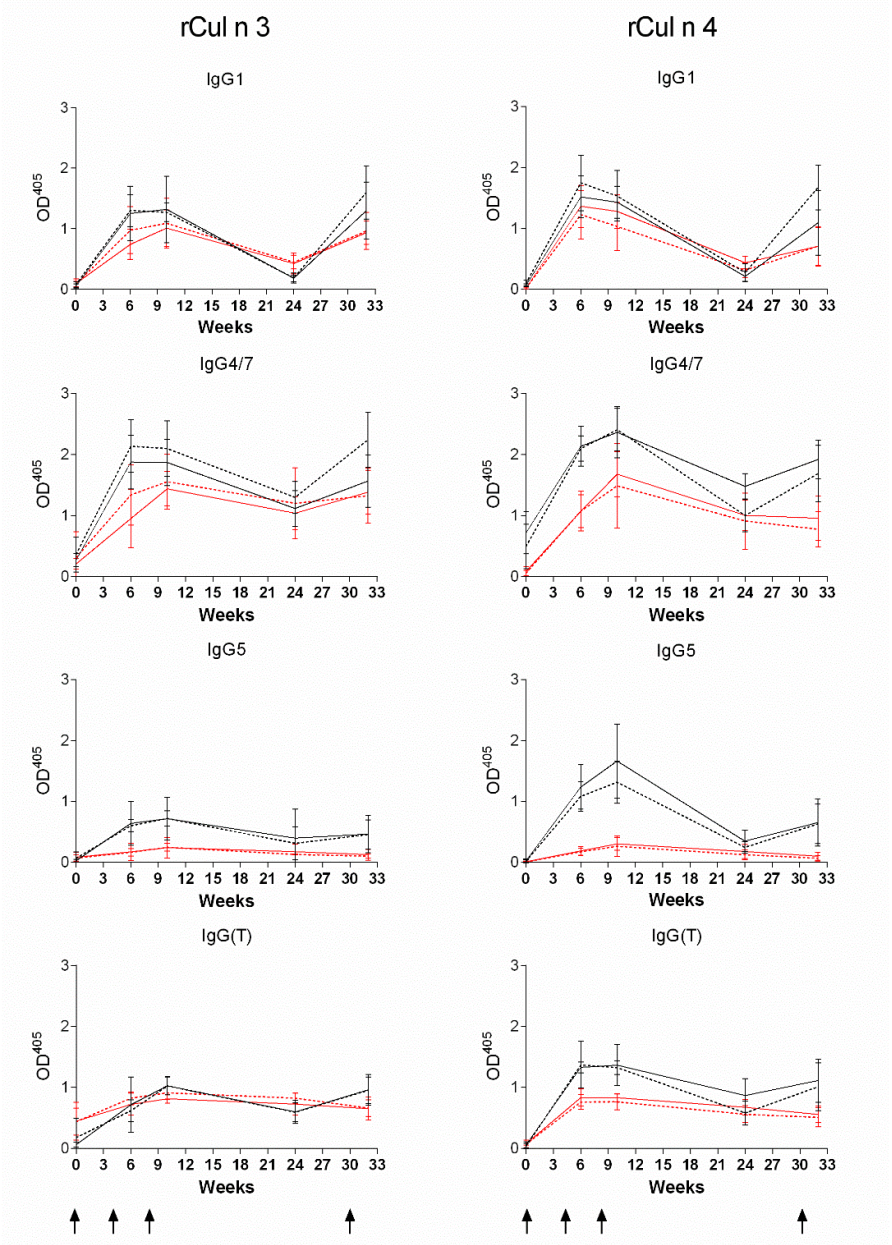

Figure 18: Time course of IgG subclass response against *E.coli* and insect cell produced allergens.

Corrected OD values and standard deviations for the two groups of horses at different time points, Alum (—/—) and Alum/MPLA (- - -/- - -). *E. coli* (black) and insect cell (red) produced allergens. The first three arrows indicate the vaccination and the last the intradermal test at week 30.

The antibody response against Bac-rCul n 3 and Bac-rCul n 4 showed a similar pattern as against *E. coli* produced allergens except to a lower extent as would be expected. The difference between the response against *E.coli* and insect cells produced allergens was greater for the rCul n 4, especially for IgG4/7 and IgG5. For IgG4/7 the increase after the vaccination was clear, but IgG5 was very low following the vaccination. The IgG4/7 background on both proteins was lower on the insect cell expressed ones, especially Bac-rCul n 4 (Figure 18).

Lastly the allergen-specific IgA was measured by ELISA at selected time points, weeks 0, 6, 10, 24 and 32. As IgA is lower than IgG in serum the samples were diluted 1:10 instead of 1:200.

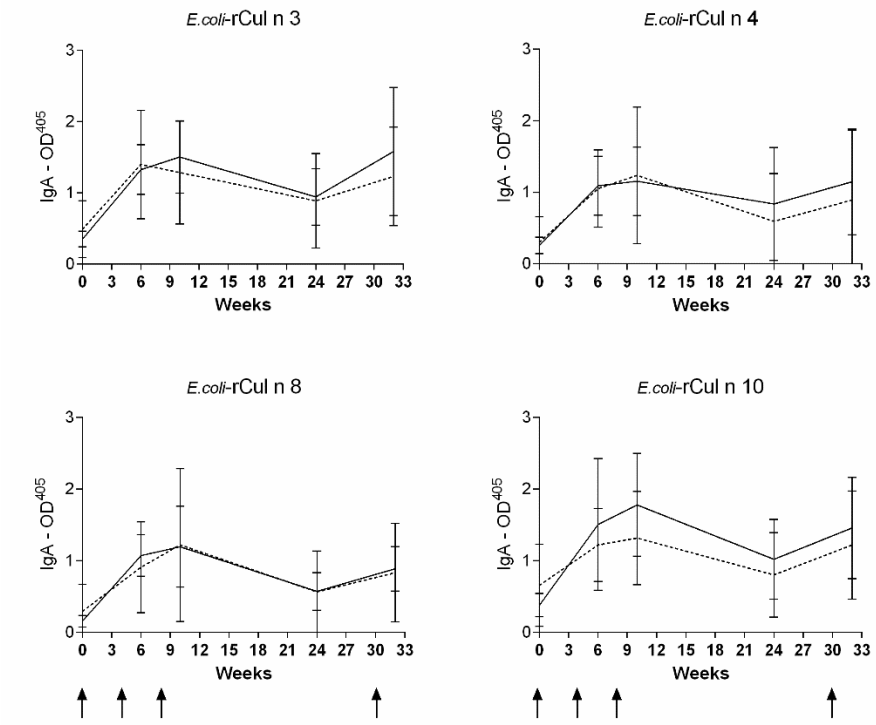

Figure 19: Time course of allergen-specific IgA response measured by ELISA. Corrected OD value and standard deviation for the two groups of horses at different time points, Alum (──) and Alum/MPLA (- - -). The first three arrows indicate the vaccination time points and the last the intradermal test at week 30.

Vaccination with the r-allergens also induced significant IgA response after the second vaccination (week 6) (Figure 19 and Table 1 in Paper II). The third vaccination only increased the response slightly; then at week 24 the response had decreased but was boosted again with the skin test. There was no significant difference between the groups.

4.2.2 Inhibitory capacity of the sera

The ability of the pre- (week 0) and postimmune (two weeks after the 3rd vaccination) sera to block the binding of IgE to the allergens was tested against the *E.coli* produced allergens as well as against Bac-rCul n 3 and Bac-rCul n 4 (Figure 20 and Figure 2 in Paper II). A pool of the sera from the groups was used for the assay.

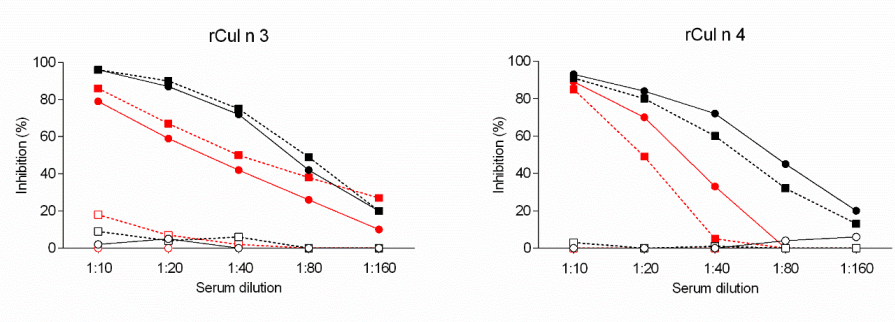

Figure 20: Inhibition of IgE binding to the rCul n 3 and rCul n 4 with pool of sera from the two groups.
Mean percent inhibition by pre- and post-vaccination sera from the two groups, diluted 1:10 -1:160 applied to an ELISA plate coated with *E. coli* expressed (black) and insect cell expressed (red) rCul n 3 and rCul n 4, prior to adding serum from IBH positive horse at a dilution of 1:5. Preimmune sera Alum horses (—○—/—○—), Alum/MPLA horses (- -□- -/- -□- -) and postimmune sera Alum horses (—●—/—●—) and Alum/MPLA horses (- - ■ - -/- - ■ - -).

The pool of the post-immune sera from both groups was able to inhibit binding of IgE, from an IBH horse to allergens in a dilution-dependent manner. The mean inhibition by the 1:10 dilution of the postimmune sera ranged from 96% to 70% depending on the allergen (Figure 2 Paper II). The post-immune sera inhibited the binding of IgE to Bac-rCul n 3 and -rCul n 4 to a slightly lower degree and had more dilution effect when tested against Bac-

rCul n 4. The pre-immune sera did not inhibit the binding of IgE to the allergens (Figure 18 and Figure 2 in Paper II).

4.2.3 Cytokine response after *in vitro* stimulation of PBMCs

To analyze the cytokine response PBMCs from the twelve horses were stimulated *in vitro* three weeks after the last vaccination with Bac-rCul n 3 as well as PBMCs from six unvaccinated control horses. The cells were stimulated for 24 h for RNA isolation and 96 h for harvesting the supernatant. IFN-γ, IL-4 and IL-10 were measured in cell supernatant with a bead-based multiplex assay and the mRNA expression with qRT-PCR (Figure 21).

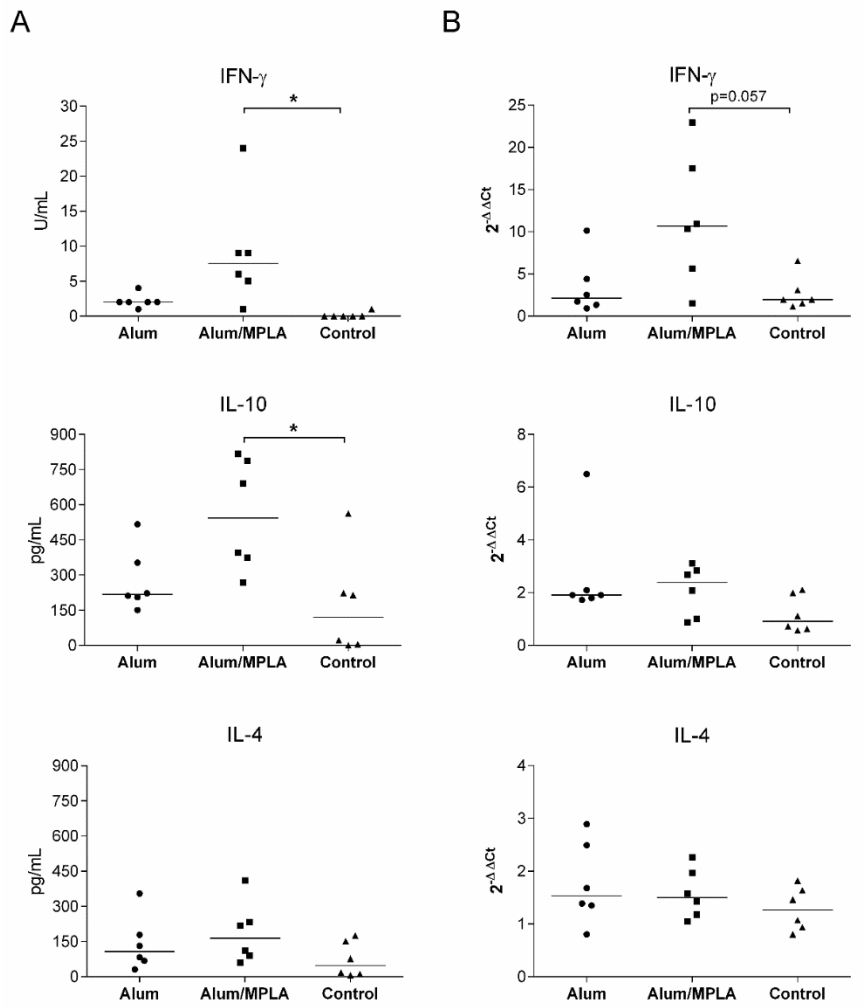

Figure 21: Detection of IFNγ, IL-4 and IL-10 after *in vitro* stimulation of PBMCs. Detection of cytokines after *in vitro* stimulation of PBMCs from the Alum and Alum/MPLA vaccinated horses and six control horses**. A)** The cytokines were measured in supernatants using a bead-based multiplex assay. **B)** mRNA expression of cytokines was measured by qRT-PCR. The values for each horse are plotted with the median. Comparison was performed using Kruskal-Wallis Multiple Comparison Z value Tests with Bonferroni correction. An asterisk (*) with line indicates statistically significant differences between the groups of horses.

One-way ANOVA was used to compare the cytokine expression at the mRNA and protein level. For the protein level statistical differences were observed for IFN-γ and IL-10 between the Alum/MPLA and the control horses, whereas no differences were observed for IL-4 (Figure 21A). When

the Alum and Alum/MPLA groups were compared using the Mann-Whitney U-test the Alum/MPLA horses produced significantly more IFNγ (Median = 7.5 versus 2 U/mL, $p<0.05$) and IL-10 (543 versus 217 pg/mL, $p<0.05$). At the mRNA level, the same indication for IFNγ expression was seen in One-Way ANOVA ($p=0.057$). The Mann-Whitney U-test revealed a significantly higher IFNγ mRNA level in the Alum/MPLA group compared to Alum (median $2^{-\Delta\Delta Ct}$ = 10.65 versus 2.1, $p<0.05$), indicating the same tendency for the mRNA and protein level.

4.2.4 Allergen-specific IgE and skin test

Allergen-specific IgE was measured in sera on the four r-allergens at different time points by ELISA (weeks 0, 2, 6, 10 16). No IgE was detected following the vaccination (Figure 22).

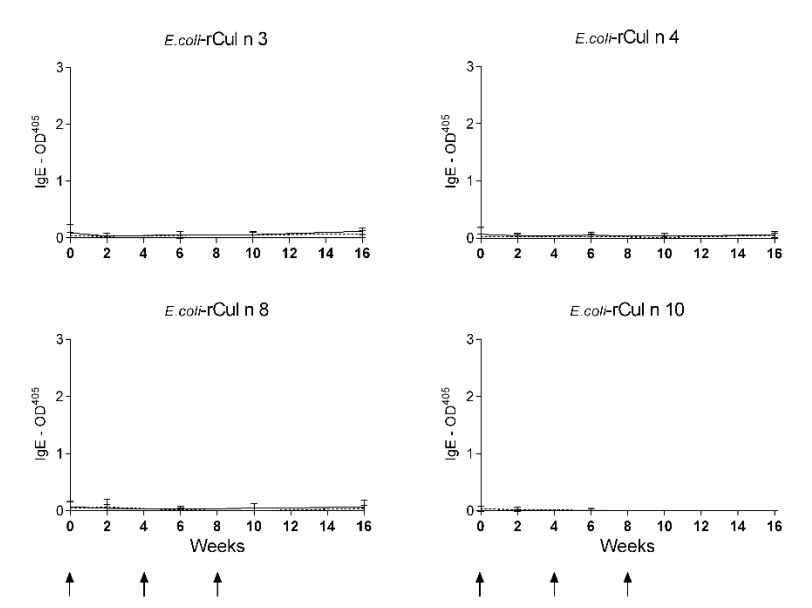

Figure 22: Time course of IgE response measured by ELISA.
Corrected OD mean values and standard deviation for the two groups at different time points, Alum (——) and Alum/MPLA (- - -). The arrows indicate the vaccination time points.

At week 30 an intradermal test was performed for analysis of sensitization. The horses were injected with the four r-allergens in the neck.

Histamine was used as the positive and dH_2O as the negative control. The horses did not react to the allergens nor the negative control but all of the horses reacted to the histamine 30 min post injection (data not shown).

4.3 Oral treatment with transgenic barley (Paper III)

4.3.1 Barley mixture and device

The Cul n 2 allergen, expressed in barley, had to be made accessible for the oral antigen presenting cells. The recombinant barley grains were ground to a powder and dissolved in dH_2O, 200, 300 and 500 mM NaCl (Figure 23A). Water was not usable to extract proteins from the barley but all concentrations of NaCl resulted in a release of the Cul n 2. The lowest concentration 200 mM NaCl was chosen for the barley mixture. The extraction was tested in 200 mM NaCl at different ratios, 1:2, 1:4, 1:8, 1:10, and Cul n 2 was extracted at all of them. The thickness of the mixture was of concern as it should stay in the horse's mouth for several hours; therefore the lowest ratio 1:2 was used for the mixture (Figure 23B).

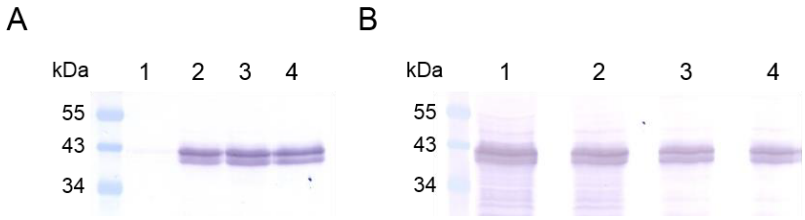

Figure 23: WB of Cul n 2 extraction.
A) In different solutions, 1: dH_2O, 2: 200 mM NaCl, 3: 300 mM NaCl, 4: 500 mM NaCl. **B)** At different ratios 1: 1/2, 2: 1/4, 3: 1/8, 4: 1:10. Primary antibody: anti-Cul n 2 mAb 1:10000 and secondary antibody: Anti-mouse-AP 1:5000.

A bit was developed to treat horses via mucosa of the mouth with the barley mixture. The mixture had to be located in the month for some time to be accessible for the oral APC. A spiral bit open at one end was the final product (Figure 24A).

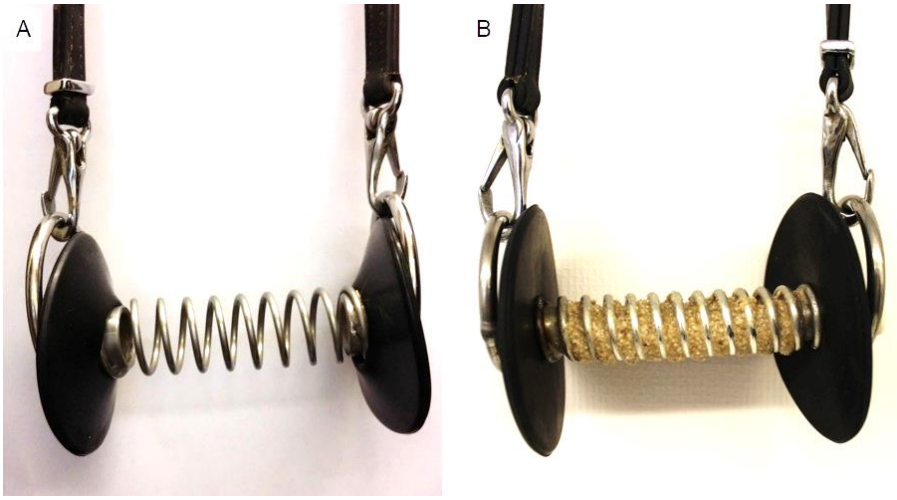

Figure 24: A spiral bit used to treat horses with barley mixture.
A) Empty bits **B)** Spiral bit filled with the barley mixture.

After mixing 50 g of barley with 100 mL 200 mM NaCl the mixture needed 2 g of a thickener, Hydroxyethylcellulose Natrosol 250 HHR PC, to stay in the bits. The bits were wrapped with parafilm and the barley mixture added with a 30 mL syringe. When the parafilm was removed the mixture stayed in the bits and they were put in the mouth of the horse.

For estimating the amount of Cul n 2 protein in 1 g barley, a semi-quantitative comparison was carried out. A known amount of Cul n 2, expressed in *E. coli,* was analyzed with WB as well as different dilutions of Cul n 2 barley extract. The amount of Cul n 2 in 1 g barley was estimated to be 100 µg (Figure 25).

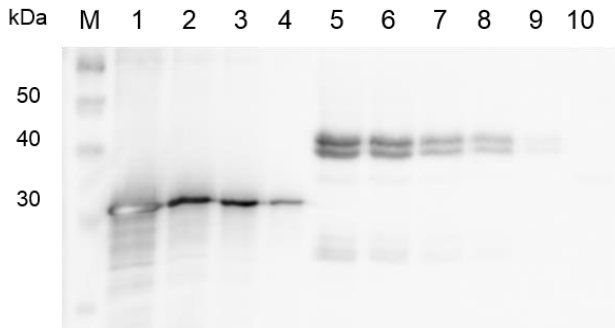

Figure 25: WB of *E. coli* expressed Cul n 2 and Cul n 2 barley extract.
M: Super signal, **1-4:** *E. coli* expressed Cul n 2,100 ng, 50 ng, 25 ng and 10 ng. **5-9:** Cul n 2 barley extraction, 1:2, 1:4, 1:8, 1:10, 1:20. **10:** Negative control. Primary antibody anti-Cul n 2 mAb 1:10000 and secondary antibody anti-mouse-HRP 1:5000.

4.3.2 Treatment and sample collection

Seven healthy Icelandic horses were included in the study; four received the Cul n 2 barley mixture and three a mixture made from normal barley. The horses were treated 6 times with 50-100 g barley at 3 to 4 week intervals. In total each horse was treated with 400 g barley. In treatments 1, 2, 3 and 5 the horses received 50 g and in 4 and 6 they got 100 g. Eight months post the last treatment they were boosted with 100 g barley (Figure 26).

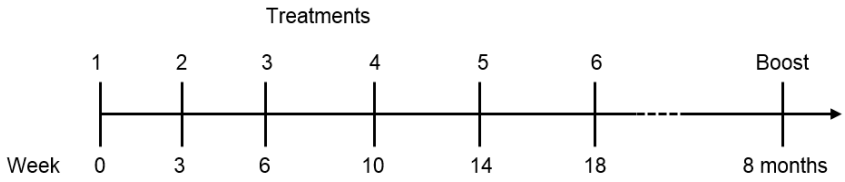

Figure 26: Time points of the treatments and the boost.

Sera and saliva were collected before (week 0) and two weeks after the last treatment (week 20), before and two weeks after the boost.

4.3.3 Specific antibody response in the sera following treatment

Allergen specific antibody response in the sera of the barley treated horses was tested on *E. coli*-rCul n 2 and *Pichia*-rCul o 2; the corresponding protein from *C. obsoletus*. IgG1, IgG4/7, IgG5 and IgE was measured in sera taken

before and after treatment and before and after the barley boost. The results are shown as ELISA increment.

