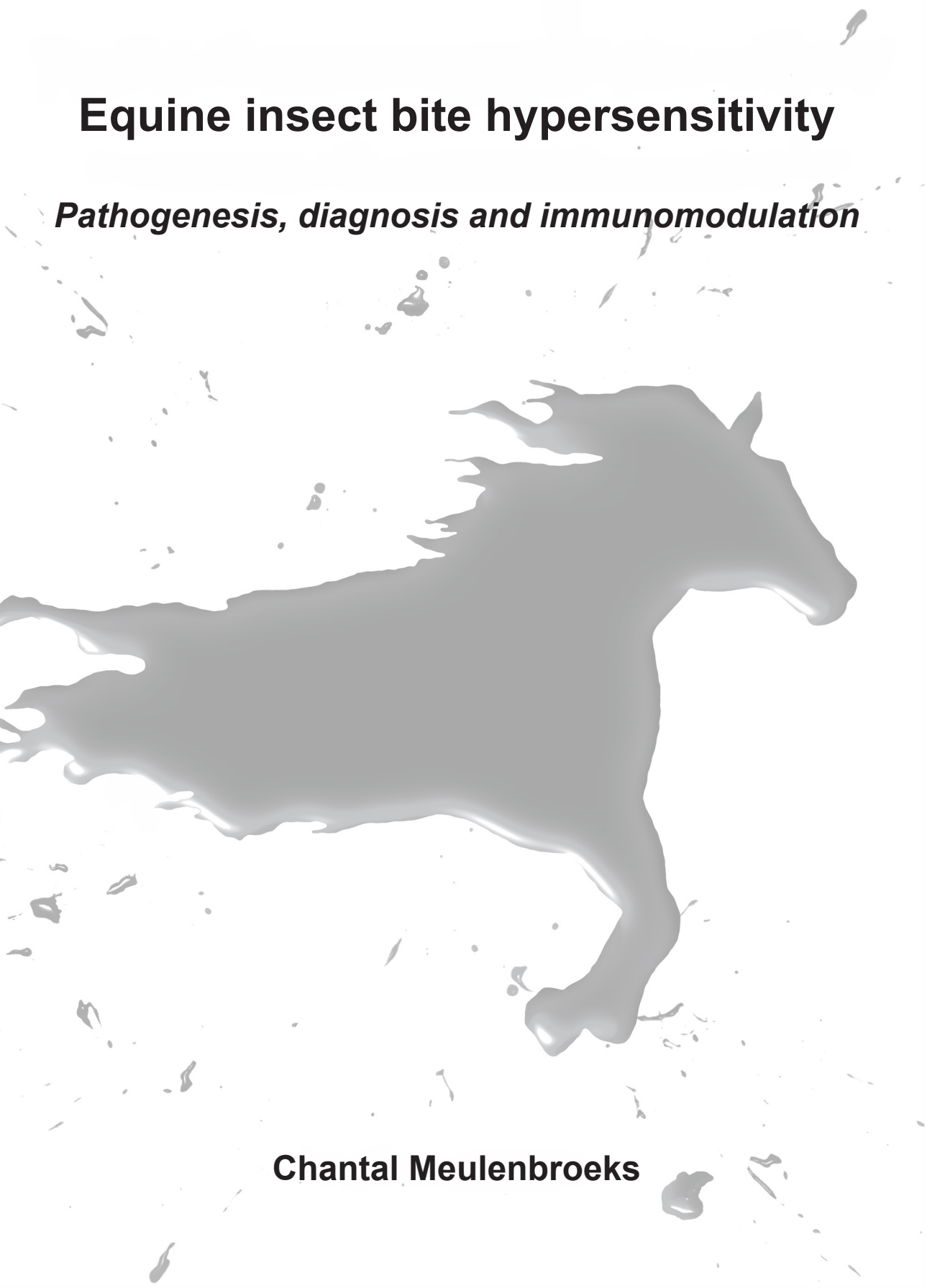
Equine insect bite hypersensitivity

Pathogenesis, diagnosis and immunomodulation



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Pathogenesis, diagnosis and immunomodulation

Staart- en maneneczeem
Pathogenese, diagnose en immunomodulatie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 5 januari 2016 des middags te 4.15 uur

door

Chantal Meulenbroeks
geboren op 18 mei 1981 te Hoogeloon, Hapert en Casteren

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Contents

Chapter 1

General Introduction

Insect bite hypersensitivity (IBH) is an IgE-mediated allergic skin disorder in horses and ponies caused by bites of *Culicoides spp*. It is also known as summer eczema, sweet itch, and Queensland itch.[1-4] The disease is observed worldwide with an onset between 2 and 4 years of age in general.[3, 5, 6] The clinical manifestations of IBH include: severe pruritus with oedema, papules, crust formation, and alopecia.[5, 7-9] Severely affected horses cannot be shown are not suitable to ride due to the great discomfort they endure and are sometimes euthanased.[10, 11] The current treatment strategies are mainly preventative methods and aim at reduction of exposure to the *Culicoides spp*. by stabling and/or using blankets and insect repellents.[11-13] Unfortunately, at present fully effective treatment or prevention methods are not available.

IBH and *Culicoides spp.*

IBH is considered to be a serious problem worldwide with the exception of Antarctica, New Zealand and Iceland[14]. Although IBH occurs in most breeds, it is more prevalent in Shetland ponies, Friesian and Icelandic horses.[15, 16] The prevalence ranges from 3-11% of the entire horse/pony population in areas in the UK, 37%[17] in parts of Germany[18], 10-60% in areas of Australia[4] and 0-71% in regions of The Netherlands[19]. On a breed level especially horses imported from Iceland have a prevalence of more than 50%.[20, 21]

IBH has a multifactorial etiology and several studies suggest that allergic individuals are genetically predisposed.[5, 15, 16, 22] *Culicoides* are small biting insects, measuring 1-3 mm in size, which belong to the family Ceratopogonidae **(Fig. 1)** and feed on blood derived from mammals and birds.[14] The 1400 species are identified by their size and wing patterns.[23] In the Netherlands about 25 *Culicoides* species can

Figure 1: *Culicoides obsoletus.* (Nathalie van der Meide)

be found, the most common being *C. obsoletus, C. impunctatus, C. pulicaris and C. dewulfi*.[24] *Culicoides,* are mainly active at sunset and sunrise during the summer months on warm, dry and windless days.[25, 26] Since *C. obsoletus* is the most abundant *Culicoides spp*. attracted by horses, [27-30] this may indicate that *C. obsoletus* is one of the primary causes of IBH worldwide.

Increased IBH prevalence in imported Icelandic horses compared to other breeds or EU-born Icelandic horses is not fully understood, but it is suggested that the lack of *Culicoides spp*. exposure before export and increased environmental pressure after export, play a role.[1, 20] When Icelandic horses imported from Iceland to Europe, get exposed to allergens between 7 and 10 months of age, they do not develop IBH more frequently than Icelandic horses born in Europe, suggesting that if immune tolerance occurs against IBH, it happens within the first 10 months after birth.[31] In these horses the risk to develop IBH increases with the age at import. In addition, Icelandic horses born in a country where *Culicoides* is present do not seem to be more susceptible to develop IBH in comparison to other breeds.[31]

IBH immunopathogenesis

The adaptive immune response plays a role in host defense against infection and is essential for health. Apparently harmless antigens like Culicoides saliva can cause hypersensitivity, which can result in chronic inflammatory processes and even death.[32]

Hypersensitivity reactions are classified into four types. IBH is a combination of Type I (IgE-mediated) and Type IV (delayed-type hypersensitivity, being T-cell mediated).[10, 33-35] IBH belongs to Type IV group 2 responses which is characterized by inflammatory responses, the presence of large amounts of eosinophils and are activated by Th2 cells.[32, 36] Recently, it was reported that Th17 cells are also involved in allergic reactions such as contact hypersensitivity and Th2 delayed type hypersensitivity[37] and thus they might play a role in IBH. IBH can be subdivided in two phases, a sensitization phase and an effector phase **(Fig. 2)**.

The sensitization phase occurs when a naïve individual is exposed to allergens, in case of IBH saliva proteins from *Culicoides spp*. A antigens that enter the skin are recognized by immature dendritic cells (DC), mainly Langerhans cells (LC) in the skin, through pattern recognition receptors such as the Toll-like receptors. LC internalize the antigen, either by phagocytosis or by receptor-mediated endocytosis.[32, 38] After internalization the antigen is processed into small peptides and upon maturation of the LC the peptides are presented on its cell surface via the major histocompatibility complex II (MHCII) **(Fig. 2)**. The LC migrate to the lymph nodes and present the antigenic peptide to the T-cell receptor (TCR) on naïve $CD4^+$ T-cells; costimulatory signals that are necessary to continue the immune response are produced by the interaction between molecules such as CD28 (B7) on T-cells and CD80/CD86 on LC **(Fig. 2)**.[39]

Early IL-4 production causes the naïve $CD4^+$ T-cells to proliferate and differentiate into T Helper 2 (Th2) cells, producing cytokines like IL-4, IL-5 and IL-13. It is still unclear what causes this early IL-4 production, but it is most likely due to the presence of thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 produced by epithelial and endothelial cells.[39, 40] The Th2 cytokine production will eventually skew the (T helper 1) Th1/Th2 balance to Th2.

The TCR of allergen-specific Th2 cells present in the lymph nodes may interact with allergen-specific B cells, of which MHC II molecules are loaded with allergenic peptides. Upon costimulation with molecules like CD40 and CD40L (CD154),[41-44] the Th2 cells expose B cells to IL-4, IL-5, IL-10, IL-13 and GM-CSF, eventually

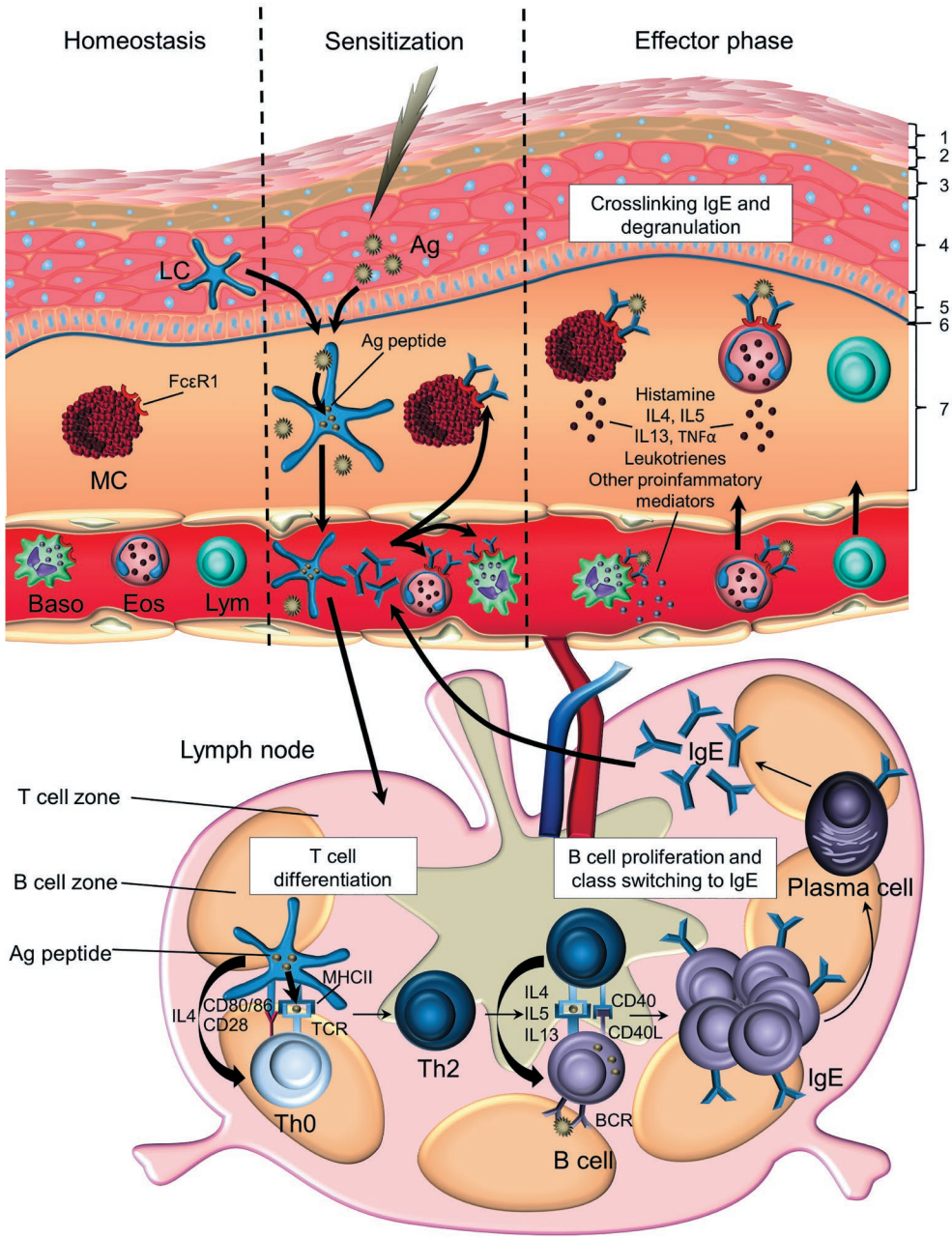

Figure 2: Simplified schematic representation of the IBH pathogenesis.
(1) Stratum corneum, (2) Stratum lucidum, (3) Stratum granulosum, (4) Stratum spinosum, (5) Stratum basale, (1-5) Epidermis, (6) Basement membrane and (7) Dermis.

resulting in clonal expansion, isotype switching to immunoglobulin E (IgE) [45] and differentiation into plasma cells.[46] The antigen-specific IgE produced spreads through the bloodstream and the lymphatic system into the skin, where it binds to the high-affinity IgE receptors (FcεR1) on mast cells and eosinophils **(Fig. 2)**. Basophils do also express the FcεR1 and can bind IgE, but they reside in the circulation where they, more rapid than mast cells, produce IL-4 and IL-13 and thus enhance inflammation. In humans, IgE has a short half-life of around 2 days when circulating in the serum, but when bound to FcεRI on mast cells, it increases to up to several months.[47] The total IgE level of horses with skin allergy is not significantly higher than in healthy horses kept in the same environment, except for imported Icelandic horses.[48, 49] However, horses suffering from IBH do have more serum *Culicoides*-specific IgE antibodies compared to healthy control horses.[34, 50-52]

Individuals are considered to be sensitized for a particular antigen when antigen-specific IgE is bound to mast cells, eosinophils and or basophils. Sensitized individuals do not show any clinical symptoms yet. There are sensitized individuals that when exposed to the antigen a second time will never develop clinical symptoms, these are considered to be non-allergic.[32] To develop allergic symptoms, a sensitized individual needs to be re-exposed to the same antigen **(Fig. 2)**. The antigen-specific IgE antibodies present on mast cells, basophils and eosinophils can bind repetitive epitopes present on the allergens and the cross-link FcεR1. Mast cells, eosinophils and basophils, responsible for the late-phase cell-mediated responses,[32] degranulate and/or release vasoactive amines (histamine and serotonin), inflammatory cytokines (TNFα, IL-4, IL-9 and IL-13), vascular permeabilization mediators (e.g. leukotrienes [53]) and autocrine molecules which attract and activate eosinophils and regulate inflammation (e.g. prostaglandins). [54] Mast cell mediators can also attract neutrophils which are phagocytes.[55] The cytokines that are released can activate mast cells causing and positive feedback loop.[54, 56-58] These combined mediators result in inflammation and pruritus.

In addition, to the cells mentioned before, $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T-cells (Treg) are suggested to play an important role in allergy in humans. Horses too have these $CD4^{+}CD25^{high}FoxP3^{+}$ Treg cells, able to suppress effector T-cells and to produce IL-10, like human Treg cells.[59] In humans, Treg cells can suppress and regulate the proliferation and cytokine production of many immune cells, including Th2 cells and APC, through different mechanism e.g. IL-10, Transforming growth factor beta (TGF-β), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Programmed cell death protein 1 (PD-1), and glucocorticoid-induced TNFR-related protein (GITR) **(Fig. 3)**.[60] have a direct and indirect suppressive effect on mast cells, eosinophils and basophils by Treg producing IL-10 and TGF-β. In addition, IL-10 derived from Treg cells, can promote B cell survival and IgG4 production leading to a reduced IgE production. Such IL-10 can also suppress allergen-specific Th2 cells by blocking the CD80/CD86-CD28 pathway and preventing allergen presentation to the Th2 cells. Additionally, it inhibits DC maturation and subsequently MHCII, CD80/CD86 and IL-4 expression leading to an impaired antigen presentation. Finally, it creates a positive feedback loop by promoting the production of IL-10-producing Treg cells.[61]

TGF-β also inhibits T-cells function and the secretion and activity of many other cytokines, including interferon-γ (IFNγ), tumor necrosis factor-alpha (TNF-α) and various interleukins. It can also decrease the expression levels of cytokine receptors,

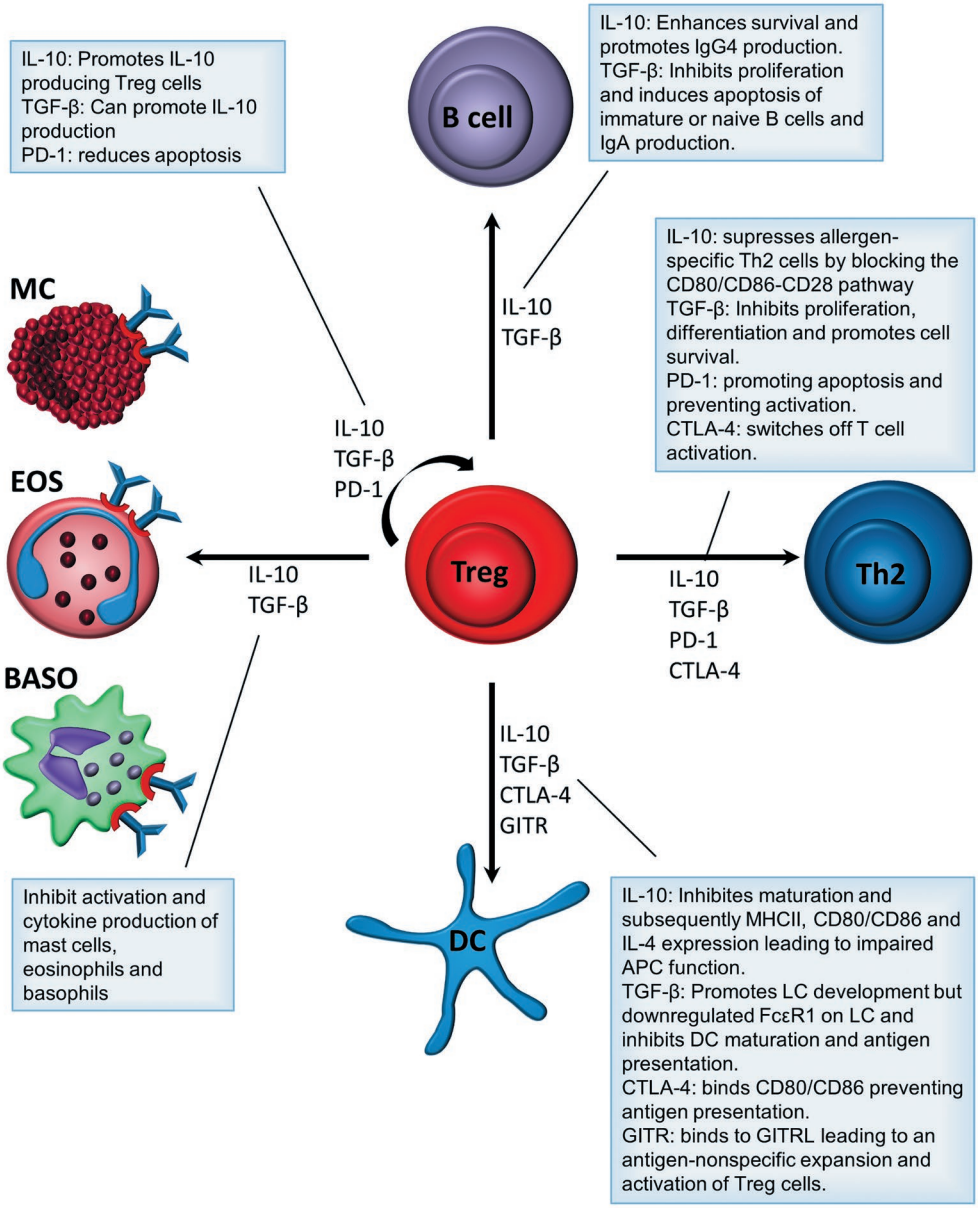

Figure 3: Potential role of regulatory T-cells (Treg) in IBH.

such as the IL-2 receptor to down-regulate the activity of immune cells [62] TGF-β1 has similar effects on B cells and inhibits proliferation and stimulates apoptosis,[63] and plays a role in controlling the expression of IgE and MHCII proteins on B cells in allergic diseases.[62, 63]

Allergens

Culicoides saliva and whole body extracts contain many proteins that have been shown to react with IgE derived from IBH-affected horses, hence may be considered as allergens.[34, 50-52, 64] However, each IBH-affected horse seems to possess a different IgE-affinity pattern for different *Culicoides* proteins, suggesting that multiple allergens are involved in the immunopathogenesis of IBH.

Two dominant IgE-binding proteins of 65 kDa and 66 kDa from respectively *C. obsoletus* (a native European species) whole body extract (WBE) and *C. sonorensis* (a North American species) saliva gland extract were found to interact primarily with serum from IBH-affected horses.[34, 65] The 66 kDa protein was further characterized and identified to be a maltase protein and named Cul s 1. Recombinant Cul s 1 was found to bind with IgE from a large group of IBH-affected horses and not control horses, confirming its allergenicity. In the same study, more candidate allergens were identified derived from the salivary glands from laboratory bred *C. nubeculosus*.[64] Two abundant proteins of 40-50 kDa showed sequence similarity to a hyaluronidase and a heavily glycosylated protein of unknown function from an EST library from *C. sonorensis*.[66] In another study, ten IgE binding proteins derived from *C. nubeculosus* were identified and expressed as recombinant allergens, and labeled Cul n 2 to Cul n 11. Forty-four out of the 45 IBH-affected horses tested, reacted with at least one of these ten allergens.[52]

In skin tests cross-reactivity of antibodies with different *Culicoides spp.* was found in Northern Germany and British Columbia with extracts and saliva of native and foreign *Culicoides spp.,* indicating the presence of species-shared allergens.[67, 68] In spite of this, it was shown that IBH-affected horses in the Netherlands had stronger IgE responses against proteins from *C. obsoletus*, the most common species found on horses, as compared to reactivity to proteins from *C. nubeculosus*.[28, 64] This may be due to low homology between IBH-relevant allergens of *C. obsoletus* and *C. nubeculosus.*[69] Recently, seven IgE-binding *C. obsoletus* complex allergens were identified and labeled Cul o 1 to Cul o 7. Cul o 1 has a homology of 78% with the previously mentioned Cul s 1.These recombinant antigens were expressed in baculovirus and *E. coli* and were able to discriminate between IBH-affected ponies and healthy controls in an IgE ELISA and in intradermal allergy tests.[69, 70] Two combinations of recombinant antigens: combi 1 (Cul o 3, 5 and 7) and combi 2 (Cul o 1, 2 and 7), were equally effective in identifying IBH-affected horses as WBE derived from the *C. Obsoletus* complex, assessing allergen-specific IgE serum levels.[70] Even though some cross-reactivity to antigens of different *Culicoides* species has been reported, the use of recombinant allergens from native *Culicoides* species might be important for diagnosis and future immunotherapy.

Diagnosis

Currently, the golden standard to diagnose IBH remains a well-documented medical history and observation of clinical symptoms in the summer, sometimes supplemented with the results of an intradermal allergy test.[71] However, there are also other tests such asan allergen-specific IgE ELISA, a basophil degranulation test, and the cellular antigen stimulation test (CAST), which may support the clinical diagnosis of IBH.

The intradermal allergy test is used to determine sensitization of skin mast cells

for a wide range of allergens in horses.[33, 72, 73] However, some studies reported low sensitivity and specificity [73], difficulty of interpretation and low reproducibility of this test. [33, 72, 73]

The basophil degranulation test, also called the histamine release test (HRT) or the functional *in vitro* test (FIT), can be used as an alternative for intradermal allergy testing.[68] It is performed on whole blood and determines the histamine release by sensitized (IgE+) basophils. In contrast, the CAST determines the sulfidoleukotrien release by peripheral blood leucocytes.[71] Although sulfidoleukotrien production was found to be highly correlated with histamine release from basophils, the sensitivity of the test decreased when horses were not exposed for several months,[71] This renders the test less useful for a diagnosis off-season.

The allergen-specific IgE ELISA can be used to determine *Culicoides*-specific IgE levels in horse serum.[70, 74, 75] A recent study has shown that the use of a pool of recombinant *C. obsoletus* antigens in this ELISA enables diagnosis of IBH with high sensitivity and specificity compared to individual recombinant allergens.[70]

Allergic contact dermatitis and UVB irradiation

Since in IBH immune responsiveness is partly displayed as delayed type hypersensitivity (DTH), it is of interest to focus briefly on interventions regarding this aspect of IBH. Allergic contact dermatitis, another example of DTH in the skin, may be studied in a DNFB (dinitrofluorobenzene) contact hypersensitivity (CHS) model in mice.[76] DNFB, like *Culicoides* allergens, is taken up by APC resident in the skin, primarily LC. In the effector phase DNFB stimulates DNFB-specific T-lymphocytes residing in the dermis to release pro-inflammatory cytokines.[77-79] The model showed that ultraviolet B (UVB) light (290-320nm) can suppress antigen-specific DNFB-mediated CHS in naïve mice.[80-82] Although, the mechanism behind this is not fully understood yet, it has been suggested that UVB suppresses mast cell degranulation in the skin.[83] Moreover, upon DNFB stimulation UVB induces antigen-specific $CD4^+CD25^+CTLA\text{-}4^+$ Treg cell (UVB-Treg) binding to the c-type lectin dectin-2 present on APC[84] which causes UVB-Treg activation, high amounts of IL-10 secretion,[85, 86] apoptosis of antigen-presenting dendritic cell by the use of the Fas/Fas-ligand system[87] and DNFB tolerance.[84] In clinical settings UVB radiation is widely used in the treatment of skin diseases in humans, such as atopic dermatitis, psoriasis, mycosis fungoides and vitiligo.[88-92] In the standard treatment protocol, patients are treated with low dose UVB light three times a week for 6 to 13 weeks before beneficial effects are observed.[93-95]

Outline thesis

The research described in this thesis is part of a larger project that aimed at improving the current understanding of the immunology, epidemiology and genetic aspects of IBH, which may eventually lead to improved diagnosis and development of intervention and or preventative strategies. The studies in this thesis focused at further elucidation of the immunopathogenesis as well as potential immune modulatory strategies for IBH. In **chapter 2** whole body extracts (WBE) from *C. obsoletus, C. nubeculosus* and *C. sonorensis* are compared for their application in diagnostic tests and to determine of which *Culicoides* species allergens should

be used in future studies. In **chapter 3** the Th1/Th2 balance, Treg cell activation and presence are examined in lesional, non-lesional and healthy skin in the IBH season compared to the off-season. In addition, in **chapter 4** we will examine the Th1/Th2 balance and Treg cell involvement in the skin of IBH-affected and non-affected ponies upon challenge with *C. obsoletus* WBE. In **chapter 5** we investigate if recombinant *C. obsoletus* WBE allergens can activate Treg and $CD4^+$ T-cells *in vitro*. The mechanism behind UVB-mediated immunosuppression will first be examined in a DNFB contact hypersensitivity mouse model (**chapter 6**), followed by a pilot experiment in which the potential immunomodulatory effect of UVB on the skin of IBH-affected horses will be studied (**chapter 7**). In the final **chapter 8** the results of our studies are discussed with reference to future diagnosis and treatment of IBH.