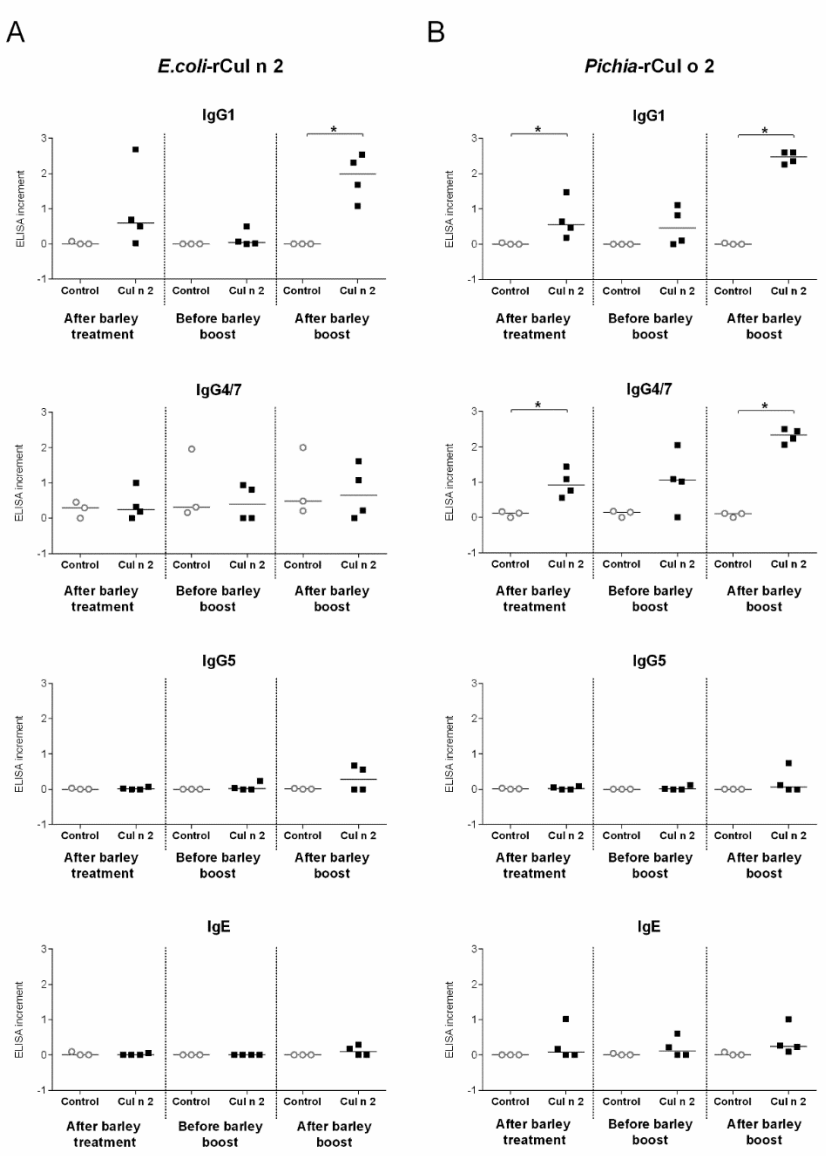

Figure 27: Allergen-specific antibody response in the sera.
A) Against *E. coli*-Cul n 2 and **B)** *Pichia*-Cul o 2 at different time points, control (○) and transgenic (■) barley treated horses. The value for each horse is plotted and shown with the median. The asterisk (*) indicates statistically significant differences.

After barley treatment, there were significant differences between the groups when tested on *Pichia*-rCul o 2 for both IgG1 and IgG4/7 compared to weak or no response against *E. coli*-rCul n 2. However, after the barley boost the Cul n 2 treated horses had significantly higher IgG1 response against both proteins and the IgG4/7 response against *Pichia*-rCul o 2. Due to a high background in the IgG4/7 response to *E.coli*-rCul n 2 before treatment no significant differences were observed for IgG4/7 when tested on *E.coli*-rCul n 2. A high IgG4/7 background had been shown before on *E. coli* expressed proteins (Paper I). Weak or no IgG5 response was detected on both proteins after treatment, but after the boost two horses responded to *E. coli*-rCul n 2 and one to the *Pichia*-rCul o 2. One horse had detectable IgE after treatment when tested on *Pichia*-rCul o 2, which decreased with time but increased with the boost. This was not seen on *E. coli*-rCul n 2 (Figure 27).

4.3.4 Specific antibody response in the saliva following treatment

Allergen specific response in the saliva of the barley treated horses was tested on *E.coli*-rCul n 2 and *Pichia*-rCul o 2. IgG1, IgG4/7 and IgG5 ELISA were measured in saliva taken before treatment and after the barley boost. The results are shown as ELISA increment.

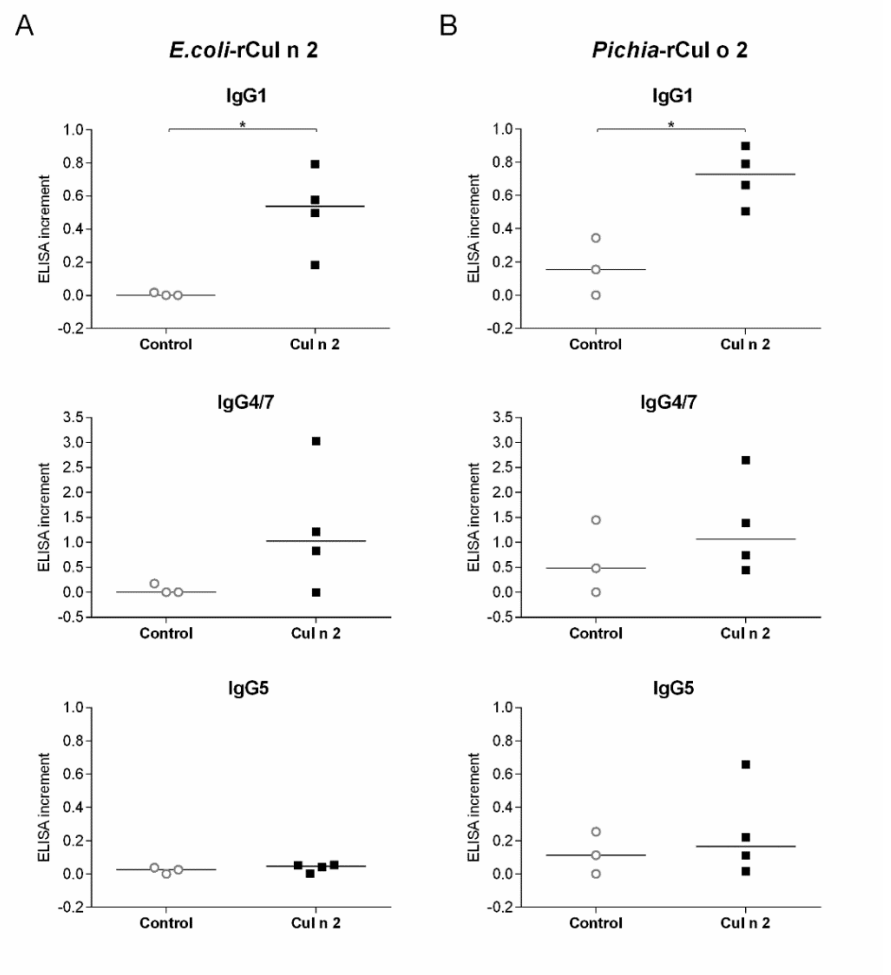

Figure 28: Allergen-specific antibody response in saliva.
A) Against *E.coli*-Cul n 2 and **B)** Against *Pichia*-Cul o 2 after barley boost for both groups, control (○) and transgenic (■) barley treated horses. The value for each horse is plotted with the median. The asterisk (*) indicates statistically significant differences.

After the barley boost the transgenic barley treated horses had a significantly higher IgG1 response in the saliva when tested on both proteins. Three out of the four transgenic barley treated horses had detectable IgG4/7 when tested on *E. coli*-rCul n 2, while the control horses were negative, but this difference did not reach significance. The four horses treated with transgenic barley responded with IgG4/7 when tested on *Pichia*-rCul o 2, but

two of the control horses as well. Only one horse had IgG5 in its saliva against *Pichia*-rCul o 2 (Figure 28).

4.3.5 Inhibitory capacity of the sera from transgenic barley treated horses

Sera taken before treatment and after the barley boost were tested in inhibition ELISA on *E. coli*-rCul n 2 and *Pichia*-rCul o 2. The sera were diluted 1:6, 1:12 and 1:24 and the percent of IgE inhibition calculated for each dilution.

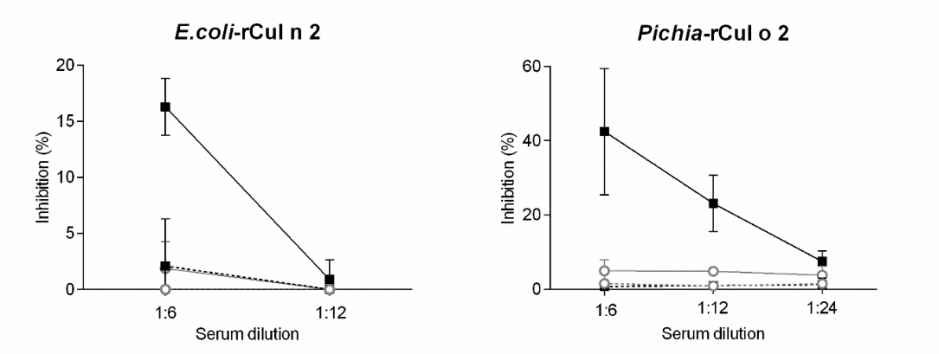

Figure 29: IgE inhibitory capacity of serum.
Sera from the control (○) and transgenic (■) barley treated horses diluted 1:6, 1:12 and 1:24, before treatment (- ○ -/- ■-) and after barley boost (—○—/—■—) on *E. coli*-rCul n 2 and *Pichia*-rCul o 2. The inhibition (%) is shown as mean and standard deviation for the groups. Notice different y-axis.

The inhibition on *Pichia*-rCul o 2 was dilution dependent as the mean inhibition was 42% (range 17%-53%) at dilution 1:6, 23% (range 12%-31%) at 1:12 and 8% (range 4%-11%) at 1:24. The inhibition on *E. coli*-rCul n 2 was lower at dilution 1:6 the mean inhibition was 17% (range 15%-20) and at dilution 1:12 no inhibition was detectable. The sera taken before treatment and from the control horses did not inhibit the binding of IgE on either protein (Figure 29).

4.3.6 Cross-reactivity between Cul n 2 and Cul o 2

The cross-reactivity between Cul n 2 and Cul o 2 was analyzed with competitive inhibition ELISA for IgG1. Sera taken after the boost from the transgenic barley treated horses were pre-incubated with different

concentrations of *E. coli*-rCul n 2, *Pichia*-rCul o 2 and a negative control (*E.coli*-rCul n 11) before being applied to an ELISA plate coated with *E. coli*-rCul n 2.

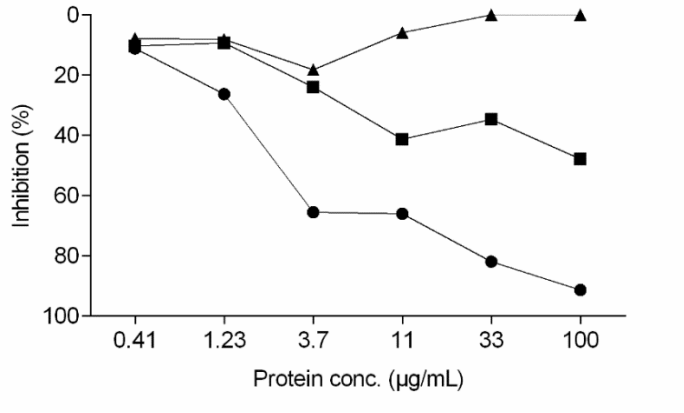

Figure 30: Competitive inhibition of IgG1 binding to Cul n 2.
Serum pool from the four transgenic barley treated horses was incubated with *E. coli*-rCul n 2 (●), *Pichia*-rCul o 2 (■) and unrelated protein (▲) as negative control before it was added to *E.coli*-rCul n 2 coated plate and IgG determined by ELISA.

The serum pool pre-incubated with 100 µg/mL of *E. coli*-rCul n 2 resulted in 91% inhibition of IgG1 binding to *E. coli*-rCul n 2. When the same pool of serum was pre-incubated with 100 µg/mL of *Pichia*-rCul o 2 48% inhibition was observed, while for the unrelated protein no inhibition was detected. The inhibition was dilution-dependent and with 0.41 µg/mL the inhibition was approximately 10% for all three proteins (Figure 30). This shows that Cul n 2 and Cul o 2 are partly cross-reactive.

4.4 An attempt to develop Cul n 4 allergy in horses (Unpublished data I)

In attempt to make healthy Icelandic horses allergic to Cul n 4, five horses were injected 5 times; 3 times s.c. and 2 times i.d. with Bac-rCul n 4 in Alum.

4.4.1 Cul n 4 serum response

Cul n 4 specific IgE response was measured by ELISA against Barley-rCul n 4 (see section 4.5) at weeks 0, 3, 6, 8, 13, 15, 16, 18, 20 and 22.

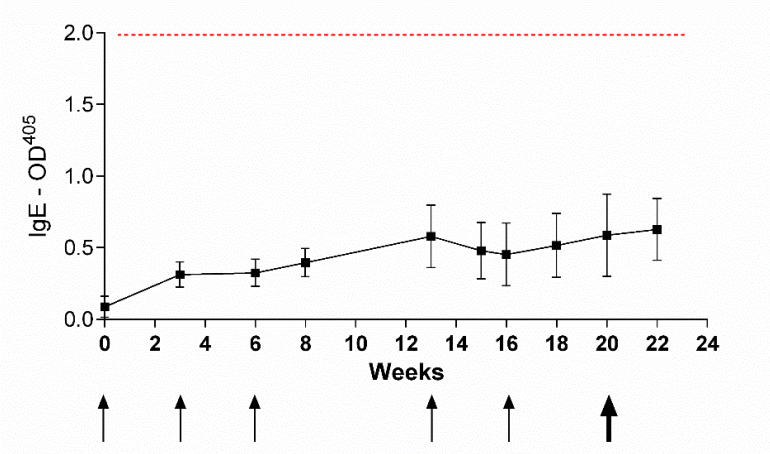

Figure 31: Time course of Cul n 4 specific IgE response measured by ELISA. Corrected OD values and standard deviations for the five horses at different time points and an IBH affected horse (- - -). The first 3 arrows indicate s.c. injections, arrows 4 and 5 the i.d. injections, and the bold arrow the skin test.

Low Cul n 4 specific IgE was detected at all time points (Figure 31). The IgE level increased slightly with the injections but did not reach the level of an IBH positive horse (OD value = 2.0).

The sera were also tested for IgG1, IgG4/7 and IgG5 response by ELISA on Barley-rCul n 4. As these horses had been injected 3 times s.c. it was interesting to see the IgG subclass profile in comparison to intralymphatic injections.

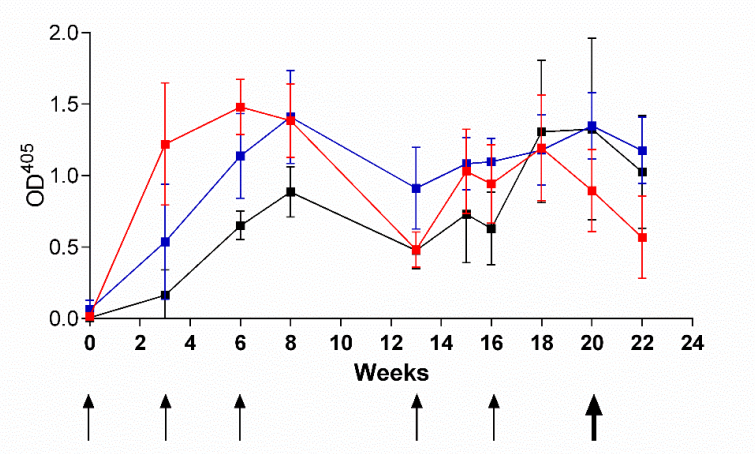

Figure 32: Time course of Cul n 4 specific IgG1, IgG4/7 and IgG5 response measured by ELISA.
Corrected OD values for IgG1 (—), IgG4/7 (—) and IgG5 (—) and standard deviations for the five horses at different time points. The first 3 arrows indicate s.c. injections, arrows 4 and 5 the i.d. injections, and the bold arrow the skin test.

With only one injection s.c. with 10 µg of Bac-rCul n 4 a strong IgG1 response was induced. The rise in IgG4/7 and IgG5 was more gradual but with the third injection a steeper rise was reached for IgG4/7. The antibody response was tested against Barley-rCul n 4; therefore the high background like seen in figure 17 on the *E. coli* produced allergens at week 0 was not a problem. The time course of the antibody response was comparable to what has been seen for i.l. and i.d. injections; with time the antibody response decreased but was boosted at week 13 when the horses were injected for the 4th time. Interestingly, a high increase in IgG5 response followed the last injection at week 16. The skin test at week 20 resulted in a decreased antibody response (Figure 32).

4.4.2 Cytokine response after *in vitro* stimulation of PBMCs

As low IgE was detected after the five injections and a different IgG profile was seen compared to i.l. injection, it was of interest to see the cytokine response of the five horses. Eleven months post the injections the horses were boosted by one injection i.d. with Bac-rCul n 4 in Alum. Two weeks after the boost PBMCs were isolated from the five horses and from five healthy

Icelandic control horses. The PBMCs were stimulated with 2 µg of Bac-rCul n 4 and Barley-rCul n 4 for 48 h and the IFN-γ, IL-4 and IL-10 measured in the cell supernatant with a bead-based multiplex assay.

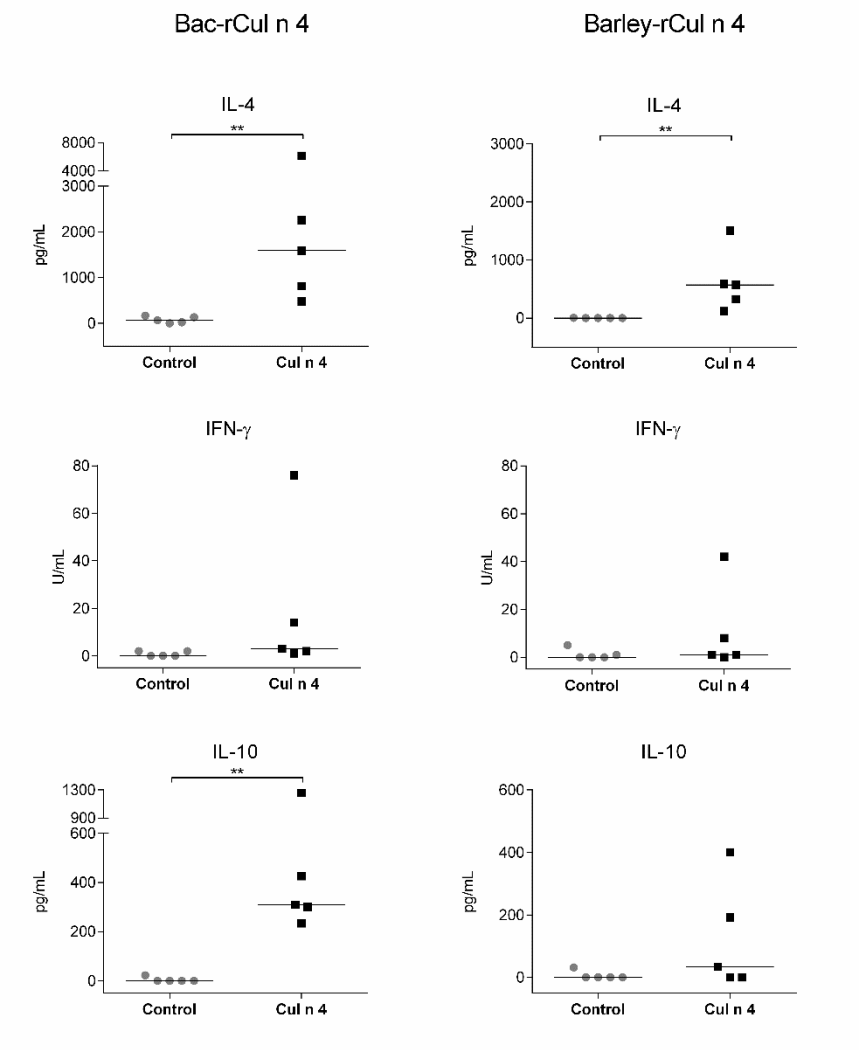

Figure 33: Detection of IL-4, IFN-γ and IL-10 after *in vitro* stimulation of PBMCs. PBMCs were isolated from Cul n 4 horses (■) and control horses (●). The cytokine level was measured in cell supernatant after *in vitro* stimulation with Bac-rCul n 4 and Barley-rCul n 4, using a bead-based multiplex assay. The value for each horse is plotted with the median. Comparison was performed using the Mann-Whitney U test. The asterisk (*) indicates statistically significant differences. ** indicate p value ≤ 0.01.

The PBMCs were stimulated with Bac-rCul n 4, the same product used for the injection and Barley-rCul n 4. The Cul n 4 horses produced significantly more IL-4 and IL-10 than the control horses after stimulation with Bac-rCul n 4 when compared with the Mann-Whitney U test. When the cells were stimulated with the Barley-rCul n 4 a significant difference between the Cul n 4 horses and the controls was only seen for the IL-4 production. For IFN-γ both groups of horses were low or negative, except for one of the Cul n 4 horses that produced IFN-γ upon stimulation with Bac- and Barley-rCul n 4 (Figure 33).

4.5 Production of *Culicoides* allergens and application in IgE ELISA (Unpublished data II)

4.5.1 Production and purification of Cul n 3 and Cul n 4

Two allergens from *C. nubeculosus*, Cul n 3 and Cul n 4 were expressed in *E. coli*, insect cells and barley seeds and purified.

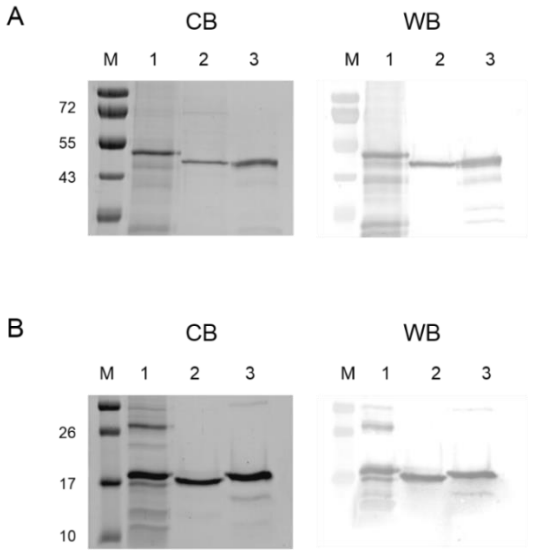

Figure 34: Expression of rCul n 3 and rCul n 4 in three expression systems.
A) rCul n 3 and **B)** rCul n 4, M) PageRuler (Thermo), 1) *E. coli*-, 2) Bac- 3) Barley-expressed allergens, coomassie blue staining (CB) and western blot (WB). Primary antibody, mouse anti-Cul n 3 and -Cul n 4 diluted 1:4000 and secondary antibody AP labelled anti-mouse diluted 1:5000 (Jackson).

The *E. coli* expressed allergens were purified under denaturing conditions, while the insect cell (Bac) and barley expressed allergens were purified under native conditions. The purified allergens were analyzed with coomassie blue staining (Figure 34A) and western blot (Figure 34B). The calculated size of Cul n 3 wss 45 kDa and was detected as a band below the 55 kDa. The Bac- and Barley-rCul n 3 were detected as a smaller band than *E. coli*-rCul n 3. The calculated size of Cul n 4 was 17 kDa and it was detected as 17-18 kDa. The Cul n 3 has a predicted secretion signal that could explain why the *E. coli* expressed protein was larger than those expressed in insect cells and barley seeds (Figure 34).

4.5.2 IgE ELISA on sera from IBH-affected and healthy horses

The serum IgE of 97 IBH-affected horses and 71 healthy controls was measured by ELISA on rCul n 3 and rCul n 4 expressed in *E. coli*, insect cells and barley for comparison of their performance to distinguish between healthy and IBH horses.

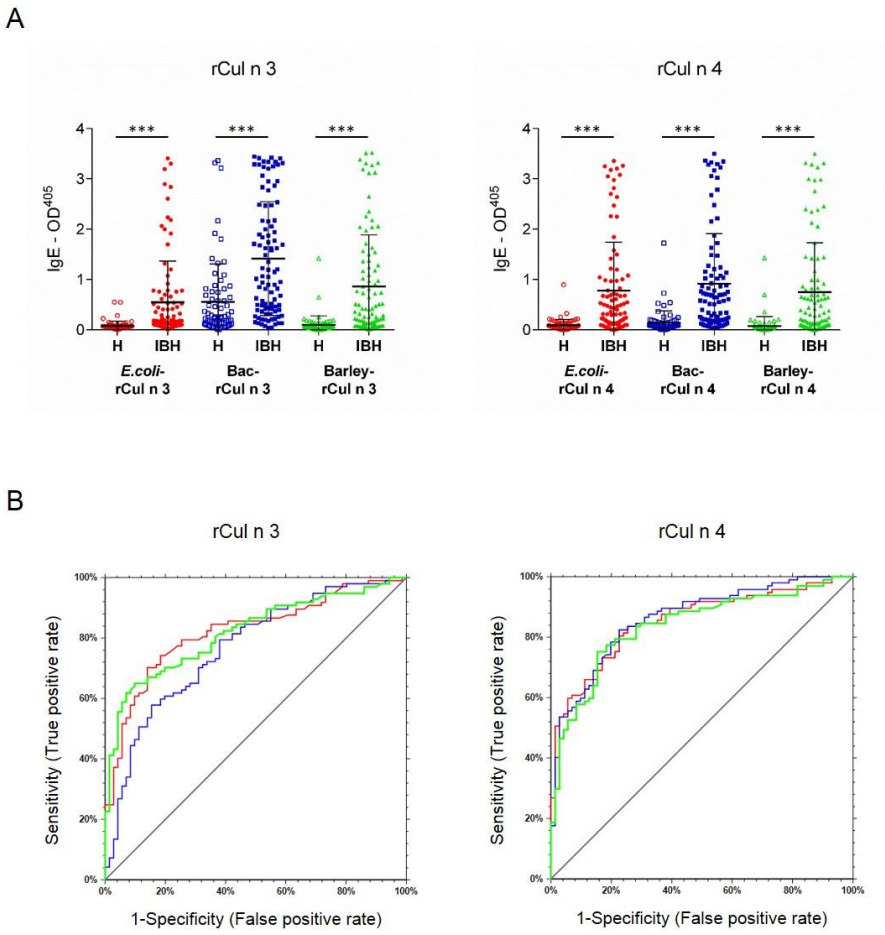

Figure 35: Performance of rCul n 3 and rCul n 4 in IgE ELISA.
Serum IgE response of 97 IBH-affected and 71 healthy controls compared on rCul n 3 and 4 from three different expression systems. **A)** rCul n 3 and rCul n 4 expressed in *E. coli* (○/●), insect cells (□/■) and barley (Δ/▲) tested by ELISA. Corrected IgE OD values for healthy (H) and IBH-affected horses, each horse plotted and shown with the mean and standard deviations for the group. The asterisks (*) indicate statistical significance in t-test. *** indicate p value ≤ 0.001. **B)** Receiver operator characteristic (ROC) curve of serum IgE levels against the *Culicoides* allergens rCul n 3 and rCul n 4 expressed in *E. coli* (—), insect cells (—) and barley (—).

Serum IgE levels of the IBH-affected horses were significantly higher on all the proteins as compared to the healthy controls (Figure 35A). For rCul n 3, the ROC analysis revealed that the performance of the ELISA was very similar for the *E. coli* and barley-produced proteins with an area under the curve (AUC) 0.82 for both, while the AUC for Bac-rCul n 3 was lower, 0.77 (Table 5 and Figure 35B). For rCul n 4 the ROC curves were relatively high and very similar for all three expression systems with values between 0.84 and 0.86 (Figure 35B and Table 5). The rCul n 4 expressed in the different expression systems not only performed equally well, but also showed a high level of correlation, demonstrated with Spearman correlation coefficients ≥ 0.849 and kappa values ≥ 0.82 (see Table 6 in Appendix).

Table 5: Diagnostic perfomance of the serum IgE ELISA with rCul n 3 and rCul n 4 produced in different expression systems.

Allergen	AUC	95% CI	Cut off (OD_{405})	Accuracy	Sensitivity	Specificity
***E.coli*-rCul n 3**	0.82	0.77-0.88	0.11	77%	70%	86%
Bac-rCul n 3	0.77	0.68-0.83	0.83	69%	60%	82%
Barley-rCul n 3	0.82	0.75-0.87	0.20	75%	62%	93%
***E.coli*-rCul n 4**	0.85	0.79-0.90	0.11	80%	84%%	75%
Bac-rCul n 4	0.86	0.80-0.91	0.15	80%	82%	77%
Barley-rCul n 4	0.84	0.77-0.89	0.09	79%	75%	85%

To further analyze the IgE data the 168 horses (IBH=97 and H=71) were split into three groups based on their origin; Icelandic-born Icelandic horses (H=23 and IBH=47), European-born Icelandic horses (H=24 and IBH=23) and European-born various breeds (H=24 and IBH=27) and the response analyzed correlated with disease status, origin of the horses and the differently expressed proteins (Figure 36).

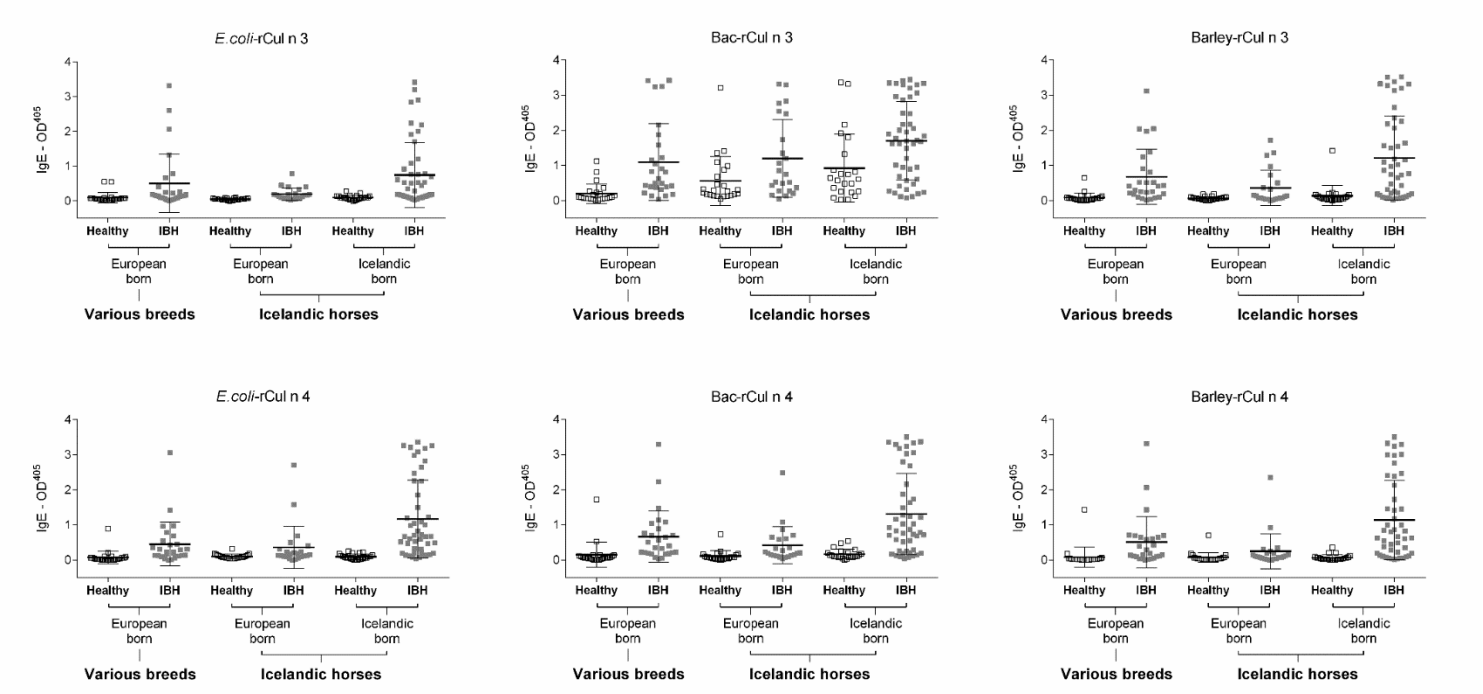

Figure 36: Serum IgE response of healthy and IBH-affected horses based on origin.
Serum IgE response of various breeds of horses, Icelandic horses, European- and Icelandic-born, compared on rCul n 3 and 4 from three different expression systems. Corrected IgE OD values for healthy (□) and IBH-affected (■) horses, each horse plotted and shown with the mean and standard deviations for the group. Linear mixed effect model was used to compare the different groups of origin and the difference between healthy and IBH-affected horses within each group

.

A significant difference in IgE response was observed between IBH-affected horses and healthy controls against all proteins in all groups except for Icelandic-born Icelandic horses against Bac-rCul n 3 and European-born Icelandic horses against *E. coli*-rCul n 4 and Barley-rCul n 4 (Table 7 in Appendix).

Interestingly, when the IgE response was compared based on origin, the IBH- affected Icelandic-born horses stand out as having significantly higher IgE levels against the rCul n 4 as compared to those born in Europe. This difference between the European- and Icelandic-born horses was not as obvious against rCul n 3 (Figure 36 and Table 8 in Appendix).

5 Discussion

5.1 The Icelandic horse

The native Icelandic horse is the only horse breed in Iceland. They were brought to the country during the early Viking settlement in the 10th century. The Icelandic horse is an isolated breed as there is no historical documentation of imports of breeding animals and since 1882 importation of horses has been prohibited by law. The Icelandic horse is an excellent riding horse and with increased popularity in the 20th century it was noted that IBH was frequently seen in these horses in Europe. Annually around 1400 horses are exported all over the world. The allergy-causing midges are not indigenous to Iceland, leading to a high prevalence of IBH in horses when exported as adults to *Culicoides* infested areas (Schaffartzik et al., 2012). The welfare of affected horses is compromised due to itching leading to rubbing, skin damage, hair loss, and sometimes secondary infections. Insect bite hypersensitivity is therefore a great animal welfare issue and a problem for the Icelandic horse industry.

5.2 Preventive vaccination against allergy

In vaccine development the main focus has been on infectious diseases and preventive vaccinations against various infectious agents are well established. However, vaccination to prevent allergic sensitization is not yet practiced in humans, although there is an increased interest as more than 25% of people in western countries suffer from allergic diseases. For an allergy vaccine pure allergens should be used as in crude extracts the allergens are in a native form with a high allergenicity risking sensitization (Valenta et al., 2012). In our study *E. coli* produced allergens purified under denaturing conditions were used for the vaccinations, lacking the correct structure of the native *Culicoides* allergens and thus lowering the risk of sensitization. Sixteen allergens from three *Culicoides* species have been identified (Langner et al., 2009; Peeters et al., 2013; Schaffartzik et al., 2011;

van der Meide et al., 2013). IBH affected-horses respond to different allergens and also to different numbers of the ones already isolated. In the study of van der Meide et al. 46% of horses responded to all the seven *C. obsoletus* allergens tested (van der Meide et al., 2014). Consequently, horses should be tested before desensitization and treated with the relevant allergens. Regarding the composition of a vaccine, it is not clear how many allergens are needed. At any rate, it is necessary to map the major allergens and distinguish between genuine sensitization and cross-reactivity. In human allergology so-called component-resolved diagnostics using microarray have been developed for defining to which specific allergenic molecules an individual is sensitized. The MeDALL allergen-chip has more than 170 allergens to evaluate IgG and IgE reactivity (Lupinek et al., 2014). This method has been tested in a preliminary experiment with allergens from biting insects and horse sera with promising results (Marti et al., 2015). A more comprehensive study is being planned. Another important factor in prophylactic immunotherapy is the time window as the administration of the vaccine has to occur before sensitization (Valenta et al., 2012). For this reason IBH and the Icelandic horses, free from the causative allergens until export, are an excellent animal model for the development and study of prophylactic immunotherapy.

5.3 Protein vaccine against insect bite hypersensitivity

Before the *Culicoides* allergens were isolated, a vaccination experiment was performed with human serum albumin (HSA). The horses were vaccinated with HSA in MPLA s.c. and intramuscularly. The IgG subclass response was in general weak. It was predominantly IgG(T) (IgG3/5), to a lesser degree IgG1 and very low IgG4/7 (unpublished data). Based on the data both the adjuvant and the injection site needed reconsideration. At that time it was not easy to obtain a Th1 focusing adjuvant. After some search and paperwork IC31® was kindly provided by Intercell (Vienna, Austria) later Valneva (Vienna, Austria). Two injection sites were compared, intralymphatic and intradermal with *Culicoides* allergens in IC31® or in PBS (Paper I).

Lymph nodes are highly organized secondary lymphoid organs. They are composed of subcapsular area, medulla, paracortex and cortex. The subcapsular area and medulla are rich in APCs and are also known as the antigen-sampling zone. These areas filter the pathogens from the lymph and the APC delivers the antigens to the B- and T-cell areas. The paracortex is rich in T cells and dendritic cells, also known as the T cell activation zone. The cortex contains B-cell follicules, also known as the B-cell activation zone (Junt et al., 2008). By intralymphatic administration the allergens are directly injected into a secondary lymphoid organ, whereby the probability of interaction between antigen and a lymphocyte specific for that particular antigen is greatly increased.

The skin has three layers, the epidermis, dermis, and the hypodermis or subcutaneous tissue. The most common vaccine injection site is into the subcutaneous tissue, mostly composed of fibroblasts and adipocytes with relatively few dendritic cells (Combadiere & Liard, 2011; Romani et al., 2010). However, the epidermis and the dermis have now gained attention as an alternative route. The epidermis has a network of dendritic cells called Langerhans cells and the dermis contains a range of specialized immune cells, such as dendritic cells, macrophages and $CD4^+$ T-helper cells. In the dermis are also the lymphatic vessels that drain the skin (Nestle et al., 2009). Intra dermal injections should therefore be more effective for obtaining a strong immune response than subcutaneous injections.

After three intralymphatic and intradermal injections over an eight week period with a total of 30 µg each of four *Culicoides* allergens in IC31®, the horses elicited an allergen specific IgG response in sera (Paper I). The IC31® adjuvant was as expected crucial. Mainly IgG1 and IgG4/7 were induced, the subclasses important for a protective immune response (Lewis et al., 2008). The capacity of IgG to block antigen-IgE binding is one of the essential factors in successful immunotherapy (Akkoc et al., 2011). The IgG antibodies induced were partly able to inhibit the IgE binding to the allergens. This was only tested in the group vaccinated intralymphically with the allergens in IC31®, the group showing the strongest antibody response. Due to low

concentration, cytokines cannot be measured directly in sera but PBMCs have to be re-stimulated *in vitro*. Before and two weeks after each vaccination PBMCs were stimulated with the four *E. coli* produced allergens used for the vaccination. The results were obscured due to the high background, probably because of bacteria protein or LPS contamination of the allergens. It was only after the third vaccination that some difference between the vaccination groups was observed. The groups receiving the allergens in IC31® showed a significantly higher IL-10 mRNA level as compared to intradermal without IC31®. Significantly more IL-4 mRNA was detected in the group vaccinated intralymphatically without IC31® as compared to intradermal with IC31®. The IC31® is Th1 focusing, yet in our experiment we were not able to detect a difference in IFNγ production between the groups; this could be blamed on faulty stimulation. For further development of a preventive vaccine more reliable cytokine measurements are needed. The intralymphatic injection resulted in a somewhat stronger antibody response therefore this route was used for the continuation. The IC31® adjuvant gave promising results and further experiments would have been interesting. However, the company providing it was not willing to continue the collaboration and adjuvants which could be purchased were therefore our next choice.

The vaccination experiment with HSA in MPLA mentioned earlier resulted in a weak and non-decisive response. Aluminum hydroxide is known to elicit a strong antibody response and is also the classical adjuvant for immunotherapy (Moingeon, 2012). AS04 adjuvant mixture (Alum/MPLA) is successfully used in anti-viral vaccine where a Th1 response is required (Garçon et al., 2011). Allergens in Alum alone or in Alum/MPLA were compared in the next experiment (Paper II). Both groups of horses (Alum and Alum/MPLA) responded with strong allergen specific antibody response after only two injections of 10 µg/injection. The IgG response was mostly IgG1 and IgG4/7 as when IC31® was used. Allergen specific IgA that has been linked to successful ASIT was induced by the vaccination (Akkoc et al., 2011). The antibodies generated were able to block the binding of IgE to allergen. The

blocking capacity was stronger than after the IC31® vaccination. For three out of the four allergens used, sera diluted 1:10 inhibited almost 100%. After the IC31® vaccination the inhibition only reached 55% with the same serum dilution. There was no induction of IgE nor were the horses sensitized. The same antibody profile was observed against two allergens expressed in insect cells. The inhibitory capacity against them was slightly lower but still showed strong inhibition, ranging from 79 to 89% at a serum dilution of 1:10. This shows that the antibodies generated were mainly directed against the relevant proteins but not against *E. coli* derived contaminants. Significantly more IL-10 and IFNγ was produced in the Alum/MPLA group as compared to non-vaccinated controls. This difference was not seen for the Alum group. Alternatively, when the Alum group was compared to the Alum/MPLA group, horses from the Alum/MPLA produced significantly more IL-10 and IFNγ. The same tendency was seen at the mRNA level although the difference did not reach significance. PBMCs were stimulated with Bac-rCul n 3, the only one of the four insect cell expressed allergens available at that time in a native form. The difference between the groups was only observed in the cytokine response, emphasizing the importance of validated *in vitro* assays for detecting cytokine production. MPLA seems to be important for focusing the immune response toward Th1 and T regulatory response. In humans, allergen-specific IgG4 and IgA, IFNγ and IL-10, along with the strong inhibitory capacity, are all important in successful ASIT (Akkoc et al., 2011).

5.4 Oral treatment with transgenic barley

Subcutaneous injection has been the golden standard in immunotherapy for more than 100 years. There is a demand for more clinically friendly non-invasive approaches (Novak et al., 2011). Barley is an excellent source of proteins with post-translation modification, endotoxin free, and the possibility of long term storage of the grains (Horvath et al., 2000; Magnusdottir et al., 2013). ORF Genetics Ltd., located in Iceland, is an innovative company that has established a method to maximize the production of recombinant proteins in barley grains. The collaboration with ORF was the foundation for the development of oral treatment and potential mass production of allergens.

Sublingual immunotherapy (SLIT) is the only alternative for subcutaneous immunotherapy (SCIT) that has been licensed for human use (reviewed in Novak et al., 2011). In sublingual immunotherapy the treatment is through the mucosa of the mouth. The mouth is an immune privileged site and, although the oral mucosa is constantly in contact with commensal and pathogenic microbes, an immune homeostasis is maintained (Novak et al., 2008). The oral mucosa has an effective immunological network, dendritic cells with strong TLR2 and TLR4 expression resulting in dominant Th1 and Th17 cells and regulatory cytokine profile, whereas inflammatory cells as mast cells and eosinophils are scarce (Allam et al., 2011; Novak et al., 2011).

ASIT using edible mucosa-targeting feed is an interesting approach to modulate the immune response. The drawback is the enzymatic digestion in the gastrointestinal tract and the large volume needed compared to pharmaceutical products. In the development of ASIT against Japanese cedar pollen allergy an attempt has been made to overcome these problems, firstly, making rice-derived recombinant proteins expressed in protein bodies, which are partly resistant to the digestion. Secondly, increasing the volume of the protein bodies by removing the starch from the milled rice powder. This formulation has only been tried in an allergy mouse model where a challenge experiment resulted in suppressed T cell response (Wakasa et al., 2015).

In collaboration with ORF Genetics Ltd. three allergens, Cul n 2, Cul n 3 and Cul n 4, were expressed in barley. We decided to develop a method to treat horses via oral mucosa (Paper III). The other option would have been to feed the horses with the transgenic barley but this would have required a much larger amount and would have created a problem exporting the transgenic whole grain for treatment of IBH horses.

Cul n 2 specific IgG1 and IgG4/7 response could be induced by treating healthy horses with allergen-expressing transgenic barley. The antibodies reacted to the corresponding allergen (Cul o 2) from *C. obsoletus* and could partly inhibit the binding of IgE to the allergen. The response was effectively boosted 8 months after the first treatment. One horse responded with allergen specific IgE. This could possibly be explained by the presence of

cross-reactive antigens in blackflies, which are present in Iceland, and midges. Cross-reactivity between salivary antigens from *S. vittatum* and *C. nubeculosus* has been demonstrated (Schaffartzik et al., 2010). The hyaluronidase from *S. vittatum* has not been identified and produced as recombinant protein, and thus we cannot test this. Although it is perturbing to see an increase in specific IgE following treatment, this can be expected if the horse is sensitized to this antigen. It is well-known in human allergies that immunotherapy at first induces a rise in allergen-specific IgE, followed by a very slow decrease (Akdis & Akdis, 2011). More relevant for a successful allergen immunotherapy is the induction of blocking IgG antibodies, induced in the same horse (Akdis & Akdis, 2011).

It proved very difficult to measure the antibody response in saliva and only after the boost was a significant difference in IgG1 response seen between the control horses and the Cul n 2 treated horses. The collection method was not optimized at the beginning of the treatment and therefore a limited amount of the pre-treatment saliva was available. The saliva itself might contain substrates interfering with the ELISA as it was usually turbid brownish or greenish.

As IgA is the mucosal antibody an attempt was made to measure it both in saliva and sera. It could not be detected, however, probably due to the weak response in general. For detection of allergen specific IgA in the Alum/MPLA experiment the sera were only diluted 1:10, indicating low serum levels. Cytokine measurements following the barley boost using *Pichia*-rCul o 2 for stimulation were negative. This might be due to too few allergen-specific T-cells and that the cytokine production was below the detection limit of the assay.

5.5 An attempt to induce allergy in horses

After having obtained a favorable immune response in healthy horses the next step was to try desensitization. Therefore an attempt was made to induce allergy in horses against Cul n 4 for subsequent barley treatment in a simple system. In an earlier study, HSA allergy was induced in horses with the protein in Alum and repeated subcutaneous injections. We failed to

repeat this with Cul n 4. The horses were low or negative in IgE and negative in the skin test, but nonetheless responded with IgG5, which has been linked to allergy (Wagner et al., 2006). Interestingly the first subcutaneous injection resulted in strong IgG antibody response. These horses were then boosted and used to test some parameters in the *in vitro* stimulation for cytokine measurements, such as titration of the proteins, and to test proteins from different expression systems. Two µg of the allergen were adequate to re-stimulate the specific T-cells for production of IFNγ, IL-4 and IL-10. The barley produced Cul n 4 stimulated efficiently, although to a somewhat lower level than the insect cell-expressed protein used for the injection.

Compared to the intralymphatic vaccination with *E. coli*-Cul n 4 in Alum more IgE, IgG5 and IL-4 were observed, indicating that the response was more towards sensitization.

5.6 Expression of recombinant allergens in barley

For development of effective diagnostic tests for IBH a good source of purified recombinant proteins is important. Serum IgE ELISA using *E. coli* expressed allergens originating from *C. obsoletus*, the most abundant *Culicoides* spp. in the area of the tested horses has shown promising results. Good specificity and sensitivity were obtained, especially with a combination of three or four allergens (van der Meide et al., 2014). We showed that the Cul n 3 and Cul n 4 allergens produced in barley seeds were comparable to the same proteins expressed in *E. coli* regarding performance in IgE ELISA. These allergens originated from *C. nubeculosus* that is not a common midge in the area of the tested horses (Casati et al., 2009). Nevertheless, the specificity and sensitivity in the ELISA was similar to that which van der Meide *et al.* observed for single allergens from *C. obsoletus*. It would have been interesting to test a combination of rCul n 3 and rCul n 4. Although one could assume that insect cells were the most appropriate expression system for producing allergens for diagnosis of IBH, the Bac-rCul n 4 did not have a better performance than the other two, and the Bac-rCul n 3 slightly worse. This is in accordance with van der Meide *et al.*, who showed that Cul o 1 expressed in insect cells did not perform better than the *E. coli* expressed in

IgE ELISA. They also detected the relativity high response of healthy horses against insect cell expressed Cul o 1 like we detected against Bac-rCul n 3 (van der Meide et al., 2013).

It was of special interest that the horses responded differently according to the origin of birth, where Icelandic-born horses responded with significantly higher IgE levels than the European-born Icelandic horses. Hellberg et al. (2006) showed that Icelandic-born horses affected with IBH responded with IgE to more *Culicoides* salivary gland proteins in immunoblot analysis than the European-born Icelandic horses. This difference in IgE response of IBH affected horses based on their origin of birth has to be analyzed further.

Recombinant allergens are also important for monitoring the immune response following vaccination. For this purpose it is crucial to not only measure the antibody response but also the cytokines. Following the vaccination with Alum and Alum/MPLA we could not detect differences in the immune response of the groups by measuring the antibodies (Paper II). As cytokines in serum are usually below the detection limits, PBMCs have to be re-stimulated *in vitro* with the allergens used for the vaccination. *E. coli* expressed allergens are not suitable for the stimulation as they tend to give too much background, probably due to endotoxin contamination (Paper I). Thus, allergens expressed in other systems need to be used. To further analyze the possible applications of barley produced allergens they should also be compared in *in vitro* stimulation of PBMCs and in intradermal tests.

6 Conclusions and future perspectives

The two vaccination studies with allergens in adjuvants gave promising results. The important IgG subclasses, IgG1 and IgG4/7 as well as IgA, were induced. The antibodies were able to block binding to allergens, both to the allergens used in the vaccination and to the same allergens expressed in a different system. The Th1 cytokine IFNγ and the regulatory cytokine IL-10 were generated following vaccination with the allergens in Alum/MPLA. A challenge experiment where vaccinated horses together with unvaccinated control horses are exported to a *Culicoides* infested areas is now crucial to test whether the horses are protected. Before that can be performed it is important to determine the major allergens causing IBH and improve the measurement of the T cell response following vaccination.