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Chapter 2

Culicoides obsoletus extract relevant for diagnostics of insect bite hypersensitivity in horses

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Abstract

Insect bite hypersensitivity (IBH) is an allergic dermatitis in horses caused by the bites of Culicoides species.

The aim of the present study was to evaluate the applicability of whole body extracts of C. obsoletus (the main species found feeding on horses in the Netherlands), C. nubeculosus (rarely found in The Netherlands) and C. sonorensis (typical for North America) for diagnosis of IBH in horses in The Netherlands.

Blood and serum samples of 10 clinically confirmed IBH affected and 10 healthy control horses were used to evaluate the IgE titers (ELISA) against the Culicoides whole body extracts of the three Culicoides species. Basophil degranulation was assessed by histamine release test (HRT) after stimulation with these extracts at 5, 0.5 and 0.05 µg/ml.

IBH affected horses had significantly higher IgE titers against C. obsoletus than against C. nubeculosus and C. sonorensis. Furthermore, C. obsoletus induced significantly higher histamine release in whole blood of IBH affected horses compared to the other extracts at 0.5 µg/ml. Western blot data revealed IgE binding to many proteins in C. obsoletus extract. This interaction was absent or weak in C. nubeculosus and C. sonorensis extracts for IBH affected horses.

Results on individual level indicate that the HRT is more sensitive than ELISA in diagnosing IBH. However, ELISA is more practical as a routine test, therefore the ELISA was further evaluated using C. obsoletus extract on 103 IBH affected and 100 healthy horses, which resulted in a test sensitivity and specificity of 93.2% and 90.0%, respectively. The IgE ELISA readings enabled the analysis of the predicted probability of being IBH affected. From an optical density 450 nm value of 0.33 onwards, the probability of IBH affected was more than 0.9. The results presented in this paper show that the use of native Culicoides spp. that feed on horse, is important for improved diagnosis and that the described ELISA based on C. obsoletus can be used routinely to diagnose IBH in countries where this species is the main Culicoides feeding on horses.

Introduction

Insect bite hypersensitivity (IBH), also called 'sweet itch' or 'summer eczema', is a seasonal recurrent allergic dermatitis in horses caused by an allergy against the bites of midges (*Culicoides* spp), or sometimes black flies (*Simulium* spp) and to an even lesser extend other insects[1-5]. IBH is found in many countries of the world with a prevalence ranging from 3 - 11.6% in areas in the UK[6, 7]; 10 – 60% in areas of Queensland, Australia[8] and 0 -71.4% in regions of The Netherlands[9].

Insect Bite Hypersensitivity is clinically characterized by strong pruritus and irritation, leading to alopecia and even secondary lesions due to scratching and rubbing. These symptoms are particularly found along the preferred feeding sites of the insect, which is the ventral midline, mane and tail region of the horse[3, 10]. Several studies indicate that the allergic reaction is predominantly IgE-mediated[11, 12]. However IgG (T) also seems to be involved[13]

Intradermal injections with *Culicoides* extracts often induce immediate and delayed type skin reactions in allergic horses[14, 15]. In Iceland, where *Culicoides* spp do not occur, IBH has never been reported[16, 17]. Several *Culicoides* species have been associated with IBH, including *C. sonorensis*, *C. nubeculosus*, *C. imicola*, *C. obsoletus* and *C. pulicari* [4, 15, 18-20]. Intradermal tests on allergic and healthy control horses in Northern Germany and British Columbia with extracts and saliva of native and exotic *Culicoides* species, showed no difference between the native and exotic *Culicoides* species, indicating the presence of species-shared allergens[21, 22]. However, intradermal skin tests in The Netherlands, with a commercial extract of *C. nubeculosus* and wild-caught *C. obsoletus,* showed the lack of cross reactivity between these *Culicoides* species[15, 23].

The aim of the present study was to evaluate three *Culicoides* species for their applicability in diagnostic tests for IBH of horses in The Netherlands: *C. obsoletus* which is most frequently found on horses in The Netherlands[24, 25], *C. nubeculosus,* widely distributed in Europe, but only occasionally detected in The Netherlands[26] and not found to be attracted to horses[24] and *C. sonorensis* which is only present in North America. Currently, *C. sonorensis* and *C. nubeculosus* are often used in studies about IBH, because they can be successfully maintained in laboratory bred colonies[27]. *C. obsoletus* however, are not available from laboratory bred colonies and have to be collected from the wild. An attempt to breed *C. obsoletus* has been made, but was not very efficient[28]. Results presented in this report show that the use of native *Culicoides* spp that feed on horse, is important for improved diagnosis and possibly, for future immunotherapy development. A diagnostic ELISA for IBH based on *C. obsoletus* is described that can be used routinely and has a high specificity and sensitivity.

Material and methods

Animals

A total of 223 horses located in different regions of The Netherlands were included in this study. Pairs of clinically confirmed IBH affected and healthy control horses

kept at the same location were formed[29]. Ten clinically confirmed IBH affected and 10 healthy control Shetland ponies were used to compare the different *Culicoides* whole body extracts in different *in vitro* diagnostic tests. The remaining 203 horses (76 Icelandic horses and 127 Shetland ponies) were used to evaluate the predictive value and test sensitivity and specificity of an ELISA using *C. obsoletus* whole body extract.

Blood samples were taken from all horses and serum was frozen in aliquots not later than 24 hours after blood sampling and stored at -20 o C until use. Blood sampling was approved by the Board on Animal Ethics and Experiments from Wageningen University and Utrecht University.

Collection of Culicoides insects

C. obsoletus insects were captured during spring and summer months using a pooter (aspirator to collect insects) **(Supp. Fig. 1)** or an "Onderstepoort" suction light trap kindly provided by Laboratory of Entomology, Wageningen University.

Horses wearing an anti-insect blanket were put outside around dawn hours on warm (> 20 °C), dry and low wind days and *Culicoides* insects were collected directly from the horses using the pooter. The insects were collected and completely frozen alive at -80 °C and stored at that temperature until preparation of the extracts. A small fraction (5%) of the insects collected with the pooter was checked under a stereo microscope to confirm the species. Identification of *C. obsoletus* was based on size and wing patterns[30] **(Supp. Fig. 2)**. The "Onderstepoort" suction light trap was operated from before dusk to far after dawn near a horse stable for 19 days at different locations in The Netherlands in the summer months of 2009. Insects were captured in 100% alcohol and frozen the next day in alcohol at -80 oC until determination. *C. obsoletus* insects were selected and separated from the other captured insects using a stereo microscope as described above. Separated *C. obsoletus* insects were used for the preparation of extracts.

Three-day-old laboratory bred *C. sonorensis* were a kind gift from Arthropod Borne Animal Diseases Research Unit Centre for Grain and Animal Health, Manhattan, US. *C. nubeculosus* insects were kindly donated by the Institute for Animal Health, Pirbright, UK. All insects were kept frozen (without alcohol) at -80 °C until preparation of the extracts.

Preparation of Culicoides protein extracts

Whole body extracts (WBE) were prepared from about 300 insects that were transferred to a 2 ml Eppendorf tube with 1 ml of PBS containing a protease inhibitor cocktail (Sigma-Aldrich, P8849) and crushed with a micro pestle. The insoluble material was removed by centrifugation at 13000 x g for 10 min at 4 °C. Supernatant was collected and filtered through sterile Millex-GV filters (Millipore) with a pore diameter of 0.22 µm and protein content of the filtrate was determined by OD280nm measurement on a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific). Samples were aliquoted, directly frozen in liquid nitrogen and stored at -80 °C until use. Quality of the protein WBE was checked by protein staining with Gelcode Coomassie blue staining (Thermo Scientific) after proteins were separated by 15% SDS-PAGE.

SDS-PAGE and Western blotting

Whole body protein extract samples, 20 µg/lane (Western blotting) or 60 µg/lane (Coomassie staining) were heated at 96 °C for 5 min with sample buffer containing dithiotreitol (DTT) and separated by SDS-PAGE (15% gel). These separated proteins were transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) by means of electrophoresis. Membranes were blocked with 5% non-fat cow's milk in Tris buffered saline (TBS)-Tween (10 mM Tris, 150 mM NaCl, pH 7.5, 0.05% (v/v) Tween 20) for 1 h at room temperature (RT) and then incubated overnight with horse sera from allergic or control horses diluted 1:10 in 5% non-fat cow's milk in TBS-Tween. Membranes were then incubated for 1.5 hour with a mAb against horse IgE (αIgE-176)[31] followed by goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS-Tween). Between each incubation step, membranes were washed three times with TBS-Tween.

Signal was detected by development with an enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham, GE Healthcare) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

Histamine release test (HRT)

The histamine release by basophils was determined by a modified method of Kaul[32]. Blood samples were collected in anticoagulant tubes (EDTA) and kept at RT in the dark until further use within 24 hours. The total blood cells were washed twice (500 x g for 10 min) with PBS to remove non-cell bound antibodies. Supernatant was discarded and the cell pellet was resuspended in PBS to its original blood sample volume. Endogenous histamine from whole body *Culicoides* extracts was removed by PD-10 Desalting columns (GE Healthcare) according to manufacturer's recommendations. The antigen induced histamine release was obtained by incubating 250 µl of washed blood cells with 250 µl of PIPES buffer (110 mM NaCl, 5 mM KCl, 40 mM NaOH, 2 mM CaCl2, 25 mM PIPES, 2 mM MgCl2) containing the histamine depleted *Culicoides* whole body extracts at final concentrations of 5 µg/ml, 0.5 µg/ml and 0.05 µg/ml at 37 °C for 60 min. Spontaneous release was obtained by incubating 250 µl of PIPES B buffer with 250 µl of washed blood cells at 37 °C for 60 min. Physical maximum release was obtained by boiling 200 µl of washed blood cells with 800 µl of PIPES buffer for 10 minutes. After incubation, all samples were chilled on ice for 5 min and pelleted by spinning down at 700 x g for 10 min. The cell-free supernatants were collected and stored at -20 °C. Subsequently, competitive RIA was carried out as per the manufacturer's instructions (LDN Nordhorn, Germany) to determine the histamine content of the supernatants.

The maximum amount of histamine obtained by boiling was set to 100%. The histamine content of each test sample was calculated from this maximum histamine release. The allergen-specific release (ASR) is calculated as:
ASR = (sample induced release – spontaneous release)/ (maximum release – spontaneous release) x 100%. Net-histamine releases that were equal or greater than 10% of the maximum release were considered as positive.

Culicoides-specific IgE ELISA

Specific IgE levels in sera of 10 IBH affected and 10 healthy control Shetland ponies,

binding the different *Culicoides* WBE, were measured by ELISA. Optimal coating concentration, serum dilution and antibody concentrations were determined prior to the experiment by titration of the different components. Costar 96-well microtitre plates were coated with 100 µl/well of 10 µg/ml *C. obsoletus*, *C. nubeculosus* or *C. sonorensis* extract, diluted in PBS, and incubated overnight at 4 °C and afterwards blocked with 150 µl of a 1.5% casein buffer (SDT, Germany) for 1.5 hour at RT. Plates were washed 5 times with PBS containing 0.05% Tween20, followed by incubation for 1.5 h at RT with 100 µl of horse serum samples diluted 1:5 in a 1.5% casein buffer. After washing, wells were incubated for 1 hour at RT with 100 µl of 2.5 µg/ml mouse monoclonal anti-equine IgE-176 (31) diluted in casein buffer. After washing the plates 5 times with PBS containing 0.05% Tween20, goat anti-mouse peroxidase conjugate (multispecies adsorbed, Serotec) diluted 1000 times in casein buffer, was applied to the wells and incubated for 1 h at RT. After 5 washes with PBS/0.05% Tween20, 100 µl tetramethylbenzidine (high sensitivity, SDT, Germany) was added to the wells and incubated for 10 min at room temperature. The reaction was stopped with 100 µl/well of 1% HCl. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices) at a wave length of 450 nm corrected for 650 nm. Based on the preliminary experiments a standard serum dilution of 1:5 was selected as suitable for comparison of OD450nm values in the IgE ELISA. The cut off level was assigned as the mean + 3 times the standard deviation (SD) of the IgE levels of the healthy control horses.

An additional 203 horse serum samples, 103 IBH affected and 100 healthy control horses, were evaluated for *C. obsoletus* specific IgE (OD450nm) values in a 384 wells plate to determine the sensitivity and specificity of this *Culicoides*-specific ELISA. The same conditions as described for the 96-wells plate were used, with 20 µl volumes per well. Distribution plots of the healthy and IBH affected horses were obtained by categorizing horses according to their OD450nm values. The first category ranged from 0 to 0.01, the second from 0.01-0.02 and the following categories each increased with 0.02 up to 0.32. Then the categories ranged from 0.32-0.35, 0.35-0.4 and subsequent categories each increased with 0.2 until the maximum OD450nm of 2.2 was reached.

Accuracy of diagnostic tests is often determined from so-called receiver-operating characteristic (ROC) curves. ROC-curves represent the trade-off between sensitivity (i.e. true positive rate) of a test and (1-specificity) (i.e. false positive rate) at all possible positivity cut-off points. The area under the curve summarizes the overall diagnostic accuracy. It takes values from 0 to 1, where a value of 0 indicates a perfectly inaccurate test and a value of 1 reflects a perfectly accurate test. A good first choice for a test cut-off value that results in a balanced optimal sensitivity and specificity is that value which corresponds to a point on the ROC-curve nearest to the upper left corner of the ROC graph.

Statistical analysis

Analysis of variance was performed on log transformed data obtained by either HRT or ELISA to determine influence of *Culicoides* species on the outcome. Factors included in the model were, *Culicoides*-species (*C. nubeculosus*, *C. sonorensis* and *C. obsoletus*), IBH-status of the horse (yes/no) and interaction between these two factors. Additionally, WBE concentration (0.05, 0.5 and 5 µg/ml) was included in the

analysis of HRT results. IBH-status within individual horses was included as random factor since the same set of horses were used in testing the three *Culicoides* types. Analysis was performed using PROC MIXED of SAS (SAS Inc, V9.2).

The relation of OD450nm value in the IgE ELISA to the IBH-status (negative or positive) was analysed with a logistic regression. The analysis was performed with the PROC LOGISTIC of SAS (SAS Inc, V9.2).

Results

Collection of C. obsoletus

Two different collection methods ("Onderstepoort" light trap and pooter) were used to determine the most selective and efficient way of collecting *C. obsoletes* from the wild.

Determination of insects collected with the "Onderstepoort" light trap revealed that many different insect species were collected in this manner, from which only a small fraction (<1%) belonged to *Culicoides* species. A total of 766 *Culicoides* midges were collected during these 19 days. The large majority of these *Culicoides* were identified as *C. obsoletus* (82%), followed by *C. dewulfii* (6.5%) and *C. punctatus* (5.0%).

Using the pooter hundreds of *Culicoides* insects were easily collected within an hour from a horse wearing an anti-insect blanket. A small part (± 5%) of the collected insects was used for identification and these were all identified as *C. obsoletus* based on size and wing patterns.

Quality of extracts of Culicoides captures by different methods

WBE were prepared from *C. obsoletus* insects collected alive with the pooter system, as well as insects collected in alcohol with the Onderstepoort light trap. Insects that were stored in alcohol were excluded in the following experiments due to substantial degradation of the proteins revealed on SDS-PAGE **(Supp. Fig. 3)**.

The extracts from laboratory bred *C. sonorensis* and *C. nubeculosus* and wild-caught *C. obsoletus* prepared in the same manner from freshly frozen insects, showed some differences in the lower molecular weight regions (indicated by arrows in **Fig. 1**) on SDS-PAGE (15% gel), but overall pattern and intensity of the protein bands were similar, with no obvious degradation, indicating similar quality of the different extracts.

IgE-specific antibodies in horse sera specific for Culicoides proteins as determined by Western blotting

Western blotting was performed to evaluate sera of clinically confirmed IBH affected and healthy control Shetland ponies for the presence of IgE specific for proteins from *C. obsoletus*, *C. sonorensis* and *C. nubeculosus*. Typical examples of 5 allergic (upper panel) and 5 healthy horses (lower panel) are shown **(Fig. 2)**.

The IgE in sera of all allergic horses reacted strongly to a number of proteins from *C. obsoletus* extract, but much weaker with proteins from *C. sonorensis* and C. *nubeculosus* extract despite the similar quality of the extracts. The antigen recognition pattern for each individual horse was different, but most IBH affected horses reacted with a protein(s) around 20 kDa. IgE from the healthy horses, except for one, hardly

recognized any proteins from any of the 3 *Culicoides* species. Proteins that did bind to the IgE in serum of healthy horses all had a Mw of 25 kDa or higher **(Fig. 2)**.

Basophil degranulation induced by Culicoides whole body extracts determined by histamine release test

For each of the 3 different *Culicoides* species the allergen-specific release (ASR) of histamine from basophils was tested at 3 WBE concentrations on whole blood samples of 10 IBH affected and 10 healthy control Shetland ponies **(Fig. 3)**, including the horses that were used for the Western blot analysis.

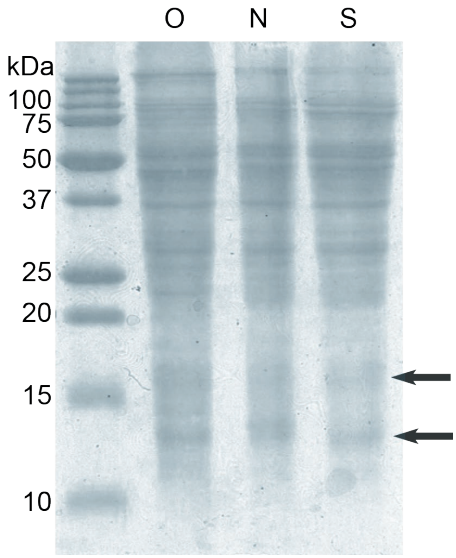

Figure 1: Coomassie staining of proteins from *Culicoides spp.* extract. *C. obsoletus* (O) (, *C. nubeculosus* (N) and *C. sonorensis* (S) whole body extracts demonstrating similar quality of the extracts.

At the highest WBE concentration of 5 µg/ml 10 out of 10 (100%) of the IBH affected horses scored positive on the *C. obsoletus* WBE, while this was 8 out of 10 (80%) for *C. nubeculosus* and *C. sonorensis*. However, at this highest WBE concentration some of the healthy horses also scored positive on all *Culicoides* species. At a concentration of 0.5 µg/ml none of the healthy control horses scored positive on any of the extracts, but at this concentration only 20% of the IBH affected horses scored positive with *C. nubeculosus* and *C. sonorensis* extract. In contrast, 100% of the IBH affected horses scored positive when the *C. obsoletus* extract was used. At this concentration the reactivity towards *C. obsoletus* was significantly higher than to *C. nubeculosus* ($p < 0.01$) and *C. sonorensis* ($p < 0.001$) **(Fig. 3)**. At the lowest WBE concentration of 0.05 µg/ml 40% of the IBH affected horses were still found to be positive with C. *obsoletus*, whereas for *C. nubeculosus* and *C. sonorensis* this was only 10% of the IBH affected horses. Also at this concentration all healthy control horses had a negative test results for all *Culicoides* extracts.

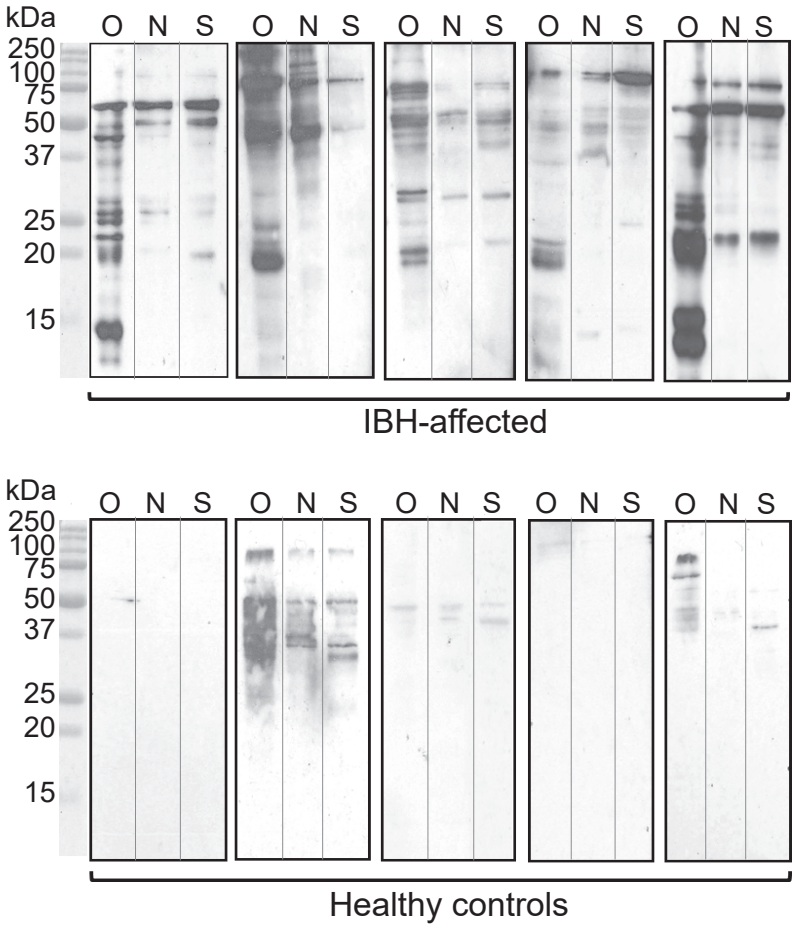

Figure 2: Immunoblot analysis of 5 IBH affected and 5 healthy horses using whole body extracts. Proteins were separated on 15% SDS–PAGE gels and transferred to nitrocellulose membranes. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. *C. obsoletus* (O), *C. nubeculosus* (N) and *C. sonorensis* (S).

IgE measurements in horse sera against Culicoides whole body extracts by ELISA

Specific IgE serum levels against WBE of the three different *Culicoides* species were determined in an indirect ELISA **(Fig.4)**.

IgE levels expressed as OD450nm values of the clinically confirmed IBH affected Shetland ponies against *C. obsoletus* extract were significantly higher compared to OD450nm values of the same IBH affected horses against the other extracts (both $p < 0.0001$). With *C. obsoletus* extract only, specific IgE serum levels of IBH affected horses were significantly higher than specific IgE levels of healthy control horses ($p < 0.0001$).

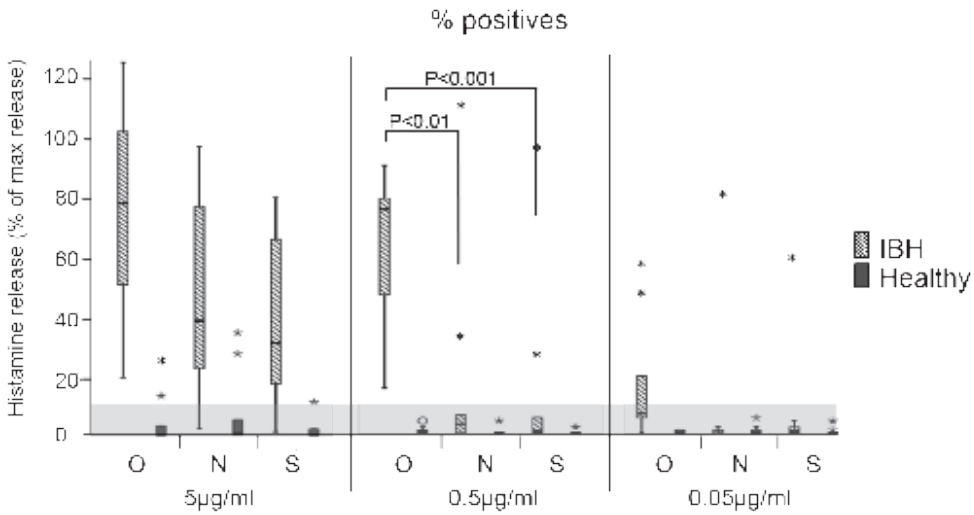

Figure 3: *Culicoides* induced histamine release as percentage of maximum release. Whole blood samples of 10 IBH affected horses and 10 healthy control horses were analyzed after stimulation with three different *Culicoides* whole body extracts (O = *C. obsoletus*, N = *C. nubeculosus*, S = *C. sonorensis*) tested at three different concentrations (µg/ml, x-axis) by a histamine release test (HRT). Results are presented in box plots. The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represents the minimum and maximum value. The stars represent the outliers. Horses with histamine release above the cut off value of 10% (highlighted in gray) were considered positive[32]. Statistical analysis was performed on log transformed data. The p values account for unequal variance.

Comparison of ELISA and HRT data for individual horses

Individual HRT **(Fig. 5A)** and ELISA **(Fig. 5B)** responses to the WBE of the different *Culicoides* species were compared for the same Shetland ponies used in **Fig. 3 and 4**. The values at a concentration of 0.5 µg/ml per extract were chosen to analyse the horses on individual level for the HRT, because at this concentration the best distinction could be made between IBH affected and healthy control horses. On individual level, for the *C. obsoletus* extract the histamine release of all IBH affected horses was higher than 10% and therefore positive[49], whereas the maximum histamine release of all healthy control horses was below 10% of the maximum histamine release. For the ELISA 6 out of 10 IBH affected horses had OD450nm values against *C. obsoletus* extract above the set cut-off level (mean + 3 times the standard deviation SD of the OD450nm values of the healthy control horses). With this cut-off level, all healthy horses were negative. In 6 out of 10 IBH cases (horses 2, 3, 4, 5, 6, and 7) the HRT and ELISA values against *C. obsoletus* WBE correlated with each other. Interestingly, the IBH affected horses with OD450nm values below the cut-off level in the ELISA (horses 1, 4, 8 and 10) did have a high positive histamine release with the HRT using *C. obsoletus* WBE. One horse had a higher histamine release after stimulation with *C. nubeculosus* and *C. sonorensis* WBE compared to *C. obsoletus*, but did have a higher IgE level against *C. obsoletus* when measured by ELISA.

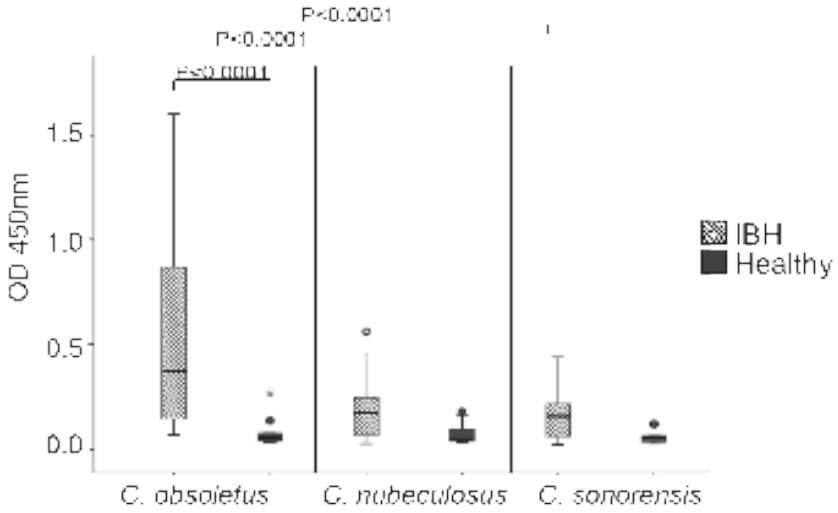

Figure 4: IgE titers with different *Culicoides spp.* IgE levels presented as OD450nm values against three different Culicoides extracts sera diluted 1:5 of 10 IBH-affected horses and 10 healthy control horses. Results are presented in box plots, for a definition see legend of Fig. 3. Statistical analysis was performed on log transformed data. The p values account for unequal variance.