Three allergens were produced in barley, and a method and a device were developed to treat horses via the oral mucosa. The treatment of healthy horses with Cul n 2 barley induced the IgG subclasses vital in protective immunity, IgG1 and IgG4/7. The antibodies were able to partly block the binding of IgE to the allergen. It would be interesting to add an adjuvant to the barley mixture, e.g. TLR-2 or TLR-4 ligands, to enhance the dendritic cell uptake of the antigens. The next step is to treat IBH affected horses. That requires production of more allergens in barley, as each horse has to be treated according to its IgE profile.

Barley produced rCul n 3 and rCul n 4 showed comparable performance in IgE ELISA as the same allergens produced in *E. coli* and insect cells. Barley is an excellent source of proteins, cost effective and can be grown on an agricultural scale. It could be a good candidate as a production system for large quantities of recombinant allergens for commercial assays.

The injection of allergens in adjuvant and the oral barley treatment are promising candidates for both prophylactic and therapeutic immunotherapy for insect bite hypersensitivity of horses.

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Research paper

Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: A pilot study

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IgG subclasses

ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of midges (*Culicoides* spp.). IgE-mediated reactions are often involved in the pathogenesis of this disease. IBH does not occur in Iceland due to the absence of *Culicoides*, but it occurs with a high frequency in Icelandic horses exported to mainland Europe, where *Culicoides* are present. We hypothesize that immunization with the *Culicoides* allergens before export could reduce the incidence of IBH in exported Icelandic horses. The aim of the present study was therefore to compare intradermal and intralymphatic vaccination using four purified recombinant allergens, in combination with a Th1 focusing adjuvant.

Twelve horses were vaccinated three times with 10 μg of each of the four recombinant *Culicoides nubeculosus* allergens. Six horses were injected intralymphatically, three with and three without IC31®, and six were injected intradermally, in the presence or absence of IC31®. Antibody responses were measured by immunoblots and ELISA, potential sensitization in a sulfidoleukotriene release test and an intradermal test, cytokine and FoxP3 expression with real time PCR following *in vitro* stimulation of PBMC.

Immunization with the r-allergens induced a significant increase in levels of r-allergen-specific IgG1, IgG1/3, IgG4/7, IgG5 and IgG(T). Application of the r-allergens in IC31® adjuvant resulted in a significantly higher IgG1, IgG1/3, IgG4/7 allergen-specific response. Intralymphatic injection was slightly more efficient than intradermal injection, but the difference did not reach significance.

Testing of the blocking activity of the sera from the horses immunized intralymphatically with IC31® showed that the generated IgG antibodies were able to partly block binding of serum IgE from an IBH-affected horse to these r-allergens. Furthermore, IgG antibodies bound to protein bands on blots of *C. nubeculosus* salivary gland extract.

No allergen-specific IgE was induced and there was no indication of induction of IgE-mediated reactions, as horses neither responded to *Culicoides* extract stimulation in a sulfidoleukotriene release test, nor developed a relevant immediate hypersensitivity reaction to the recombinant allergens in skin test. IL-4 expression was significantly higher in

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horses vaccinated intralymphatically without IC31®, as compared to horses intradermally vaccinated with IC31®. Both routes gave higher IL-10 expression with IC31®.

Both intralymphatic and intradermal vaccination of horses with recombinant allergens in IC31® adjuvant induced an immune response without adverse effects and without IgE production. The horses were not sensitized and produced IgG that could inhibit allergen-specific IgE binding. We therefore conclude that both the injection routes and the IC31® adjuvant are strong candidates for further development of immunoprophylaxis and therapy in horses.

1. Introduction

Equine insect bite hypersensitivity (IBH), also called summer eczema (SE), is an allergic recurrent seasonal dermatitis of horses. IgE-mediated, type I hypersensitivity with release of histamine and other inflammatory mediators from basophils and mast cells are often involved in IBH. However, in some cases cell-mediated, type IV hypersensitivity may also contribute to the pathogenesis. The eczema is caused by bites of insects mainly of the genus *Culicoides* (biting midges) (Fadok and Greiner, 1990; Quinn et al., 1983; Schaffartzik et al., 2012) and characterized by pruritic dermatosis affecting mainly the mane and tail area causing discomfort and often suffering in affected individuals (Bröstrom et al., 1987; Townley et al., 1984). The prevalence of the disease depends on the geographic region and exposure to insect bites. IBH is found almost worldwide, but one of the exceptions is Iceland where *Culicoides* spp. are not indigenous. However, Icelandic horses foaled in Iceland and exported to the European continent are more frequently affected than Icelandic horses or horses from other breeds born in an environment where *Culicoides* are present (Bröstrom et al., 1987; Halldordsottir and Larsen, 1991). The frequency of IBH in horses exported as adults and not protected from *Culicoides* bites was more than 50% after two years or more in heavily *Culicoides* infested areas (Bjornsdottir et al., 2006) while only 5–10% of Icelandic horses foaled on the European continent developed the disease (Bröstrom et al., 1987; Halldordsottir and Larsen, 1991). The reasons for this increased incidence of IBH in horses born in Iceland and exported to Europe or North America as adults are not known; environmental or epigenetic factors may contribute to this response (Marti et al., 2008). Interestingly, it has been demonstrated that the prevalence of IBH in horses imported from Iceland is influenced by the age at import, *i.e.* at first exposure to *Culicoides* allergens. Horses imported as weanlings at an age of 7–10 months did not develop IBH more frequently than Icelandic horses born in Europe (Sommer-Locher et al., 2012), suggesting that early life exposure to the causative allergens is required to prevent development of IBH. This may be explained by a higher capacity of young horses to develop a regulatory T cell immune response. A recent study has demonstrated that significantly higher number of functionally mature regulatory T cells can be induced *in vitro* in foals compared to adult horses (Hamza et al., 2015).

The allergens causing IBH originate in the *Culicoides* salivary glands of the blood-feeding females (Hellberg et al., 2006; Wilson et al., 2001). Different *Culicoides* species dominate in different geographical areas and IBH-affected horses have been shown to react in skin tests to *Culicoides* extracts made from both native and exotic species (Anderson et al., 1993). The first salivary gland proteins that bind IgE from IBH affected horses to be identified and produced as recombinant proteins were derived from laboratory-produced species. These comprised one protein from *Culicoides sonorensis* (Langner et al., 2009) and eleven from *Culicoides nubeculosus* (Schaffartzik et al., 2010, 2011). These two species are not very common in Europe, but the salivary proteins from them bind IgE from IBH horses with variable frequency. Recently, seven salivary gland proteins originating from *Culicoides obsoletus*, the main midge feeding on horses in the Netherlands, have been identified and expressed (van der Meide et al., 2013).

Our findings regarding the immune response and pathogenesis in IBH suggest that Th2 type, IgE-mediated immune reactions are involved to a much stronger extent in Icelandic horses than in some other breeds, and after export there is an imbalance between Th1, Th2 and T regulatory cells (Treg) (Hamza et al., 2008, 2010, 2013; Heimann et al., 2011). It should therefore be possible to rebalance the Th1:Th2:Treg responses and restrain the inflammatory mechanisms by strengthening the Treg response specific to the allergens using immunotherapy.

In a pilot study, attempts to shift the immune response in horses toward Th1 with subcutaneous and intramuscular injections of proteins in the Th1 adjuvant Monophosphoryl-lipid A, were only partly successful (Marti et al., 2008). Therefore, both the injection method and the adjuvant part had to be reconsidered.

Data from experimental animals and from clinical trials have shown that intralymphatic allergen administration strongly enhanced specific immunotherapy. It required reduced allergen dose, shorter duration and fewer injections as compared to the classical subcutaneous method (Senti et al., 2011). Injection into the submandibular lymph nodes is however, impractical in horses and hence intradermal injections were considered as an alternative. The skin has an extended local network of several types of professional antigen presenting cells and easy access to the skin-draining lymph nodes (Combadiere and Liard, 2011).

The IC31® is an adjuvant that combines the antimicrobial peptide $KLKL_5KLK$ and a synthetic oligodeoxynucleotide (ODN1a). It stimulates the immune system *via* the TLR9/MyD88-dependent pathway. IC31® induces potent Th1 immune response in mice (Schellack et al., 2006) and it has been shown *in vitro* that IC31® modulates the cytokine profile of human dendritic cells which is important for

protection against intracellular pathogens (Lingnau et al., 2007; Szabo et al., 2013). To our knowledge, this adjuvant has not been used in horses before.

The aim of this pilot study was to obtain a first set of data on an effective injection route with a small amount of recombinant allergens in a suitable adjuvant to vaccinate horses. This could then be used as a basis for future studies on a preventive immunization strategy against insect bite hypersensitivity.

2. Materials and methods

2.1. Animals

Twelve healthy Icelandic horses aged 5–8 years were vaccinated, and three additional horses used as controls in the skin test. They were maintained according to the Icelandic animal care guidelines for experimental animals. The horses were housed and fed hay during winter and during summer they were kept out at pasture. The experiment was performed in accord with a permit from the national animal research committee of Iceland.

2.2. Vaccination and sample collection

Twelve horses nos. 1–12 were vaccinated with four recombinant *C. nubeculosus* allergens (rCul n 1, rCul n 2, rCul n 5 and rCul n 9) produced in *Escherichia coli* and purified (Schaffartzik et al., 2011). These allergens have previously been shown to be relevant allergens for IBH, as allergic horses have significantly more often serum IgE specific for these recombinant allergens than healthy control horses and they induce immediate type reaction in intradermal tests in IBH-affected horses (Schaffartzik et al., 2010, 2011). The horses were vaccinated three times (week 0, 5 and 9) with 10 μg of each r-allergen in a mixture of 136 μL. Six horses were immunized with IC31® adjuvant (250 μL), provided by Valneva Austria GmbH, Vienna and six without (250 μL PBS, instead of IC31®). The horses were assigned randomly into four groups. The veterinarian that immunized and monitored the horses was blinded to the use of adjuvant. Horses nos. 1–3 were immunized intradermally without adjuvant and nos. 4–6 intradermally with IC31®, at four different locations on the neck. Horses nos. 7–9 were immunized in the submandibular lymph nodes without IC31® and nos. 10–12 intralymphatically with IC31®.

Blood was collected by jugular venipuncture at week 0, every other week for 15 weeks, and then monthly until week 30. Serum was stored at −20 °C until used. EDTA blood was taken before, 24 h and 48 h after each vaccination. Differential count of leukocytes was carried out within 4 h from bleeding. Body temperature and site reaction, *i.e.* swelling of lymph nodes estimated by palpation and skin reaction measured with vernier caliper, was recorded before, immediately after, 1 h, 4 h, 24 h and 48 h after each vaccination.

2.3. Serological tests

2.3.1. Immunoblot

Immunoblots with recombinant proteins. The allergens, rCul n 1, rCul n 2, rCul n 9 (1 μg) and rCul n 5 (2 μg) were separated by SDS-PAGE under denaturing conditions and transferred to PVDF transfer membrane (Millipore). After blocking with TBS-T (Tris buffered saline containing 0.1% Tween 20) containing extra 2% Tween 20, the membranes were cut into strips and incubated overnight (o.n.) at 4 °C on a rotator with sera diluted 1:2000 in TBS-T. The following day, the strips were washed a few times with TBS-T before incubation with rabbit anti-horse IgG-AP conjugate (Sigma) diluted 1:2000 in TBS-T. The strips were rewashed in TBS-T and bound antibodies detected with BCIP/NBT (Roche) diluted 1:50 in alkaline phosphatase buffer (0.1 M Tris–HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). As controls, strips were incubated with TBS-T alone or with protein specific antibodies (Schaffartzik et al., 2011).

Immunoblots with C. nubeculosus salivary gland extract (SGE). SDS-PAGE of *C. nubeculosus* SGE and Western blots were performed as described in Hellberg et al. (2006). Strips all derived from the same blot were incubated with the pre-immune sera and sera taken 2 weeks after the last immunization (week 11), diluted 1:30. Furthermore, for identification of protein bands in SGE corresponding to the recombinant proteins, mouse antibodies specific for the recombinant proteins were also incubated on SGE strips (Schaffartzik et al., 2011).

2.3.2. ELISA

Antibody levels (IgG subclasses and IgE) in sera of immunized horses (weeks 0, 2, 7, 11, 19 and 30) were measured by ELISA. Sera from five unvaccinated horses living in Iceland were added as negative controls and sera from five IBH-affected horses of the Icelandic breed living in Switzerland with high IgE to the recombinant allergens were included on each of the plates as positive controls. All samples were tested in duplicates. In all steps 100 μL were added to each well except for the blocking step and for the phosphatase substrate, where 200 μL/well were added. ELISA plates (Thermo Scientific) were coated with recombinant allergens (rCul n 1, rCul n 2, rCul n 5 or rCul n 9) (Schaffartzik et al., 2010, 2011), 2 μg/mL, diluted in 0.2 M Carbonate–Bicarbonate buffer, pH 9.4 (Thermo scientific) for 2 h at 37 °C. Plates were then washed with 150 mM NaCl (Merck) and 0.05% Tween 20 (Sigma). Non-specific binding sites were blocked with 5% dried milk powder and 5% Tween 20 in PBS (Calbiochem®), for 1 h at 37 °C. The sera (weeks 0, 2, 7, 11, 19, 30) were diluted in blocking buffer, 1:5 for IgE and 1:200 for IgG subclass detection and incubated overnight (o.n.) at 4 °C. After washing, monoclonal antibodies specific for equine IgE (1 μg/mL, clone 3H10 (Wilson et al., 2006)) or for equine IgG1 (1 μg/mL CVS45), IgG1/3 (1 μg/mL clone 159 (Goodman et al., 2012)), IgG4/7 (1 μg/mL; CVS 39), IgG5 (1 μg/mL; clone 416 (Goodman et al., 2012)) IgG6 (1 μg/mL, clone 267 (Goodman et al., 2012)) or IgG(T) (Serotec) were added to the plate and incubated for 2 h at RT on a shaker. After washing alkaline-phosphatase-conjugated goat-anti mouse IgG with minimal cross-reactivity to horse serum proteins (Jackson ImmunoResearch) diluted 1:2000 in blocking buffer was added to each well and incubated for 1½ h at RT on a shaker. After a final wash, the plates were developed with a solution of a 1.5 mg/mL phosphatase substrate (Sigma) in 10% diethanolamine (Fluka), pH 9.8.

Absorbance was measured at 405 nm after 2 h for IgE and 30 min for the IgG subclasses. Analysis of the ELISA results was performed as described in Peeters et al. (2013). Briefly, after blank correction on the optical density (OD_{405}) values the correction factor for each plate were calculated by using the average OD values of the positive control samples (sera from horses living in Switzerland and from two samples of the immunized horses that were included on each plate). Further analyses of the data were performed using the corrected OD_{405} values.

2.4. Competitive inhibition ELISA

The ability of sera from horses vaccinated intralymphatically with IC31® (i.l.+ group) to block IgE-binding of serum from IBH-affected horses to the recombinant allergens was tested.

The ELISA plates were coated and then blocked as described in Section 2.3.2. The sera from the i.l.+ group, week 0 (as negative control) and week 11 were serially twofold diluted from 1:5 to 1:40 and 50 μL/well were added in duplicates to the plates. After 1 h incubation at 37 °C, 50 μl/well of serum from an IBH-affected horse with high IgE to the respective allergens was added at a final dilution of 1:5. Plates were incubated at 4 °C o.n. after washing, anti IgE was added followed by an alkaline-phosphatase-conjugated goat-anti mouse IgG with minimal cross-reactivity to horse serum proteins (Jackson ImmunoResearch) and developed as described in Section 2.3.2. Percentage of inhibition for each dilution of the week 0 and week 11 sera was calculated with the following formula:

$$\%\ \text{Inhibition} = 100 - \left(\frac{\text{OD of IBH serum after inhibition with serum from immunized horse}}{\text{OD of IBH serum}} \times 100\right)$$

2.5. Stimulation of PBMC

Before immunization (week 0) and 2 weeks after the 3rd immunization (week 11), PBMC were isolated by density gradient centrifugation as described by Hamza et al. (2007) using Ficoll-Hypaque procedure. PBMC, 2×10^6 in 2 mL, were cultured with ConA, as positive control (10 μg/mL), or 10 μg/mL of rCul n 1, rCul n 2, rCul n 5 or rCul n 9 or were left unstimulated for 24 h.

All cell cultures were performed in RPMI medium with glutamax (Invitrogen) supplemented with horse serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL), non-essential amino acids (1%), MEM vitamins (100 μM), sodium pyruvate (1 mM), 2-mercaptoethanol (50 μM). After 24 h PBMC were harvested, lysed and homogenized in RA1 lysis buffer and stored at −80 °C until they were processed for total RNA isolation.

2.6. Total RNA isolation, reverse transcription of RNA and QRT-PCR

Total RNA isolation was performed using a commercial kit (Macherey-Nagel) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry (NanoDrop® ND-1000). cDNA synthesis was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Briefly, 0.69–1.84 μg of total RNA and random hexamers were incubated at 65 °C for 5 min and subsequently chilled on ice. Then, 5× reaction buffer, dNTPs, RiboLock RNase inhibitor and RevertAid reverse transcriptase were added and incubated for 5 min at 25 °C and 50 min at 42 °C. Reaction was stopped by incubating for 5 min at 70 °C.

cDNA was stored at −80 °C until used for subsequent quantification of mRNA coding for IFN-γ, IL-4, IL-10 and FoxP3 using TaqMan procedure and the same primers and probes as in Heimann et al. (2011). All samples were run blind by the operators for qRT-PCR in order to exclude bias by the operator. Duplicate cDNA samples were amplified for 45 cycles (15 s at 95 °C and 60 s at 60 °C) in an AB7300 real-time PCR instrument (Applied Biosystems, CA, USA) using Maxima Probe qPCR Mastermix (Fermentas). Cycle threshold (Ct) values of cytokine gene expression were normalized to the 18s rRNA content (ΔCT) as described in Lanz et al. (2013). No significant difference was detected in any of the measured parameters in the un-stimulated cells between the groups of horses. Therefore, the number of transcript copies in each sample was calculated in relation to un-stimulated cells according to ($2^{-\Delta\Delta CT}$) Method. The values obtained after immunization (week 11) were expressed in relation to the values obtained before immunization, *i.e.* week 11/week 0 and named relative IFN-γ, IL-4, IL-10 or FoxP3 mRNA expression.

2.7. Sulfidoleukotriene release test

Before vaccination and six weeks after the last vaccination, a sulfidoleukotriene (sLT) release test was performed according to Baselgia et al. (2006). In short, ACD-B anticoagulated blood samples (Vacuette) were mixed, and 20 min later, after sedimentation of the erythrocytes, the leukocyte-rich plasma was collected, transferred into a propylene tube and centrifuged at 130 × *g* for 10 min at RT. The supernatant containing most of the platelets was discarded and the pellet resuspended in 3 mL of stimulation buffer (Bühlmann Laboratories AG). Resuspended leukocytes were incubated at 37 °C with *C. nubeculosus* extract (2 and 6 μg/mL) prepared from laboratory-bred *C. nubeculosus* as described by Marti et al., 1999 (Marti et al., 1999) or concanavalin A (ConA, 20 μg/mL) used as a positive control or with buffer alone to determine spontaneous sLT release. After 40 min incubation, plates were centrifuged at 1000 × *g* at 4 °C and the supernatant transferred into a new 96-well microtiter tissue culture plate and kept at −20 °C until assayed. sLT determination was carried out using the CAST 2000-ELISA following the manufacturer's instruction (Bühlmann Laboratories AG). For all further evaluation

values of the net stimulation were used, *i.e.* the spontaneous sLT release was subtracted from the values obtained with ConA or with *C. nubeculosus* extract, as performed previously (Baselgia et al., 2006). The cut offs defined in a previous study (Baselgia et al., 2006) were used to define a positive test result.

2.8. Intradermal test

Intradermal tests were performed six months after the first vaccination on vaccinated horses and on three healthy control horses, as described in Schaffartzik et al. (2011). The recombinant allergens (rCul n 1, 2, 5 and 9) were diluted to a concentration of 10 μg/mL and 1 μg/mL in dH_2O or PBS depending on the solubility of the protein. The skin test area, in the lateral neck (opposite site to the vaccination) was clipped and 100 μL of the recombinant allergens, of dH_2O and PBS used as negative controls, and of 0.2 mg/mL histamine diluted in dH_2O or PBS as positive control, were injected intradermally. Diameters of the wheal reaction at the injection sites were measured after 30 min.

2.9. Statistical analyses

The statistical analyses on the IgG subclass and cytokine data were performed using the statistical software program NCSS 8 (NCSS Statistical Software, 329 North 1000 East, Kaysville, UT 84037, USA). Descriptive statistics were run on the IgG subclasses and IgE ELISA results. Shapiro Wilk test showed that after log transformation of the data, IgG(T) levels were normally distributed (Shapiro Wilk $W \geq 0.99$, $p > 0.05$). According to the Shapiro Wilk W test, the log-transformed IgG1, IgG1/3, IgG4/7 and IgG5 antibody levels were strictly taken still not normally distributed (Shapiro Wilk $W \geq 0.96$, $p < 0.01$). However, because there was no severe violation of normality according to the visual evaluation of the normality plots, and because the repeated measures ANOVA is robust to minor violations of multivariate normality (http://www.ats.ucla.edu/stat/sas/library/repeated_ut.htm), repeated measures ANOVA was performed for determination of the effects of the administration route (intradermal *versus* intralymphatic) of the adjuvant (with or without IC31®) on antibody levels in function of time (week 0 to week 30) and of the 4 r-allergens (rCul n 1, 2, 5 and 9) used for immunization on these variables. Separate models were run with levels of IgG1, IgG1/3, IgG4/7 and IgG5 as outcome variables, respectively. Interaction terms of significant variables were also included in the models. Mauchly's test was used to check for the assumption of circularity of within-subject covariance matrices. Because this assumption was not always fulfilled, the Geisser–Greenhouse correction was used for the within-subject F test. $p \leq 0.05$ was considered significant. For significant factors in any of the repeated measures ANOVA models, significant subgroup differences were identified with a pairwise multiple comparison test with a Bonferroni correction of the significant level.

IgG6 and IgE values could not be analyzed with the repeated measures ANOVA because of severe violation of normality, even after log-transformation (Shapiro Wilk $W < 0.91$, $p < 1 \times 10^{-10}$).

As even after log transformation the cytokine data were not normally distributed, the non-parametric ANOVA Kruskal–Wallis multiple-Comparison Z-Value Test was used to compare cytokine (IFN-γ, IL-4, IL-10) and FoxP3 mRNA expression by stimulated PBMC from horses of the four immunization groups (immunized in lymph node with [i.l.+] or without [i.l.−] or intradermally with [i.d.+] or without [i.d.−] IC31®). The Bonferroni test was used to correct for multiple comparisons. On each occasion $p \leq 0.05$ was considered significant.

A linear mixed effect model was used to compare the mean diameter (from the four different injection locations) of skin reaction in RStudio (version 0.98.1091, http://www.rstudio.com/). The model used adjuvant, time and vaccination as categorical variables and horse as a random factor. The horses received the allergens with or without adjuvant, the skin reaction was measured at five different time points post injection and the horses were vaccinated three times.

3. Results

3.1. Clinical examination

The immunization was well tolerated, no rise in temperature, local swelling or soreness up on palpation of submandibular lymph nodes was observed. Diameter of skin reaction was measured at five different time points (0, 1, 4, 24 and 48 h) post injection of allergens alone or with IC31®. Four hours after injection the reaction was significantly greater in the horses that received the allergens in IC31® ($p = 0.002$) but significant difference was not observed at other time points (data not shown). Leukocyte count was within normal range following vaccinations (data not shown).

3.2. Specific antibody response of vaccinated horses

3.2.1. Total IgG response

The total IgG response two weeks after the third vaccination was measured by immunoblot of the allergens used for vaccination. Horses that were vaccinated with the recombinant allergens in IC31® responded much more strongly than those vaccinated with allergens alone. This was seen regardless of whether they were injected intradermally or intralymphatically. There was no clear difference between the two injection routes (supplementary data, Table S1 and Figure S1).

Supplementary table and figure related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.05.002.

3.2.2. IgG subclass response

Sera from vaccinated horses, taken before, two weeks after each of the three vaccinations and after the skin test were tested for IgG subclass responses against rCul n 1, rCul n 2, rCul n 5 and rCul n 9 in ELISA.