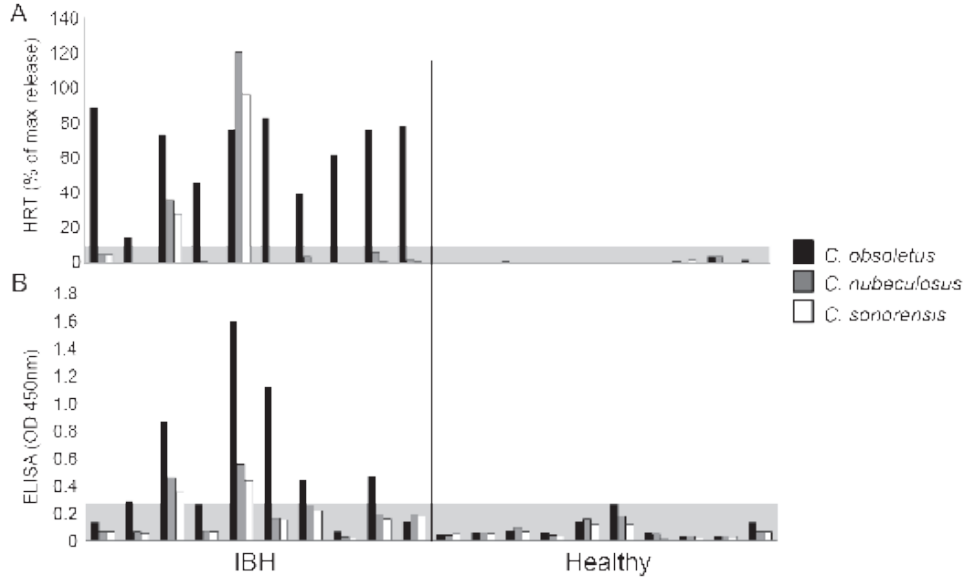

Figure 5: Comparison between histamine release test (HRT) and ELISA of individual horses. A) IgE levels are presented as OD450nm values. B) Histamine release after stimulation with whole body extract at a concentration of 0.5 µg/ml, is presented as percentage of maximum release. The cut-off of each test is highlighted in light gray and corresponds to 10% for the HRT and mean + 3 times standard deviation of the C. obsoletus values of the healthy control horses for the ELISA.

ELISA test sensitivity and specificity

The results described in section 3.6 indicate that the HRT outperforms the ELISA as a diagnostic test for IBH, but the ELISA is more practical as a routine test. It is much less laborious and can be performed on serum samples, while the HRT requires fresh full blood. Therefore the ELISA using *C. obsoletus* extract was further evaluated with sera of 103 clinically confirmed IBH affected horses and 100 healthy control horses (76 Icelandic horses and 127 Shetland ponies).

When categorizing the healthy and IBH affected horses according to their OD450nm values from the IgE ELISA, two distributions with equal variance were observed which are nearly baseline-separated **(Figure 6A)**. Although the distribution curve of healthy and IBH affected horses overlapped somewhat, the IBH-affected horses had higher serum IgE levels against *C. obsoletus* WBE compared to healthy control horses ($p < 0.0001$) **(Fig. 6B)**.

The pattern of the ROC-curve **(Fig. 6C)** indicates that the sensitivity sharply increases already at low false positive rates. The sensitivity of the test is therefore high over a large range of cut-off points. The accuracy of the test as evaluated by the area under the curve (AUC) is high and amounted to 0.97, indicating that high sensitivity is achieved with a high specificity. The point on the ROC-curve nearest to the upper left corner of the curve corresponds with a sensitivity of 93.2% and a specificity of 90.0% and is obtained at an OD450nm cut-off value of 0.2.

Logistic regression was performed to analyse the relation of IgE OD450nm values to the IBH-status. The response IBH-affected or healthy was regressed on OD450nm values.

Fig. 6D shows the predicted probabilities of both IBH-outcomes (i.e. affected or healthy) related to OD450nm values. With IgE-values close to zero the probability of being healthy is much higher than the probability of having IBH; up to an IgE value (OD450nm value) of 0.07 (true for 70% of the healthy horses) the probability of being IBH negative is approximately 10 times higher than being IBH positive and 5 times higher for a value of 0.12 (true for 85% of the healthy horses). At the inflection point at an OD450nm value of approximately 0.2 the probability being IBH-positive or negative is equal. From an OD450nm value of 0.33 onwards (true for 75% of the IBH affected horses) the probability of IBH-positive is more than 0.9.

Discussion

In this study we evaluated three different *Culicoides* whole body extracts for their applicability for *in vitro* diagnosis of IBH horses in The Netherlands. We show that *C. obsoletus* (a species found feeding on horses in The Netherlands) whole body extract is much better for *in vitro* diagnosis of IBH by ELISA and HRT, than *C. nubeculosus* and *C. sonorensis* (not found feeding on horses in the Netherlands) whole body extracts. An IgE ELISA with *C. obsoletus* whole body extract performed with 103 IBH affected horses and 100 healthy horses located in the Netherlands demonstrated a high sensitivity and specificity (93.2% and 90.0%, respectively) and can thus be used as a valuable test to diagnose horses for IBH in countries where *C. obsoletus* is the main species found feeding on horses.

This study describes an easy and selective method of catching *Culicoides* spp.

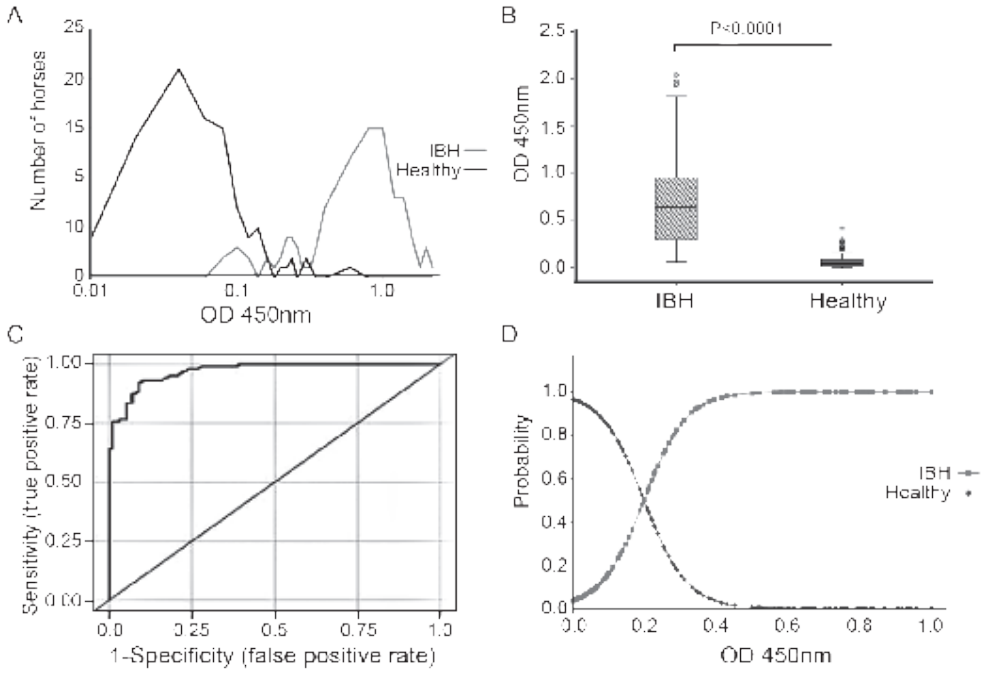

Figure 6: Validation of the C. obsoletus-specific IgE ELISA on serum samples of 100 healthy and 103 IBH-affected horses. (A) Distribution plots of the healthy and IBH-affected horses categorized for their OD450nm ELISA reading. (B) Box plot of IgE levels of the healthy and IBH-affected horses presented as OD450nm values The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represents the minimum and maximum value. The stars represent the outliers. The p value accounts for unequal variance. (C) ROC curve of C. obsoletus-specific IgE ELISA on sera samples of 100 healthy and 103 IBH-affected horses. The area under the curve, indicating diagnostic accuracy of the test was 0.97. (D) Estimated probability to be IBH-affected in relation to the OD450450nm value. Determined by logistic regression analysis of the obtained ELISA data.

attracted to horses by using a pooter. Although this method is initially more labour intensive than an “Onderstepoort” suction trap, it is a selective way of collecting preferentially those *Culicoides* species attracted to horses. This obviates the need for the labour intensive selection of the desired *Culicoides* species out of a large majority of unwanted insects that is required when using a light trap. The biggest advantage of the pooter method is that the insects are caught in a gentle way that keeps them alive, preventing substantial protein degradation as is observed for insects captured in alcohol by the “Onderstepoort” light trap.

The majority of *Culicoides* spp that were caught by both capturing methods were found to be *C. obsoletus:* over 80% when using the light trap and nearly 100% when directly collected from the horse by the pooter. This is in agreement with earlier studies performed in The Netherlands that also found *C. obsoletus* to be the main *Culicoides* species attracted to horses[24, 25]. In other countries, such as England[5] Ireland[19], Japan[33] and United States[4], *C. obsoletus* was also found to be the most

important *Culicoides* species attracted to horses.

Comparison of three *Culicoides* extracts demonstrates that in The Netherlands horses with IBH have more specific IgE directed against whole body extracts of *C. obsoletus* than against *Culicoides* species that do not feed on these horses. Although cross-reactivity between different *Culicoides* species has been reported[21, 22] our study shows weaker IgE-binding in Western blot and ELISA to proteins of non-indigenous *Culicoides* species compared to native *C. obsoletus*, which was also observed before[5]. This indicates the importance of using extracts from native *Culicoides* species feeding on horses for reliable diagnostics of IBH.

The binding of IgE from allergic horses with *C. sonorensis* and *C. nubeculosus* proteins might be due to cross reactivity between these proteins and the *C. obsoletus* antigens and maybe also proteins of other insect species that the horses were exposed to. The horses in this study might have been exposed to the native *C. nubeculosus*, but our as well as another study performed in The Netherlands[24] did not find any *C. nubeculosus* insects to be attracted to horses. Although *C. sonorensis* and *C. nubeculosus* antigens have previously been successfully used in different diagnostic tests[34-36] our study clearly shows the importance of using an extract of a *Culicoides* species to which horses have been actually exposed to for diagnosis. Western blot data revealed many IgE-binding proteins in *C. obsoletus* extract that were absent in *C. nubeculosus* and *C. sonorensis* extracts. Interestingly, a protein of around 20 kDa from *C. obsoletus* extract was found to be bound by IgE from almost all clinically confirmed IBH affected horses, whereas this was not observed for *C. nubeculosus* and *C. sonorensis* extracts and also not for IgE from healthy horses. This makes this protein an interesting candidate allergen for further characterization.

The *C. obsoletus* insects collected from the wild using the pooter were all female species, since females need blood to reproduce and were trying to feed on the horse when captured. Although WBE of laboratory-bred insects were of both sexes, it is unlikely that this explains the lower allergen-reactivity by *C. nubeculosus* and *C. sonorensis* in this study. Other studies found *Culicoides* extracts made from males only, to be just as effective in stimulating horse basophils[37] and non-salivary antigens from the thorax of *Culicoides* spp. have shown IgE reactivity with IBH-affected horses[11]. One of the IBH affected horses reacted even slightly stronger in the HRT with *C. nubeculosus* and *C. sonorensis* than with *C. obsoletus* WBE, and therefore rules out a lack of antigens of lower protein quality in the *C. nubeculosus* and *C. sonorensis* WBE. This was also demonstrated by the similar pattern and intensity of the protein bands of all three WBE on SDS-PAGE.

Surprisingly, some horses had low IgE-binding in the ELISA but a high histamine release in response to *Culicoides* extract stimulation in the HRT. IgG (T) has also been observed to bind to skin mast cells and therefore might play an important role in the histamine release reaction in IBH[13]. Therefore, the observation that some horses have low IgE-binding in ELISA but a high histamine release in response to *Culicoides* extract stimulation, might be due to cross-linking of allergen-specific IgG(T) instead of IgE.

The results in this study indicate that the histamine release test (HRT) might be more sensitive and reliable for diagnosis of IBH than the ELISA. However, this comparison was made on a relatively small number of horses (10 IBH affected and 10 healthy control horses). When tested with a large number of horses, the IgE

ELISA, resulted in a high specificity (90.0) and sensitivity (93.2) and proved to be the method of choice for routine screening, because it is more robust, easier to perform and more economical than the HRT. The test clearly discriminates between IBH-affected and healthy controls such that there is little overlap of distributions. The accuracy of the test as evaluated by the area under the curve (AUC) of the ROC-graph is high (0.97), indicating that high sensitivity is achieved with a high specificity. At an OD450nm cut-off value for positivity of 0.2 the test has a sensitivity of 93.2% and a specificity of 90.0%. However, this cut-off value does not take the actual OD450nm value of an individual horse into account, apart from being lower or higher than the determined cut-off value for positivity. Therefore logistic regression analysis was performed to determine the relation of the IgE OD450nm values to the IBH-status. For most horses the ELISA can determine with 90-100% probability the correct IBH-status of the individual tested horse. For those OD450nm values where reliability is less, e.g. around the inflection point at OD450nm value of 0.2, the horse owner can choose for an additional HRT to obtain a more conclusive diagnosis. Western blot analysis on *C. obsoletus* whole body extracts using serum of healthy horses showed some IgE reactivity of these horses against proteins with Mw above 25 kDa. Therefore, the use of selected recombinant proteins from *C. obsoletus* might further improve the sensitivity of the ELISA described in this study.

At present, treatment of IBH is based on insect avoidance by stabling, use of anti-midge blankets (pyjamas) or insect repellents and suppression of symptoms by the use of corticosteroids. Specific immunotherapy might be possible and some immunotherapy trials have been carried out with *Culicoides* whole body extracts, but with varying results[38, 39]. The use of purified allergens could further improve diagnostics, but could also be a benefit for immunotherapy. Collecting insects is very time consuming and for immunotherapy over 10,000 insects were necessary per horse[38], therefore recombinant allergens from an infinite source would be a sensible alternative for whole body extracts.

Currently, allergens have been identified and produced from species that are not present or common in The Netherlands, e.g. *C. sonorensis* and *C. nubeculosus* [34-36]. Implementation of future immunotherapy in horses will depend on the availability of correct allergens and therefore the use of allergens from *Culicoides spp* to which horses have been exposed, which for The Netherlands is mostly *C. obsoletus*, might be crucial.

In conclusion, our results show that horses with IBH in the Netherlands have much more IgE antibodies against *C. obsoletus* proteins compared to *C. sonorensis* and *C. nubeculosus* proteins which can be routinely detected in different diagnostic tests. The developed ELISA to identify sensitization against *C. obsoletus* allergens provides a valuable diagnostic test to discriminate IBH affected from healthy control horses in The Netherlands, but will also be valuable in other countries were *C. obsoletus* is mostly found feeding on horses.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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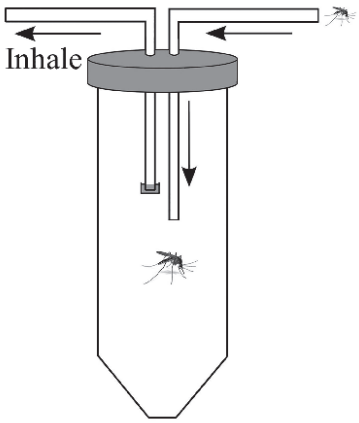
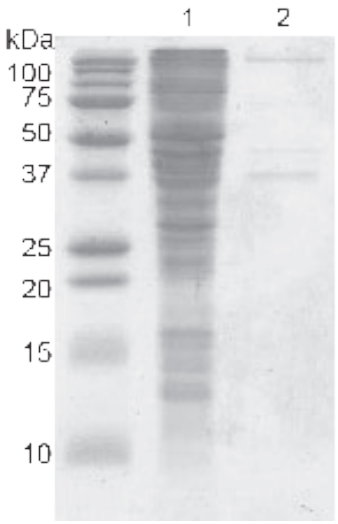
Supplementary data



Supp. Fig 1: Pooter used to collect Culicoides from horses. Two separate straws are inserted in two holes into a 50 ml tube. One straw has a fine mesh covering the end inside of the tube. This straw is used to inhale while aiming the other one at the insect to suck it into the tube

Supp. Fig 2: *C. obsoletus* insect. Individual example of a female C. obsoletus used in this study and identified on size and wing pattern according to Campbell and Pelham-Clinton (1960).



Supp. Fig 3: Protein saparation of *C. obsoletus* extract. Coomassie staining of proteins from *C. obsoletus* extract (50 µg), from insects collected alive with the pooter (1) and from insects collected in alcohol with the “Onderstepoort” light trap (2).

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Chapter 3

Seasonal differences in cytokine expression in the skin of Shetland ponies suffering from insect bite hypersensitivity

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Abstract

Insect bite hypersensitivity (IBH) in horses is a seasonal, IgE-mediated, pruritic skin disorder primarily caused by *Culicoides spp*.

We hypothesize that a mixed Th2/Th1-type immune status, off season, alters into Th2-dominated immune reactivity in the skin of IBH-affected ponies in the IBH season.

To study these immune response patterns *Culicoides*-specific IgE levels, skin histopathology and cytokine and transcription factor mRNA expression (IL4, IL10, IL13, IFNγ, FoxP3 and $CD3_{\zeta}$) in lesional and non-lesional skin of ponies affected by IBH in the IBH season were compared with those of the same animals off season and those in skin of healthy ponies in both seasons.

The present study revealed a significantly higher histopathology score in lesional skin of affected ponies than in non-lesional skin and skin of healthy ponies in the IBH season. *C. obsoletus*-specific IgE serum levels of ponies with IBH were significantly higher than those in healthy ponies in both seasons. Interestingly, *C. obsoletus*-specific IgE serum levels within each group were the same in the IBH season and off season. The expression of IL4, IL13 and IFNγ mRNA in skin biopsies in the IBH season showed a significant increase compared to off season in both skin derived from healthy control ponies (n=14) as well as in lesional and in non-lesional skin from IBH-affected animals (n=17). This apparently general up-regulation of cytokine expression during the IBH season directly correlated with an increased CD3ζ mRNA expression in the skin, indicating an overall increased T cell influx during the summer months. The only significant difference observed between lesional skin from IBH-affected animals as compared to skin from healthy control animals in the IBH season was a lower expression of IL13/$CD3_{\zeta}$ in the affected animals. FoxP3 and IL10 levels were unaffected, except for a lower expression of FoxP3 in healthy control skin in the IBH season as compared to off season, In addition, the increased level of *C. obsoletus*-specific IgE did not correlate with higher histological scores in LE skin.

In summary, our data indicate a general immune activation in the skin of both healthy and IBH-affected ponies during the IBH season that potentially obscures the Culicoides-specific immune reaction pattern, even in lesional skin of IBH-affected animals.

Introduction

Equine insect bite hypersensitivity (IBH) is a seasonally recurrent, pruritic skin disorder mainly caused by the bites of female *Culicoides* spp.[1-5]. In Europe all horses are exposed to *Culicoides* bites and develop IgG antibodies to salivary antigens. About 5 percent of horses develop clinical signs of allergy with elevated *Culicoides*-specific IgE[6-8]. Approximately 95 percent of the *Culicoides spp*. found around IBH horses in the Netherlands and other European countries, such as Sweden is *C. obsoletus*[2, 5, 9]. Clinically, at onset IBH is characterized by intense pruritus with papules and wheals, which develop into serous crusts, scaling and alopecia on the mane, tail and in some cases the ventral midline, when the disease becomes chronic[10-12]. Lesions are only observed during the *Culicoides* season and are absent off season. The diagnosis of IBH is based on these characteristic seasonal clinical manifestations as well as skin test reactivity to the geographically relevant *Culicoides* antigen [13]. Histologically, IBH lesions are characterized by a mixed perivascular to diffuse cellular infiltrate consisting of mainly CD4+ T cells [14] and, especially in acute lesions, eosinophils[15, 16].

Expression levels of IL13 mRNA, but not of IL4 or IL5 mRNA, have been described to be significantly elevated in lesional and non-lesional skin of IBH-affected horses[17]. On the other hand, IL10 mRNA levels were lower in lesional compared to non-lesional skin, but this difference was not statistically significant [17]. An increase in IL4 mRNA production with a concomitant decrease in IFNγ mRNA production by cultured equine PBMC was observed during the IBH season[18-20].

No differences in total serum IgE levels were found between healthy horses and animals affected by IBH[21]. However, *Culicoides*-specific IgE serum titers have been reported to be higher in horses with IBH[6-8, 17] as compared to healthy horses. Correlations between *Culicoides*-specific IgE titers and histological scores for IBH have not been reported so far, nor have differences in these disease parameters between the IBH season and off season been investigated.

In order to better understand the pathogenesis of IBH we investigated the differences in skin pathology and cytokine expression between IBH “on and off” season. We hypothesize that the immune balanse (a mixed Th2/Th1-type) off season alters into a Th2-dominated immune response in the skin of ponies with clinical manifestations of IBH, due to exposure to *C. obsoletus.* Therefore we have compared *Culicoides*-specific IgE serum levels, parameters of dermatopathology, the mRNA expression of cytokines (IL4, IL10, IL13, IFNγ), the transcription factor FoxP3 and the T cell receptor linked $CD3_{\zeta}$ in lesional and non-lesional skin of ponies affected by IBH during the IBH season with those of the same animals off season, as well as of healthy control ponies in both seasons.

Our results show significantly higher histopathology scores in lesional skin of affected ponies than in non-lesional skin in the IBH season. The same result was observed when comparing lesional skin of IBH ponies and skin from control ponies in the IBH season. *Culicoides*-specific IgE serum levels were higher in IBH-affected ponies compared to control animals, but within the group of IBH-affected ponies they were equally high in each season. In addition, in both IBH skin and control skin there was an upregulation of $CD3_{\zeta}$, IL4, IL13 and IFNγ during the IBH season compared to the situation off season. Except for a down regulation of FoxP3 in healthy control skin in the IBH season compared to off season, FoxP3 and IL10 levels were unaffected.

Material and Methods

Animals

A total of 31 privately-owned Shetland ponies were included in the study. Ponies with IBH (n=17, age range 3-19 years) were selected on the basis of clinical symptoms and history, i.e. recurrent, seasonal, pruritic skin lesions located at the mane, tail and ventral midline with remission off season. Control ponies (n=14, age range 2-20 years) were randomly chosen from the same stables as the ponies with IBH and had no clinical symptoms or history of IBH. Ponies in both groups were dewormed and vaccinated at regular intervals and did not receive any corticosteroid treatment.

All animal experiments were approved by the Animal Ethics Committee of the Utrecht University.

Collection and processing of blood and skin samples

To ensure that ponies were not exposed to *Culicoides spp*. recently, off season samples were taken in March 2011 before the start of the IBH season when the average maximum temperature was 11.1 °C and midnight temperatures were down to -2.5°C. During the *Culicoides* season in August 2011 (with an average maximum temperature of 20.2°C) to insure maximum exposure to *Culicoides* the same ponies were sampled again.

Skin biopsies of 4 mm were taken under local anesthesia with 2% lidocaine (B. Braun, AG Melsungen, Deutschland). Biopsies of healthy and non-lesional skin were collected from the dorsolateral neck, whereas chronic lesional skin biopsies were taken from the crest. There were no acute lesions present at the time of sampling.

Three biopsies were taken per sampling site: the first two were snap-frozen in liquid nitrogen and stored at -70°C until used for RNA isolation. The third biopsy was fixed in 4% neutral buffered formaldehyde for 24-48 h and paraffin-embedded for histopathology.

Blood samples (20 ml) were collected from the jugular vein of each pony in off and IBH seasons into heparin tubes. Serum was separated after centrifugation and frozen at -20°C until analyzed for *Culicoides*-specific IgE levels.

C. obsoletus whole body extract preparation

Whole body extract (WBE) was prepared from about three hundred female *C. obsoletus* insects, which were frozen at -80 °C without any liquid. They were suspended in a 2ml Eppendorf tube with 1ml of PBS containing protease inhibitor cocktail according to the manufacturer's specification (Sigma-Aldrich, St. Louis, MO, USA) and crushed with a micropestle. The sample was centrifuged at 14000 rpm for 10 min at 4°C to remove any insoluble material, the supernatant was filtered through a sterile 0.22µm pore diameter Millex-GV filter (Millipore, Amsterdam, NL) and the protein content of the filtrate was measured at an OD of 280nm with a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). This WBE was snap-frozen in liquid nitrogen en stored at -80°C[22].

Culicoides-specific serum IgE levels

According to Van der Meide *et al.*[22] a costar 96-well microtiter plate was coated with 100µl per well of 10µg/ml *C. obsoletus* WBE in PBS and incubated overnight at 4°C.

The next day, the plate was washed 3 times with 0.05% Tween20 in PBS and each well was incubated with 150µl blocking buffer containing 1.5% casein (SDT, Baesweiler, Germany) for 1.5 hours at RT. The wells were washed 5 times with 0.05% Tween20 in PBS and subsequently incubated with the serum samples diluted 1:5 in 1.5% casein buffer in duplicate. After washing, wells were incubated with 100µl (2.5µg/ml) mouse monoclonal anti-equine IgE-176 (Wagner *et al.*[8]) for 1 hour at RT. Again the wells were washed 5 times. Subsequently, wells were incubated for 1 hour at RT with 1:1000 goat anti-mouse IgG peroxidase conjugate (AbD Serotec, Düsseldorf, Germany) in casein buffer. After washing 5 times wells were incubated with 100µl high sensitivity tetramethylbenzidine (SDT, Baesweiler, Germany) for 10 min at RT. The reaction was stopped by adding 100µl of 1% HCL per well. Absorbance was measured with a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Berkshire, UK) at a wavelength of 450nm corrected for the OD measured at 650nm.

Histological examination of skin samples

Paraffin-embedded biopsies were cut in 4 µm sections and stained with haematoxylin-eosin (HE) for routine histopathology. A semi-quantitative grading system (0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) was used to assess the degree of epidermal (both hyperplasia and hyperkeratosis) and dermal (infiltration of both mononuclear cells and eosinophils) changes. The overall histological grade, defined as the sum of the grades for each of these parameters, was determined for each biopsy. All slides were analyzed by the same European College of Veterinary Pathologists-certified pathologist, who was blind with regard to the group and time assignment of biopsies.

Quantitative Real-time reverse transcription-polymerase chain reactions

Frozen skin samples were disrupted and homogenized in TRIzol reagent (Invitrogen, Breda, NL) using a Biopulverizer (Biospec #59012N, Biospec Inc., Bartlesville, OK) and polytron (PT 1200 E, Kinematica AG, Lucerne, CH). TRIzol manufacturer's instructions were followed until the water-phase was obtained after the chloroform step. Subsequently, total RNA was extracted using RNeasy columns (Qiagen, Venlo, NL) and eluted with 30 µl of RNase free water. The RNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). One µg of total RNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories, Veenendaal, NL) according to manufacturer's instructions. A 5′ nuclease assay using TaqMan probes was employed for qRT-PCR amplification. Expression of the following genes was assessed: IL4, IL10, IL13, IFNγ, FoxP3 and $CD3_{\zeta}$. Reactions were performed in 25 µl volumes containing 5 µl cDNA, 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Austin, TX, USA), 0.9 µM relevant primers and 0.25µM fluorescence-labeled probes and milliQ. The qRT-PCR were performed in an iCycler (Bio-Rad laboratories, Veenendaal, NL) with amplification conditions of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 62, 60 or 57 °C (for details see **Table 1**). Each reaction was ran in duplicate and a no-template control was included to exclude contamination of reagents with cDNA on every plate. PCR efficiencies were calculated using a relative standard curve derived from a cDNA pool of equine PBMC stimulated with pokeweed

mitogen (Sigma–Aldrich, St. Louis, MO, USA) for 24h. Relative expression of IL4, IL10, IL13, IFNγ, FoxP3 and $CD3_{\zeta}$ was calculated using the Pfaffl method[23] using the housekeeping gene 18s ribosomal RNA (18s rRNA) as a reference gene. To asses the role of T cells in IBH the ratios of IL4, IL10, IL13, IFNγ, FoxP3 with $CD3_{\zeta}$ were determined.

Table 1: Primers and probes and the PCR conditions used for qRT-PCR.

Gene	cDNA (µl)	T (°C)	reference	Sequences (5'-3')
IL4	2.5	60	Ainsworth *et al*.[31]	
IL10	5	60	Primers: Swiderski *et al*.[32]	Probe: 6FAM-GGCTGAGAACCACGGCCCAG-MGBNFQ
IL13	2.5	60	Heimann *et al*.[17]	
FoxP3	2.5	59	NM_001163272.1	Forward: CCAGCTATGATCAGCCTTCC Reverse: GAAGACCTTCTCGCATCCAG Probe: 6FAM-GCTGGCAAATGGCGTCTGCA-MGBNFQ
IFNγ	2.5	60	Ec03468605_g1; Applied Biosytems	
$CD3_{\zeta}$	2.5	60	Debrue *et al*. [33]	
18S	0.25	60	4352930E; Applied Biosytems	

Statistical analyses

Statistical analyses were carried using GraphPad Prism 4.00 (Graphpad Software, San Diego, CA). As our data were not normally distributed, correlation between *C. obsoletus*-specific IgE levels and total LE skin histological scores was examined using a non-parametric correlation (Spearman) test. The Mann–Whitney *U* test (+) was used to compare values from IBH-affected ponies with those from healthy controls. The non-parametric paired Wilcoxon Signed-Rank Test (WSR Test) (*) was used for comparison of paired data (lesional/non-lesional and IBH season/off season). Results were considered significant at $p \leq 0.05$.

Results

C. obsoletes-specific IgE levels

C. obsoletus-specific IgE serum levels of ponies with IBH were significantly higher than those in control ponies in both seasons (P=0.0001 and P= 0.0002, respectively) **(Fig 1A)**. However, within each group there was no significant difference between the levels in the IBH season and off season (IBH vs IBH P=0.2676 and Co Vs Co P=0.0537).

Histopathology

HE-stained sections of 14 control ponies (Co) and non-lesional skin (NL) of 16 IBH ponies, sampled off season, were examined by light microscopy according to the semi-quantitative grading system. Since some of the ponies were sold during the summer and one died of an unrelated cause, 11 of the control and 15 IBH-affected ponies were available for examination during the IBH season. Control skin histology scores did not differ between the seasons, nor did control skin and NL skin (**Tables 2 and 3**). The average total histological score was significantly higher (P=0.0039)

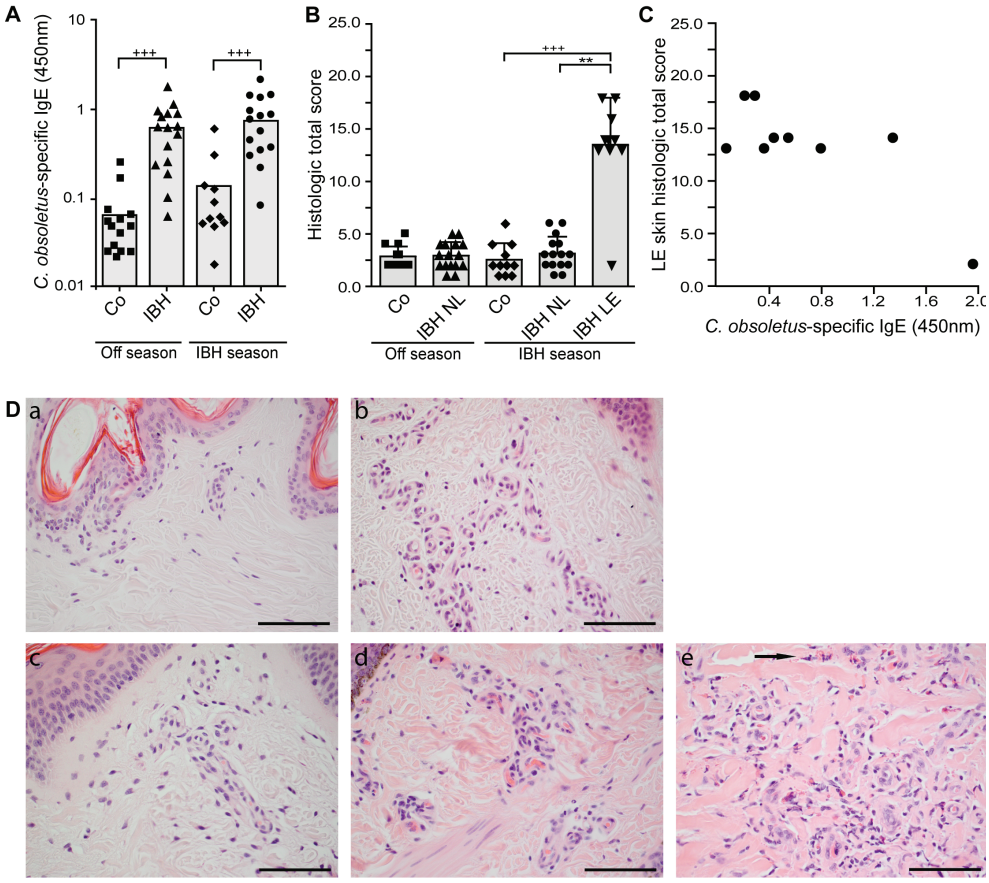

Figure 1: C. obsoletus-specific IgE serum levels and histology in the off season and in the IBH season. IgE serum levels and histological scores of skin of 14 control and 16 IBH ponies (off season) and 11 control and 15 IBH ponies (IBH season) were examined. Histology scores of lesional skin of 9/15 IBH ponies were determined. (A) C. obsoletus-specific IgE serum levels as determined by ELISA assay. The bars represent the mean of all samples in the group. (B) Mean histological scores were determined according to a semi-quantitative grading system (0-4) for acanthosis, hyperkeratosis, lymphocyte infiltration and eosinophilic granulocytes (C) C. obsoletus-specific IgE serum levels plotted against the lesional skin's total histological sores (P=0.4366). (D) Representative examples of HE stained section of: a) Off season control skin with few perivascular mononuclear cells in the superficial dermis (average total histological grade 3). b) Off season non-lesional (NL) skin with mild perivascular inflammation (average total histological grade 3). c) IBH season control skin (Co) with minimal perivascular inflammation of the superficial dermis (average total histological grade 3). d) IBH season non-lesional skin (NL) with mild perivascular inflammation (average total histological grade 3). e) IBH season lesional skin (LE) with pronounced dermal inflammation and moderate numbers of eosinophils (arrow) (average total histological grade 14). Original magnification 400x. The bar corresponds to 50 µm.

in LE skin compared to NL skin from the same ponies as well as compared to that of the control animals (P=0.0006) **(Fig. 1B and D)**. More specifically, LE skin had higher numbers of infiltrating mononuclear cells and eosinophils **(Tables 2 and 3)**. In addition, acanthosis and hyperkeratosis were found only in LE skin.

The total histological scores of LE skin of the IBH ponies did not correlate with their *Culicoides*-specific IgE values (P=0.4366) **(Fig. 1C)**.

Cytokine expression levels in the skin

The mRNA expression levels of $CD3_{\zeta}$, a marker for T cells, normalized against 18s ($CD3_{\zeta}$/18s) were significantly increased in the IBH season in healthy control skin (P=0.0039) compared to off season; the same observation was made in NL skin of ponies with IBH (P=0.0001) **(Fig. 2)**. However, there was no difference between NL and LE skin in the IBH season nor between Co and NL skin in the off season **(Fig. 2)**.

The ratio IL4/$CD3_{\zeta}$, as determined by IL4/18s divided by its corresponding $CD3_{\zeta}$/18s value, both in control and affected ponies showed no difference between

Table 2: Off season semi-quantitative histological scores (average and range between brackets) from healthy ponies and ponies with IBH.

	Healthy (n=14)	Non-lesional (n= 16)
Acanthosis	0 (0-0)	0 (0-1)
Hyperkeratosis	0 (0-1)	0 (0-1)
Upper dermis: lymphocytes	1 (1-2)	1 (1-2)
Upper dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)
Middle dermis: lymphocytes	1 (0-2)	1 (0-2)
Middle dermis: eosinophilic granulocytes	0 (0-0)	0 (0-1)
Deep dermis: lymphocytes	0 (0-0)	0 (0-0)
Deep dermis: eosinophilic granulocytes	0 (0-0)	0 (0-0)
Average total histological score	3 (2-5)	3 (1-5)

0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe

Table 3: IBH season semi-quantitative histological scores (average and range between brackets) from healthy ponies and ponies with IBH.

IBH season	Healthy (n=11)	Non-lesional (n=15)	Lesional (n=10)
Acanthosis	0 (0-0)	0 (0-1)	2 (1-2)
Hyperkeratosis	0 (0-0)	0 (0-0)	1 (0-3)
Upper dermis: lymphocytes	1 (1-2)	1 (1-2)	2 (1-4)
Upper dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-3)
Middle dermis: lymphocytes	1 (0-2)	1 (0-1)	2 (0-3)
Middle dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-3)
Deep dermis: lymphocytes	0 (0-1)	0 (0-1)	2 (0-3)
Deep dermis: eosinophilic granulocytes	0 (0-1)	0 (0-1)	1 (0-2)
Average total histological score	3 (1-6)	3 (1-6)	14 (2-18)

0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe

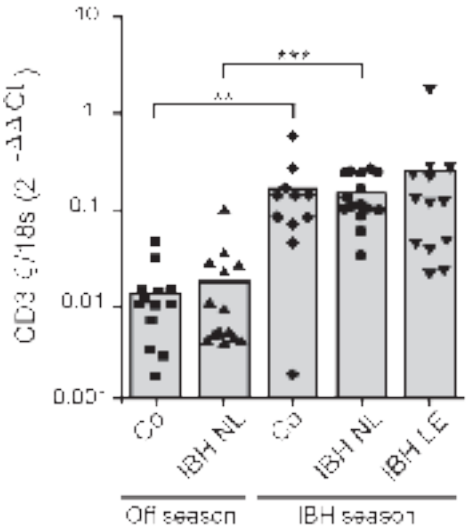

Figure 2: Expression of CD3$_\zeta$ mRNA, a T cell receptor marker. Off season 13 control and 14 IBH ponies were used. In the IBH season 11 of the 13 control ponies were examined and all 14 IBH ponies were examined of which 13 had lesional skin 1 IBH ponies was added in the course of the experiment. The mRNA expression of CD3$_\zeta$ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.

seasons **(Fig. 3)**. There was also no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season. When comparing only IL4/18s expression an increase was observed when comparing NL skin as well as control skin off season and during the IBH season (respectively P=0.004 and P=0.0273). In the IBH season no difference was found between healthy, NL and LE skin **(supp. Fig. 1)**.

IL13 mRNA expression off season was not detectable, whereas in the IBH season IL13/CD3$_\zeta$ was lower in LE skin compared to healthy control skin (P=0.0239), but not compared to NL skin **(Fig. 4)**. The same results were obtained when only 18s was used for normalization **(supp. Fig. 2)**.

The IFNγ/CD3$_\zeta$ analysis showed a significant increase when comparing Co off season and Co IBH season (P=0.002), NL off season versus NL IBH season (P=0.0002). However, there was no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season **(Fig. 5)**. The same results were observed when IFNγ/18s levels were compared **(supp. Fig. 3)**.

IL10/CD3$_\zeta$ mRNA expression levels did not differ between off season and the IBH season **(Fig. 6)**. Moreover, there was no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season. Analysis of IL10/18s shows a tendency for increased IL10 expression in the IBH season **(supp. Fig. 4)**.

Analysis of FoxP3/CD3$_\zeta$ mRNA expression showed a significant down regulation between Co off season and Co skin in the IBH season (P=0.0078) **(Fig. 7)**. However, there was no difference between NL off season and NL skin in the IBH season. The same was observed for NL and LE skin in the IBH season. Finally, there was

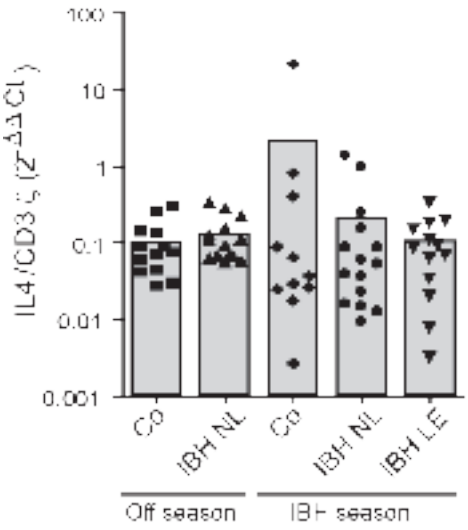

Figure 3: Expression of IL4/CD3$_\zeta$ mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL4 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

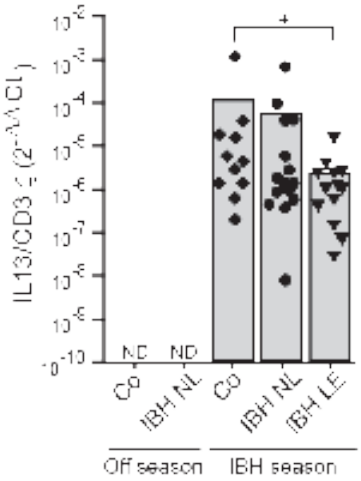

Figure 4: Expression of IL13/CD3$_\zeta$ mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL13 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

no difference in FoxP3 expression levels between on and off season normalized against only 18s. There was also no difference between Co and NL skin in the off season and Co, NL and LE skin in the IBH season **(supp. Fig. 5)**.

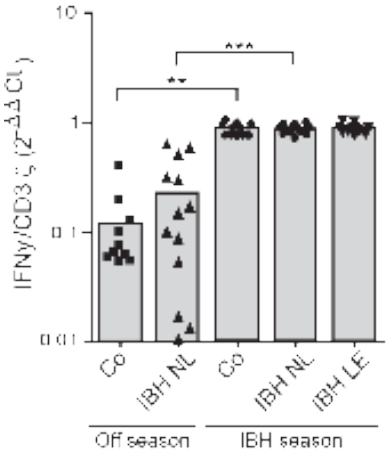

Figure 5: Expression of IFNγ/CD3$_\zeta$ mRNA, a Th 1 cytokine. The skin of 10 control and 13 IBH ponies were used during off season. In the IBH season all 10 control ponies plus 1 extra were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IFNγ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

Discussion

IBH is an IgE-mediated, pruritic skin disorder caused primarily by *Culicoides spp*. Differences in cell types present in the skin and as a potential consequence in functional immunological aspects between IBH "on and off" season may be indicative for processes underlying the disease. In general, IBH is thought to be a Type-1 hypersensitivity with a potential Th2 skewed immune responsiveness[17, 18].

In the present study, we examined the skin-infiltrating cells by histopathological analysis as well as cytokine expression by PCR in the off and IBH season in the same pony to determine Th1 versus Th2 immune responsiveness. Additionally, specific IgE serum levels were measured to determine correlation between allergen-specific IgE levels and the presence of histological manifestations.

A study by Wagner *et al.*[8] provided the first direct evidence that IgE mediates

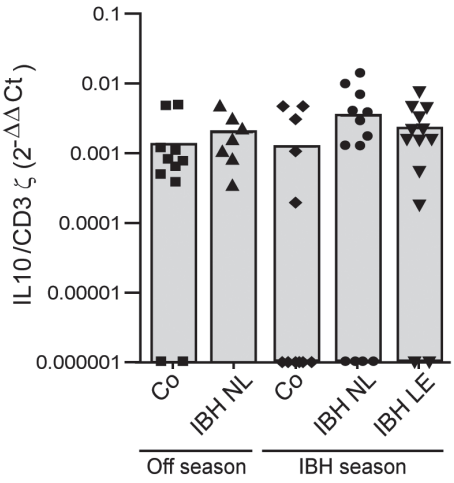

Figure 6: Expression of IL10/CD3$_\zeta$ mRNA, an immune suppression cytokine. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of IL10 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in that group.

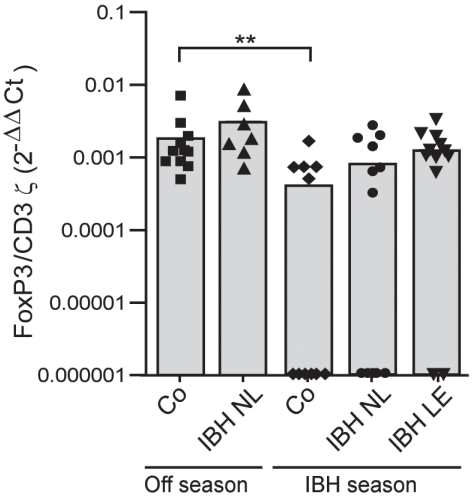

Figure 7: Expression of FoxP3/CD3$_\zeta$ mRNA, a Treg cell marker. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of FoxP3 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the CD3$_\zeta$/18s value of the same sample. The bars represent the mean of all samples in the group.

classical Type-I hypersensitivity in horses and plays a major role in the pathogenesis of IBH. In addition, *C. nubeculosus*-specific IgE serum levels were found in IBH-affected horses[17]. Such a finding is comparable with the situation in other animals such as the dog, where significantly higher flea-specific IgE levels where found in cases of flea allergy dermatitis (FAD) than in healthy dogs[24, 25].

The *C. obsoletus* WBE-specific IgE titers in IBH ponies are higher than those in the control ponies in the off season as well as in the IBH season, suggesting that in IBH there is a Th2 skewing of the immune system. There was no difference between the specific IgE serum titers off season and in the IBH season within each group. IgE titers being stable throughout the year indicate continuous production of IgE by plasma cells, which instead of memory B cells do not need to be reactivated to produce IgE.

Next to clinical symptoms, there are currently two major tests to diagnose IBH in ponies and horses i) the intradermal skin test using WBE, ii) a culicoides-specific serum IgE ELISA. The intradermal skin allergy test is based on degranulation of mast cells in vivo, causing tissue swelling, due to cross-linking of *Culicoides*-specific IgE by the allergen and the ELISA assay is based on the binding of *Culicoides* specific serum IgE to *Culicoides* extract coated plates. Since Culicoides-specific IgE is the most important factor in both these tests and IgE titers seem to stay stable between seasons it is likely that these tests can be done off season as well as in the IBH season.

The higher histological scores for LE skin suggests a Th2 response of the immune system. There was however, no difference between healthy control skin and NL skin of IBH ponies in the IBH season. This might be explained by the fact that only mild clinical symptoms were found in all IBH ponies potentially due to the relatively cold IBH season, and consequently less active *C. obsoletus* midges than in an average season.

Remarkably, there was no correlation between the allergen specific IgE titer and the total histological score of LE skin from the same pony. This indicates that levels of free serum IgE can not be used as markers for the severity of IBH. An explanation for the discrepancy may be that circulating allergen-specific IgE levels do not reflect the amount of mast cell- or eosinophil-bound IgE, which more likely contributes to the inflammatory response. In addition, Wagner *et al.*[8] suggest that IgG(T), which can also activate skin mast cells, may play a role in IBH[8]. Also in dogs with FAD, flea-specific IgG levels are higher than in healthy dogs[25].

To further assess our hypothesis that a mixed Th2/Th1-type immune status, off season, alters to a Th2-dominated immune response in the skin of IBH-affected ponies, hence local immune response parameters, skin mRNA expression levels of IL4, IL10, IL13, $CD3_{\zeta}$, FoxP3 and IFNγ were determined.

The $CD3_{\zeta}$ mRNA expression in skin of IBH-affected and control ponies was determined to assess differential T cells presence. A significant increase in T cells was observed in Co skin in the IBH season as compared to off season. The same was seen in NL skin. However, there was no significant difference between Co and NL skin in the off season, nor in all types of skin in the IBH season, which suggests that the immune system in the skin is activated. This activation might be due to other seasonal factors such as bites of mosquitoes and warble flies. It might even be preferential to perform experiments during the off season to exclude additional

effects from other insect bites, which might interfere with the assays.

To address Th2 skewing of the immune response in IBH, we examined mRNA expression of the Th1 type cytokine IFNγ and of the Th2 type cytokines IL4 and IL13. These mRNA levels were subsequently assessed relative to the corresponding $CD3_{\zeta}$ mRNA levels since these cytokines are mainly secreted by T cells. There was no difference between NL and LE skin in the IBH season in expression of IL4, which was increased in the IBH season in both controls and ponies with IBH when only normalised with 18s **(sup. Fig.1)**. Correcting for the number of T cells ($IL4/CD3_{\zeta}$) abolished the significant increase of IL4 in Co and IBH skin **(Fig. 2)**. This indicates that the increase in IL4 mRNA expression is due to increased numbers of T cells, which is in line with the study of Heimann et al (2011)[17] where difference in IL4/18s expression between healthy horses and horses with IBH were observed neither. Similar observations have been reported for example in healthy dogs and dogs with FAD[26]. The lack of altered IL4 expression by T cells and the general increase of IL4/18s suggests that other factors may contribute to the development of clinical manifestations.

In the present study, a substantial expression of IL13 mRNA was observed during the IBH season suggesting again a general immune activation in all types of skin. In the IBH season a significant lower expression of $IL13/CD3_{\zeta}$ was observed in chronic lesional skin as compared to control skin. IL13 as a Th2 cytokine plays a role in the effector phase of allergic reactions [27]. Hence, IL13 mRNA levels are expected to increase in acute lesional skin as shown by Heimann et al (2011)[17], but maybe not in chronic lesional skin, which was confirmed by our results. A similar situation has been described for FAD dogs[26]. A down regulation of IL13 was observed after *in vitro* stimulation of allergic skin exposed to fleas for 4 days with flea antigen[26] which is coherent with our observation *in vivo*. Hence, T cells do not seem to alter IL13 production in our study. However, we do need to take into account that chronic lesional skin was examined and that Heimann *et al.*[17] did find a significant increase in IL13 in more acute lesional skin indicating a Th2 skewing of the immune response.

Our study showed an overall significant elevation of IFNγ in the IBH season as compared to off season confirming a general activation of the immune system in the skin. Contrary to elevated IFNγ levels in human chronic AD lesions [28], there were no differences observed in IFNγ mRNA expression between control, non-lesional and chronic lesional skin of ponies in the IBH season **(Fig. 5)**, which is in line with earlier reports by Heimann *et al.*[17]. Our combined results indicated a mixed Th2/Th1 cytokine expression in both seasons, but at a higher level during the IBH season.

In addition, to the Th1 and Th2 cytokines, Treg cells have been suggested to play a major role in allergies in humans[29]. We therefore decided to examine FoxP3 and IL10 mRNA expression. FoxP3 expression relative to that of $CD3_{\zeta}$ only showed a significant decrease in healthy skin in IBH season compared to the off season. This suggests that there is a decrease of Treg cells in the skin of healthy ponies in the IBH season. This yet remains to be explained. There was no difference in FoxP3 expression between healthy and NL skin off season. Furthermore, there was no difference between healthy, NL and LE skin in the IBH season **(Fig. 7)**. When examining the FoxP3/18s data no significant differences could be found. This suggests that numbers of FoxP3 positive cells in the skin do not differ between healthy and IBH-affected ponies. This is in contrast with the study of Heimann et al

(2011)[17], which reported a significant decrease in FoxP3 in NL and LE skin compared to healthy skin suggesting a decrease in Tregs. The reason for these differences might be that they have omitted horses with low histological scores from further analysis. In line with our results a study by Hamza et al (2012)[17] showed that the number of $CD4^+$ $CD25^{+hi}$ $FoxP3^+$ T cells levels in PBMC of healthy horses was similar to that in IBH-affected horses. In addition, when $CD4^+$ $CD25^+$ T cells were stimulated with *C. nubeculosus* extract, expression of FoxP3 was significantly higher in healthy controls compared to IBH-affected horses[30]. Our results do not address the reactivity of Treg cells in the skin of IBH-affected ponies compared to healthy ponies.

There was no difference in IL10/$CD3_\zeta$ between seasons. Moreover, there was also no difference between control, non-lesional and chronic lesional skin in the IBH season **(Fig. 6)**, which is similar to the observations of Heimann *et al.*[17]. Our current approach does not confirm a regulatory role for IL10, a longitudinal study may give more inside into the kinetics.

In summary, our data of the same ponies in the off season versus the IBH season showed a general increase of $CD3_\zeta$ expression in the IBH season. IL4/18s, IL13 and IFNγ levels were also higher in the IBH season compared to the off season. The increased level of *C. obsoletus*-specific IgE and number's of cutaneous eosinophils, although not correlated in LE skin, both suggest a Th2 type reactivity of the immune system of IBH-affected ponies. However, based on the IL4, IL13 and IFNγ parameters the Th2/Th1 balance seems to be maintained. Moreover, the role of regulatory T cells (FoxP3 and IL10) could not be confirmed in the present study. These overall results suggest that the immune system in the skin for healthy and IBH-affected ponies is activated in the IBH season in general.

Our data warrant a careful re-evaluation of studies describing the immune status of the skin, taking general seasonal influences in account.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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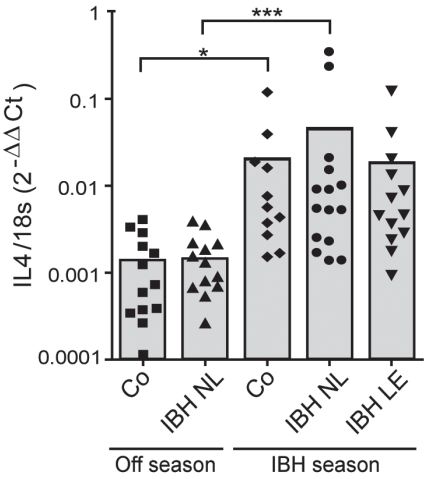
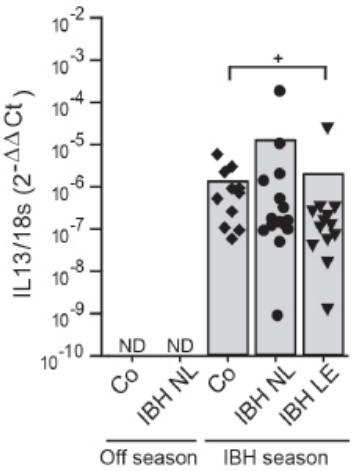
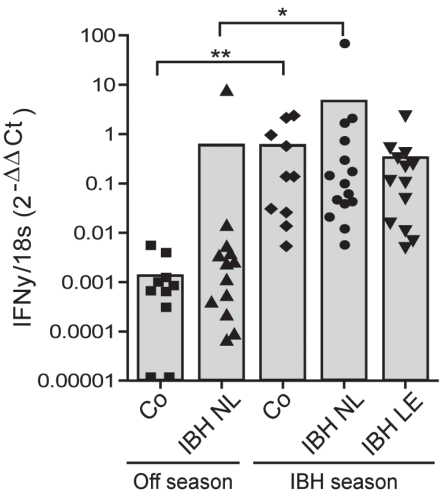
Supplementary data



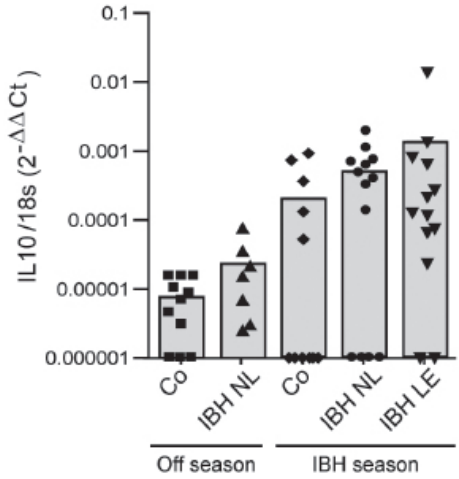
Supp. Figure 1: Expression of IL4 mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL4 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.



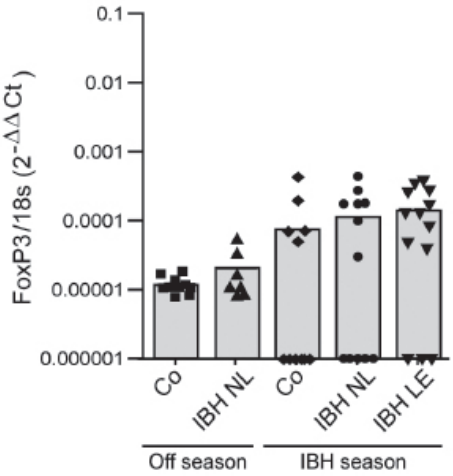
Supp. Figure 2: Expression of IL13 mRNA, a Th 2 cytokine. The skin of 13 control and 13 IBH ponies were used during off season. In the IBH season 11 of the 13 control ponies were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IL13 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.