The repeated measures ANOVA showed that immunization with 10 μg of each of the four r-allergens resulted

in a highly significant increase in antibody levels (Fig. 1), although the time course and degree of the increase varied depending on the IgG subclass and the specific r-allergen (Table 1 and Fig. 1). For IgG1 and IgG1/3 subclasses, a significant increase was already observed two weeks after the 1st immunization. These antibody levels increased further after the 2nd and 3rd immunization, and then decreased until week 19, where they were significantly lower than at week 7 and 11, *i.e.* two weeks after the 2nd and 3rd immunization, respectively (Table 1). The intradermal test performed at week 28 boosted the antibody response. At week 30, the IgG1 and IgG1/3 response reached similar levels as at week 7 and 11, except for Cul n 9. The repeated measures ANOVA further revealed that use of IC31® resulted in significantly higher IgG1 and IgG1/3 levels, while there was only a tendency for intralymphatic compared to intradermal immunization to induce a higher IgG1/3 response ($p = 0.07$). Although an increase in IgG1 and IgG1/3 levels was observed for all four r-allergens, the antibody responses to rCul n 9 and rCul n 5 were significantly higher than to rCul n 1(IgG1, IgG1/3) and rCul n 2 (IgG1/3).

For IgG4/7, the results were similar but not as clear as for the two subclasses described above. Even before immunization relatively high (mean OD value 0.68–1.48) values were obtained by ELISA with the four rCul n allergens. Immunization nonetheless induced an increase in IgG4/7 levels, but conversely to IgG1 and IgG1/3, this increase was only statistically significant at week 7, *i.e.* two weeks following the 2nd immunization. As for IgG1 and IgG1/3, IgG4/7 levels also decreased until week 19 and then increased following the IDT (Fig. 1), but these variations were not as clear as for IgG1 and IgG1/3 and did not reach statistical significance. Immunization with adjuvant again resulted in a higher, although not significant ($p = 0.07$), antibody response; nor did the immunization route influence the IgG4/7 response significantly. The different r-allergens also induced different IgG4/7 levels ($p = 0.052$), with again rCul n 5 and rCul n 9 resulting in higher titers than rCul n 1 and Cul n 2.

The measured IgG5 and IgG(T) levels were lower than the IgG1, IgG1/3 and IgG4/7 levels, but a significant increase following the 2nd immunization (week 7) could be observed and the increase and decrease of IgG5 and IgG(T)levels followed a similar pattern as the other IgG subclasses. However, for IgG5 and IgG(T) no significant effect of adjuvant or administration route could be observed in the repeated measures ANOVA, although intralymphatic immunization with IC31® led to the highest IgG5 and IgG(T) responses (Fig. 1). The strongest IgG5 response was seen against rCul n 9, which was significantly higher than against the three other r-allergens. For IgG(T), rCul n 5 and rCul n 9 induced the highest response (Table 1).

IgG6 antibody levels were very low and no increase could be observed following immunization (data not shown).

3.2.3. IgE response

Sera from vaccinated horses taken before, two weeks after each of the three vaccinations, and after the skin test, were tested for IgE response against rCul n 1, rCul n 2, rCul n 5 and rCul n 9 by ELISA. No IgE production could be detected at any time point (supplementary data, Figure S2). The OD values of IBH affected horses, used as positive controls, were between 2 and 2.8.

Supplementary figure related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.05.002.

3.3. Inhibition of allergen-specific IgE-binding by sera from horses vaccinated intralymphatically with IC31®

The sera from the horse group with the strongest IgG responses, *i.e.* those vaccinated intralymphatically with the allergens in IC31® were tested for their IgE blocking capacity. While preimmune sera led to no or only marginal inhibition of IgE-binding, addition of the postimmune sera resulted in a mean inhibition of 54.4–18.4%, depending on the allergen tested (Fig. 2).

3.4. Binding of allergen-specific IgG antibodies from vaccinated horses to C. nubeculosus salivary gland extract

Sera taken before immunization and two weeks after the last vaccination were tested on salivary gland extract from *C. nubeculosus* by immunoblotting. IgG1 from vaccinated horses bound to proteins of the correct size, namely Cul n 1 and Cul n 9, as shown by comparison with the binding of mouse antibodies specific for the corresponding r-proteins (Schaffartzik et al., 2011). Binding to Cul n 2 and Cul n 5 cannot be distinguished because of the similar size of these two protein bands. The immunoblot also showed that IgG binding of post-vaccination sera to Cul n 1 and Cul n 9 was usually stronger in the IC31® vaccinated group compared to horses vaccinated without IC31®. This difference was especially clear for the Cul n 1 protein (Fig. 3).

3.5. Cytokine and FoxP3 mRNA expression of the vaccinated horses

The relative cytokine expression by stimulated PBMC was compared between the four immunization groups. Testing of the results for the single r-allergens or stimulant showed no significant differences in the One-Way ANOVA for any of the cytokines or FoxP3. As this was probably due to the small number of horses per group, the data of the 4 r-allergens was then pooled to enhance the power of the analyses (Fig. 4). No significant differences between the immunization groups could be found for relative IFN-γ and FoxP3, while IL-4 expression was significantly higher in the i.l.– group compared to the i.d.+ group. Interestingly, horses immunized with IC31® either intralymphatically or intradermally had a significantly higher IL-10 expression than those immunized intradermally without IC31® (i.d.–). IL-10 expression in the i.l.+ group was also higher than in the i.l.– group, but the difference did not reach statistical significance (Fig. 4).

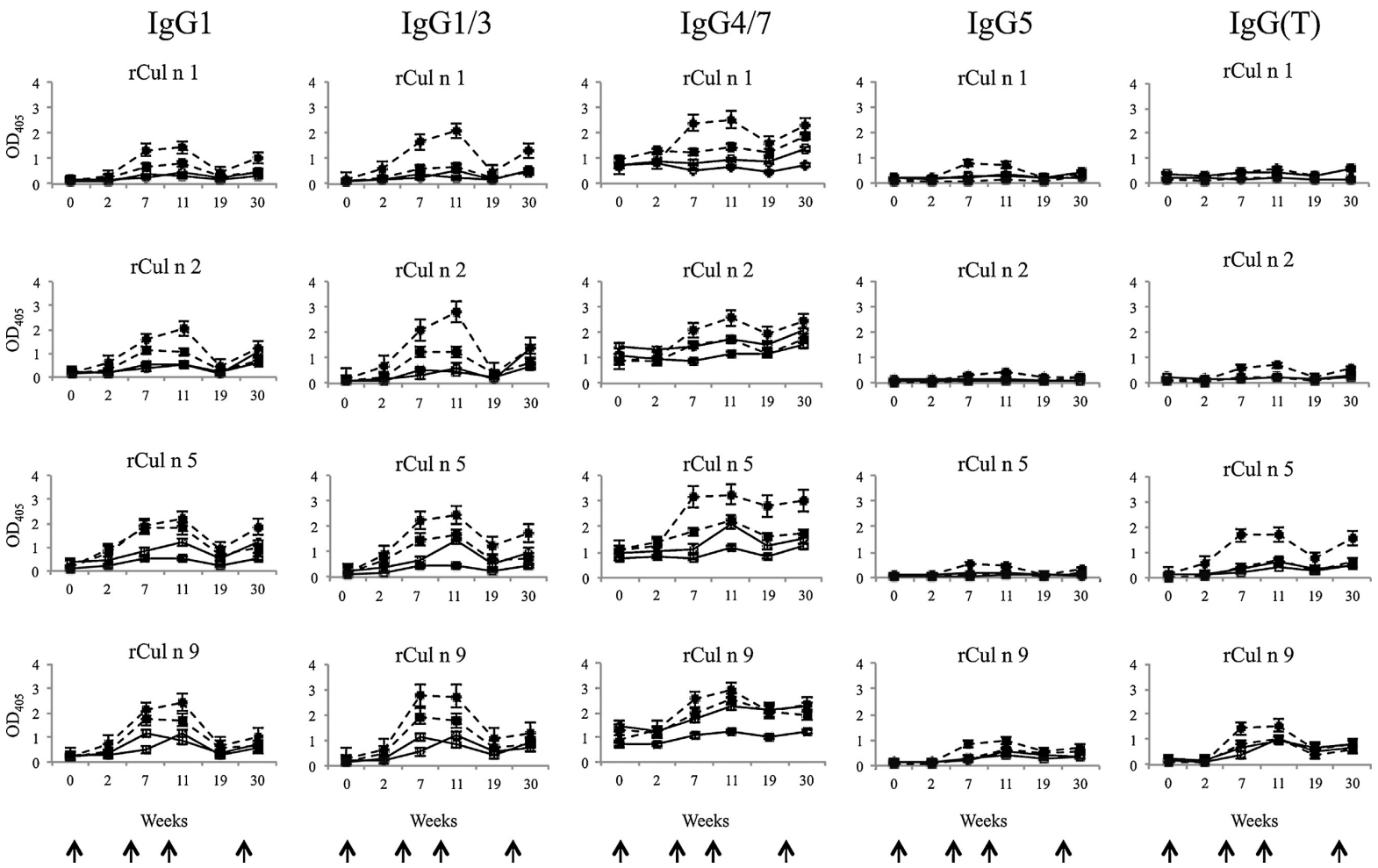

Fig. 1. Time course of IgG subclass response measured by ELISA. Corrected OD mean values and standard error for the three horses in each group at different time points. Intradermal injection without adjuvant (—□—), intradermal injection with IC31® adjuvant (- - ■ - -), intralymphatic injection without adjuvant (—○—), intralymphatic injection with IC31® adjuvant (- -●- -). The first three arrows indicate the vaccination time points and the last the intradermal test.

Table 1
Factors influencing the IgG subclass responses following immunization of horses with recombinant *Culicoides nubeculosus* (Cul n) allergens. Results of the repeated measures ANOVAs.

	log IgG1		log IgG1/3		log IgG4/7		log IgG5		log IgG(T)	
	Mean (SE)	*p* value	Mean (SE)	*p* value	Mean (SE)	*p* value	Mean (SE)	*p* value	Mean (SE)	*p* value
Adjuvant (IC31®)		$p \leq 0.01$		$p \leq 0.01$		$p = 0.07$		ns		ns
No	2.49 (0.06)		2.47 (0.06)		2.99 (0.06)		2.00 (0.13)		2.38 (0.08)	
Yes	2.79 (0.06)		2.81 (0.06)		3.17 (0.06)		2.11 (0.13)		2.42 (0.08)	
Localization		$p = 0.17$		$p = 0.07$		ns		ns		ns
Intradermal	2.57 (0.06)		2.55 (0.06)		3.03 (0.06)		1.94 (0.13)		2.31 (0.08)	
Intralymphatic	2.70 (0.06)		2.74(0.06)		3.12 (0.06)		2.17 (0.13)		2.48 (0.08)	
Allergen		$p \leq 0.001$		$p \leq 0.0001$		$p = 0.052$		$p \leq 0.001$		$p \leq 0.01$
Cul n 1	2.47 (0.04)	[a]Cul n 5, Cul n 9	2.50 (0.04)	[a]Cul n 5, Cul n 9	2.93 (0.06)		2.06 (0.08)	[a]Cul n 9	2.37 (0.05)	
Cul n 2	2.60 (0.04)		2.55 (0.04)	Cul n 5, Cul n 9	3.10 (0.06)		1.94 (0.08)	Cul n 9	2.22 (0.05)	Cul n 5, Cul n 9
Cul n 5	2.75 (0.04)	Cul n 1	2.74 (0.04)	Cul n 1, Cul n 2	3.14 (0.06)		1.86 (0.08)	Cul n 9	2.47 (0.05)	Cul n 2
Cul n 9	2.73 (0.04)	Cul n 1	2.78 (0.04)	Cul n 1, Cul n 2	3.15 (0.06)		2.37 (0.08)	Cul n 1, 2, 5	2.53 (0.05)	Cul n 2
Week after 1st immunization		$p \leq 0.00001$		$p \leq 0.000001$		$p \leq 0.000001$		$p \leq 0.000001$		$p \leq 0.000001$
W0	2.19 (0.04)	[a]W2-30	2.09 (0.04)	[a]W2-30	2.88 (0.03)	[a]W7, 11, 19, 30	1.70 (0.07)	[a]W7, 11, 19, 30	2.06 (0.05)	W7, 11, 19, 30
W2	2.46 (0.04)	W0, 7, 11,30	2.44 (0.04)	W0, 7, 11, 30	2.95 (0.03)	W7, 11, 19, 30	1.74 (0.07)	W7, 11, 19, 30	2.04 (0.05)	W7, 11, 19, 30
W7	2.87 (0.04)	W0, 2, 19	2.89 (0.04)	W0, 2, 19	3.11 (0.03)	W0, 2	2.15 (0.07)	W0, 2	2.54 (0.05)	W0, 2
W11	2.94 (0.04)	W0, 2, 19	2.97 (0.04)	W0, 2, 19	3.22 (0.03)	W0, 2	2.38 (0.07)	W0, 2	2.71 (0.05)	W0, 2, 19
W19	2.51 (0.04)	W0, 7, 11,30	2.55 (0.04)	W0, 7, 11, 30	3.11 (0.03)	W0, 2	2.14 (0.07)	W0, 2	2.43 (0.05)	W0, 2, 11
W30	2.85 (0.04)	W0, 2, 19	2.91 (0.04)	W0, 2, 19	3.21 (0.03)	W0, 2	2.23 (0.07)	W0, 2	2.62 (0.05)	W0, 2
Interactions										
Adjuvant*week		≤0.001		≤0.001		≤0.05		≤0.05		≤0.0001
Allergen*week		≤0.00001		≤0.000001		0.07		≤0.0001		≤0.00001
Localization*week		ns		≤0.05						
Localization*week*allergen										

[a] Significantly different from . . . in Bonferroni corrected (all-pairwise) multiple comparison test.

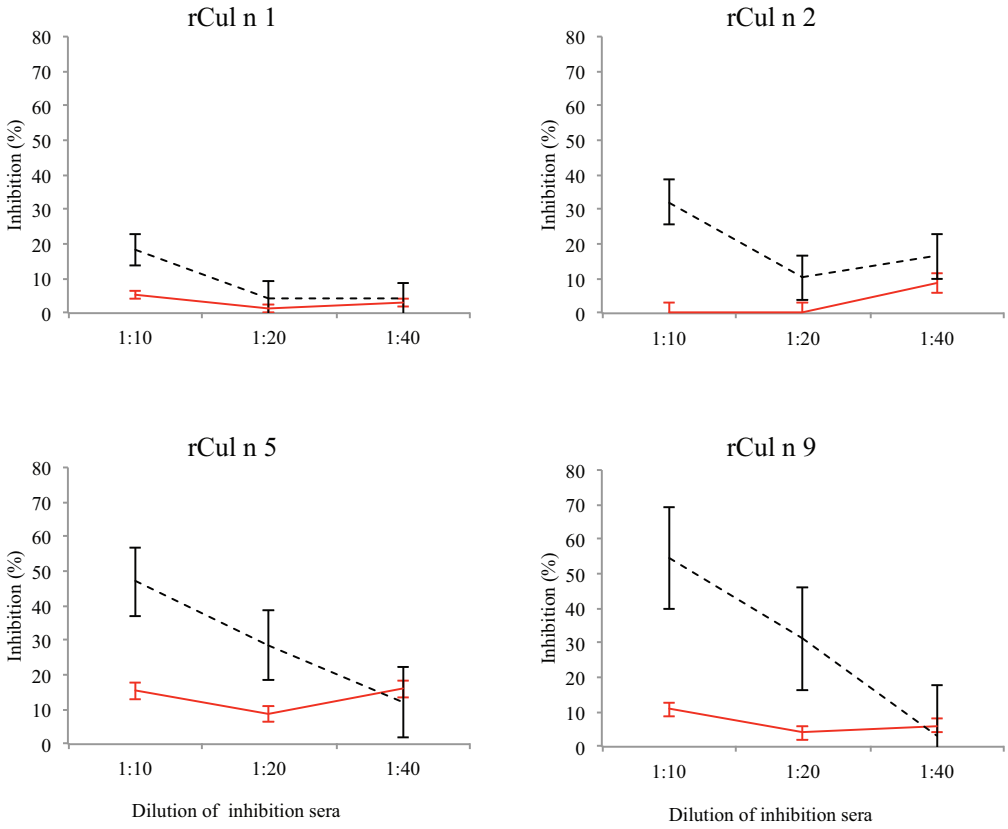

Fig. 2. Inhibition of IgE binding to allergens with sera from i.l.+IC31® vaccinated horses. Binding of IgE from serum of an IBH positive horse to rCul n 1, rCul n 2, rCul n 5, rCul n 9 was inhibited by postimmune sera (week 11) of i.l.+IC31® vaccinated horses. Mean % inhibition by pre- (whole red line) and post- (broken black line) vaccination sera from horses nos. 10–12, diluted 1:10–1:40 applied to an ELISA plate coated with allergens, prior to adding serum from an IBH positive horse at a 1:5 dilution.

3.6. In vitro sulfidoleukotriene release following C. nubeculosus extract stimulation of leukocytes

Sensitization to the recombinant allergens was investigated with *in vitro* sulfidoleukotriene release test prior to vaccination and six weeks after the 3rd vaccination. Sulfidoleukotriene production was detected after stimulation of PBL from all horses with Con A, used as positive control, but not after stimulation with the four recombinant allergens independent

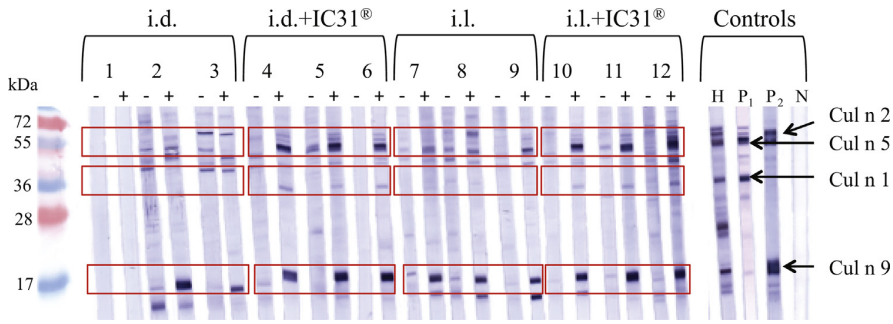

Fig. 3. Binding of IgG1 serum antibodies from vaccinated horses (1–12) to the relevant allergens (Cul n 1, Cul n 2, Cul n 5, Cul n 9) in *C. nubeculosus* salivary gland extract (SGE) separated by SDS-PAGE. PVDF membrane strips incubated with pairs of serum (1/30 dilution), week 0 (−), two weeks after the third vaccination (+). The control strips incubated with; serum (1/5 dilution) from IBH-affected horse for detection of IgE (H), specific polyclonal mouse antibodies (1/500 dilution) against Cul n 1 and Cul n 5 (P_1), Cul n 2 and Cul n 9 (P_2), TBS-T as negative control (N). Horses vaccinated: 1–3 intradermally without adjuvant (i.d.), 4–6 intradermally with IC31® (i.d.+IC31®), 7–9 intralymphatically without adjuvant (i.l.), 10–12 intralymphatically with IC31® (i.l.+IC31®).

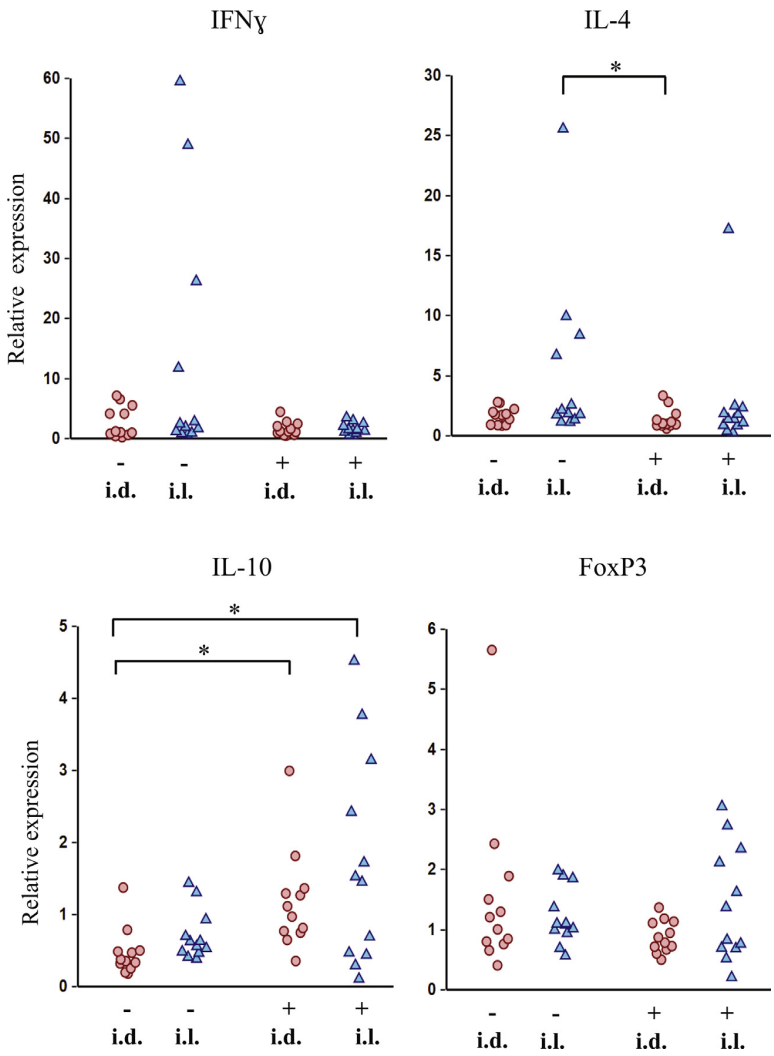

Fig. 4. Relative cytokine and FoxP3 expression after *in vitro* stimulation of PBMC. Expression levels were assessed by quantitative RT-PCR and normalized to 18S rRNA using the ddCt method. Comparison between vaccination groups was performed with One-Way ANOVA ($^*p \leq 0.05$).

of injection site and injection material (data not shown).

3.7. Intradermal test (IDT)

The ability of the recombinant allergens to induce type I hypersensitivity reactions in the vaccinated horses and three healthy controls was tested *in vivo* by IDT. All horses responded strongly to histamine but did not react to any of the allergens 30 min post-injection (data not shown).

4. Discussion

IBH is a severe problem in Icelandic horses exported to the European continent, but does not occur in horses living in Iceland, because the causative agent of this allergy, *Culicoides* spp., are not endemic in Iceland (Schaffartzik et al., 2012). This provides an opportunity for an experimental setup for developing a preventive vaccination strategy against IBH. Horses could be vaccinated in Iceland, where they are not exposed to the allergens, and then transported to highly infested *Culicoides* areas where at least 50% of them would be expected to develop IBH (Bjornsdottir et al., 2006).

Allergen specific immunotherapy using subcutaneous injections has been practiced in humans for treatment of allergies for over 100 years (Fitzhugh and Lockey, 2011). This has also been tried in IBH-affected horses using whole body extract of *Culicoides*, with contradictory results (Anderson et al., 1996; Barbet et al., 1990; Ginel et al., 2014). Whole body *Culicoides* extract can be expected to contain various substances not relevant for IBH, and therefore it is

difficult to determine the dosage and frequency of injections needed to standardize the treatment. Today many of the causative allergens of IBH have been identified and produced as recombinant proteins. This provides the opportunity to develop an allergen specific immunotherapy against IBH (Langner et al., 2009; Schaffartzik et al., 2011; van der Meide et al., 2013).

Immunotherapy as presently practiced, using subcutaneous injection of increasing doses of allergen over a very long period of time (years), has low compliance in humans and probably also in horses. A more practical and efficient immunotherapy is thus required.

In the present study we have tested two injection (intralymphatic and intradermal) methods to raise an immune response that could protect against IBH. The intralymphatic route has been shown in humans to need shorter time, less protein and fewer injections, and was therefore an obvious choice (Senti et al., 2009). We have compared the intralymphatic to the intradermal injection route. Epicutaneous immunization has been shown to be more effective than subcutaneous or intramuscular applications and required 5 to 10-fold less antigen (Briggs et al., 2000; Kenney et al., 2004; Redfield et al., 1985).

We used four allergens originating from the salivary glands of *C. nubeculosus*, Cul n 1 (antigen 5-like protein), Cul n 2 (hyaluronidase), Cul n 5 (unknown protein) and Cul n 9 (D7-related protein). Three (Cul n 1, 2 and 9) of the allergens are known salivary proteins from other insects. Antigen 5-like protein is a major allergen in vespid venoms (King and Spangfort, 2000) and hyaluronidase in bee venom (Soldatova et al., 1998). D7 related proteins that belong to the superfamily of pheromone/odorant binding proteins are widespread in blood-sucking Diptera and are immunogenic in humans (Calvo et al., 2006; Doucoure et al., 2013; Valenzuela et al., 2002). The recombinant proteins were produced and purified from *E. coli* (Schaffartzik et al., 2010, 2011). They were injected either alone or with IC31® adjuvant that induces a Th1 type response in mice (Olafsdottir et al., 2012; Pattacini et al., 2012) and in humans when used with *Mycobacterium tuberculosis* antigens (van Dissel et al., 2010).