Supp. Figure 3: Expression of IFNγ mRNA, a Th 1 cytokine. The skin of 10 control and 13 IBH ponies were used during off season. In the IBH season all 10 control ponies plus 1 extra were examined and all 13 plus 2 extra IBH ponies were examined of which 13 had lesional skin. The mRNA expression of IFNγ was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.



Supp. Figure 4: Expression of IL10 mRNA, an immune suppression cytokine. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of IL10 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.



Supp. Figure 5: Expression of FoxP3/CD3ζ mRNA, a Treg cell marker. The skin of 11 control and 7 IBH ponies were used during off season. In the IBH season all control ponies were examined and all 7 plus 6 extra IBH ponies were examined, all having lesional skin. The mRNA expression of FoxP3 was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23]. The bars represent the mean of all samples in the group.

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Chapter 4

Allergen-specific cytokine polarization protects Shetland ponies against *Culicoides obsoletus*-induced insect bite hypersensitivity

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Abstract

The immunological mechanisms explaining development of an allergy in some individuals and not in others remain incompletely understood. Insect bite hypersensitivity (IBH) is a common, seasonal, IgE-mediated, pruritic skin disorder that affects considerable proportions of horses of different breeds, which is caused by bites of the insect *Culicoides obsoletus*. We investigated the allergen-specific immune status of individual horses that had either been diagnosed to be healthy or to suffer of IBH. Following intradermal allergen injection, skin biopsies were taken of IBH-affected and healthy ponies and cytokine expression was determined by RT-PCR. In addition, allergen-specific antibody titers were measured and cytokine expression of *in vitro* stimulated, allergen-specific CD4 T-cells was determined. 24 h after allergen injection, a significant increase in mRNA expression of the type-2 cytokine IL-4 was observed in the skin of IBH-affected Shetland ponies. In the skin of healthy ponies, however, an increase in IFN-γ mRNA expression was found. Analysis of allergen-specific antibody titers revealed that all animals produced allergen-specific antibodies, and allergen-specific stimulation of CD4 T-cells revealed a significant higher percentage of IFNγ-expressing CD4 T-cells in healthy ponies compared to IBH-affected ponies. These data indicate that horses not affected by IBH, in contrast to the so far established dogma, are not immunologically ignorant but have a Th1-skewed allergen-specific immune response that appears to protect against IBH-associated symptoms. To our knowledge this is the first demonstration of a natural situation, in which an allergen-specific immune skewing is protective in an allergic disorder.

Introduction

Following the seminal discovery by Mosmann and Coffman that CD4 T-cells can differentiate into different subtypes[1], hypersensitivity reactions became associated with different CD4 T-helper (Th) subtypes. Th1 cells, as characterized by the expression of the cytokine IFN-γ, have been associated with type IV hypersensitivity reactions, which are T-cell mediated, delayed type hypersensitivity responses. Th2 cells, as characterized by the expression of the cytokines IL-4, IL-5, and IL-13, have been associated with classical, allergic type-I hypersensitivity reactions; reactions that are associated with an IgE-mediated degranulation of mast cells. Nevertheless, it rapidly was recognized that a more mixed reactions of both types of immune responses persists in most allergic individuals. In mouse models, it was shown that the treatment of allergic animals with type-1 inducing CpG-ODN can ameliorate disease symptoms[2]. However, mainly due to a lack of a truly natural, experimental model systems, knowledge of how these two types of immune responses develop in conjunction with each other during the immune response to an allergen, and how these dynamic interactions contribute to, or prevent the development of allergic disorders, is still largely lacking[3]. Most interestingly, even in a clinical trial in which immune-stimulatory CpG-ODN sequences coupled to allergens were administered, the treatment-induced amelioration of symptoms was not correlated with intracellular levels of IL-4 or IFN-γ in activated CD4T cells[4].

To determine how an underlying, allergen-specific immune skewing may contribute to the development of allergies, we chose a natural, experimental model system in horses. Considerable proportions of horses of different breeds suffer from an IgE-mediated allergic reaction to *Culicoides spp*., a disorder commonly called insect bite hypersensitivity (IBH)[5-7]. Diagnosis of IBH is predominantly based on the assessment of clinical symptoms in the IBH season and a positive skin test with *Culicoides spp.* extract. *Culicoides*-specific IgE titers are reported to be higher in IBH-affected ponies compared to healthy ponies[8-11], and histology of IBH lesional skin showed pronounced eosinophilia[12] and IgE- positive mast cells in acute lesional IBH skin[13]. Therefore, IBH has generally been considered a type-I allergic hypersensitivity reaction. Nevertheless, also an enhanced infiltration of $CD4^+$ T cells and a pronounced type-IV delayed type hypersensitivity component has been described in skin test responses[14].

To our knowledge, no systematic, longitudinal studies have been performed so far that would have given insight into the local immune response during an allergen-induced IBH challenge. To address this aspect of immune responsiveness, we challenged healthy and IBH sensitive ponies by injection of *C. obsoletus* whole body extract into the skin and collected biopsies at different time points thereafter. Our results revealed that IBH-affected ponies show a clear IL-4 characterized type-2 skewing of the immune response upon intra-cutaneous allergen injection. Moreover, contrary to general assumption, healthy ponies, were not immunologically ignorant to *Culicoides*-specific antigens, but showed a type-1 skewed immune response characterized by IFN-γ expression, which correlated with protection against IBH-associated symptoms.

Methods

Study population

Sixteen Shetland ponies were included in the study during the IBH off-season and 19 during the IBH-season, which reflected winter and summer respectively. IBH-affected ponies (n=10 and n=8 respectively, age range 2-10 years) had each been diagnosed by a certified veterinarian and had a history of recurrent, seasonal, pruritic clinical signs of the skin at the mane and tail with remission in the off-season. Control ponies (n=6 and n=11, respectively, age range 4-15 years) were randomly selected from the same stables and had been diagnosed to have no clinical signs or history of IBH. None of the ponies was treated with immunosuppressive drugs prior to or during the experiments.

All animal experiments were approved by the Animal Ethics Committee of the University of Utrecht.

C. obsoletus whole body extract preparation

Whole body extract (WBE) was prepared as previously described before[15]. In brief, whole body extract (WBE) was prepared from about three hundred life female *C. obsoletus* insects, which were frozen at -80 °C. After crushing insects with a micro-pestle in 1ml of PBS containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), samples were centrifuged at 14 000 rpm for 10 min at 4°C. Supernatant was filtered, snap-frozen in liquid nitrogen and stored at -80°C, until use as WBE.

Diagnostic skin test

In horses, it is a common and accepted practice to diagnostically relate allergen-induced swelling to histamine-induced swelling and therefore all ponies were injected intra-dermally with 0.1 ml PBS (T=0), 0.1 ml 1:1000 histamine solution (positive control) and 0.1 ml 1 mg/ml *C. obsoletus* WBE. The developing swelling was then measured 30 min post injection. The relative wheal diameter (RWD) was calculated by subtracting the average value of the histamine and PBS wheal diameter from the corresponding *C. obsoletus* wheal diameter. RWD=*C. obsoletus* WD – ((histamine WD + PBS WD)/2).

Collection and processing of blood and skin samples

Prior to injection, blood was collected form each pony. For the determination of *C. obsoletus*-specific antibody isotypes titers, serum was separated and stored at -20°C. Skin biopsies (4 mm) of WBE injection sites and controls were taken 5 min, 20 min and 24 h after allergen injection, under local anesthesia with 2% lidocaine (B. Braun, AG Melsungen, Germany). Three biopsies were taken per time point, whereby each site was separated far enough from the next to prevent any influence from the one injection to the other. Of the three biopsies taken per time point, two were snap-frozen in liquid nitrogen and stored at -80°C until used for RNA isolation. The third skin biopsy was fixed in 4% neutral buffered formaldehyde and paraffin-embedded for histopathology.

C. obsoletus-specific *antibody titers*

ELISA was performed and analyzed as described previously[15]. In brief, microtiter plates were coated with 10 µg/ml *C. obsoletus* and incubated overnight at 4°C. After washing the plates and blocking, diluted serum samples (1:5, 1:50 and 1:500) were added in duplicate. After 1.5 h, plates were washed and incubated for 1 h with HRP-labeled, goat anti-horse isotype specific antibodies: IgGa (AAI35P), IgGb (AAI36P), IgGc (AAI37P) or IgG(T) (AAI38P) (AbD Serotec, Düsseldorf, Germany) diluted 1:1000 in casein buffer. The microtiter plates were washed with PBS-Tween and developed with tetramethylbenzidine at RT. The reaction was stopped with a 1% HCL solution. Absorbance was measured with a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Berkshire, UK) at a wavelength of 450 nm corrected for the OD measured at 650 nm. The values used for further analysis were calculated by subtracting the OD_{450} of the serum-free control from the OD_{450} of serum samples.

Histological examination of skin samples

Paraffin-embedded biopsies were cut in 4 µm sections and stained with either haematoxylin-eosin (HE) for routine histopathology or toluidine blue (TB) for mast cell analysis. Sections were graded according to a semi-quantitative grading system (0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) as previously described[12]. All HE slides were analyzed microscopically in a blinded fashion towards the experimental grouping of biopsies. Total number of mast cells was determined by counting these cells in three representative fields per slide at 400 x magnification.

mRNA expression in skin biopsies

Snap-frozen skin samples were cut and homogenized with beads for 30 min at a frequency 25/s in 0.5 ml TRIzol reagent (Invitrogen, Breda, NL) using a Mixer Mill 301 (Retsch Verder NV, Vleuten, NL). Next 0.1 ml 100% chloroform was added to each sample and incubated at RT for 3 minutes. A water-phase was obtained by centrifuging 15 min at 14000 rpm at 4 ˚C. The mRNA was extracted from the water-phase using an RNeasy kit (Qiagen, Venlo, NL) according to the manufacturer's specification. The mRNA concentrations were measured with a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). A concentration of 1 µg total mRNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories, Veenendaal, NL) according to the manufacturer's instructions.

QPCR was performed as described previously[12]. All probes were designed with Primer3 (version 0.4.0); for details see **Supp. Table 1**. Relative expression for each gene was calculated by the Pfaffl method[16] using the housekeeping gene 18s ribosomal RNA (18s rRNA) as a reference.

In vitro differentiation of monocyte-derived dendritic cells

Monocytes were isolated from PBMC using mouse anti-human CD14 (biG 10, Biometec) and anti-mouse IgG microbeads using an LS column (Miltenyi Biotec) according to the manufacturers' specifications. Cells were then differentiated for two days in the presence of purified, *E.coli* expressed recombinant horse IL-4 (50 ng/ml) and horse GM-CSF (50 ng/ml). After 2 days, medium was refreshed with new IL-4 and GM-CSF. The culture was maintained for an additional 3 days prior to antigen

exposure. Differentiation and maturation was examined by FACS analysis using mouse anti-human CD206 (3.29B1.10, Beckman Coulter) and mouse anti-human CD86 (IT2.2, Biolegend), and their respective isotype controls.

Antigen-specific T-cell stimulation

PBMC were stimulated with 10 µg/ml WBE or left untreated (mock). After 2 days, medium was refreshed with medium containing 2 ng/ml recombinant equine IL-2 (Kingfisher Biotech). Three days later, cells were harvested and mixed with matured autologous monocyte derived DC that had been activated for 24 h with 1 µg/ml LPS either in the presence or absence of 10 µg/ml WBE. Cells were then incubated for 6 h in the presence of 5 µM monensin (Sigma). Cells were then stained for CD3 and CD4 using the mouse anti-equine CD4 (clone CVS4) and intracellularly with mouse anti-bovine IFNg (CC302, MCA1783F, AbD Serotec), and mouse anti-equine IL-4 Alexa 647 (12H8). Cells were analyzed by flow cytometry and data analyzed using FlowJo software (Tree Star).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.00 (Graphpad Software, San Diego, CA). To compare unpaired samples form IBH-affected ponies with those from healthy ponies, Mann–Whitney *U* test (+) was used. To compare paired data, non-parametric, paired Wilcoxon Signed-Rank Test was used. Results were considered significant when $p \leq 0.05$.

Results

Increased influx of leucocytes in animals suffering from IBH

To investigate the allergen-specific immune balance in the skin of ponies that either suffered from IBH or had been diagnosed to be healthy **(Fig. 1A)**, biopsies were taken at different time points following *C. obsoletus* allergen injection in the skin. In previous studies, we had found an overall, basal activation of the immune system in the skin of horses in summer[12]. To ascertain maximal detection of allergen-specific immune responses in comparison to basal immune activation in the skin, all samples were collected during winter, when *Culicoides spp.* are absent. Histological analysis of the skin following allergen injection was performed using H&E-stained slides from 10 IBH-affected and 6 healthy ponies. Slides were graded by a certified expert, who was blinded for the experimental set-up. In none of the samples, signs of acanthosis or hyperkeratosis could be detected, indicating that the biopsy sites all represent acute lesions **(Supp. Table 2)**. As shown in **Figure 1B**, both healthy and IBH-affected ponies showed an increased lymphocyte influx into the skin upon allergen-injection over time. Influx into the skin of IBH-affected animals was significantly stronger than that into the skin of unaffected animals **(Fig. 1B)**. Eosinophilic granulocytes were found predominantly in the middle dermis of the skin, and consistently more eosinophils were found in the skin of IBH-affected than in the skin of healthy ponies **(Supp. Table 2)**.

Mast cells have been shown before to play an important role in the immune-pathogenesis of IBH[13, 17]. As shown in **Figure 1C**, also the number of mast cells

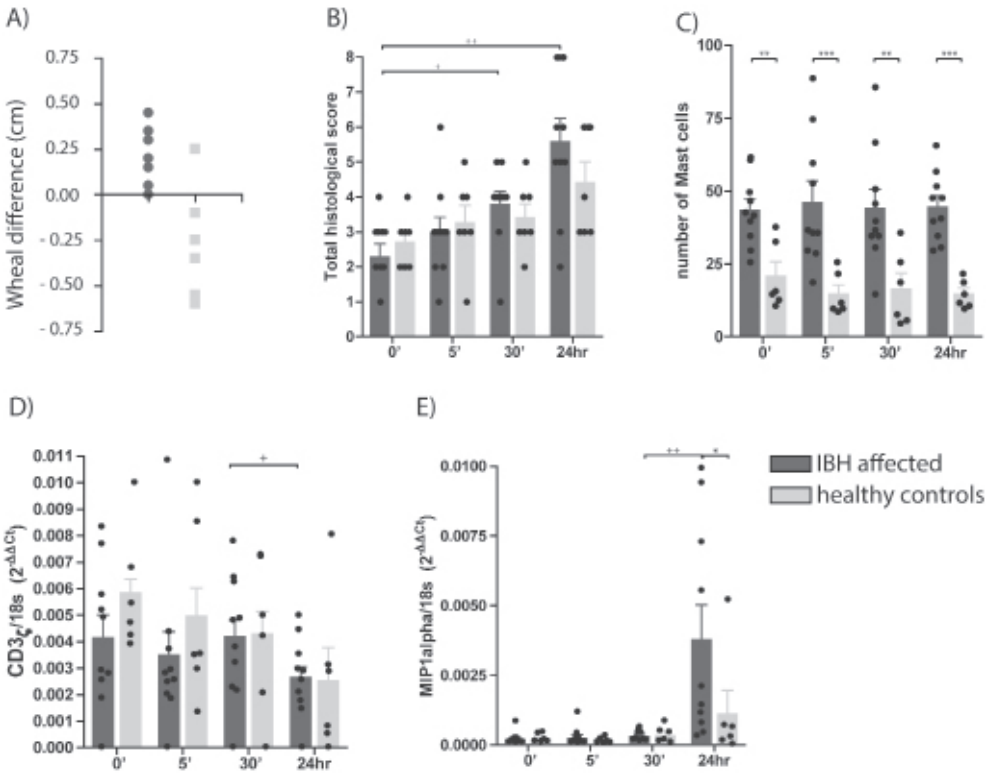

Figure 1: Delayed-type hypersensitivity responses in ponies following allergen injection in the skin. Immune reactions of 6 healthy (light bars) and 10 IBH-sensitive animals (dark bars) to allergen injection in winter was determined at the site of injection at different time points after injection. A) relative skin swelling in animals, 30 min following *C. obsoletus* WBE compared to histamine injection; B) total histological score of the skin; C) number of mast cells; D) total CD3 mRNA expression; E) total MIP1α mRNA expression. Bars represent average +SEM; dots represent individual animals.

in the skin of IBH-affected animals was significantly higher than that in the skin of unaffected animals. Frequency of mast cells in the skin following injection stayed consistent over time, in both groups, suggesting that there is no substantial allergen injection-induced efflux of mast cells into the draining lymph nodes[18].

In contrast to mast cells, however, T-cells appeared to be emigrating from the skin. mRNA expression of $CD3_\zeta$, a T cell marker, in snap-frozen skin biopsies declined in the skin of all treated animals, but was most pronounced in the skin of IBH-affected animals (P=0.0391) **(Fig. 1D)**. MIP1α, a chemokine and activation marker, however, showed the direct opposite development. In all animals MIP1α mRNA expression was up-regulated 24 h after allergen injection, most pronounced in the skin of IBH-affected animals (P=0.0039) **(Fig. 1E)**. MIP1α is expressed by a wide range of different activated leukocytes, such as macrophages and activated T-cells. Expression of MIP1α was most pronounced 24 h after allergen injection. It can be assumed that most of the injected allergen will have been taken up and been processed at that time point. Therefore these data suggest that the main source of MIP1α at the site of injection must have been cells belonging to the adaptive immune

response that were reacting to processed epitopes presented by local antigen presenting cells. Such data further suggest that un-specifically activated by-stander T-cells may at that time point have emigrated the site of inflammation, while allergen-specific T-cells have immigrated and have become activated.

IBH-affected animals have a type 2-biased immune response, while unaffected animals have a type-1 biased immune response.

To analyze the immune response following allergen injection in the skin in more detail, we assessed cytokine expression in the biospies taken at different time points after injection. We found the Th2 cytokine marker, IL-4, significantly (P=0.0039) upregulated 24 h after allergen injection. The expression was significantly (P<0.0001) higher in the skin of IBH-sensitive than in the skin of unaffected animals **(Fig. 2B)**. Also IL-5, another Th2 cytokine marker, showed a significant (P=0.0137) upregulation in IBH skin 24 h after allergen injection (data not shown). In contrast to IBH-sensitive animals, in the skin of healthy animals the Th1 cytokine IFNγ was significantly (P=0.0313) upregulated 24 h post injection **(Fig. 2A)**. In effect, the balance of these two cytokines was substantially shifted towards an IFN-γ-dominated response in healthy animals, while IL-4 was more prominent in animals with IBH **(Fig. 2C)**. Other cytokines, such as IL-10, did not show any up- or down-regulation between the different time points (data not shown). The expression of FoxP3, a transcription factor specific for regulatory T-cells, did not differ between time points; nor was it different between the skin of healthy and IBH-sensitive animals (data not shown).

Taken together, these data show that ponies sensitive to IBH have a strong type-2 biased immune response, while animals protected against IBH-associated pathology show a bias towards a type-1 immune response 24 h after allergen injection.

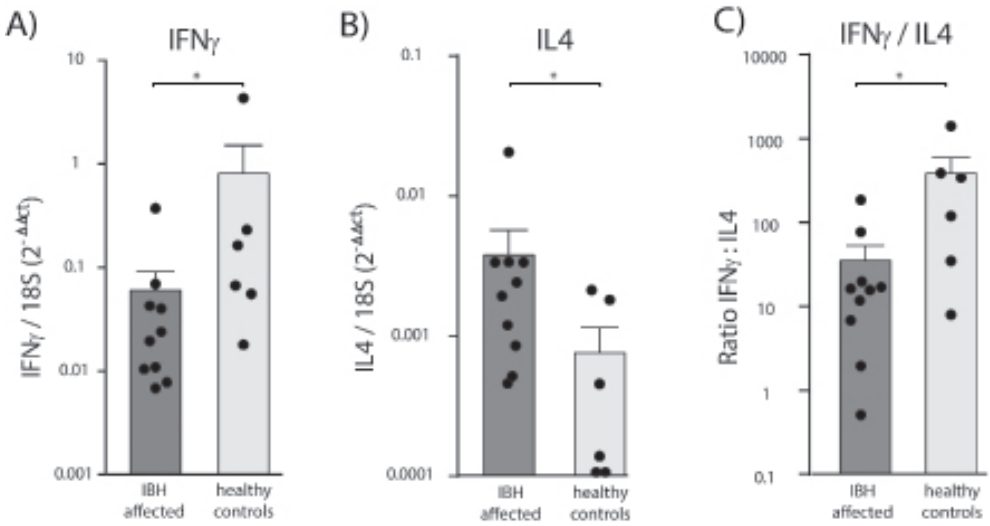

Figure 2:Type-1 immune response skewing in the skin of healthy ponies following allergen injection. Cytokine expression at the site of injection, 24 h after allergen injection, was determined by qRT-PCR. A) IFN-γ and B) IL4 mRNA expression as ratio to 18S. C) Ratio IFN-γ / IL4 expression. Bars represent average +SEM; n = 6 and n = 10, for healthy and IBH-sensitive animals, respectively; dots represent individual animals.

Healthy ponies have Culicoides-specific antibody titers similar to IBH-affected animals.

To determine whether both IBH-sensitive as well as healthy ponies may have developed antigen-specific immune responses against *C. obsoletus* antigens, we measured *C. obsoletus*-specific IgE, IgGa, IgGb, IgGc and IgG(T) antibody levels in the blood of the animals in the off-season. As shown in **Figure 3**, all animals, whether sensitive to IBH or not, had clearly detectable levels of *C. obsoletus*-specific antibodies. As described before [10], the average *C. obsoletus*-specific IgE serum level was elevated for IBH-sensitive compared to healthy animals **(Fig. 3)**, and significant differences between IBH-sensitive and healthy animals were measured for *C. obsoletus*-specific IgGa antibodies (P=0.042), an isotype that correlates with the human IgG1 and is associated with type-2 immune responses in horses [19]. No substantial differences, however, were found for *C. obsoletus*-specific IgGb, IgGc and IgG(T) antibodies in serum between IBH and healthy ponies **(Fig. 3)**.

These data show that all ponies have been exposed to allergens and that all animals have developed *C. obsoletus*-specific antibody responses.

Allergic animals have a Th2-biased allergen-specific immune response, while protected animals have a Th1-biased response

To determine, whether the above-described bias in immune responses is reflected in the underlying allergen-specific CD4 T-helper cell differentiation, we determined the allergen-specific T-cell response in ponies. To this end, CD4 T-cells were isolated from the blood of ponies in summer during the allergy season. Isolated CD4 T-cells were then *in vitro* expanded by PBMC exposed to allergen. Expanded allergen-specific CD4 T-cells were then re-stimulated with allergen-exposed monocyte derived

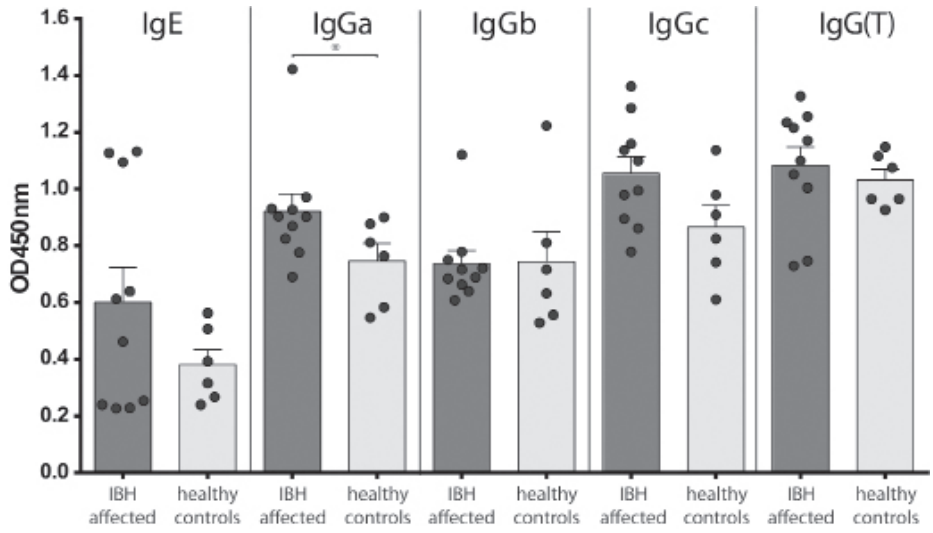

Figure 3: Healthy horses have allergen-specific serum antibodies. Allergen-specific antibody titers, for different isotypes, were determined in the serum of healthy (dark bars) and IBH-sensitive animals (dark bars) by ELISA. Bars represent average +SEM; n = 6 and n = 10, for healthy and IBH-sensitive animals, respectively; dots represent individual animals.

dendritic cells (DC) and cytokine expression was determined by intracellular cytokine staining. Background activation was determined by activation of expanded T-cells by DC that had not been exposed to allergen. As shown in Figure 4, CD4 T-cells expanded from healthy horses was significantly (P=0,0058) higher in frequency than IFNγ-producing allergen-specific CD4 T-cells from animals that suffered from IBH. Despite the fact that healthy horses had also elevated IL4 responses, the ratio of IFNg- versus IL4-producing T-cells was in comparison to IBH affected animals

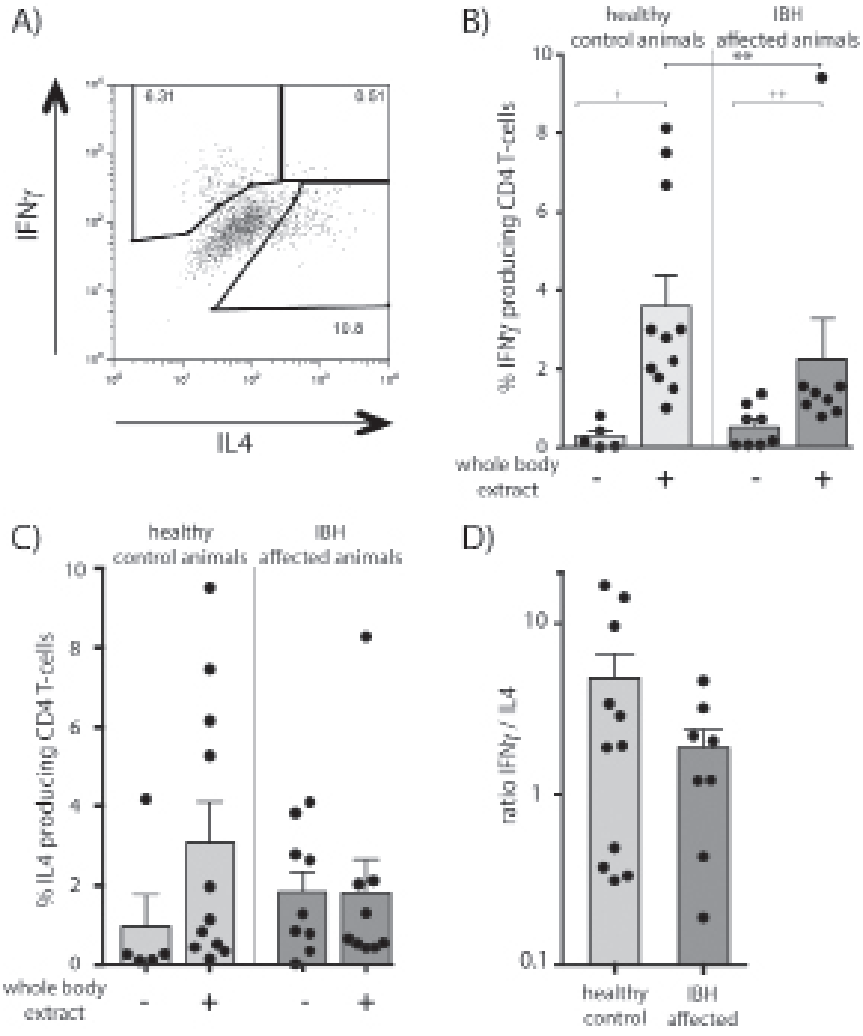

Figure 4: Increased frequencies of allergen-specific Th1 cells in healthy compared to IBH-affected ponies. Allergen-specific CD4 T-cells were *in vitro* expanded in summer from PBMC of 11 healthy and 8 IBH-affected animals, by stimulation with WBE. Cytokine expression of expanded CD4 T-cells was then determined by exposure to monocyte-derived DCs in the presence or absence of allergen. A) representative FACS blot; B) IFN-γ^+ and **C**) IL4$^+$ CD4 T-cells; D) ratio IFN-γ/IL4 CD4 T-cells. Bars represent average +SEM; dots represent individual animals.

substantially skewed towards IFNg-producing cells in healthy animals **(Fig. 4 D)**.

Taken together, these data indicate that an underlying Th1 bias of the allergen-specific immune response correlates with a protection of animals from the allergen-induced IBH-associated pathology.

Discussion

Allergies are wide spread immunological disorders affecting a considerable proportion of the populations. A complex interaction of genetic as well as environmental factors determines whether an allergen-exposed individual becomes allergic or not. Generally, it is assumed that individuals either react with an IgE-associated immune response or remain immunological ignorant or tolerant upon exposure to the allergen. Moreover, is it assumed that in healthy individuals mainly cells that generally are involved in the induction of peripheral tolerance, such as regulatory T-cells, induce tolerance also against allergens. Nevertheless, it remains still largely unknown why such an immunological tolerance is induced in non-allergic individuals but not in allergic individuals.

IBH is an IgE-mediated, naturally occurring allergic skin disease in horses caused by bites of the insect*, Culicoides spp.*. IBH is affecting horse breeds differently and within affected breeds variable fractions of local horse populations suffer from it[20]. IBH therefore appeared a highly attractive model system to determine, why some animals that were exposed to the allergen developed IBH, while others remained unaffected. To address the underlying mechanism that determines why some animals are affected by IBH and others are not, we injected into animals known to be affected by IBH, as well as in animals known to be not affected by IBH, whole body extracts of *Culicoides spp.* - a method known to reliably reflect naturally evoked hypersensitivity reactions in the skin[15]. Our data show that an underlying allergen-specific Th1 immune response correlated with protection, while an underlying allergen-specific Th2 immune response led to the development of IBH. Thus, in contrast to general consensus, healthy animals were not tolerant to the allergen, but responded in a non-pathogenic way to exposure.

In recent years, clinical studies in patients suffering from a number of different types of allergies, such as allergies to cow's milk[21], bee venom[22] or birch pollen[23], have shown that an amelioration of symptoms and the induction of tolerance in allergic individuals is associated with an increase of allergen-specific IgG4 antibodies. These antibodies compete with the existing allergen-specific IgE antibodies for the allergen and thereby prevent the degranulation of mast cells[24]. This shift away from IgE to IgG4 antibodies is supposed to be mediated by IL-10 producing T-cells with regulatory function[24]. In this context, it is interesting to mention that the group of Marti described an increased frequency of $CD25^{high}$ allergen-specific FoxP3-expressing regulatory T-cells in healthy horses. The expression of FoxP3 was significantly higher in regulatory T-cells derived from healthy than in cells derived from IBH-affected horses, when *in vitro* stimulated by allergen[25].

The suppressive capacity of FoxP3-expressing regulatory T-cells *in vivo* is influenced by mast cells[26, 27]. While the activation of mast cells enhances the suppressive capacity of local regulatory T-cell populations[26, 27], IgE-crosslinking

induced degranulation of mast cells diminishes the suppressive capacity of regulatory T-cells[28]. This effect is most likely mediated via the local secretion of histamine[29]. In line with these finding, it was recently demonstrate in a peanut-allergy model that mast cell-derived IL-4 contributes to the priming of allergen-specific Th2 cell responses, while IgE activated mast cells impaired regulatory T-cell induction[30]. Under allergic situations either IgE blockade or inhibition of mast cell signaling promoted regulatory T-cell induction and restored regulatory T-cell versus Th2 cell balance. This balance ultimately led to immune tolerance[30]. Similarly, a recent study using CpG treatment in a chronic mouse lung inflammation model[31], demonstrated that the treatment-induced amelioration of disease was less dependent on the induction of IFNg expression but was more associated with an enhanced regulatory T-cell response.

In line with these findings, we would -based on the data presented here- suggest a scenario in which individuals that do not suffer from an allergic reaction may in first instance not be ignorant to an allergen, but develop a type-1 skewed allergen-specific immune response. In those individuals that react with a type-1 biased allergen-specific immune response, regulatory T-cell populations can suppress local allergic immune responses and induce allergen-specific immune tolerance. Those individuals, however, who react with a type-2 biased immune response, allergen-specific IgE crosslinking may induce degranulation of basophils and mast cells, which renders the local regulatory T-cell population non-functional. This may allow the local inflammation to perpetuate itself, finally leading to the observed allergen-specific epitope spreading and allergen-induced immunopathology. To test such a hypothesis, further research should establish whether these two independent observations in horses, i.e. of differential immune bias of IBH-sensitive and unaffected animals as described here, and the enhanced regulatory function of FoxP3 expressing regulatory T-cells in unaffected animals, as described by Hamza *et al*.[25], are connected with each other via an IgE and mast cell degranulation mediated mechanism.

Thus, taken together, our data cannot fully explain why some individuals react with a type-2 immune response to allergens in the first place. Nevertheless, our data suggest a novel way of how to prevent the development of allergic diseases, for instance in individuals in danger of developing occupational allergic diseases. Such persons could, for instance, be immunized prior to exposure to the allergen in a way that skews the allergen-specific immune response towards a type-1 immune response. Further, should a therapeutic inhibition of mast cell degranulation as well as of type-2 immune responses, accompanied by the induction of allergen-specific type-1 biased allergen-specific immune responses, ameliorate symptoms of allergic individuals and protect treated individuals from developing allergies. Our data further suggest that the natural occurring allergic immune response of horses to *Culicoides obsoletus*-derived allergens could be a valuable model system to test such novel therapeutic approaches; in particular so, since it is well established that the immune response to *Culicoides* allergens in horses at the T cell level closely resembles those in human allergies[32].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary data

Supp. Table 1: Primers and probes and the PCR conditions used for qRT-PCR.

Gene	cDNA (µl)	Ta (°C)	Reference	Probe sequences (5'-3')
IL4	2.5	60	Ainsworth et al., 2003[33]	
IFNγ	2.5	60	Ec03468605_g1; Applied Biosytems	
MIP1α	2.5	60	Ec03469406; Applied Biosytems	
CD3ζ	2.5	60	Debrue et al., 2005[34]	6FAM-CGAGAACCAGCGGCGGAGAGG-MGBNFQ
18S	0.25	60	4352930E; Applied Biosytems	

Supp. Table 2: HE-semi-quantitative histological scores (average and range between brackets) from healthy and IBH affected ponies.

	Healthy (n=6)				IBH (n=10)			
	0'	5'	30'	24hrs	0'	5'	30'	24hrs
Acanthosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Hyperkeratosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Upper dermis: lymphocytes	1.2 (1-2)	1.3 (1-2)	1.3 (1-2)	1.5 (1-2)	0.9 (0-1)	1.2 (1-2)	1.3 (1-2)	1.2 (1-2)
Upper dermis: Eosinophilic granulocytes	0 (0-0)	0.3 (0-1)	0.2 (0-1)	0.3 (0-1)	0.4 (0-1)	0.4 (0-1)	0.6 (0-1)	0.6 (0-2)
Middle dermis: lymphocytes	0.7 (0-1)	0.8 (0-1)	0.8 (0-1)	1.0 (1-1)	0.8 (0-1)	0.9 (0-1)	0.9 (0-1)	1.1 (1-2)
Middle dermis: Eosinophilic granulocytes	0 (0-0)	0 (0-0)	0.2 (0-1)	0.7 (0-2)	0.1 (0-1)	0.2 (0-1)	0.4 (0-1)	1.2 (0-2)
Deep dermis: lymphocytes	0.8 (0-1)	0.8 (0-1)	1.0 (1-1)	1.0 (1-1)	0.1 (0-1)	0.3 (0-1)	0.5 (0-1)	0.9 (0-2)
Deep dermis: Eosinophilic granulocytes	0 (0-0)	0 (0-0)	0 (0-0)	0.2 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)	0.7 (0-2)
Average total histological score	2.7 (2-4)	3.3 (1-5)	3.4 (2-5)	4.4 (3-3)	2.3 (0-4)	3 (1-6)	3.8 (1-5)	5.6 (2-8)

0 = absent, 1= minimal, 2= mild, 3 = moderate, and 4 = severe.

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Chapter 5

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

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Abstract

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

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

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

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

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

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

Introduction

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

Various studies have shown that IBH is an IgE-mediated allergy that resembles type I allergies in humans.[4, 5] In addition, there is evidence for the involvement of Th1-, Th2- and Treg-type cytokines in IBH in Icelandic horses.[6-10] It has been shown that peripheral blood mononuclear cells (PBMC), stimulated with *Culicoides* whole body extract (WBE), from IBH-affected horses, produced higher levels of IL-4 and lower levels of IFN-γ than those from healthy horses, suggesting a bias towards a Th2 immune responsiveness.[6] PBMC from affected horses also produced less IL-10 and TGFβ1, indicating that IBH is associated with a reduced regulatory immune response.[7] Furthermore, upon stimulation of PBMC with *Culicoides* whole body extract (WBE) the expression of FoxP3 by $CD4^{+CD25+}$ cells was significantly higher in healthy than in IBH-affected horses. Moreover in skin biopsies of healthy Icelandic horses, significantly higher levels of mRNA for Foxp3 were found compared to skin of IBH-affected horses.[10] In contrast, another study showed no significant differences in Foxp3 mRNA expression levels between skin biopsies of IBH-affected and healthy Shetland ponies.[11]

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

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

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

In the present study we used a Dendritic Cell (DC)/Peripheral Blood Mononuclear

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

Material and methods

Animals

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

Production of recombinant allergens

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

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

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

The sequences of horse GM-CSF and horse IL-4 (Genbank, accession number NM_001082519.1 and NM_001081882.1) encoding the mature form (without signal sequence) of the proteins were ordered as synthetic genes (GeneArt, Life Technologies, Bleiswijk, Netherlands) codon-optimized for expression in *E.coli* and with a BamHI and HindIII restriction site at the 5'and 3'-ends, respectively. The

products obtained were cut with BamHI and HindIII and cloned downstream of the hexahistidine encoding tag into the corresponding restriction sites of a pET15b (Novagen)-derived vector, pET15bNew. The isolated pET15bNew plasmids encoding IL-4 or GM-CSF were used to transform *E. coli* BL21 CodonPlus (DE3) (Agilent Technologies, Amstelveen, Netherlands). Expression, purification and refolding of the insoluble (inclusion bodies) horse GM-CSF was performed as described for recombinant carp chemokines.[19] The horse IL-4, produced using the same approach as described for GM-CSF, was present in the soluble protein fraction of the bacterial lysate, which was kept on ice. After addition of 0.4 M NaCl, 25 mM imidazole and 1% Triton X114 it was purified by incubation with chelating sepharose fast flow (Amersham-Biosciences GE Healthcare, Eindhoven, Netherlands) charged with Ni^{2+} according to manufacturer's instructions. The Ni2+ beads were subsequently washed with 40 column volumes ice cold Phosphate Buffered Saline (PBS), containing an additional 0.4 M NaCl, 25 mM imidazole and 1% Triton X114 to remove contaminating proteins and Lipopolysaccharide (LPS). The Ni^{2+} beads were then washed with 5 volumes of 40% isopropanol in PBS to remove the triton-X114 and subsequently washed with 10 column volumes of PBS containing an additional 0.4 M NaCl. The his6-tagged, recombinant horse IL-4 was finally eluted with the same buffer containing 250 mM imidazole. Protein-containing fractions were pooled and dialyzed extensively against PBS and, after filter sterilization (0.2 µm), stored at -20°C.

Production and FITC labelling of the monoclonal anti-equine CD4

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

PBMC collection

At the end of the IBH season heparin blood (140 ml) was collected from the jugular vein of each pony with Intraflon 2 catheters (16G Ø1.6 mm; Vycon, France) under local anaesthesia with 2% lidocaine (B. Braun, Melsungen, Germany). The blood was mixed with 140 ml PBS, containing 2% horse serum. Of this mixture 35 ml was carefully pipetted into a SepMate™-50 (STEMCELL technologies, Grenoble, France) tube with Histopaque-1077 (Sigma-Aldrich, Zwijndrecht, Netherlands) that was centrifuged at RT and 1300g for 20 min. The PBMC fraction harvested from the interphase was then washed twice with PBS containing 2% horse serum (Gibco Life Technologies, Bleiswijk, Netherlands) and left on ice until further use.

Monocyte-derived dendritic cells (DC)

After PBMC isolation, 75% of the cells were labelled with a cross-reactive mouse anti-human CD14 (a monocyte marker, cross reactive to horse according to the manufacturer) (biG 10, Biometec, Greifswald, Germany) diluted 1:100 and incubated at RT for 15 min. Cells were then washed twice with Macs buffer (PBS with 2%

horse serum and 2 mM EDTA; Gibco Life Technologies, Bleiswijk, Netherlands) incubated with anti-mouse IgG microbeads (Miltenyi Biotec, Leiden, Netherlands) and isolated via LS columns (Miltenyi Biotec, Leiden, Netherlands) according to the manufacturer's specifications. The monocyte depleted cell fraction was added to the 25% remaining PBMC to be used for antigen-specific T-cell generation.

To produce DC the CD14-positive cells (monocytes) were transferred into complete medium (RPMI 1640 (Gibco Life Technologies, Bleiswijk, Netherlands), 10% horse serum, 1% Glutamax (Gibco Life Technologies, Bleiswijk, Netherlands), 1% Pen/Strep (Gibco Life Technologies, Bleiswijk, Netherlands) and 30μM β-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands)) containing horse IL-4 (50 ng/ml) and horse GM-CSF (50 ng/ml) to induce monocyte differentiation into DC and plated into a 24-wells plate (1 x 10^6 cells/well), and incubated at 37°C and 5% CO_2 for two days.

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

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

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

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

After 2 days 0.5 ml medium was carefully removed and replaced by 0.5 ml fresh complete medium with 2 ng/ml recombinant equine IL-2 (kingfisher Biotech, Saint Paul, MN, USA). After 3 more days PBMC were harvested and washed with complete

medium. These cultures were then plated into a 96 well v-bottom plate (0.5 X10^6 cells/well) for antigen-specific T-cell stimulation. Matured antigen-loaded (Pokeweed mitogen (PWM), P1, P2 or mock) DC were washed with complete medium and added (0.05 X10^6 cells/well) to their corresponding antigen stimulated PBMC in a ratio of 1:10 (DC:PBMC). After one hour of recovery at 37˚C and 5% CO_2 additional antigen (PWM, P1, P2 or Mock) and 2 µl of monensin (Sigma-Aldrich), to block secretion of produced cytokines, were added and cultures were incubated at 37˚C and 5% CO_2 for 6 h. Subsequently, cells were harvested and washed two times with PBS buffer and left on ice. Cell suspensions were sampled for FACS analysis and the remaining cells were re-suspended in 0.5 ml Trizol (Invitrogen Life Technologies, Bleiswijk, Netherlands) and frozen at -20°C for mRNA extraction.

Flow cytometry

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

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

To identify the $CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}4^+$ cells, labelling was first performed with mouse anti-equine CD4 and cells were subsequently fixed. After fixation and permeabilization, rat anti-human CD3 Pacific Blue (CD3-12, AbD Serotec, Puchheim, Germany) and secondary labelling was performed for CD4 with anti-mouse PE (e-Biosciences, Vienna, Austria) and intercellular staining with cross-reactive mouse anti-bovine IFN-γ FITC (CC302, MCA1783F, AbD Serotec, Puchheim, Germany), and mouse anti-equine IL-4 Alexa 647 (12H8; Wagner Lab, Cornell University, USA). Mouse IgG1 FITC and mouse IgG1 Alexa 647 isotype controls (GM4992 and MG121, Life technologies, Bleiswijk, Netherlands) were used to determine unspecific staining. Cells were washed and flow cytometry was performed with a FACS canto II. Data were analysed using FlowJo software. For the $CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}4^+$ fraction only FACS measurements with more than 200 events in the final gate were used for analysis, except for the $CD3^+CD4^+CD25^{high}FoxP3^+IL\text{-}10^+$ staining a lower value of

100 events was accepted otherwise too many samples would be lost for analysis.

QPCR

Messenger RNA extraction from the frozen Trizol samples was performed as previously described.[11] In short, 0.1 ml 100% chloroform was added to 0.5 ml of the Trizol samples and incubated at RT for 3 minutes. After centrifugation (15 min at 14000 rpm at 4 ˚C) the water-phase was used to extract mRNA using an RNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's specifications. The mRNA concentrations were measured with a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, Netherlands). All mRNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories, Veenendaal, Netherlands) according to manufacturer's instructions. QPCR was performed for the following genes: 18s, $CD3_{\zeta}$, IFN-γ, IL-4, IL-10 and FoxP3, as described previously.[11] Relative expression of all cytokines were normalized against 18s using the Pfaffl method[23] and a fixed point from the standard curve was used as calibrator. Subsequently mRNA expression levels were assessed relative to the corresponding $CD3_{\zeta}$ expression, to relate the cytokine expression to the T-cells in the DC/PBMC co-cultures.

Statistical methods

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

Results

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

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

Maturation of the cultured DC one day after addition of antigen and LPS as determined by FACS analysis was approximately 50% as indicated by CD86 expression on the DC **(supp. Fig 1)**.

To determine the effect of recombinant allergen-specific stimulation of T-cells, mRNA expression of CD3ζ, IFN-γ, IL-4, IL-10 and FoxP3 was measured with RT-QPCR and normalized against 18s rRNA levels. The normalized values of IFN-γ,

IL-4, IL-10 and FoxP3 were divided by the normalized CD3ζ **(supp. Fig. 2)** values to correct for the number of T-cells in the DC/PBMC co-cultures.

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

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

IL-10 mRNA was only significantly upregulated between mock and PWM

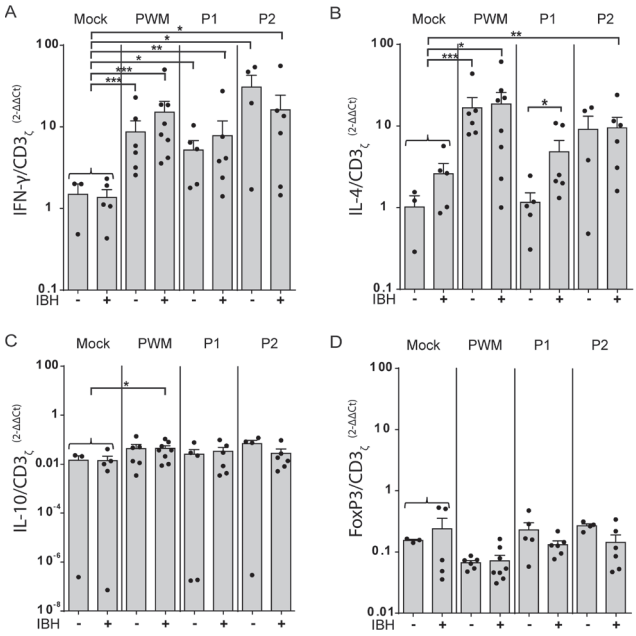

Figure 1: Expression of IFN-γ, IL-4, IL-10 and FoxP3 mRNA in Dendritic Cell / Peripheral Blood Mononuclear Cell (DC/PBMC) co-cultures stimulated with C. obsoletus-specific antigens. DC/PBMC co-cultures derived from IBH and control ponies were stimulated with pools of recombinant *C. obsoletus* antigen (P1= Cul o 1, Cul o 2, Cul o 5; P2= Cul o 3, Cul o 5, Cul o 7), Pokeweed Mitogen (PWM) = (positive control)) or left unstimulated (mock) for 6 h in the presence of monensin. The mRNA expression of IFN-γ, IL-4, IL-10 and FoxP3 was determined by qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[23] and subsequently divided by the $CD3_\zeta$/18s value of the same sample. The dots represent individual ponies and the bars represent the means of all samples in the groups. Significant differences are indicated as followed * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001. A) Expression of IFN-γ /$CD3_\zeta$ (Th1 cytokine). B) Expression of IL-4/$CD3_\zeta$ (Th2 cytokine). C) Expression of IL-10/$CD3_\zeta$ (immune regulatory cytokine). D) Expression of FoxP3/ $CD3_\zeta$ (Treg cell marker).

(p=0.0345) stimulated T-cells from IBH-affected ponies **(Fig.1C)**.

There were no significant changes in FoxP3 mRNA expression between T-cells exposed to the different stimuli compared to the mock **(Fig.1D)**.

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

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

Flow cytometry was used to determine the percentages of CD4 positive cells expressing IFN-γ or IL-4 in the DC/PBMC co-cultures **(Fig 2A-C)**. The percentage of IFN-γ expressing T-cells was significantly upregulated in DC/PBMC co-cultures from control ponies stimulated with P1 ($p<0.0001$), P2 ($p<0.0001$) and PWM ($p<0.0001$) compared to mock stimulation. T-cells from IBH-affected ponies also expressed significantly more IFN-γ when stimulated with PWM ($p<0.0001$), P1 ($p<0.0001$) and P2 ($p<0.0001$) compared to mock **(Fig. 2A)**. There were no significant changes in percentage IL-4 expressing T-cells between mock-stimulated T-cells and the different stimuli or between T-cells from IBH-affected and control ponies **(Fig.2B)**.

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

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

We found a significant increase of $CD25^{high}$FoxP3-positive cells in T-cells from control ponies stimulated with P1 ($p=0.0006$), P2 ($p=0.0367$) and PWM ($p=0.0010$) compared to unstimulated T-cells **(Fig. 3A)**. The IBH-affected ponies showed significant differences between unstimulated T-cells and P1 ($p=0.0051$) and PWM ($p=0.0189$) stimulation. Additionally, all $CD3^{+}CD4^{+}CD25^{high}FoxP3^{+}$ cells expressed IL-10 **(Fig 3B)**.

Discussion

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

To examine the presence of antigen specific T-cells and a possible difference in Th1/Th2 balance between IBH-affected and control ponies, IFN-γ and IL-4 expression levels in allergen stimulated and unstimulated (mock) cultures were compared. QPCR showed that IFN-γ mRNA was significantly upregulated in DC/PBMC co-cultures from both control and IBH-affected ponies stimulated with the

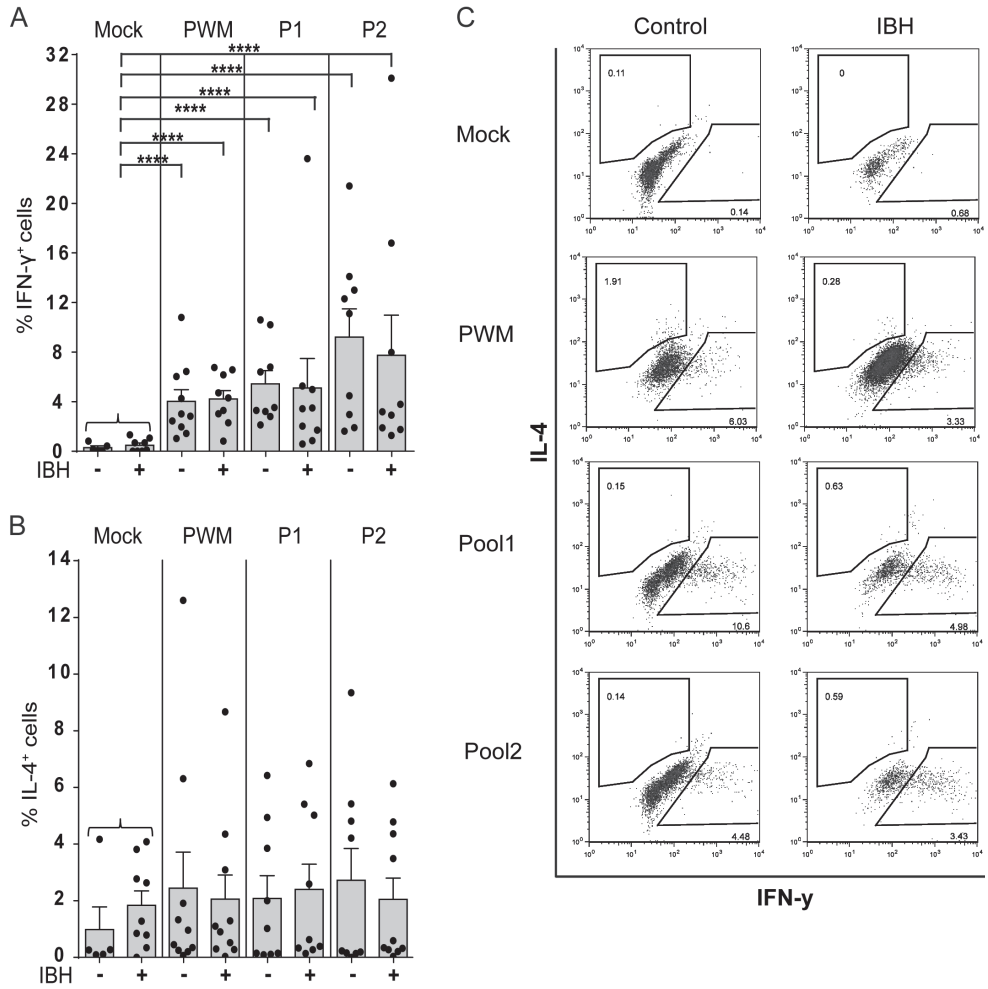

Figure 2: Percentages of IFN-γ and IL-4 expressing CD3+CD4+ T-cells in Dendritic Cell / Peripheral Blood Mononuclear Cell (DC/PBMC) co-cultures stimulated with *C. obsoletus*-specific antigens as determined by flow-cytometry (FACS). DC/PBMC co-cultures derived from IBH and control ponies were stimulated with pools of recombinant *C. obsoletus* antigen (P1= Cul o 1, Cul o 2, Cul o 5; P2= Cul o 3, Cul o 5, Cul o 7), Pokeweed Mitogen (PWM) = (positive control)) or left unstimulated (mock) for 6 h in the presence of monensin. Subsequently, all cells were stained for analysis by flow cytometry. Live cells were gated for $CD3^+$ and the $CD3^+$ population was used for $CD4^+$ gating. Subsequently, the $CD4^+$ population was used for IFN-γ and IL-4 analysis. The dots represent individual ponies and the bars represent the mean of all samples in the group. Significant differences are indicated as followed * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001. A) IFN-γ expression. B) IL-4 expression. C) Representative FACS plots for all different stimuli.