In our study, the administration of 3 doses of only 10 μg of each recombinant allergen in combination with IC31® gave a strong IgG antibody response with both injection routes. The intralymphatic application resulted in a somewhat stronger antibody production than intradermal injection, detected both by WB (Figure S1) and ELISA (Fig. 1). This IgG response was without induction of IgE antibodies and consequently also no sensitization of the horses as demonstrated with an *in vitro* sLT release assay with *Culicoides* extract and by intradermal tests. Ideally, an IDT and sLT release assay should be performed at the same time on a horse allergic to *Culicoides* proteins as positive controls. However, as horses in Iceland are not exposed to bites of *Culicoides* and are not sensitized to these allergens, this could not be done. Nevertheless, IDT and sLT release assays have been and are performed with the same proteins by our group in Switzerland (Baselgia et al., 2006; Schaffartzik et al., 2011).

The antibodies generated after vaccination bound not only to the r-proteins (Figure S1), but also to the respective proteins extracted from the salivary glands of *C. nubeculosus*. This was more pronounced in horses that received the r-allergens in IC31® (Fig. 3).

Horses have seven IgG subclasses (Wagner et al., 2004) and antibodies have been raised against all of them (Keggan et al., 2013). The IgG subclasses differ in function, with IgG1, IgG3, IgG4 and IgG7 being the equine immunoglobulins that bind complement (Lewis et al., 2008) and are important in protection against viral and bacterial infections (Goodman et al., 2012; Lopez et al., 2002; Nelson et al., 1998; Sheoran et al., 1997; Soboll et al., 2003; Svansson et al., 2009). They are therefore the IgG subclasses which it is vital to raise in Th1-mediated vaccinations. The antibody response measured with IgG(T) specific antibody, which binds to both IgG3 and IgG5, is on the other hand not associated with protective immunity in viral infections (Goehring et al., 2010; Goodman et al., 2012; Soboll Hussey et al., 2011), but has been linked to sensitization (Wagner, 2006). This suggests that the proposed association of IgG(T) with allergic conditions is more due to the IgG5 than IgG3 subclass detected by the anti IgG(T) antibodies.

The antibody response data are presented without subtracting the pre-vaccination values to show the background response that is especially pronounced for IgG4/7. This is true for the twelve vaccinated horses and the five controls, but slightly lower for rCul n 1 than the other three (Fig. 1.) The IgG4/7 antibodies represent the most prevalent isotype in horse serum and are important for secondary immune responses and in latent infections, whereas IgG1 is mainly produced in primary infections and is rather short lived (Goodman et al., 2012; Svansson et al., 2009; Wagner, 2006). The only biting insects known to bite mammals in Iceland are *Simulium vittatum* (Johannsson, 1988). However, it has been shown that the antigen 5-like proteins from *S. vittatum* (Sim v 1) and *C. nubeculosus* (Cul n 1) share IgE-binding epitopes (Schaffartzik et al., 2010) and 50% of IBH-affected Icelandic horses also react to salivary gland extracts of *S. vittatum* (Baselgia et al., 2006). Therefore the response of the untreated horses could be due to IgG4/7 cross-reactivity to the salivary gland proteins from *S. vittatum*.

A repeated ANOVA was performed because it allowed analysis of the effects of the different factors on antibody levels as function of time, and also enhanced the power of the analysis. We are aware that the number of horses per group in this pilot study was very small, but statistically significant differences are probably relevant. This analysis showed that for all tested IgG subclasses there was a significant increase in antibody levels following immunization. The IgG1 and IgG1/3 response occurred very early, as antibody levels were already significantly higher than in the preimmune sera two weeks after the 1st immunization but they also decreased quite rapidly. This is in accordance with previous studies (Goodman et al., 2012; Svansson et al., 2009; Wagner, 2006). Immunization with the r-allergens also induced antibodies of the IgG4/7 and IgG5 but a significant increase in these IgG subclasses was only seen two weeks after the 2nd immunization.

The repeated measure ANOVA also showed that the use of IC31® lead to a higher antibody production. The increased response was mainly seen for IgG1 and IgG1/3

and was statistically significant. IgG4/7, IgG5 and IgG(T) levels were also higher in the horses immunized with IC31® but the difference did not reach statistical significance.

Antibodies were induced against all four r-allergens, at least in the horses immunized with IC31®. However, there were significant differences in the level of the responses between the r-allergens. Overall, rCul n 5 and rCul n 9 induced a better antibody response than rCul n 1 and rCul n 2 (Table 1). The antibodies generated against rCul n 5 and rCul n 9 also displayed the best IgE-blocking activity (Fig. 2). Interestingly, Figs. 1 and 3 suggest that the effect of IC31® was stronger for the weaker antigens. Repeated immunization without IC31® resulted in an increase in Cul n 9 and Cul n 5 specific antibodies, but hardly any increase in Cul n 1 and Cul n 2 specific antibodies. This is particularly well illustrated for Cul n 1 in Fig. 3. After the 3rd immunization IgG antibodies specific for the 35-kDa protein band corresponding to Cul n 1 can only be detected in the sera of the horses immunized with IC31®. This is independent of the immunization route. Use of IC31® seems especially important to improve the antibody response against weak antigens. Table 1 and Fig. 1 also suggest that the time course of the antibody response is influenced by IC31®, as shown by significant interaction between the adjuvant and the week (Table 1). Fig. 1 shows that the peak of the antibody response is nearly reached after the 2nd immunization with IC31® while the response without IC31® is usually still low after 2nd immunization and only increases, if at all, after the 3rd vaccination. Finally, intralymphatic immunization leads to a higher antibody response than intradermal immunization. This difference was weaker than the effect of IC31® and did not reach the level of significance, probably because of the small number of horses included. An interesting observation is that the intradermal test boosted the antibody response (Fig. 1 and Table 1).

Due to the small group sizes, the cytokine expression data from the PBMC stimulation with the four r-allergens was pooled for statistical analysis. No difference between the groups was seen in IFN-γ and FoxP3 expression. IL-4 expression was significantly higher in horses vaccinated intralymphatically without adjuvant as compared to those that received the allergens in IC31® intradermally. Most interestingly, horses that were vaccinated with the adjuvant using either route had higher IL-10 expression than those immunized intradermally without IC31® (Fig. 4). Although this indicates that vaccination with IC31® favors Th1 and T regulatory responses, a larger group of horses is needed in order to obtain more significant results. In addition, purified r-allergens produced in *E. coli* tended to give background in stimulations of PBMC taken from both pre-vaccination and control horses. Allergens produced in insect cells should be evaluated and also an earlier time point for stimulation than two weeks after vaccination.

Effective immunotherapy in humans elicits an increase in IL-10 production leading to an increase in allergen-specific IgG4 and often a decrease in IgE. The IgG4 antibodies then block IgE binding to allergens, inhibit IgE facilitated allergen presentation to T cells and allergen-induced boost of memory IgE production (Cavkaytar et al., 2014; Focke-Tejkl et al., 2014; Larche et al., 2006; Ozdemir et al., 2011). We tested the sera from the horse group with the strongest IgG response, *i.e.* those vaccinated intralymphatically with the allergens in IC31®, for IgE blocking capacity. Indeed these sera could inhibit the binding of IgE from an IBH horse (Fig. 2).

In summary, vaccination of horses previously not exposed to the allergens, both intralymphatically and intradermally with r-allergens in IC31® adjuvant, was well-tolerated and induced immune responses without IgE production. The horses produced IgG that could inhibit r-allergen-specific IgE-binding. Furthermore, the use of IC31® led to increased production of the regulatory cytokine IL-10. We therefore conclude that both injection routes and the IC31® adjuvant are strong candidates for further development of immunotherapy in horses.

Conflicts of interest

Andreas Meinke is an employee of Valneva Austria GmbH.

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Short communication

A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A

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ABSTRACT

Insect bite hypersensitivity (IBH) is an IgE-mediated dermatitis of horses caused by bites of *Culicoides* insects, not indigenous to Iceland. Horses born in Iceland and exported to *Culicoides*-rich areas are frequently affected with IBH.

The aims of the study were to compare immunization with recombinant allergens using the adjuvant aluminum hydroxide (Alum) alone or combined with monophosphoryl lipid A (MPLA) for development of a preventive immunization against IBH.

Twelve healthy Icelandic horses were vaccinated intralymphatically three times with 10 μg each of four recombinant *Culicoides nubeculosus* allergens in Alum or in Alum/MPLA.

Injection with allergens in both Alum and Alum/MPLA resulted in significant increase in specific IgG subclasses and IgA against all r-allergens with no significant differences between the adjuvant groups. The induced antibodies from both groups could block binding of allergen specific IgE from IBH affected horses to a similar extent. No IgE-mediated reactions were induced. Allergen-stimulated PBMC from Alum/MPLA horses but not from Alum only horses produced significantly more IFNγ and IL-10 than PBMC from non-vaccinated control horses.

In conclusion, intralymphatic administration of small amounts of pure allergens in Alum/MPLA induces high IgG antibody levels and Th1/Treg immune response and is a promising approach for immunoprophylaxis and immunotherapy against IBH.

1. Introduction

Equine insect bite hypersensitivity (IBH), or summer eczema, is an allergic recurrent seasonal dermatitis of horses. It is caused by bites of insects mainly of the genus *Culicoides* (biting midges) (for review Schaffartzik et al., 2012) and characterized by papules and intense pruritus affecting the feeding sites of the midges (Bröstrom et al., 1987). IBH affects all horse breeds and is found almost worldwide with the exception of places where *Culicoides* species are not indigenous, as in Iceland (Illies, 1978). However, IBH is a severe problem in Icelandic horses exported from Iceland to the European continent. Over 50% of these horses develop the disease two years or more after importing into heavily *Culicoides* infested areas (Bjornsdottir et al., 2006), while Icelandic horses foaled in Europe develop the disease with a similar prevalence as most other horse breeds (Bröstrom et al., 1987). Presently, there is no satisfactory treatment of IBH (Schaffartzik et al., 2012), and thus preventive immunization may be an attractive option for horses exported from Iceland to countries where *Culicoides* are present. In humans, prophylactic immunotherapy to prevent allergic sensitization is not yet practiced but has been considered (Valenta et al., 2012). The allergen extracts mostly used in allergen immunotherapy are not suited as they risk inducing sensitization to other proteins in the mixture. For prophylactic treatment it is essential to use well-defined pure allergens and to treat before sensitization occurs (Valenta et al., 2012). IBH in Icelandic horses is thus an interesting model for development and study of prophylactic immunotherapy

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as Iceland is free of the causative agent and the horses are only sensitized after export. Furthermore, *Culicoides* allergens have been identified and characterized at the molecular level and are available as pure recombinant proteins (Mueller et al., 2016).

According to our studies on the immune response and pathogenesis of IBH, Icelandic horses exported from Iceland to Europe develop a stronger Th2 polarized immune response than their disease status matched counterparts born in Switzerland (Hamza et al., 2007). Further studies demonstrated an imbalance between the Th2/Treg immune response in IBH, using both *Culicoides* stimulated PBMC and skin biopsies (Hamza et al., 2008, 2013; Heimann et al., 2011). These findings indicate that a preventive immunization against IBH should aim at inducing an allergen-specific Treg and/or Th1 immune response.

Due to the high cost of production and purification of recombinant proteins and the risk of side effects when using a high dose of allergens (Klimek and Pfaar, 2013) it is of great importance to have an efficient route of injection and a vigorous adjuvant to be able to use small amounts of the allergens. In a previous study we could show that when using the Th1 adjuvant IC31® (Schellack et al., 2006) intralymphatic injection gave a slightly stronger immune response than intradermal injections (Jonsdottir et al., 2015). Based on these findings and on results from immunotherapy in humans (Senti et al., 2008), it was of interest to test further this injection route with other adjuvants.

Despite being a Th2 focusing adjuvant, Alum is the classical adjuvant used in immunotherapy for humans (Moingeon, 2012). However, when T-cell-mediated immunity is necessary additional components are needed (Brewer, 2006). Monophosphoryl lipid A (MPLA) is a detoxified derivative of lipopolysaccharide (LPS) that binds to Toll like receptor 4 (TLR4) and retains most of the adjuvant capacity of LPS (Evans et al., 2003; Qureshi et al., 1982). MPLA is used in vaccine formulations and has been registered for use in humans (Casella and Mitchell, 2008). Since MPLA promotes primarily a Th1 type of response (Puggioni et al., 2005), it is being applied as an adjuvant in immunotherapy and has been shown to enhance specific IgG1 and IgG4 antibodies, and reduce allergy symptoms (Drachenberg et al., 2001; Mothes et al., 2003). The mixture of aluminum hydroxide and MPLA (AS04) is being used successfully in viral vaccines (Garçon et al., 2011).

The aim of our study was to compare the immune response induced by intralymphatic immunization with pure recombinant allergens in Alum alone or a mixture of Alum and MPLA in the search for optimal conditions for prophylactic immunotherapy against equine IBH.

2. Materials and methods

2.1. Animals

Twelve healthy Icelandic horses, 7–10 years of age, were vaccinated. In addition three healthy horses were used as controls in the skin test and six horses as control in the *in vitro* stimulation of PBMC, age 5–13 years. All horses were living in Iceland. The experiment was performed in accordance with a permit from the National Animal Research Committee of Iceland (no. 0113–16).

2.2. Vaccination and sample collection

Twelve horses were vaccinated into the submandibular lymph nodes with four *Escherichia coli*-expressed recombinant *Culicoides nubeculosus* allergens (rCul n 3, rCul n 4, rCul n 8 and rCul n 10) (Schaffartzik et al., 2011). The horses were vaccinated three times (week 0, 4, 8) with 10 μg of each allergen. The horses were randomly divided into two groups. Six horses were vaccinated with the allergens in 500 μg aluminum hydroxide gel (Alhydrogel® 2%, Invivogen) and the other six with allergens in a mixture of Alum (500 μg) and 50 μg MPLA (Avantilipids). The total volume per vaccination was 400 μL. Blood was collected by jugular venipuncture at week 0, every other week for 16 weeks, and then monthly until week 32. Serum was stored at −20 °C until used. A differential count of leukocytes from EDTA blood was carried out (Jonsdottir et al., 2015).

2.3. Production of Cul n 3 in insect cells

Cul n 3 was expressed in insect cells because pilot experiments had shown with *E. coli* derived Cul n 3 the cytokine production by PBMC was extremely low or absent, probably because this *E. coli* expressed protein precipitated in medium. The *Cul n 3* gene was amplified from a λZAPII cDNA library, made from salivary glands of *C. nubeculosus* (Schaffartzik et al., 2011), with primers (Fw: 5′- ATGCATAATTTTGCAGGGAT-3′ and Re: 5′-CGCATATGTGGTCAAAGTAG-3′) designed based on the gene sequence of Cul n 3 (GenBank Accession No. HM145951). The protein was expressed in insect cells according to Bac-to-Bac® HBM-TOPO® Secreted Expression system. The Baculovirus was amplified in Sf-9 cells and the protein expressed in High five cells.

The Bac-rCul n 3 protein was purified under native conditions with HIS-Select™ HF Nickel Affinity Gel (Sigma) in conformity with the manufacturer's protocol and dialyzed against PBS. The protein was analyzed with Coomassie blue staining (Wong et al., 2000) and detected with Cul n 3 specific polyclonal antibodies diluted 1:4000 (Schaffartzik et al., 2011) in WB (Fig. 1A) (Jonsdottir et al., 2015).

2.4. Serological tests

2.4.1. ELISA

Sera from six unvaccinated horses living in Iceland and from five IBH-affected horses living in Switzerland were included on each plate, as negative and positive controls, respectively. Allergen-specific antibody levels were determined by ELISAs as described in Jonsdottir et al. (2015) with the following changes. Plates were coated with 2 μg/mL of the recombinant allergens rCul n 3, rCul n 4, rCul n 8, rCul n 10 and Bac-rCul n 3. Sera (weeks 0, 2, 6, 10, 16) were added at a dilution of 1:5 for IgE detection, 1:10 for IgA (weeks 0, 6, 10, 24, 32) and 1:200 for IgG subclasses (weeks 0, 2, 6, 10, 16, 24, 32). Specific monoclonal antibodies were used for IgG subclasses and IgE detection as described by Jonsdottir et al. (2015) and for IgA, a monoclonal equine IgA specific antibody in a 1:250 dilution (Serotec) was applied.

2.4.2. Competitive inhibition ELISA

Pools of sera from the Alum group and Alum/MPLA group, containing the same amount of serum from each of the six horses, were used to analyze the ability of sera from vaccinated horses to block the IgE-binding of serum from an IBH-affected horse to the recombinant allergens. Pools of preimmune sera (negative control) and postimmune sera (two weeks after the third vaccination) were used in the competitive inhibition ELISA performed as described by Jonsdottir et al. (2015). The percentage of inhibition for each dilution of the pre- and postimmune serum pools was calculated.

2.5. Stimulation of PBMC and determination of cytokines

PBMC were isolated from the horses by Ficoll-Hypaque in conformity with Hamza et al. (2007) three weeks (week 11) after the 3rd vaccination, and from six unvaccinated control horses. PBMC were stimulated for 24 h (for RNA isolation) or for 4 days (for analysis of the supernatant) with ConA (10 μg/mL) as a positive control

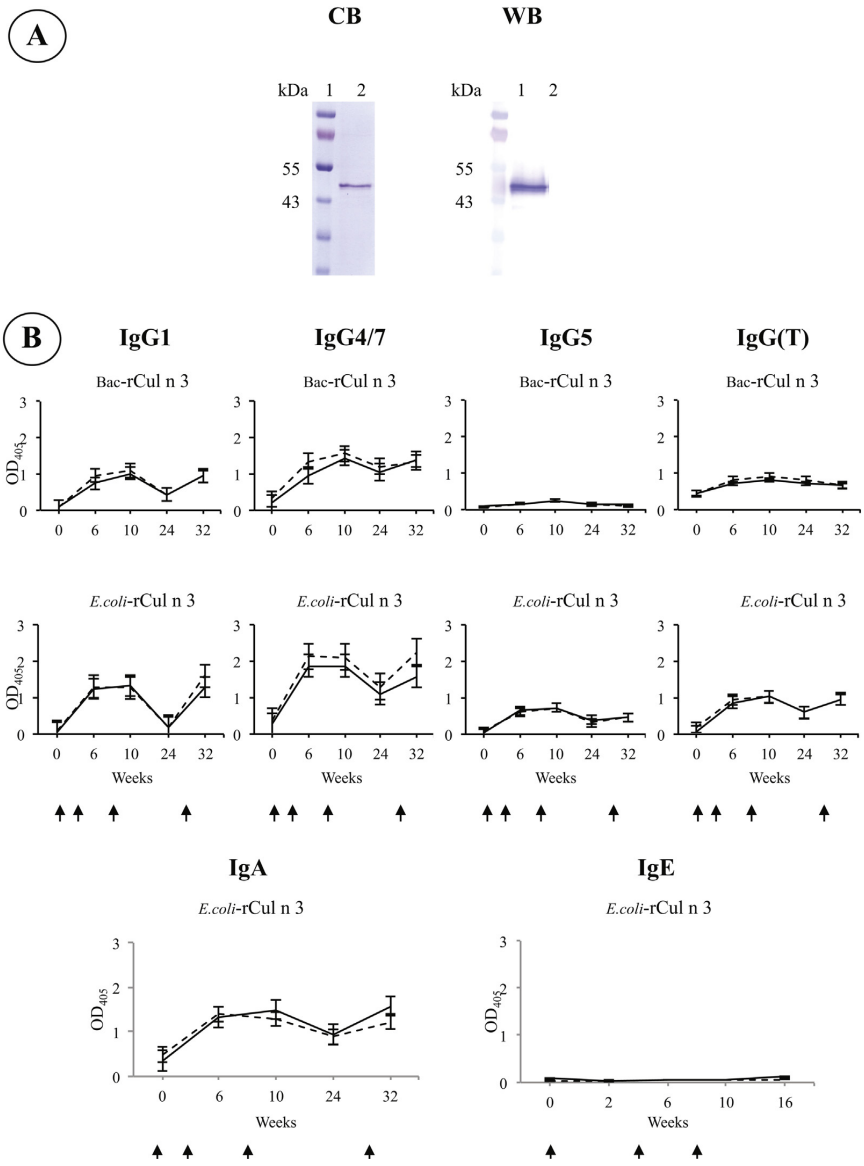

Fig. 1. (A) Purification and detection of Bac-rCul n 3 produced in High five insect cells. Coomassie blue staining (CB) and Western blot (WB) of purified Bac-rCul n 3 in PBS, detected with Cul n 3 specific polyclonal antibody (1:4000). (B) Cul n 3 specific antibody response. Time course of IgG subclass, IgA and IgE response against *E. coli*-rCul n 3 measured by ELISA and comparison of IgG subclass response against Bac-Cul n 3. Corrected OD mean values and standard error for the six horses in the two groups at different time points, Alum (——) and Alum/MPLA (- - - -). The first three arrows indicate the vaccination time points and the last the intradermal test.

and Bac-rCul n 3 (2 μg/mL) or cultured in medium alone. The stimulation was performed in accordance with Jonsdottir et al. (2015). After 24 h PBMC were harvested, lysed and homogenized in RA1 lysis buffer (Macherey–Nagel) and stored at −80 °C until used for total RNA isolation. After 4 days' incubation, cell supernatants were harvested and stored at −80 °C until cytokine measurement.

Determination of IL-4, IFNγ and IL-10 in cell supernatants was performed at the Department of Population Medicine and Diagnostic Sciences, Cornell University, with bead-based multiplex assays (Wagner and Freer, 2009). The values for the medium alone were subtracted and the results shown as mean and standard errors for each group as pg/mL for IL-4 and IL-10 and U/mL for IFNγ. Total RNA isolation, Reverse Transcription of RNA and Q RT-PCR for Cytokine mRNA expression was performed as described previously (Jonsdottir et al., 2015).

2.6. Skin test

Skin tests were performed according to Schaffartzik et al. (2011) at week 30. In short, horses were injected in the lateral neck with 100 μL of the allergens rCul n 3, 4, 8 and 10 diluted to 1 μg/mL and 10 μg/mL. Histamine 0.2 mg/mL was used as the

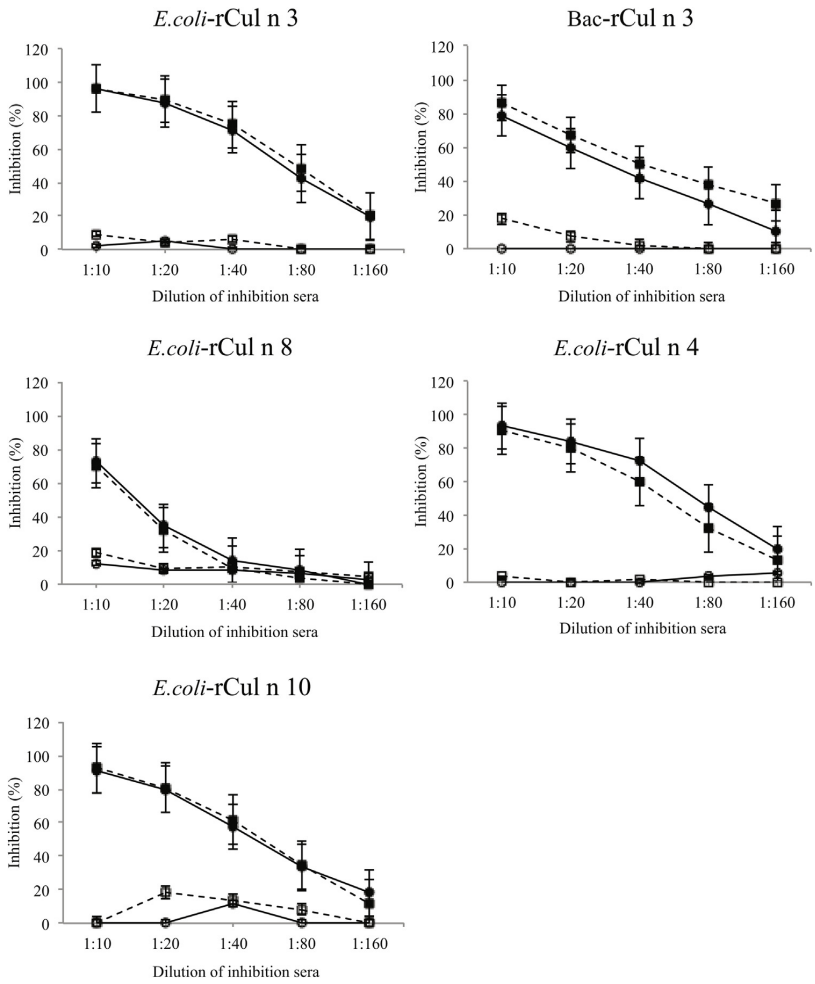

Fig. 2. Inhibition of IgE binding to the allergens with pool of sera from the two vaccination groups. Mean percent inhibition by pre and post vaccination sera from the two groups, diluted 1:10–1:160 applied to an ELISA plate coated with allergens, prior to adding serum from an IBH positive horse at a dilution 1:5. Preimmune sera Alum horses (—○—), preimmune sera Alum/MPLA horses (- -□- -), postimmune sera Alum horses (—●—), postimmune sera Alum/MPLA horses (- -■- -).

positive control. Diameters of the wheal reaction at injection site were measured 30 min post injection.