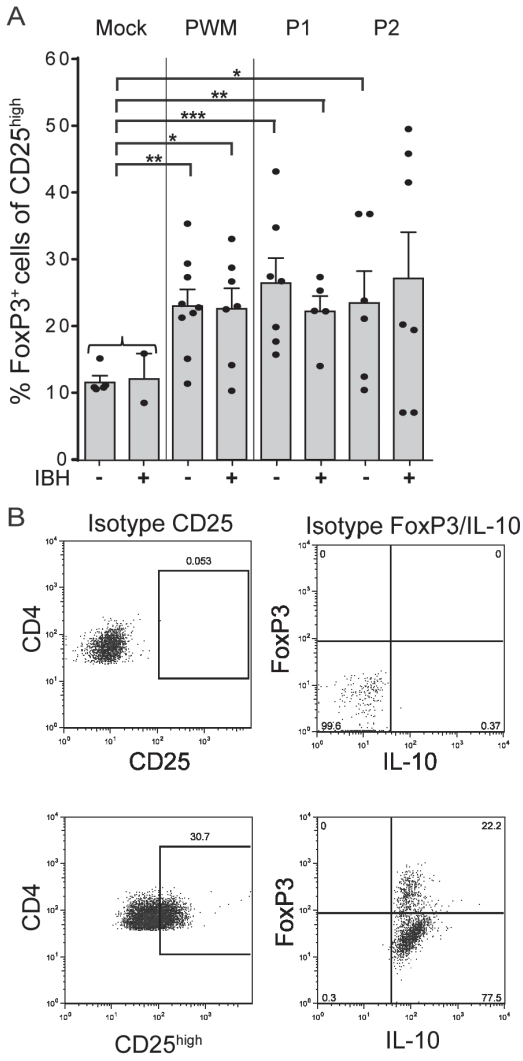

Figure 3: Percentage of FoxP3 and IL-10 expressing $CD3^+CD4^+CD25^{high}$ cells in Dendritic Cell / Peripheral Blood Mononuclear Cell (DC/PBMC) co-cultures stimulated with *C. obsoletus*-specific antigens as determined by flow-cytometry. DC/PBMC co-cultures derived from IBH and control ponies were stimulated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

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

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

In addition, to the Th1\Th2 balance, Treg cells have been suggested to play a major role in the control of allergies in humans[24] and horses.[18] For this reason we examined the FoxP3 and IL-10 mRNA expression in the DC/PBMC co-cultures by QPCR and the percentage of $CD3^{+}CD4^{+}CD25^{high}$ expressing FoxP3 and IL-10 cells by flow cytometry. The mRNA levels for IL-10 and FoxP3 were much lower than those for IFN-γ and IL-4 and no significant differences in FoxP3 and IL-10 mRNA expression were observed between unstimulated and P1, P2 or PWM stimulated DC/PBMC co-cultures (Fig. 1). However, in contrast to these QPCR data performed on the total DC/PBMC co-cultures, flow cytometry analysis of the $CD3^{+}CD4^{+}CD25^{high}$ subpopulation in these cultures, did reveal an increase of FoxP3 and IL-10 producing

cells after stimulation with PWM and with the recombinant allergen pools P1 and P2. This indicates that P1 and P2 are able to stimulate the development of antigen-specific Tregs. The overall FoxP3 and IL-10 data suggest that P1 and P2 can activate Treg cells to produce the immune suppressive cytokine IL-10 *in vitro*.

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

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

The authors want to thank all Shetland pony owners for their cooperation and Peter W.T. Stolk (Stolk Equine Consultancy, Leersum, The Netherlands) for performing all the clinical procedures in this study. We are grateful for the mouse anti-equine CD4 producing CVS4 hybridoma which was generously provided by David P. Lunn (Colorado State University, USA). We would also like to thank Bettina Wagner for providing the mouse anti-equine IL-4 Alexa 647 antibody (Cornell University, USA).

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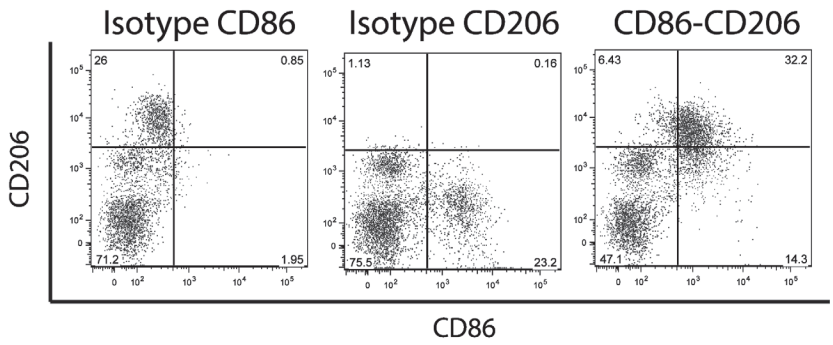
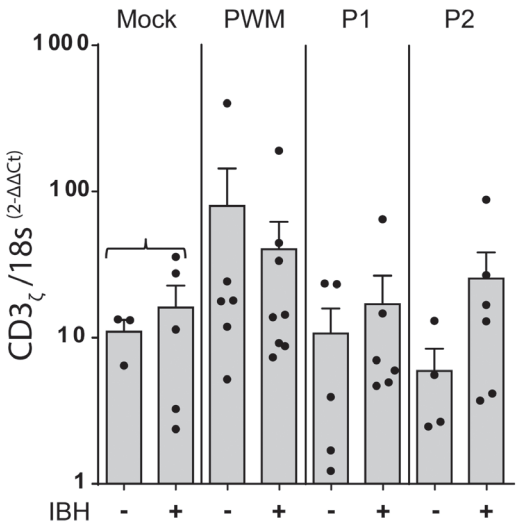
Supplementary data



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



Supp. Figure 2: Expression of CD3ζ mRNA, a T cell receptor marker in antigen-stimulated DC/PBMC co-cultures. Antigen-specific T-cells derived from IBH and control ponies were stimulated with mature unloaded (Mock) or antigen-loaded, monocyte-derived DC (P1= Cul o 1, Cul o 2, Cul o 5; P2= Cul o 3, Cul o 5, Cul o 7; or PWM= (positive control)) for 6 h in the presence of monensin. The mRNA expression of $CD3_{\zeta}$ was determined by qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method [23]. The dots represent individual ponies and the bars represent the mean of all samples in the group.

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Chapter 6

Basophil-derived Amphiregulin is essential for UVB irradiation-induced immune suppression

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Abstract

Ultraviolet-B (UVB) irradiation (290-320nm) is used to treat skin diseases like psoriasis and atopic dermatitis, and is known to suppress contact hypersensitivity (CHS) reactions in mouse models. Regulatory T cells (Treg cells) have been shown to be responsible for this UVB-induced suppression of CHS. The EGF-like growth factor Amphiregulin (AREG) engages the EGF-R on Treg cells and, in different disease models, it was shown that mast cell-derived AREG is essential for optimal Treg cell function *in vivo*. Here we determined whether AREG plays a role in UVB-induced, Treg cell-mediated suppression of CHS reactions in the skin. Our data show that AREG is essential for UVB-induced CHS suppression. In contrast to general assumption, however, were mast cells dispensable for UVB-induced immune suppression, while basophil-derived AREG was essential. These data reveal a novel function for basophils in the homeostasis of immune responses in the skin. Basophils thus fulfill a dual-function; they contribute to the initiation of effective type-2 immune responses and, by enhancing the suppressive capacity of local Treg cell populations, also to local immune regulation in the skin.

Introduction

UVB-irradiation treatment is in wide clinical use for the treatment of skin disorders, such as psoriasis[1]. Depending on the intensity and the wavelength, UVB irradiation can induce a wide range of different physiological responses. At low dose, UVB irradiation induces immune suppression in mice[2, 3]. FoxP3 expressing regulatory T cells (Treg cells) have been shown before to play a central role in the process of local resolution of inflammation and the induction of peripheral tolerance [4], and it was shown that also this UVB-irradiation induced form of antigen-specific immune tolerance is Treg cell mediated and can adoptively be transferred into naive mice[5, 6]. Although it is well established that inflammatory conditions can influence Treg cell function, the precise signals that enhance or dampen Treg cell function during different forms of inflammation remain poorly understood. We recently have shown that the functionality of local Treg cells and their capacity to resolve local inflammation is determined by signals received via the Epidermal Growth Factor Receptor (EGF-R)[7]. EGF-R is highly expressed in Treg cells[7], which is induced via STAT5 signaling[8]. The EGF-like growth factor Amphiregulin (AREG) enhances the suppressive capacity of Treg cells in *in vitro* suppression assays, and mast cell-derived AREG is essential for optimal Treg cell function *in vivo*[7].

Here we tested whether UVB-mediated immune suppression is dependent on AREG. Using UVB exposure induced immune suppression in either C57BL/6 *wt* or AREG gene-deficient mice, and transfer experiments, we show that UVB-induced immune suppression is AREG-dependent. In contrast to general assumption, however, we find that not mast cell-derived AREG but basophil-derived AREG is essential for UVB induced suppression of CHS.

Material & Methods

Mice

C57BL/6 mice were purchased form Charles River and Harlan. C57BL/6 FoxP3-GFP transgenic, c-kit$^{w\text{-}sh}$, CD4cre x EGF-R$^{flox/flox}$, AREG gene-deficient, C57BL/6.SJL (CD45.1) and CD45.1.2 mice (F1 of C57BL/6 x C57BL/6.SJL) were bred in house under specific pathogen-free conditions. AREG gene-deficient mice had been backcrossed for at least 14 times onto C57BL/6. Mice were used between 7 and 17 weeks of age. All animal experiments had been approved by the Committee on Animal experiments of Utrecht University prior to their performance.

UVB irradiation and induction of contact hypersensitivity

TL20W/12 lamps (Philips Eindhoven, the Netherlands) were used for the UVB irradiation. This lamp has about 60% output in the UVB range and an emission spectrum ranging from 280 nm to 350 nm with a maximum around 310 nm. Relative spectral distribution measurement of the UVB source was performed with a calibrated standard UV-visible spectrometer (model 752, Optronic Laboratories Inc., USA). Throughout the experiments irradiation of the mice was monitored with a UVB detector device of Waldmann (Waldmann, Villingen-Schwenningen, Germany), which was calibrated with the above-mentioned spectrometer. A 4 x 2.5 cm field on

the backs of mice was shaved. Mice were then either irradiated with the broadband UVB irradiation for 4 consecutive days or left untreated. The UVB intensity has been measured before each experiment. Twenty-four hours after the last UVB irradiation, all mice were sensitized to DNFB by painting 25µl 0.3% DNFB, dissolved in a 4:1 acetone / olive oil mixture, onto their shaved backs. 10 days after sensitization, mice were challenged on the ear with 20µl 0.1% DNFB, dissolved in a 4:1 acetone / olive oil mixture. Challenge was repeated 24 h later and ear thickness measured 24 h later, using a digital micrometer ("Quick Mini", Mitutoyo, Kawasaki, Japan). Ear swelling was calculated by subtracting the thickness of the ear prior to challenge from the thickness of the ear after 48 h of challenge. Average swelling of both ears was used for further analysis.

Adoptive transfer of immune response

5 days after UVB irradiation donor wt C57BL/6 or FoxP3-GFP transgenic mice were sacrificed by cervical dislocation, the skin draining lymph nodes (inguinal, axillary and brachial lymph nodes) excised and $1x10^7$ SDLN cells or $4x10^5$ FACS-sorted Treg cells transferred into naïve hosts via i.v. injection. Induction of contact hypersensitivity was performed as described above. FACS-based cell sorting was performed on a BD Influx Cell sorter.

Antigen-specific activation of in vitro differentiated basophils

BM was isolated from C57BL/6 mice and differentiated in the presence of WEHI cell supernatant containing recombinant murine IL3 for 7 days. Cells were then incubated with anti-CD49-PE, anti-c-kit-APC antibodies and FITC-conjugated IgE antibody specific for DNP (H1 DNP-epsilon 26.82) (35) FITC-labeled cells (Fc-e-R positive) were FACS-sorted based on CD45b expression (basophils) and lack of c-kit expression (to exclude mast cells). Purified basophils were activated in the presence of 100 ng/ml DNP-BSA or PMA/ionomycine, or were left untreated.

Generation of mixed BM chimeras

Mixed BM chimeric mice were generated as previously described (36). In short, BM donor cells from C57BL/6 *wt* and RORα gene-deficient or from C57BL/6 *wt* and Mcpt8cre transgenic mice were mixed 1:1 and 10^7 cells were transferred *i.v.* into lethally irradiated (10 Gy) C57BL/6 *wt* acceptor mice. Eight weeks after BM transfer, reconstituted mice were subjected to UVB-induced immune suppression was induced as described above.

Sample collection and Flow cytometry

Cells were isolated from spleen and ear draining lymph nodes (EDLN), red blood cells removed and cell surface staining of CD4. CD45.1 and CD45.2 was performed for at least 20 min at 4 ˚C in the presents of Fc-block (2.4G2). Intercellular staining for FoxP3 was performed according to the manufacturer's specifications using a FoxP3 intracellular staining kit (eBioscience). Samples were measured using a FACS CantoII (BD Biosciences) and analyzed with FlowJo software (Tree star).

Quantitative real-time reverse transcription-polymerase chain reaction

Total mRNA was extracted from the ear draining lymph nodes (EDLN) after

challenge using TRIzol (Invitrogen) and further treated as previously described (37). RT-PCR for IL-4, IFNγ, FoxP3, CD3ε and 18s were performed according to the manufacture instructions using TaqMan Gene Expression Assays (Applied BioSystems) Mm00445259, Mm00801778, Mm00475162, Mm00599684 and 4352930E respectively.

Statistics

Statistical analysis between two groups was performed with a two-tailed Mann-Whitney U test using statistical software (GraphPad Prism 6.0). P values < 0.05 were considered to be significant. In the Figures depicts * a P value < 0.05, ** a P value < 0.01 and *** a P value < 0.001

Results

UVB exposure of 400 J/m²/day leads to optimal suppression of CHS in C57BL/6 mice

Exposure to UVB irradiation can induce a wide range of different inflammatory reactions. Low levels of UVB irradiation induce immune suppression in mice[2, 3]. To determine the precise intensity at which UVB irradiation induces immune suppression in C57BL/6 mice, mice were exposed to UVB irradiation, ranging over different intensities, then sensitized with the hapten DNFB and, 10 days later, challenged with DNFB on the ear. In untreated mice, such a challenge induces a CHS reaction that leads to ear swelling, which is a typical form of delayed-type hypersensitivity (DTH). As shown in **Figure 1A**, UVB irradiation within a specific window of intensity induced immune suppression, reaching a maximum at a range of 400 J/m²/day. UVB irradiation exposure of a higher intensity diminished this suppression **(Fig. 1A)**. Prior UVB irradiation treatment (400 J/m²/day) enhanced the portion of FoxP3 expressing T-cells in the ear-draining lymph nodes (EDLN) of challenged mice **(Fig. 1B)**, which

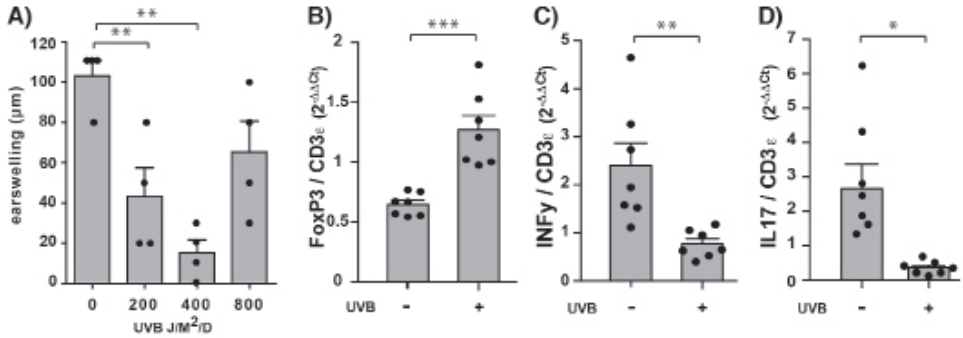

Figure 1: UVB irradiation suppresses contact hypersensitivity induced ear swelling in mice. C57BL/6 *wt* mice were exposed to different intensities of UVB irradiation and were then sensitized with DNFB. 10 days later mice were challenged on both ears and A) ear swelling was measured after 48 h of challenge. Ear draining lymph nodes (EDLN) were extracted for QPCR analysis of CD3ε, FoxP3, IFNγ and IL17 mRNA expression. Bars represent average +SEM; n = 4-7 mice per group and dots represent individual mice.

directly correlated with diminished IFNγ- and IL17-expression in the EDLN (**Fig. 1C-D**, respectively). These data show that UVB irradiation exposure at 400 J/m^2/day induces the most optimal suppression of CHS in C57BL/6 mice.

Lack of UVB irradiation induced immune suppression in AREG-/- mice

UVB mediated suppression is transferrable and has been shown to be Treg cell mediated[5, 6]. Since we have shown that Treg cells, for optimal function, are dependent on AREG-induced EGF-R signaling, we tested the role of AREG in UVB-induced immune suppression. To this end, we exposed both C57BL/6 *wt* and AREG gene-deficient mice to UVB irradiation, sensitized them and challenged them 10 days later on the ear. In agreement with an earlier study that showed that AREG is not essential for the induction of CHS in mice[9], both non-UVB-treated *wt* and AREG-deficient control mice developed a clear CHS reaction upon challenge. UVB irradiation exposure, however, induced immune suppression in *wt* mice, whereas UVB irradiation treatment had no immune tolerizing effects in AREG gene-deficient mice **(Fig. 2A-B)**. These data show that UVB mediated immune suppression is dependent on AREG expression.

Efficient suppression of CHS by Treg cells is dependent on AREG expression

AREG enhances Treg function *in vivo* and in *in vitro* suppression assays[7]. To determine whether this UVB-induced and AREG dependent form of immune suppression is Treg cell mediated, we performed cell transfer experiments from UVB-treated animals into animals that had not been exposed to UVB. First, we transferred

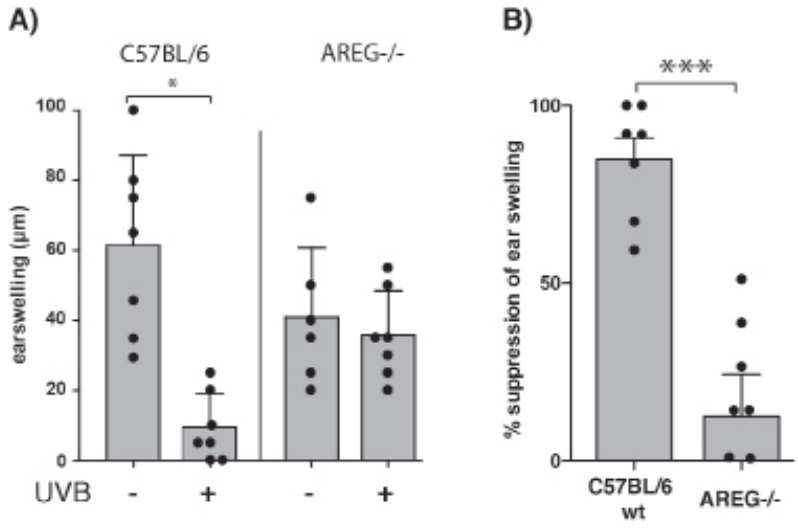

Figure 2: UVB irradiation suppresses ear swelling in *wt* but not in AREG$^{-/-}$ mice.
C57BL/6 *wt* and AREG$^{-/-}$ mice were either irradiated or left untreated. All mice were sensitized with DNFB and challenged 10 days later. A) Ear swelling was measured and B) % of suppressed ear swelling in UVB exposed versus unexposed control animals was determined. Bars represent average +SEM; n = 7 mice per group and dots represent individual mice.

cells isolated from the skin-draining lymph nodes (SDLN, i.e. inguinal, axial and brachial lymph nodes) of UVB-exposed C57BL/6 animals into *wt* C57BL/6 or AREG gene-deficient mice. Tracing of the transferred cells in the spleen of recipient mice, based on expression of the CD45.1 and CD45.2 congenic markers, showed that similar amounts of cells had been transferred **(Fig. 3A)**. These transferred cells conferred their suppressive capacity onto *wt*, but not onto AREG gene-deficient recipient mice **(Fig. 3B)**. Since, after challenge, frequencies of transferred Treg cells in the ear draining lymph nodes of *wt* and AREG-/- recipient mice were similar **(Fig. 3C)**, we concluded that AREG expression in recipient mice was essential for efficient UVB-mediated immune suppression.

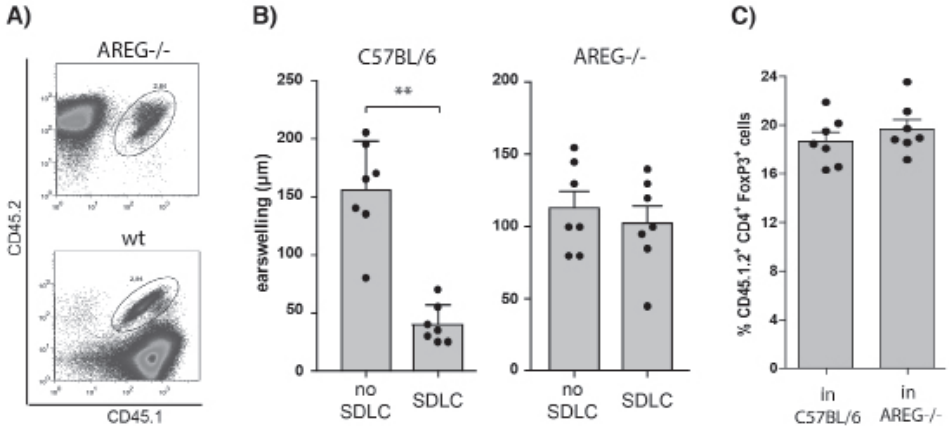

Figure 3: Transferred skin draining lymph node cells confer the ability to suppress CHS onto *wt* but not AREG$^{-/-}$ mice. Naïve CD45.1.2 BL/6 *wt* mice were irradiated and sensitized. 5 days later cells from the skin-draining lymph nodes (SDLN) were transferred into naïve CD45.1 BL/6 *wt* or into naïve AREG$^{-/-}$ mice (CD45.2). Recipient mice were sensitized and challenged 10 days later. A) Frequency of transferred lymphocytes in the spleen of C57BL/6 and AREG-/- recipient mice was determined, B) ear swelling was measured in recipient mice and in control mice that had not received SDLN, and C) the frequency of Treg cells in the ear draining lymph nodes of recipient mice was determined. Bars represent average +SEM; n = 7 mice per group and dots represent individual mice.

To verify that Treg cells mediated the observed immune suppression, Treg cells were purified from UVB-exposed FoxP3-GFP transgenic mice by FACS sorting and were transferred into *wt* C57BL/6 or AREG gene-deficient mice. As shown in **Figure 4A**, transfer of Treg cells was sufficient to induce suppression of CHS in *wt*, but not in AREG gene-deficient mice. Thus, Treg cells contribute to UVB-induced immune suppression.

To determine whether the requirement for AREG in this model was mediated through a direct effect on Treg cells, we transferred similar amounts of cells isolated from the SDLN of either UVB-exposed *wt* C57BL/6 or CD4cre x EGF-R$^{fl/fl}$ mice (a mouse strain that lacks EGF-R expression specifically on T-cells) into *wt* C57BL/6 mice. Transferred cells derived from UVB-treated *wt* mice suppressed CHS significantly (P = 0.007) better in recipient mice than cells derived from UVB-

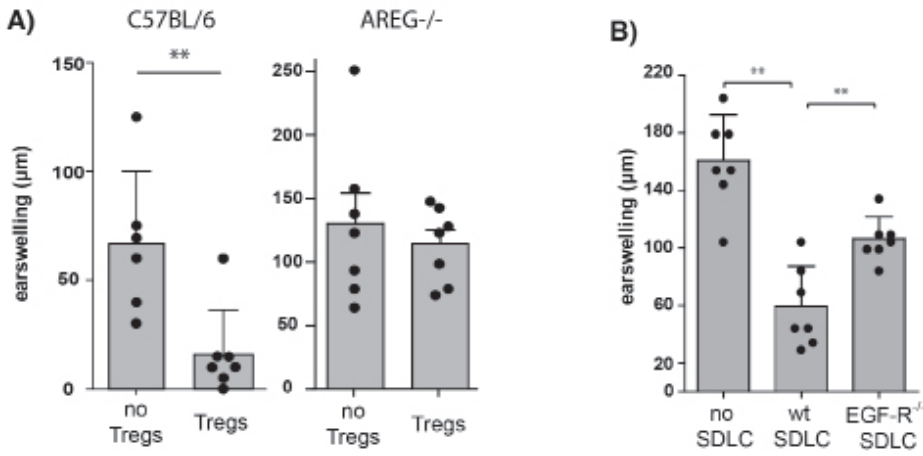

Figure 4: Suppression is transferred by Treg cells whose function is dependent on AREG. A) Naïve FoxP3-GFP transgenic mice were irradiated and sensitized. Five days later, Treg cells were purified from the skin-draining lymph nodes (SDLN) and spleen and transferred into naïve C57BL/6 *wt* or AREG$^{-/-}$ mice. Recipient and control mice that had not received Treg cells were sensitized and challenged 10 days later. Ear swelling was measured. B) C57BL/6 *wt* or CD4cre x EGF-R$^{fl/fl}$ mice were irradiated and sensitized. Five days later, lymphocytes derived from the skin-draining lymph nodes (SDLN) were transferred into naïve C57BL/6 *wt* mice. Recipient mice were sensitized, challenged 10 days later and ear swelling in recipient mice versus control mice was determined. Bars represent average +SEM; n = 7 mice per group and dots represent individual mice.

treated mice that lacked EGF-R expression in T-cells **(Fig. 4B)**. These data show that AREG acts directly on Treg cells and enhances their suppressive capacity in this hypersensitivity model.

Suppression of CHS is dependent on basophil-derived AREG

To determine the physiologically relevant source of AREG that enables Treg cells to suppress CHS, we induced UVB-induced immune suppression in the mast cell deficient mouse strain c-kit$^{w\text{-}sh}$. To our surprise, c-kit$^{w\text{-}sh}$ mice were perfectly capable of suppressing CHS upon UVB exposure **(Fig. 5A)**. These findings are in contrast to the findings of Hart *et al*.[10] who reported that mast cells were essential for UVB-induced immune suppression. This group however used a mast cell deficient mouse strain with a mutation in the c-kit receptor (W^f/W^f), which in addition to mast cell deficiency also displays basophil deficiency[11]. Not only mast cells have been reported to produce AREG, but also human basophils[12] and murine[13] and human ILC2 cells[14]. All these cell types are known to play an important role in inflammatory conditions in the skin. We therefore first tested whether also murine basophils express AREG. To this end, we differentiated basophils *in vitro* and labeled them with FITC-labeled IgE antibodies. Basophils were then FACS-sorted based on IgE binding as well as CD49b and c-kit expression. IgE bound cells that expressed CD49b but not c-kit (i.e. basophils) were then activated by antigen-specific crosslinking or via PMA/Ionomycin activation. Either way of activation induced expression of AREG in basophils **(Supp. Fig. 1)**. These data demonstrate that activated mouse basophils express AREG.

To test whether basophils and / or ILC2 are involved in the suppression of CHS in mice that have been tolerized by UVB exposure, we tested mice that either lack basophils, i.e. Mcpt8cre transgenic mice[15], or lack ILC2, i.e. RORα gene deficient mice[16] for their involvement in UVB irradiation induced immune suppression. Since both cell types play a central role also in the initiation of immune responses and in the attraction of other leukocytes to site of inflammation, and since RORα gene-deficient mice have a lethal phenotype that is not connected to the immune system (so called "staggerer"), we established mixed bone marrow chimeric (BMX) mice with a 1:1 mixture of bone marrow (BM) derived from AREG gene-deficient and either Mcpt8cre transgenic or RORα gene-deficient mice. Due to homeostatic expansion, frequencies of basophils and ILC2 in these newly established BMX mice will be the same at eight weeks after reconstitution. However, in BMX mice that received Mcpt8cre transgenic BM all basophils will be derived from the AREG-/- donor, while in mice that received RORα gene-deficient BM, all ILC2 will be derived from AREG-/- donors. Eight weeks after re-constitution, we detected similar frequencies of basophils in the spleen of all BMX mice (CD49b$^+$ cells, **Fig. 5B**), which demonstrates that all basophils in the spleen of BMX mice that received a mix of ARGE-/- and Mcpt8cre transgenic BM were derived from the AREG gene-deficient BM donor.