2.7. Statistical analyses

Descriptive statistics were run on the IgG subclasses, IgA and IgE ELISA results using the statistical software NCSS 8 (NCSS Statistical Software, 329 North 1000 East, Kaysville, Utah 84037, USA). Antibody levels were log transformed because they were not normally distributed. Allergen-specific logIgG subclasses and logIgA levels were then analyzed using Proc Mixed of SAS (SAS Inst. Inc., Cary, NC). Week of measurement was included as a repeated effect, with the individual horse as the subject. Separate models were run with levels of log IgG1, IgG1/3, IgG4/7, IgG5 and IgG(T) as outcome variable, respectively. The statistical model included effect of adjuvant (Alum or Alum/MPLA), recombinant allergen (rCul n 3, 4, 8, 10), and time (weeks after immunization: 0, 2, 6, 10, 16, 24, 32), and their interactions. Non-significant interactions were removed from the final model.

As the cytokine values were not distributed normally, the non-parametric ANOVA Kruskal–Wallis Multiple-Comparison *Z*-Value Test was used to compare the differences on cytokine mRNA levels and in cytokines present in the supernatants in horses immunized with Alum, Alum/MPLA and non-immunized control horses. The Bonferroni correction was used to correct for multiple comparisons. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Clinical examination

The vaccinations in the submandibular lymph nodes were well tolerated. No rise in temperature or pain on palpation was observed, but mild local swelling occurred in a few cases. Leukocyte counts were within the normal range following vaccinations (data not shown). No difference was observed between vaccination with Alum or a mixture of Alum and MPLA.

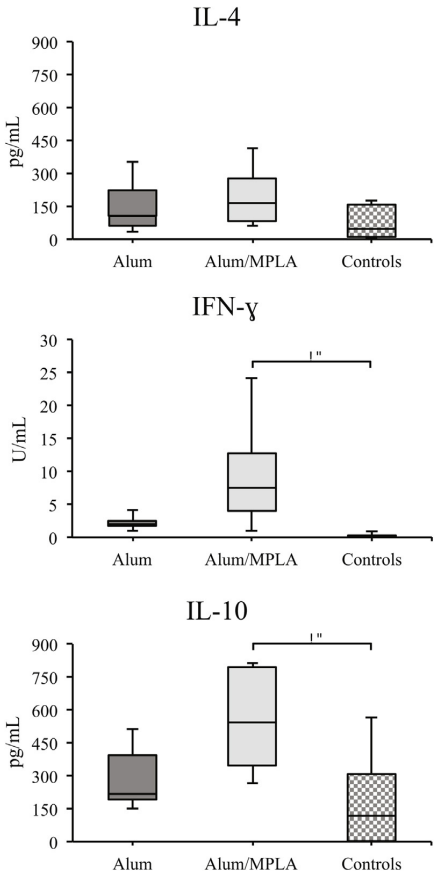

Fig. 3. Detection of IL-4, IL-10 and IFNγ after *in vitro* stimulation of PBMC from the Alum and Alum/MPLA vaccinated horses and six control horses. The cytokines were measured in supernatants using bead-based multiplex assays. Comparisons were performed using Kruskal–Wallis Multiple Comparison *Z* value Tests with Bonferroni corrections. An asterisk (*) with line indicates statistically significant differences between the groups of horses.

3.2. Specific antibody response of vaccinated horses

Immunization with 10 μg each of the four recombinant allergens led to a significant increase in all tested IgG subclasses as soon as two weeks after the first vaccination (Table 1), both in the Alum and Alum/MPLA groups, with further increase until week 10, *i.e.* 2 weeks after the 3rd vaccination (Fig. 1B). Antibody levels then decreased until week 24 but were still significantly higher than before vaccination (Table 1). Interestingly, the intradermal test performed at week 30 boosted the specific antibody responses (Fig. 1B).

Comparison of the two groups of horses vaccinated with Alum or Alum/MPLA, showed a similar IgG subclass response in both groups (Table 1 and exemplified for Cul n 3 in Fig. 1B).

Analysis of the data revealed significant differences in the IgG subclass levels between the different allergens. This confirms results from the previous study with the IC31® adjuvant (Jonsdottir et al., 2015).

The Bac-rCul n 3 antibody response showed a similar pattern as when the *E. coli*-Cul n 3 was used in the ELISA (Fig. 1B). As expected the measured antibody increase was usually lower on Bac-rCul n 3 than on *E. coli*-rCul n 3 that was used for the vaccination. These results confirm that the antibody response is mainly raised against the Cul n 3 protein and not against the *E. coli* contamination of the recombinant proteins used for immunization (purity of 95%).

As a protective role of IgA has been suggested in allergic diseases such as asthma (Gloudemans et al., 2013) and an increase in serum IgA is seen in allergen specific immunotherapy (Jutel et al., 2003), we also determined allergen-specific IgA levels in the sera. The horses in both groups had developed a significant IgA response against all four allergens (Table 1) already after the second immunization, as exemplified for rCul n3-specific IgA in Fig. 1B. The serum IgA levels were much lower than IgG as the sera could only be diluted 1:10 instead of 1:200 in the ELISA. No significant effect of MPLA could be observed on the IgA response compared to Alum alone.

Importantly immunization with the recombinant allergens did not induce sensitization as no IgE response was detected after the vaccinations, as exemplified for rCul n 3 in Fig. 1B. Nor did the horses respond to the *Culicoides* allergens in intradermal tests, irrelevant of the adjuvant used (data not shown).

3.3. Inhibition of allergen-specific IgE-binding by sera from the vaccinated horses

An important factor in immunotherapy is the induction of IgG antibodies that can block the binding of IgE to the relevant allergens (Flicker and Valenta, 2003). In the present study pools of the pre- and postimmune sera (two weeks after third vaccination) from the two groups of horses were tested in an inhibition ELISA. The inhibition of IgE-binding to rCul n 3, rCul n 4 and rCul n 10 was over 90% in the 1:10 serum dilution and still 60% or more in the 1:40 dilution. Inhibition of binding to rCul n 8 was lower, around 70% in the 1:10 serum dilution and the 1:40 dilution had no inhibitory activity. This was regardless of the use of MPLA (Fig. 2). In our previous vaccination study (Jonsdottir et al., 2015) with the adjuvant IC31®, the blocking activity of the 1:10 diluted sera was lower, only reaching 18 to 54%, depending on the allergen used. This result accords with higher antibody titers observed in the present study. However, we cannot distinguish whether this difference was due to the adjuvants or the allergens, as different recombinant *Culicoides* allergens were used. The inhibitory activity of the sera was also tested against Bac-rCul n 3 showing that the antibodies generated though immunization with *E. coli* expressed Cul n 3 were also able to efficiently inhibit IgE-binding to Bac-rCul n 3, although to a slightly lower extent (Fig. 2).

3.4. IL-10, IFNγ and IL-4 protein expression in PBMC stimulated in vitro with Bac-rCul n 3

To further characterize the type of immune response induced by these adjuvants, PBMC were stimulated *in vitro* with Bac-rCul n 3 and the cytokine production determined. In contrast to the antibody response, there was a significant difference between the adjuvant groups in the cytokine responses. Upon stimulation of PBMC with Bac-rCul n 3 the horses vaccinated with the allergens in Alum/MPLA produced significantly more IFNγ and IL-10 than the non-vaccinated controls, whereas this difference was not observed between the horses vaccinated with Alum and the control horses (Fig. 3). The same tendency was seen at the cytokine mRNA levels (data not shown). When the two vaccinated horse groups were compared without the controls the Alum/MPLA horses produced significantly more IFNγ and IL-10 than the Alum horses. These findings suggest that MPLA is important to obtain the preferable Th1/Treg cytokine profile and shows that in horses, like humans, a combination of Alum and MPLA (AS04) induces a higher level of IFNγ as compared to Alum alone (Didierlaurent et al., 2009).

Table 1
Mixed linear regression model of association between log-transformed levels of IgG subclasses and adjuvant and allergens with time (weeks after immunization) as repeated effect.

	log IgG1			log IgG1/3			log IgG4/7			log IgG5			log IgG(T)			log IgA		
Effect	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value
Adjuvant																		
Alum/MPLA[a]	0.23	0.05	**<.01**	1.27	0.21	**<.001**	0.01	0.02	.60	−0.04	0.05	.48	−0.06	0.03	<.05	−0.06	0.03	.08
Allergen																		
Cul n 3[b]	0.20	0.03	**<.0001**	1.75	0.21	**<.0001**	−0.27	0.07	**<.001**	1.12	0.19	**<.0001**	0.20	0.04	**<.0001**	0.22	0.05	**<.0001**
Cul n 4[b]	0.26	0.03	**<.0001**	1.70	0.21	**<.0001**	0.01	0.07	.87	0.27	0.19	.18	0.23	0.04	**<.0001**	0.06	0.05	.21
Cul n 10[b]	0.12	0.03	**<.001**	1.29	0.21	**<.0001**	−0.02	0.07	.75	0.57	0.19	**<.01**	0.19	0.04	**<.0001**	0.23	0.05	**<.0001**
Week																		
2[c]	1.25	0.05	**<.0001**	2.94	0.21	**<.0001**	0.42	0.07	**<.0001**	2.00	0.20	**<.0001**	1.04	0.05	**<.0001**	nd		
6[c]	1.46	0.05	**<.0001**	3.12	0.21	**<.0001**	0.54	0.07	**<.0001**	2.43	0.20	**<.0001**	1.32	0.05	**<.0001**	0.60	0.05	**<.0001**
10[c]	1.47	0.05	**<.0001**	3.13	0.21	**<.0001**	0.53	0.07	**<.0001**	2.49	0.20	**<.0001**	1.36	0.05	**<.0001**	0.64	0.05	**<.0001**
16[c]	0.95	0.05	**<.0001**	2.44	0.21	**<.0001**	0.47	0.07	**<.0001**	2.08	0.20	**<.0001**	1.25	0.05	**<.0001**	0.34	0.05	**<.0001**
24[c]	0.61	0.05	**<.0001**	2.18	0.21	**<.0001**	0.28	0.07	**<.0001**	1.67	0.20	**<.0001**	1.07	0.05	**<.0001**	nd		
32[c]	1.33	0.05	**<.0001**	2.95	0.21	**<.0001**	0.50	0.07	**<.0001**	2.01	0.20	**<.0001**	1.27	0.05	**<.0001**	0.55	0.05	**<.0001**
Interactions																		
Adjuvant × week									ns			ns			ns			ns
Alum/MPLA × 2[d]	−0.15	0.07	**<.05**	−1.23	0.29	**<.0001**												
Alum/MPLA × 6[d]	−0.20	0.07	**<.01**	−1.25	0.29	**<.0001**												
Alum/MPLA × 10[d]	−0.21	0.07	**<.01**	−1.23	0.29	**<.0001**												
Alum/MPLA × 16[d]	−0.25	0.07	**<.01**	−1.39	0.29	**<.0001**												
Alum/MPLA × 24[d]	−0.23	0.07	**<.01**	−1.40	0.29	**<.0001**												
Alum/MPLA × 32[d]	−0.07	0.07	.28	−1.10	0.29	**<.0001**												
Allergen × week			ns			**<.0001**			**<.05**			**<.05**			ns			ns
Adjuvant × allergen × week			ns			**<.05**			ns			ns			ns			ns

nd = not done.

[a] *vs* Alum: these significant differences for IgG1 and IgG1/3 and the resulting significant interaction between adjuvans × week, were due to the fact that mean IgG1 and IgG1/3 levels at week 0 in the Alum/MPLA group were slightly but consistently higher than in the Alum group (data not shown).

[b] *vs* Cul n 8.

[c] *vs* week 0.

[d] *vs* Alum × corresponding week.

Notably, in our study Th1/Treg biased immune response induced by MPLA could not be detected in the antibody response.

4. Conclusion

In conclusion, our study confirms that also in horses, intralymphatic immunization enables the use of a small amount of allergens and relatively few injections for procuring a potent response. Importantly, because of the induction of a Th1/Treg immune response, Alum/MPLA seems an interesting combination for preventive allergen immunization. For further development of immunoprophylaxis and therapy against equine IBH these results need to be confirmed by determining the cytokine response using additional allergens and in allergen immunotherapy trials with IBH affected horses.

Conflicts of interest

No conflict of interest.

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Paper III

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Oral administration of transgenic barley expressing a *Culicoides* allergen induces specific antibody response

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Summary

Background: Insect bite hypersensitivity is an immunoglobulin (Ig)E-mediated dermatitis of horses initiated by bites of midges of the genus *Culicoides*. *Culicoides* spp. are not indigenous to Iceland and the prevalence of insect bite hypersensitivity is much higher in horses born in Iceland and exported as compared to Icelandic horses born in a *Culicoides* rich environment. Immunotherapy is therefore needed.
Objectives: The aim of the study was to express an allergen from *Culicoides* in barley grain and investigate whether an immune response could be obtained in healthy Icelandic horses by oral treatment with transgenic barley expressing the allergen.
Study design: In vivo experiment.
Methods: The allergen was expressed in barley grain with the Orfeus technique. A device was developed to treat horses orally with barley flour. Four Icelandic horses were treated with transgenic barley and 3 with control barley, in total 500 g in 7 feedings. Serum and saliva samples were collected for measuring specific antibodies.
Results: The allergen Cul n 2, a hyaluronidase originating from the salivary gland of *Culicoides nubeculosus*, was expressed in barley. Horses treated with the transgenic barley mounted a Cul n 2 specific IgG1 and IgG4/7 response in serum and saliva. The serum response was significantly different between the transgenic and control barley treated horses for both subclasses and the saliva response for IgG1. The induced serum antibodies bound to the corresponding allergen from *Culicoides obsoletus*, rCul o 2 and were able to partially block binding of Cul n 2 as well as Cul o 2 specific IgE from insect bite hypersensitivity affected horses.
Main limitations: Small number of horses.
Conclusion: This study shows that specific antibody response can be induced in horses not exposed to *Culicoides*, using oral treatment with transgenic barley expressing an allergen. Further studies will determine whether this approach is a useful alternative for prevention and treatment of equine insect bite hypersensitivity.

Keywords: horse; insect bite hypersensitivity; *Culicoides* allergen; oral immunotherapy; barley grain

Introduction

Insect bite hypersensitivity or summer eczema is a dermatitis of horses caused by Th2 type immunoglobulin (Ig)E mediated hypersensitivity reaction to *Culicoides* spp. (biting midges). The midges are not found in Iceland and therefore the disease does not occur there [1]. All breeds of horses can be affected, but Icelandic horses born in Iceland and exported to a *Culicoides* infested environment are more strongly affected than most other breeds [2]. The causative allergens of insect bite hypersensitivity originate in the salivary glands of *Culicoides* spp. Several allergens from 3 *Culicoides* species have been isolated and the proteins expressed in *Escherichia coli* and some in insect cells [3]. The hyaluronidase of *Culicoides* is one of the allergens causing insect bite hypersensitivity. It has been isolated from *Culicoides nubeculosus* (Cul n 2) [4] and *Culicoides obsoletus* (Cul o 2) [5].

The rising demand for therapeutic proteins has greatly enhanced research on plant-based protein expression because of their eukaryotic protein machinery, improved safety and lower cost. Barley grain is an excellent source of proteins and ideal for stable storage [6]. This is important since cultivation costs are low in proportion to total production costs, and purification constitutes the bulk of the expense [7,8]. Noninjection routes of allergen specific immunotherapy are becoming more and more applicable [9]. The mucosa of the mouth is an immune privileged site constantly bathed in various antigens derived from food and microbes. Consequently it has an effective immunological network that can enforce tolerogenic mechanisms [10]. The sublingual route has been established for human use as a valid noninvasive alternative to the subcutaneous route [11]. We were interested in investigating whether oral treatment of horses with transgenic barley expressing an allergen was able to induce an antibody response and could be potentially used in the future for preventive and therapeutic oral immunotherapy against insect bite hypersensitivity.

Materials and methods

Generation of Cul n 2 specific monoclonal antibody

A Cul n 2 specific monoclonal antibody (mAb) was generated with the hybridoma technique [12,13] against rCul n 2 expressed in *E. coli* (*E. coli*-rCul n 2) [4]. Ig positive hybrids were tested on *E. coli*-rCul n 2 in western blot (WB) according to Jonsdottir *et al.*[14] except for the secondary antibody that was AP-labeled goat anti-mouse[a] , dilution 1/5000.

Expression of Cul n 2 in barley grains

The sequence encoding Cul n 2 (GenBank: HM145950.1) was codon optimised for expression in barley[b] and synthesised[c]. The gene had N-terminal $(HQ)_6$-tag and the expression cassette was under the control of 0.45 kb D-hordein promoter. The expression and production were performed as described before [7]. Two barley lines (No. 19 and 70) were used for stable production. The presence of Cul n 2 protein in the lines was confirmed by WB, as described above with the anti-Cul n 2 mAb, 1/20,000. The amount of Cul n 2 in the barley grain was estimated semiquantitatively using SynGene – GeneTools to compare a known amount of Cul n 2 (expressed in *E. coli*) to the protein bands in the Cul n 2 barley extract. The samples were analysed in WB in different dilutions, using the Cul n 2 specific

Fig 1: Spiral bit used for oral treatment with barley.

mAb, HRP-labeled anti-mouse antibody[a] and detected with ChemiFast Chemiluminesence[d] and captured with GENE GNOME SynGene bioimaging[d].

Barley mixture and device for oral treatment

The barley grain was ground to a fine powder in a coffee grinder before 200 mmol/L NaCl was added in the ratio 1:2 and mixed. A thickener, Hydroxyethylcellulose Natrosol 250 HHR PC[e] (CAS: 9004-62-0), 2 g per 50 g barley, was added and stirred for 1 h at RT. The mixture was kept at 4°C overnight. For feeding the barley mixture to the horses, a device in a form of a spiral bit was developed (Fig 1).

Animals

Seven healthy Icelandic horses, aged 6–8 years, all located in Iceland and thus not exposed to *Culicoides*, were included in the study. During winter the horses were housed and fed hay and in summertime horses were kept out on pasture. The horses were kept in accordance with the Icelandic animal care guidelines for experimental animals.

Oral treatment and sample collection

Seven horses were treated orally with the barley mixture 6 times with 50–100 g barley grain, equivalent to 5–10 mg of rCul n 2, each time over a period of 20 weeks. In total, they received 400 g of barley grain. Four were treated with transgenic barley expressing Cul n 2 and 3 with normal Golden Promise barley [7]. The horses were boosted with 100 g barley 8 months after the last treatment using the same procedure. The bits were filled using a 30 mL syringe 4–5 times and the treatment lasted 4–6 h. Before treatment and 2 weeks after each treatment blood and saliva were collected. Blood was collected as described previously [14] and saliva was collected with a lacerated tampon, placed in the bit and kept in the mouth of the horses for 2 h. The tampon was placed on a pipette tip in a conical tube and spun at 170 × ***g*** and 4°C. The saliva was collected, spun again at 20,800 × ***g*** for 5 min and kept at −80°C until used.

Enzyme-linked immunosorbent assay

Serum enzyme-linked immunosorbent assay: Allergen specific IgG1, IgG4/7, IgG5, IgE and IgA was measured by enzyme-linked immunosorbent assay (ELISA) in the sera on *E. coli*-rCul n 2 [4] and rCul o 2 produced in *Pichia* (*Pichia*-rCul o 2) [15] by VTU technology[f] and kindly provided by Boehringer Ingelheim Animal Health GmbH. The plates were coated with 2 μg/mL of each *r*-protein diluted in 0.2 mol/L Carbonate-Bicarbonate buffer[b], pH 9.4 for 2 h at 37°C. Plates were then washed and nonspecific binding sites blocked with 5% dried milk powder and 5% Tween 20 in phosphate-buffered saline[g] , for 1 h at 37°C. The sera taken before and after treatment (after 400 g barley), and before and after the barley boost, were diluted in blocking buffer, 1:50 for detection of IgG subclasses, 1:10 for IgA and 1:5 for IgE detection, applied to the plates and incubated overnight at 4°C. After washing, mAb 1 μg/mL specific for equine IgE (clone 3H10 [16]) or for IgG1 (CVS45), IgG4/7 (CVS39), IgG5 (clone 416) [17] or IgA (1:250, clone K129.5G5[h]) were added to the plates and incubated for 2 h at RT. After washing, alkaline-phosphatase-conjugated goat-anti mouse IgG[a] diluted 1:2000 in blocking buffer was added and incubated for 1½ h at RT. Plates were developed with a solution of a 1.5 mg/mL phosphatase substrate[i] in 10% diethanolamine[i], pH 9.8 and absorbance measured at 405 nm. The results are presented as an ELISA increment where for each horse the OD value before treatment was subtracted from the OD values after barley treatment, before and after the barley boost.

Saliva ELISA: For detection of allergen specific IgG1, IgG4/7, IgG5 and IgA in the saliva, the same ELISA was performed as for the sera except that the saliva was diluted 1:10 and only tested before treatment and after the boost. The results are presented as ELISA increment.

Competitive inhibition ELISA for demonstration of blocking capacity of the induced antibodies

The ability of the horse sera to block IgE-binding of insect bite hypersensitivity-affected horses to *E. coli*-rCul n 2 and *Pichia*-rCul o 2 was tested in an inhibition ELISA [14]. The ELISA plates were coated with *E. coli*-rCul n 2 or *Pichia*-rCul o 2 and blocked as described above. The sera taken before the treatment and after the barley boost were diluted 1:6, 1:12 and 1:24 and added in duplicates to the plates. After 1 h incubation at 37°C, serum from an insect bite hypersensitivity-affected horse with high Cul n 2 and Cul o 2 specific IgE was added and the ELISA carried out for IgE detection as described above. The percentage of inhibition was calculated for both time points and all dilutions using the following formula:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{OD of insect bite hypersensivity serum after inhibition with serum from barley treated horse}}{\text{OD of insect bite hypersensitivity serum}} \times 100 \right)$$

Competitive inhibition ELISA to demonstrate cross-reactivity between Cul n 2 and rCul o 2

E. coli-rCul n 2, *Pichia*-rCul o 2 and a negative control recombinant protein (*E. coli*-rCul n 11 [4]) were serially diluted 3-fold (0.41–100 μg/mL) in phosphate-buffered saline and incubated with a serum pool for 30 min at RT. The serum pool consisted of the after-boost sera (final dilution 1:50) of the 4 horses treated with Cul n 2 transgenic barley. ELISA plates were coated with *E. coli*-rCul n 2 and blocked as described above. The preincubated sera were added to the coated plates and incubated at 4°C overnight. Then anti-equine IgG1 antibodies were detected as described above (Serum ELISA). The percentage of inhibition was calculated by setting the absorbance obtained for the serum pool preincubated without recombinant proteins in the fluid phase as 0% inhibition.

Data analysis

Statistical analyses were carried out using the software program NCSS 10[j] . A Mann Whitney *U* test was used to compare antibody increments

between the transgenic and control barley treated horses at the different time point. Overall, P≤0.05 were considered significant.

Results

Generation of monoclonal antibody against *E. coli* produced rCul n 2

The mAb generated against *E. coli*-rCul n 2 bound to the corresponding protein band in immunoblot both in the form of hybridoma supernatant and ascites (Fig 2a).

Production of rCul n 2 in barley grain

The screening of the Cul n 2 transgenic barley generations was performed with Cul n 2 specific poly- and monoclonal antibodies (data not shown). The HQ-tag specific antibody did not react with any of the Cul n 2 grain samples and the protein detected with the Cul n 2 specific antibodies was around 40 kDa, whereas the predicted size should be 50 kDa. We thus assume that a truncated form of the protein was expressed, lacking a part of the amino terminal and thereby the HQ-tag. The expression of Cul n 2 in two T3 generation barley lines, as detected by the Cul n 2 antibodies, is shown in Figure 2b. The amount of rCul n 2 in 1 g of barley grain was estimated at around 100 μg (Fig 2c). The recombinant Cul n 2 was released from the ground grain in 200 mmol/L NaCl in the ratio 1:2, the mixture used for the oral treatment (Fig 2d). The rCul n 2 expressed in barley gives a double band in SDS-PAGE (Fig 2b–d). This might be due to post-translational modification in the barley as opposed to *E. coli* [7].

Allergen specific response following oral treatment with Cul n 2 transgenic barley

After barley treatment 3 of the 4 Cul n 2 horses showed an IgG1 response on *E. coli*-rCul n 2 and *Pichia*-rCul o 2, and all 4 horses were efficiently boosted (Fig 3). The difference between the transgenic and control barley treated horses was significant after the boost on both allergens and also after treatment on *Pichia*-rCul o 2. A very high IgG4/7 background (data not shown) on the *E. coli*-rCul n 2 allergen made it impossible to distinguish a response even after the boost. However, all the transgenic barley treated horses responded significantly higher on *Pichia*-rCul o 2 as compared to the control horses, both after treatment and the boost. There was no IgG5 response after treatment and none or only a very low one after the barley boost. No IgE response was detected on *E. coli*-rCul n 2 and 3 of the 4 transgenic barley treated horses were also IgE negative on *Pichia*-rCul o 2. However, one of the transgenic barley treated horses showed some IgE response after the barley treatment (Fig 3).

The IgG subclass response was also measured in the saliva before treatment and after the barley boost. There was a significant IgG1 response to both *E. coli*-rCul n 2 and *Pichia*-rCul o 2 (Fig 4). Three out of the 4 transgenic barley treated horses had a higher IgG4/7 response than the controls when tested on the *E. coli*-rCul n 2, but not on the *Pichia*-rCul o 2 due to background (Fig 4). No IgG5 response could be detected in the saliva on *E. coli*-rCul n 2 but one horse responded on *Pichia*-rCul o 2 after the barley boost (Fig 4).

Inhibition of Cul n 2 specific IgE binding by sera from horses treated with Cul n 2 transgenic barley

The IgE blocking capacity of the sera was tested before treatment and 2 weeks after the barley boost. The sera after the boost from the transgenic barley treated horses were able to inhibit binding of serum IgE from an allergic horse to *Pichia*-rCul o 2. The mean inhibition was 42% (range 17–53%), 23% and 7.5% at serum dilution 1:6, 1:12 and 1:24, respectively. Inhibition was seen to a lower degree with *E. coli*-rCul n 2 with a mean inhibition of 16% (range 14–20%) at serum dilution 1:6, but further dilutions of the sera did not inhibit IgE binding (data not shown). Inhibition capacity of sera before treatment and sera from the control horses was ≤5% (Fig 5).

Competitive inhibition ELISA to demonstrate cross-reactivity between Cul n 2 and Cul o 2

A competitive inhibition ELISA was performed to confirm that the observed increase in Cul o 2 binding equine IgG1 antibodies after treatment with the transgenic barley was due to cross-reactivity between these 2 proteins. A concentration-dependent inhibition of IgG1-binding to *E. coli*-rCul n 2 was observed following preincubation of the horse sera with *E. coli*-rCul n 2 and *Pichia*-rCul o 2 (Fig 6). However, while inhibition with *E. coli*-rCul n 2 reached 90% at a concentration of 100 μg/mL, inhibition with the same

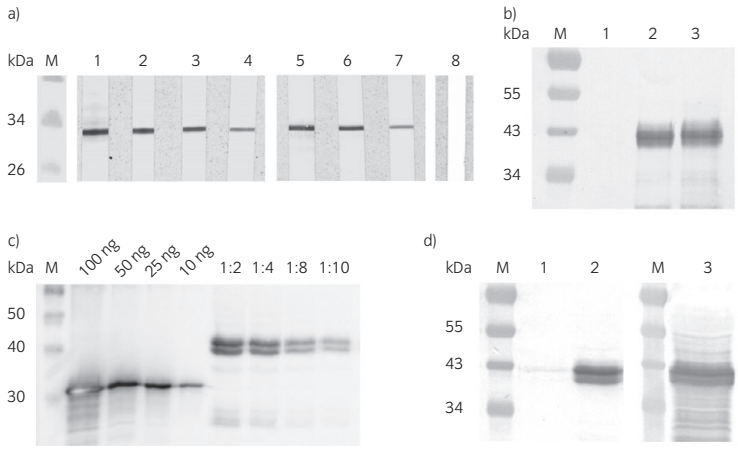

Fig 2: Western blots. a) Reactivity of Cul n 2 specific monoclonal antibody. *Escherichia coli* produced Cul n 2 (1 μg/blot), M: Page Ruler[b], 1–4: ascites 1/5000, 1/50.000, 1/200.000, 1/500.000, 5–7: hybridoma supernatant 1/20, 1/200, 1/2000 and 8: Goat-anti mouse-AP alone. b) Production of Cul n 2 in barley grains. M: Page Ruler[b], 1: Unmodified barley (Golden promise), 2: Cul n 2 barley line no 19, 3: Cul n 2 barley line no 70. c) Semiquantitative analysis of Cul n 2 protein in extract of barley line 70. M: Super Signal marker[b], known amount of Cul n 2, expressed in *E. coli* (100–10 ng) compared to different dilutions of barley extract (1:2–1:10). d) Extraction of Cul n 2 from barley. M: Page Ruler[b]. Barley flour dissolved in: 1: dH_2O ratio 1/8, 2: 200 mmol/L NaCl ratio 1/8, 3: 200 mmol/L NaCl ratio 1/2. b–d) Detected with Cul n 2 specific monoclonal antibody (ascites 1/20.000).

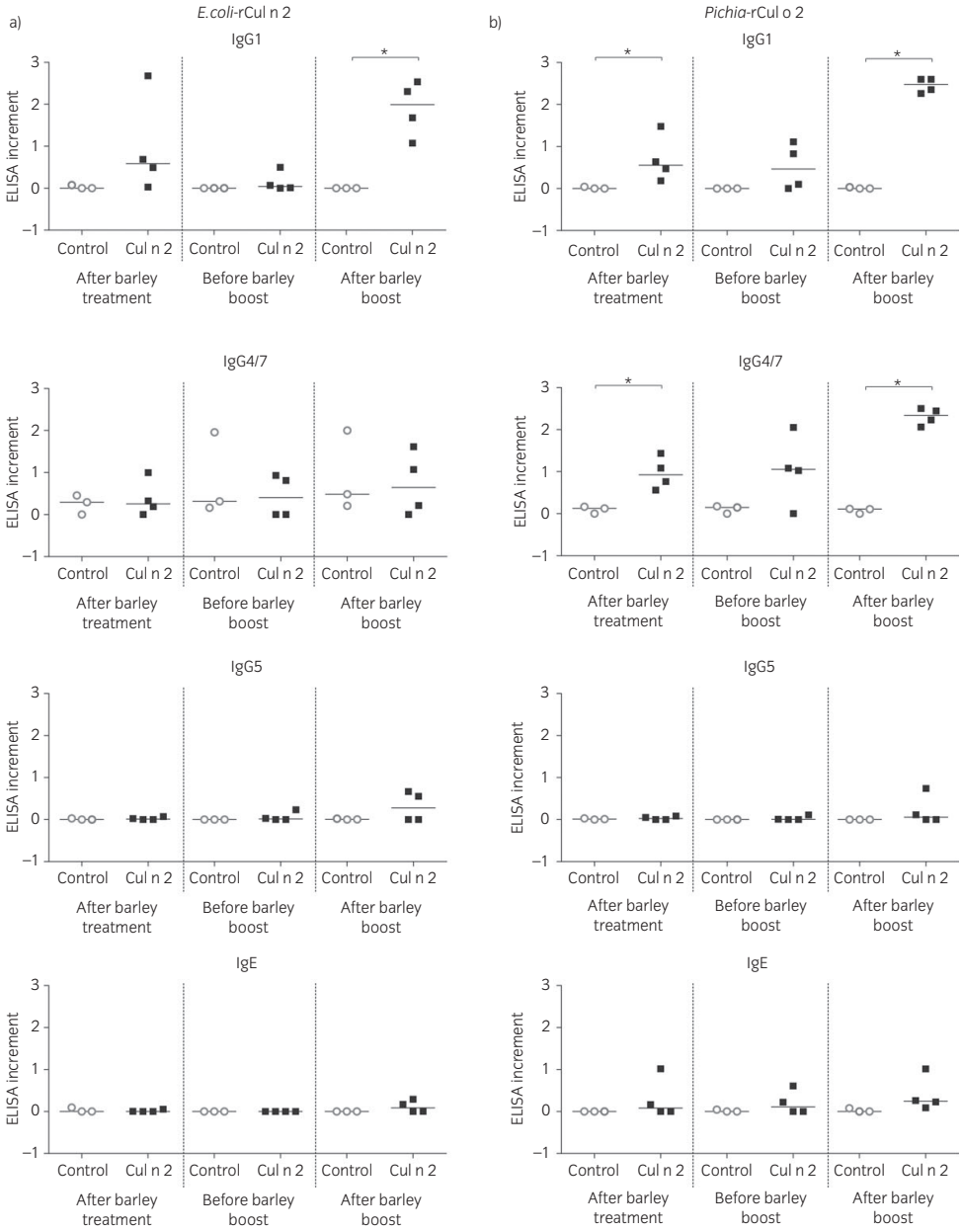

Fig 3: Specific serum antibody response. a) *Escherichia coli*-rCul n 2 and b) *Pichia*-Cul o 2. Immunoglobulin G subclass and immunoglobulin E response at 3 different time points, control horses (○) and Cul n 2-treated horses (■). Results shown as enzyme-linked immunosorbent assay (ELISA) increment for each horse (○/■) with median for the groups. An asterisk (*) indicates statistically significant differences.

amount of *Pichia*-rCul o 2 only resulted in a 50% inhibition, indicating that the allergens are not fully cross-reactive. Pre-incubation with a negative control recombinant protein (*E. coli*-rCul n 11) inhibited only marginally and not in a concentration-dependent manner.

Discussion

The mouth is an immune privileged site [10] and, in human oral mucosa, there is abundance of dendritic cells with strong TLR2 and TLR4

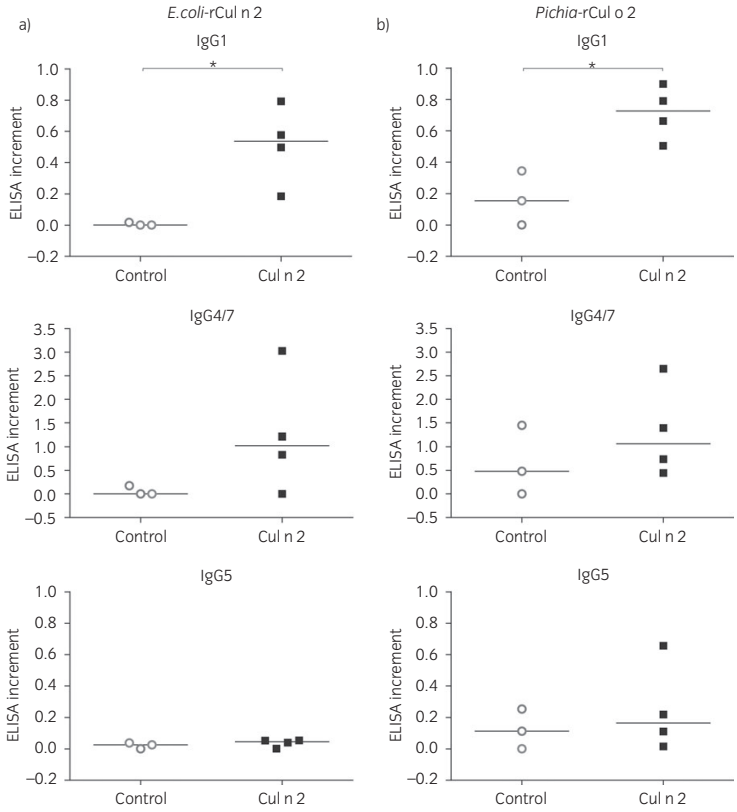

Fig 4: Specific saliva antibody response a) *Escherichia coli*-rCul n 2 and b) *Pichia*-Cul o 2. Immunoglobulin G subclass response after the barley boost, control horses (○) and Cul n 2-treated horses (■). Results shown as enzyme-linked immunosorbent assay (ELISA) increment for each horse (○/■) with median for the groups. An asterisk (*) indicates statistically significant differences.

expression, which induce a predominantly Th1 and Th17 cells as well as regulatory immune response. There is also negligible presence of inflammatory cells, such as mast cells and eosinophils. Consequently, allergic reactions are rare in the mouth despite heavy trafficking of food proteins and microbes [10]. However, healthy individuals have measurable IgG response to food proteins, especially if consumed in large quantities [18,19]. Sublingual immunotherapy or allergy treatment via the mucosa of

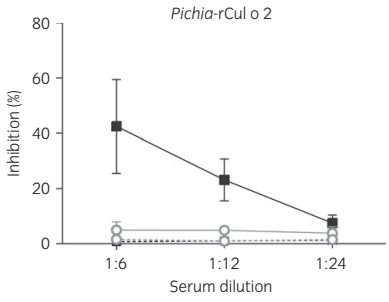

Fig 5: Inhibition of immunoglobulin E binding to *Pichia*-rCul o 2. Serum from control (○) and transgenic barley treated (■) horses diluted 1:6, 1:12 and 1:24, before treatment (··■··/··○··) and after the boost (–○–/–■–) applied to an enzyme-linked immunosorbent assay plate coated with *Pichia*-rCul o 2 prior to adding serum from an insect bite hypersensitivity positive horse at dilution 1:5. The inhibition (%) is shown as mean and s.d. for the groups.

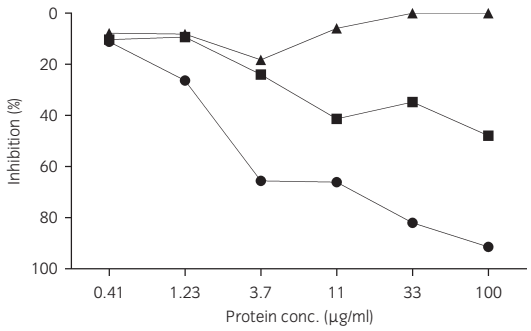

Fig 6: Competitive Inhibition of equine immunoglobulin G1-binding to solid phase coated Cul n 2. A pool of sera (after boost) of the 4 barley Cul n 2-treated horses was incubated with increasing amounts of *Escherichia coli*-rCul n 2 (●) or *Pichia*-rCul o 2 (■) or an unrelated protein (▲) as negative control. Pre-incubated serum samples were transferred to wells coated with *E. coli*-rCul n 2 and residual equine immunoglobulin G1-binding determined by enzyme-linked immunosorbent assay.

··■··

the mouth has given promising results against pollen allergy in humans [20]. We were interested in testing a similar approach against insect bite hypersensitivity in horses. For this purpose, we expressed allergens in barley and developed a device to treat horses. To test the device and the concept, healthy Icelandic horses were treated with transgenic barley grains expressing one allergen and the immune parameters measured.

A flour mixture was made where the rCul n 2 allergen was released from the barley (Fig 2d) and the induction of an immune response was evaluated by measuring Cul n 2 specific antibodies. Furthermore, as many different *Culicoides* species can induce insect bite hypersensitivity, we also wanted to evaluate whether the oral treatment with an allergen derived from one *Culicoides* species would result in an immune response against the corresponding allergen derived from another species. This was possible as the hyaluronidase from both *C. nubeculosus* (*E. coli*-rCul n 2) and from *C. obsoletus* (*Pichia*-rCul o 2) were available. The amino acid identity between Cul n 2 and Cul o 2 is 75% [5].

The immunoglobulin classes of horses are IgM, IgD, IgG, IgE and IgA and 7 IgG subclasses [21] with different functions. IgG1 and IgG4/7 are thought to be essential for protection against viral and bacterial infections while the IgG3 and IgG5 have been associated with Th2 response and extracellular pathogens [22]. After a treatment period of 20 weeks, the transgenic barley treated horses had a significantly higher IgG1 response against *Pichia*-rCul o 2 than the controls, whereas the IgG1 response on *E. coli*-rCul n 2 did not reach significance. This was also the case for IgG4/7, probably due to the fact that the pre-treatment sera showed very high IgG4/7 background on the *E. coli*-rCul n 2, used in the ELISA. A high IgG4/7 background to *E. coli* expressed proteins has been observed previously in sera from some horses [14] and may be due to IgG4/7 antibodies against trace amounts of contaminating *E. coli* proteins present in the recombinant protein (purity of 95% [4]). No IgG4/7 background reactivity was seen on the *Pichia*-rCul o 2, as this protein was expressed in yeast (data not shown), and a significant difference was observed between the transgenic barley treated horses and control horses after treatment when tested on *Pichia*-rCul o 2 (Fig 3). Interestingly, both Cul n 2 specific IgG1 and IgG4/7 could be effectively boosted with one barley dose 8 months after the last treatment, and in most transgenic barley treated horses IgG1 and IgG4/7 responses were higher after the boost than after the initial treatment. These IgG1 and IgG4/7 responses were particularly clear when tested on *Pichia*-rCul o 2 (Fig 3). To prove that this increase in Cul o 2-specific antibodies was due to cross-reactivity, a competitive inhibition ELISA was done exemplarily for IgG1. Indeed, antigen-specific binding of treatment-induced rCul n 2-IgG1 was inhibited by *Pichia*-rCul o 2, although, as can be expected, to a lower extent than by rCul n 2, which was the immunogen (Fig 6).

In the saliva, a significant difference in IgG1 response was observed between the transgenic barley treated horses and the controls on both *E. coli*-rCul n 2 and *Pichia*-rCul o 2. The IgG4/7 results were less clear; 3 out of 4 transgenic barley treated horses responded on *E. coli*-rCul n 2 but only one differed from the control horses on *Pichia*-rCul o 2 (Fig 4). The IgG5 response both in serum and saliva was very low or absent. An attempt was made to measure allergen specific IgA but we were not able to show difference between the controls and the transgenic barley treated horses (data not shown). However, one of the transgenic barley treated horses showed a low IgE response in the serum on *Pichia*-rCul o 2 that increased with the boost. This was not seen with *E. coli*-rCul n 2 (Fig 3). *Simulium vittatum* (black flies) are indigenous in Iceland [23]. A cross-reactivity between *Culicoides* and *Simulium* salivary antigens has been shown previously [24]. Although sensitisation to *Simulium* has not been noted in horses in Iceland, it cannot be excluded that the horse was sensitised prior to the treatment. Consequently, the generation of IgE could be expected as it is well-known in human allergy that allergen specific immunotherapy often induces a raise in allergen specific IgE at the beginning of treatment, followed by a very slow IgE decrease [25].

Changing the balance towards an allergen specific Th1 response and generating regulatory T cells are crucial factors in successful immunotherapy [26]. Another important immune parameter of induced clinical tolerance is IgG antibodies that have the capacity to inhibit IgE-binding to the specific allergen [27]. Sera from the transgenic barley treated horses were able to partly block the binding of IgE to both *E. coli*-rCul n 2 and *Pichia*-rCul o 2, indicating that the immune response we aimed for had been induced.

Conclusion

We have developed a device and a method for the oral treatment of horses with transgenic barley expressing allergens. Healthy Icelandic horses responded to treatment with barley expressing a *C. nubeculosus* allergen with a specific IgG response in the blood and saliva. The antibodies induced could partly inhibit IgE-binding to the allergen as well as to the corresponding allergen from another *Culicoides* species. This pilot study indicates that oral treatment with barley expressing allergens could be a promising option for both prophylactic and therapeutic treatment against insect bite hypersensitivity and deserves further study.

Authors' declaration of interests

Einar Mäntylä declares ownership of shares in ORF Genetics Ltd.

Ethical animal research

The experiments were performed in accordance with a permit from the National Animal Research Committee of Iceland, no. 2013-03-02.

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Authorship

S. Jonsdottir contributed to the study design, study execution, data analysis, interpretation of data, writing and final submission of the manuscript. V. Svansson contributed to the study design, study execution, interpretation of data and revision of the manuscript. S. Stefansdottir contributed to the study execution. E. Mäntylä contributed to the production of the barley and revision of the manuscript. E. Marti contributed to the study design, study execution, data analysis, interpretation of data and writing of the manuscript. S. Torsteinsdottir contributed to the study design, study execution, data analysis, interpretation of the data and writing of the manuscript. All authors approved the final manuscript.

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Appendix

Table 6: Comparison of the IgE ELISA with rCul n 3 and rCul n 4 produced in different expression systems.

Expression systems	Spearmann correlation coefficient		Kappa*	
	Cul n 3	Cul n 4	Cul n 3	Cul n 4
Barley/*E.coli*	0.73	0.88	0.86	0.86
Barley/Bac	0.65	0.86	0.75	0.86
Bac/*E.coli*	0.60	0.85	0.71	0.82

*Agreement between binary IgE ELISA results (positive/negative) with the r-allergens expressed in different expression systems was assessed by use of Kappa statistic (κ), which evaluates the proportion of agreement that occurred beyond that expected due to chance alone. Values of Kappa between 0.4 and 0.5 indicate a moderate level of agreement, those between 0.5 and 0.6 can be regarded as acceptable, and those between 0.6 and 0.8 are good. If Kappa=1, there is perfect agreement.

Table 7: Difference between IBH and healthy horses within each group of origin.
PostHoc test to analyze the difference in IgE response against rCul n 3 and rCul n 4 between IBH-affected and healthy horses within each group of origin. The difference was analyzed for each expression system.

		rCul n 3			rCul n 4		
Expression system	**Group**	**Esti. diff**	**p-value**	**Significant**	**Esti. diff**	**p-value**	**Significant**
E.coli	European born Icelandic	0,881	0,017	*	0,501	0,229	ns
	Icelandic born Icelandic	1,260	≤ 0,001	***	1,746	0	***
	European born various breeds	0,924	0,01	**	1,257	≤ 0,001	***
Insect cells	European born Icelandic	0,718	0,092	ns	0,869	0,003	**
	Icelandic born Icelandic	0,779	0,007	**	1,520	≤ 0,001	***
	European born various breeds	1,587	≤ 0,001	***	1,297	≤ 0,001	***
Barley seeds	European born Icelandic	0,896	0,014	*	0,550	0,149	ns
	Icelandic born Icelandic	1,810	≤ 0,001	***	1,868	0	***
	European born various breeds	1,554	≤ 0,001	***	1,284	≤ 0,001	***

Table 8: Difference between groups of origin.
PostHoc test to analyze the difference in IgE response against rCul n 3 and rCul n 4 between the groups of origin. The difference was analyzed for each expression system.

			rCul n 3			rCul n 4		
Contrast			Esti. diff	p-value	Significant	Esti. diff	p-value	Significant
E.coli	H	Euro. born Icelandic - Ice. born Icelandic	-0,404	0,655		0,136	0,994	
		Euro. born Icelandic -Euro. born various breeds	-0,244	0,980		0,504	0,053	.
		Ice. born Icelandic - Euro. born various breeds	0,160	1,000		0,369	0,338	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-0,784	0,088	.	-1,110	≤ 0,001	***
		Euro. born Icelandic -Euro. born various breeds	-0,287	0,995		-0,252	0,984	
		Ice. born Icelandic - Euro. born various breeds	0,496	0,590		0,857	0,007	**
Bac	H	Euro. born Icelandic - Ice. born Icelandic	-0,422	0,596		-0,313	0,549	
		Euro. born Icelandic -Euro. born various breeds	0,944	0,001	***	0,052	1,000	
		Ice. born Icelandic - Euro. born various breeds	1,366	≤ 0,001	***	0,365	0,349	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-0,483	0,696		-0,963	0,003	**
		Euro. born Icelandic -Euro. born various breeds	0,076	1		-0,376	0,850	
		Ice. born Icelandic - Euro. born various breeds	0,559	0,415		0,588	0,175	
Barley	H	Euro. born Icelandic - Ice. born Icelandic	-0,282	0,948		0,007	1	
		Euro. born Icelandic -Euro. born various breeds	-0,059	1,000		0,179	0,959	
		Ice. born Icelandic - Euro. born various breeds	0,223	0,991		0,171	0,970	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-1,196	≤ 0,001	***	-1,311	≤ 0,001	***
		Euro. born Icelandic -Euro. born various breeds	-0,718	0,289		-0,555	0,432	
		Ice. born Icelandic - Euro. born various breeds	0,478	0,641		0,756	0,028	*