Eight weeks after reconstitution, mice were tested for UVB-induced suppression of CHS. BMX mice that lacked AREG expression in ILC2 showed a high variability of responses but an overall significant suppression of CHS **(Fig. 5C)**. However, BMX mice that lacked AREG expression in basophils did not show any substantial

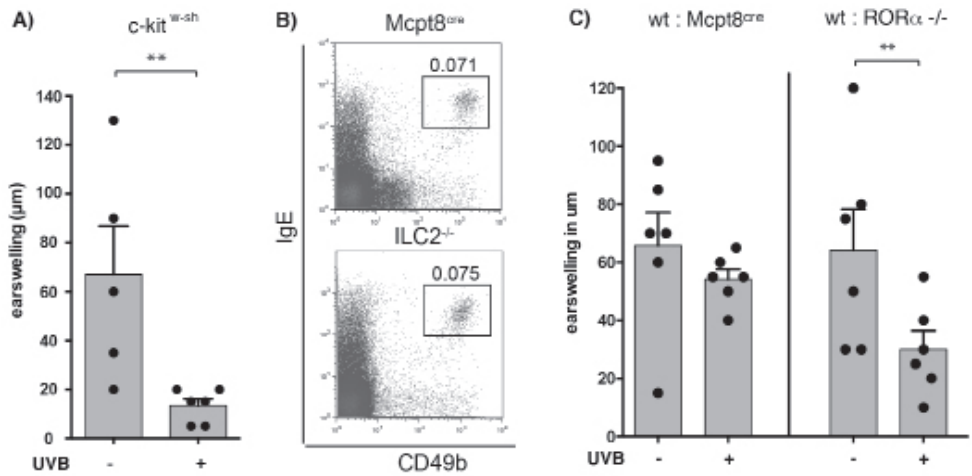

Figure 5: Basophil-derived AREG is essential for UVB irradiation induced immune suppression. A) Mast cell deficient c-kit$^{w\text{-}sh}$ mice were irradiated and then sensitized with DNFB. 10 days later mice were challenged on both ears and ear swelling was measured after 48 h. B & C) C57BL/6 mice were irradiated with 10 Gy gamma irradiation and re-constituted with a 1:1 mixture of bone marrow (BM) derived from AREG$^{-/-}$ and Mcpt8cre transgenic or derived from AREG$^{-/-}$ and RORα$^{-/-}$ mice. B) 8 weeks after reconstitution, frequency of basophils in the spleen was measured by flow cytometry. C) Reconstituted mice were irradiated with UVB light and then sensitized. 10 days later mice were challenged on both ears and ear swelling was measured after 48 hrs. Bars represent average +SEM; n = 5-6 mice per group and dots represent individual mice.

suppression of CHS, at all **(Fig. 5C)**.

Taken together, these data show that, in the skin, basophil-derived AREG is essential for Treg cell-mediated suppression of CHS in mice.

Discussion

In contrast to published data[10], our data reveal that not mast cells but basophils play an essential role in UVB-induced immune suppression in mice. These results reveal a novel function of basophils in the regulation of immune responses in the skin. So far basophils have been best known for their early production of IL4[17], for their contribution to Th2 cell differentiation[17-19], for their association with the pathogenesis of allergic[15, 20] and atopic dermatitis[21] and with the induction of anaphylaxis in specific mouse models[22]. Also their unique contribution to protective immunity against helminth infections[23] and ticks[24] is well established. Recently, however, it became apparent that basophils are of high importance also in the regulation of skin homeostasis[25]. For instance, basophils mediate the differentiation of inflammatory monocytes into alternatively activated, so called M2 macrophages at the site of inflammation. This differentiation of inflammatory monocytes into M2 macrophages within allergic skin lesions significantly contributes to the alleviation of allergic skin inflammation[25]. Here we reveal a novel function of basophils during skin inflammation, i.e. to enable local Treg cells to suppress CHS. The unexpected discovery of this immune regulatory function for basophils is in line with a number of other findings that indicate a substantial contribution of type-2 immune responses to immune suppression and tissue repair[26, 27].

Predominantly mast cells have so far been associated with immune suppressive functions in the skin. So is, for instance, in a skin transplant model the collaboration between Treg cells and mast cells essential for the induction of immune tolerance[28]. Also in a chronic UVB irradiation model, it has been shown that mast cell-derived IL10 plays an important role in the dampening of sunburn associated inflammation in the skin[29]. In addition, have we shown that mast cell-derived AREG is essential for optimal Treg cell function in BM transplantation-induced skin rejection, in T cell-mediated colitis and in a tumor vaccination model[7]. All these examples are forms of slowly developing, chronic inflammation. Thus it is tempting to speculate that a chronic form of inflammation allows mast cells to immigrate, proliferate and differentiate at the site of inflammation. In CHS, however, antigen-specific Treg cells are induced by UVB-exposure of the back of a mouse, while acute inflammation is induced in the ear of a mouse. Basophils are one of the first cells that infiltrate the site of inflammation, but, with a half-life of about two days, are also relatively short lived[30]. Remarkably, our data indicate that AREG derived from ILC-2 appears to have a less strong influence on Treg cell function in UVB-induced immune suppression of CHS than that derived from basophils **(Fig. 4A)**. Substantial numbers of ILC2 can be found in non-inflamed skin[31] and it will be interesting to investigate in future studies why basophils contribute significantly better to the Treg cell-mediated suppression of CHS than ILC2 do.

Given these observed differences in the different types of inflammation, it is tempting to speculate that mast cells and basophils fulfill similar functions at

different stages of inflammation. While mast cells carry type-2 responses during later phases of inflammation, basophils may play a more important role during early phases of local inflammatory responses. In this regard it is interesting to note that mast cells can not only enhance Treg cells function[7], but can also suppress it. For instance, degranulation of mast cells temporarily abolishes Treg cell function *in vivo*[32]; a process possibly mediated via histamine mediated suppression of Treg cell function[33]. Similarly, exposure of basophils to T-cell derived IL-3 induces AREG expression[12]. The amounts of AREG expressed by basophils are much higher when stimulated by IL-3 than by IgE cross-linking, which was in direct negative correlation to IL-4 expression and histamine release by basophils[12]. Thus, it is attractive to speculate that also in the case of basophils, the type of inflammation determines whether basophils enhance or diminish the suppressive capacity of local Treg cell populations. In this regard, it also is very interesting to note that a developmental and functional heterogeneity within basophil populations has been revealed recently[34]. One type of basophils, so called IL-3-elicited basophils, is highly sensitive to IgE crosslinking and degranulates and releases histamine upon IgE mediated activation. The other type of basophils, so called TSLP-elicited basophils, is less sensitive to IgE mediated cross-linking but readily produces cytokines upon exposure to IL-3 or IL-33[34]. TSLP is a predominant epithelial cell-derived cytokine expressed in the skin. It therefore would be interesting to see whether these two different basophil populations might play opposing roles in Treg cell-mediated regulation of local inflammation in the skin, which would further emphasize the special role the skin plays in immune regulation.

Conflict of Interest

The authors state no conflict of interest

Acknowledgments

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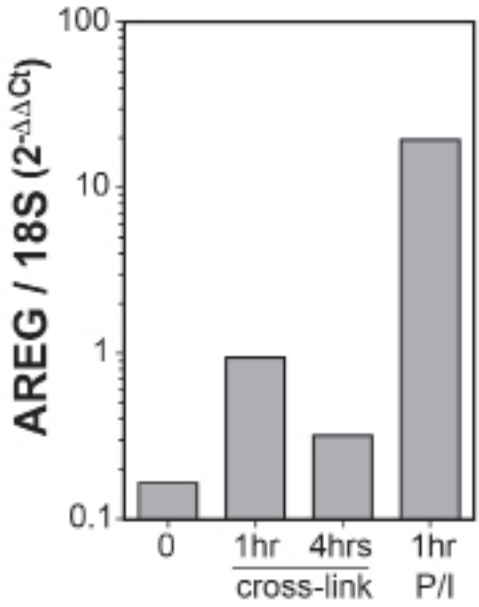
Supplementary data



Supp. Figure 1: Activated mouse basophils express Amphiregulin.
Basophils were in vitro differentiated from BM cells in the presence of recombinant IL3. Differentiated cells were stained with fluorescence labeled IgE antibodies and with antibodies to detect CD49b and c-kit expression, and IgE-positive, CD49b positive, c-kit negative cells (the basophil population) were purified by FACS-sort. Cells were then activated by FcεR cross linking or with PMA/Ionomycin. AREG expression was measured by real time-PCR. scDNA levels were equalized to 18S expression.

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Chapter 7

The effect of UVB-irradiation on *C. obsoletus*-sensitive skin of horses and ponies prior to intradermal challenge - a pilot study

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Abstract

Insect bite hypersensitivity (IBH) is an allergic pruritic dermatitis in horses which is predominantly caused by the bites of *Culicoides spp*. In IBH a Th2 response is most prevalent and the initial of 'allergic reaction at onset' is IgE-mediated. To date there is no satisfactory treatment method.

Currently UVB light therapy has been partially successful in treatment of human patients with allergic contact dermatitis. In addition, UVB-induced immunosuppression of contact dramatis in mice is mediated by Treg cells induced by basophil-derived amphiregulin (AREG). Hence, in the present pilot study we investigate the effect of UVB-irradiation on the skin of horse with IBH.

In this pilot study 6 ponies were treated with UVB light followed by a challenge with *C. obsoletus* whole body extract (WBE). Skin swelling was measured 30 min, 24h and 48h post challenge and skin biopsies were taken for histopathology and assessment of expression of mRNA of IFNy, IL-4, IL-10, FoxP3 and AREG at the latter two time points.

A significantly smaller wheal size was found in the UVB-irradiated skin as compared to non-irradiated skin 30 min after WBE challenge. This suggests that less inflammatory mediators where released in UVB-irradiated skin. Because there was no increase in expression of mRNA of IL-10, FoxP3 and AREG in UVB-exposed skin 24h post challenge, it could not be proven that AREG-mediated Treg cell activation occurs in IBH skin after UVB irradiation. However since this pilot study only accounts for the current experimental conditions, other test variables need to be tested in the future. The downregulation of IL-4 and IFNy mRNA found in UVB-irradiated skin 48h post challenge compared to 24h, which was absent in non-irradiated skin, indicated a downregulation of both Th2 and Th1 immune responsiveness as a result of irradiation. These results warrant further studies on the effects of UVB treatment and its mechanism in horses with IBH.

Introduction

Insect bite hypersensitivity (IBH) is a skin disease in horses and ponies which manifests initially with papules followed by severe itch. The itch results in self-inflicted chronic lesions with hair loss, scaling, crusting and thickening of the skin.[1] To date, the most common treatment strategies for IBH are itch-relieving ointments, blankets, insecticides and glucocorticosteroids, which control symptoms rather than curing the disease. Prolonged use of topical corticosteroids as well as systemic immunosuppressant drugs (e.g., corticosteroids) can result in severe cutaneous and systemic side effects.

Although theoretically antigen-specific immunotherapy (ASIT) is a promising treatment modality, little is known of the immunopathogenesis, and moreover the results of ASIT using *Culicoides spp*. whole body extract, are contradictive so far.[2-4]

An alternative treatment of IBH may be phototherapy with UVB light. UVB radiation is widely used in the treatment of skin diseases in humans, such as psoriasis, atopic dermatitis (AD), mycosis fungoides and vitiligo.[5-9] Human AD shares general features of barrier dysfunction and skin infection with other diseases such as allergic contact dermatitis. Whereas a Th2 response is observed in acute AD, a mixed immune response (Th2/Th1/Th22) is common in chronic AD.[10] The exact mechanism by which UVB therapy works in AD is not known.

Although, UVB therapy can be beneficial, there are some short-term side effects like erythema, dry skin with pruritus, occasional blistering, and an increased frequency of recurrent herpes simplex eruptions.[11] Long-term side effects include photoageing and possible carcinogenesis.[11]

The first beneficial clinical effects of low dose UVB light therapy are generally seen after 6 to 8 weeks of treatment with three exposures a week.[12-14] In a previous study we found that naïve mice irradiated with UVB light were protected against induction of contact hypersensitivity (CHS) also known as contact dermatitis. This protection was initiated due to the production of amphiregulin (AREG) by basophils, which contributed to activation of Treg cells, responsible for a systemic allergen-specific suppressive effect.[15]

In another study we showed that 24h after challenge with WBE, IBH-affected ponies upregulate IL-4 mRNA in the skin, whereas healthy controls upregulate IFNy[16]. It was concluded that upon challenge with WBE, the immune system of healthy individuals responds by skewing the Th1/Th2 balance to a more Th1 response with subsequent reduction of clinical symptoms. Taken these results into consideration, we wanted to examine if UVB-irradiation can either upregulate IFNy or downregulate IL-4.

We hypothesize that local UVB-irradiation has a beneficial effect on IBH, either by increasing amphiregulin production and subsequent activation of Treg cells, or by skewing the immune response towards a Th1 responsiveness.

In order to investigate such potential effects of local UVB irradiation, expression of the cytokines IL-4, IL-10, FoxP3, AREG and IFNy were examined, as well as skin swelling induced by WBE challenge and histopathology of UVB-irradiated or non-irradiated skin of IBH-affected horses.

Methods

Animals

Six IBH-affected ponies/horses (one Dutch Warmblood, three Shetland ponies, one miniature horse and one mini Appaloosa with an age range of 3-22 years) were included in the study during the IBH off-season. The animals had a history of recurrent, seasonal, pruritic clinical signs of the skin at the mane and tail with remission in the off-season. None of the ponies were treated with immunosuppressive drugs prior to or during the experiments.

All animal experiments were approved by the Animal Ethics Committee of the University of Utrecht.

C. obsoletus whole body extract preparation

Whole body extract (WBE) was prepared as previously described by Van der Meide et al.[17]. In brief**,** WBE was prepared from about 300 living, female *C. obsoletus* insects, which were immediately frozen at -80 °C. After crushing insects with a micro-pestle in 1ml of PBS-containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), samples were centrifuged at 14 000 rpm for 10 min at 4°C. Supernatant was filtered (0.22µm pore diameter), snap-frozen in liquid nitrogen and stored at -80°C.

UVB radiation, WBE challenge and assessment

Seven small squares (4 x 4 cm) were shaven on the neck of each of the animals for 0,1ml intradermal injections with: 1) 1:1000 histamine solution (positive control); 2) PBS 24h; 3) PBS 48h; 4) 1 mg/ml *C. obsoletus* WBE 24h; 5) 1 mg/ml *C. obsoletus* WBE 48h; 6) 1 mg/ml *C. obsoletus* WBE 24h and UVB irradiation; 7) 1 mg/ml *C. obsoletus* WBE 48h and UVB irradiation. To insure that the UVB negative injection sites were not subjected to UVB irradiation due to possible systemic effect injections 1-5 were performed before 6 and 7. After 30 min, 24h and 48h relative wheal diameter (RWD) was measured for each of the conditions 1-5 according to standard procedures.[18] The RWD was calculated by subtracting the average value of the histamine and PBS wheal diameter from the corresponding *C.obsoletus* wheal diameter. RWD=*C. obsoletus* WD – ((histamine WD + PBS WD)/2).

Fourty-eight hours post challenge, two squares of the UVB-negative sites, two squares were irradiated with broad band (290-320 nm) UVB light (400J/m^2/d) (TL12 lamp, Philips Eindhoven, The Netherlands) for 4 consecutive days. Due to the size of the lamp only 1 square could be irradiated at a time. The other square was covered with a stack of 5 paper towels during irradiation to prevent double exposure.[19] The UVB lamp used has approximately 60% output in the UVB range and an emission spectrum ranging from 280 nm to 350 nm with a maximum around 310 nm. Relative spectral distribution measurement of the UVB source was performed with a calibrated standard UV-visible spectrometer (model 752, Optronic Laboratories Inc., USA). Throughout the experiments irradiation of the animals was monitored with a UVB detector device (Waldmann, Villingen-Schwenningen, Germany), calibrated with the above-mentioned spectrometer.

Twenty-four hours after the last UVB irradiation, in all ponies each irradiated site was intradermally injected with 0,1ml of 1 mg/ml *C. obsoletus* WBE. After 30 min, 24h and 48h RWD was measured.

Collection and processing of blood and skin samples

Three skin biopsies (4 mm) of each PBS and WBE (with and without UVB irradiation) injection sites were taken under local anesthesia with 2% lidocaine (B. Braun, AG Melsungen, Germany) at 24h and 48h post challenge. Two biopsies of each injection site were snap-frozen in liquid nitrogen and stored at -80°C until used for RNA isolation. The other one was fixed in 4% neutral buffered formaldehyde for 24-48h and paraffin-embedded for histopathology.

Histological examination of skin samples

Paraffin-embedded biopsies were cut in 4 µm sections and stained with haematoxylin-eosin (HE) for routine histopathology. A semi-quantitative grading system (0=absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) was used to assess the degree of epidermal (hyperplasia and hyperkeratosis) and dermal (infiltration of both mononuclear cells and eosinophils) changes. The overall histological score, defined as the sum of the scores for each of these parameters, was determined for each biopsy. All slides were analyzed by the same European College of Veterinary Pathologists-certified pathologist, who was blinded with regard to the group and time assignment of biopsies.

Quantitative Real-time reverse transcription-polymerase chain reactions

Frozen skin samples were minced and homogenized in TRIzol reagent (Invitrogen, Breda, NL) using a Biopulverizer (Biospec #59012N, Biospec Inc., Bartlesville, OK) and polytron (PT 1200 E, Kinematica AG, Lucerne, CH). TRIzol manufacturer's instructions were followed until the water-phase was obtained after the chloroform step. Subsequently, total RNA was extracted using RNeasy columns (Qiagen, Venlo, NL) and eluted with 30 µl of RNase free water. The RNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Thermo Scientific, Etten-Leur, NL). One µg of total RNA was used to produce cDNA with an iScript cDNA Synthesis Kit (Bio-Rad laboratories, Veenendaal, NL) according to manufacturer's instructions. A 5′ nuclease assay using TaqMan probes was employed for qRT-PCR amplification. QPCR was performed for the following genes: 18s, CD3ζ, IFNγ, IL-4, IL-10 and FoxP3 as described previously.[20] Expression of AREG was assessed with primers described by SM Lindbloom et al 2008[21] and the probe sequence 5'-CCCTTGGGTCTCGACTATGA-3' was designed using Primer3. Reactions were performed in 25 µl volumes containing 5 µl cDNA, 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Austin, TX, USA), 0.9 µM relevant primers and 0.25µM fluorescence-labeled probes and milliQ. The qRT-PCR were performed in an iCycler (Bio-Rad laboratories, Veenendaal, NL) with amplification conditions of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 62, 60 or 57 °C. Each reaction was ran in duplicate and a negative control was included to exclude contamination of reagents with cDNA on every plate. PCR efficiencies were calculated using a relative standard curve derived from a cDNA pool of equine PBMC stimulated with pokeweed mitogen (Sigma–Aldrich, St. Louis, MO, USA) for 24h. Relative expression of IL-4, IL-10, IFNγ, FoxP3, AREG and $CD3_{\zeta}$ was calculated using the Pfaffl method[22] using the housekeeping gene 18s ribosomal RNA (18s rRNA; 4352930E, Applied Biosystems) as a reference gene. The relative expression values of cytokine that are

primarily produced by T-cells in the skin (IL-4, IL-10, IFNγ and FoxP3) were divided by the relative $CD3_{\zeta}$ value from the same skin sample to be able to express on a per T-cell basis.

Statistical analyses

Statistical analyses were carried using GraphPad Prism 4.00 (Graphpad Software, San Diego, CA). As data were not normally distributed, the non-parametric paired Wilcoxon Signed-Rank Test (WSR Test) (*) was used for comparison of all paired data. Results were considered significant at $p \leq 0.05$.

Results

Relative wheal diameter after UVB irradiation

To determine the effect of prior UVB irradiation on the wheal diameter induced by injection of WBE, the RWD was calculated for different time points **(Fig. 1)**. At time point 30 min the RDW in the UVB-irradiated skin of 6 ponies was significantly (p=0.0313) smaller compared to that in non-UVB-irradiated skin from the same ponies. At 24h this difference was no longer significant. After 48h the wheal formation had totally disappeared.

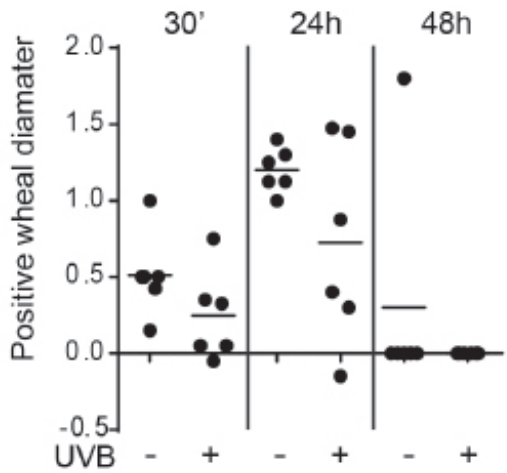

Figure 1: Relative Wheal diameter (RDW) of UVB-irradiated and non-irradiated skin challenged with WBE from *C. obsoletus* at 30 min, 24h and 48h post challenge. Non-irradiated skin from IBH-affected ponies was injected with WBE, PBS and histamine. In addition, UVB-irradiated skin in the same ponies was injected with WBE. The absolute wheal diameter was measured at 30 min, 24h and 48h post challenge. The RWD was calculated by subtracting the average value of the histamine and PBS absolute wheal diameter from the corresponding WBE absolute wheal diameter. RWD=*C. obsoletus* WD – ((histamine WD + PBS WD)/2). Lines represent the mean of all samples in the group.

Table 1: H&E-semi-quantitative histological scores (average and range between brackets) from IBH-affected ponies under different conditions at different time points.

	PBS		WBE		UVB + WBE	
	24h	48h	24h	48h	24h	48h
Acanthosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Hyperkeratosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Upper dermis:						
lymphocytes	0.2 (0-1)	0.8 (0-1)	1.0 (1-1)	0.7 (0-1)	1.2 (1-2)	1.0 (0-2)
eosinophilic granulocytes	0 (0-0)	0 (0-0)	1.0 (0-2)	0.3 (0-1)	1.0 (0-2)	0.8 (0-2)
Mid dermis:						
lymphocytes	0.2 (0-1)	0.3 (0-1)	0.7 (0-1)	1.0 (0-2)	1.2 (1-2)	0.8 (0-2)
eosinophilic granulocytes	0 (0-0)	0 (0-0)	0.8 (0-2)	0.7 (0-2)	1.0 (0-2)	1.2 (0-2)
Deep dermis:						
lymphocytes	0 (0-0)	0 (0-0)	0.3 (0-1)	0.7 (0-2)	1.3 (0-2)	0.8 (0-2)
eosinophilic granulocytes	0 (0-0)	0 (0-0)	0.5 (0-2)	0.7 (0-2)	1.2 (0-2)	1.2 (0-2)
Total histological score	**0.3 (0-1)**	**1.2 (0-2)**	**4.3 (1-6)**	**4.0 (0-8)**	**6.8 (2-12)**	**5.8 (1-12)**

0 = absent, 1= minimal, 2= mild, 3 = moderate, and 4 = severe.

Histopathology scores of UVB-irradiated skin 24h after WBE challenge as compared to non-irradiated skin

Before challenge no visual differences were observed between UVB-irradiated and non-irradiated skin. To assess the effect of UVB light treatment on the histological parameters of inflammation after challenge, scores for individual parameters were determined and the total histological scores were calculated. When analyzing the individual histology parameters 24h and 48h after challenge, no indications of acanthosis and hyperkeratosis, characteristic for chronic lesions, were found **(table 1)**.

At 24h post challenge a significant higher total histology score was found in

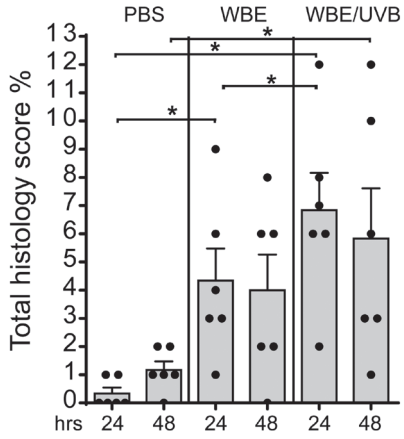

Figure 2: Total histology scores of UVB-irradiated and non-irradiated skin challenged with WBE from *C. obsoletus* and PBS from six IBH-affected ponies. Non-irradiated skin was injected with WBE and PBS and biopsies were taken at 24h and 48h post challenges (PBS, WBE). In addition, UVB-irradiated skin in the same ponies was injected with WBE and biopsies were taken at 24h and 48h post challenge (WBE/UVB). The mean total histological scores were determined according to a semi-quantitative grading system (0-4) for acanthosis, hyperkeratosis, lymphocytes and eosinophilic granulocytes. The bars represent the mean of all samples in the group.

UVB-irradiated (p=0.0156) and non-irradiated (p=0.0156) WBE-challenged skin compared to PBS-challenged skin **(Fig. 2 + table 1)**. In addition, at 48h after challenge a significant higher total histological score was found in UVB-irradiated WBE-challenged skin (p=0.0313) compared to PBS-challenged skin, but no difference between non-irradiated WBE-challenged and PBS challenged skin. Furthermore, 24h post challenge a significant higher total histology score was found in UVB-irradiated WBE-challenged skin compared to non-irradiated WBE-challenged skin. Finally, the average scores of lymphocytes and eosinophilic granulocytes infiltration in the skin followed the same tendency as the total histological scores **(table 1)**.

Cytokine expression in the skin after WBE challenge

To determine whether UVB irradiation can modulate the immune responsiveness, we examined mRNA expression of CD3ζ, IFN-γ, IL-4, IL-10, FoxP3 and AREG in non-irradiated WBE-challenged, UVB-irradiated WBE-challenged and PBS-challenged skin, at 24 and 48h post challenge.

The T-cell marker CD3ζ, was only upregulated in UVB-irradiated WBE challenged skin (p=0.0313) 48h after challenge compared to 24h **(Fig. 3A)**.

Cytokine analysis was done after calculation of their expression ratios with CD3ζ (IFNγ/CD3ζ, IL-4/CD3ζ, IL-10/CD3ζ and FoxP3/CD3ζ).

IFNγ/CD3ζ was significantly upregulated 48h after WBE challenge (p=0.0313) compared to PBS **(Fig. 3B)**.No difference was found between UVB-irradiated WBE challenged and PBS challenged skin. IFNγ/CD3ζ was significantly downregulated 48h after WBE challenge in UVB-irradiated skin compared to non-irradiated skin (p=0.0313). There were no significant differences in IFNγ/CD3ζ mRNA expression between the other conditions.

IL-4/CD3ζ was significantly higher in UVB-irradiated, WBE-challenged skin 24h after challenge compared to non-irradiated, PBS-challenged skin (p=0.0313) **(Fig. 3C)**. There was no significant increase of IL-4/CD3ζ in skin challenged with WBE compared to PBS. Although IL-4/CD3ζ mRNA was higher in UVB-irradiated, WBE-challenged skin compared to non-irradiated WBE-challenged skin (p=0.0469), IL-4/CD3ζ expression in UVB-irradiated skin challenged with WBE was significantly downregulated 48h after challenge compared to 24h (p=0.0156).

FoxP3/CD3ζ, a Treg cell marker, showed no significant differences except for a significant decrease in UVB-irradiated skin with WBE challenge at 48h after challenge (p=0.0469) compared to non-irradiated WBE challenge **(Fig. 3D)**.

IL-10/CD3ζ, an immune suppressive cytokine produced amongst others by Treg cells, was downregulated in PBS-challenged skin 48h after challenge compared to 24h after challenge (0.0469) **(Fig. 3E)**. In addition, IL-10/CD3ζ was also significantly lower in WBE-challenged skin 24h after challenge compared to skin challenged with PBS (p=0.0469). There were no significant differences found between the other conditions and time points.

AREG, a Treg cell activation cytokine, mRNA expression levels appeared to be stable in all conditions tested **(Fig. 3F)**.

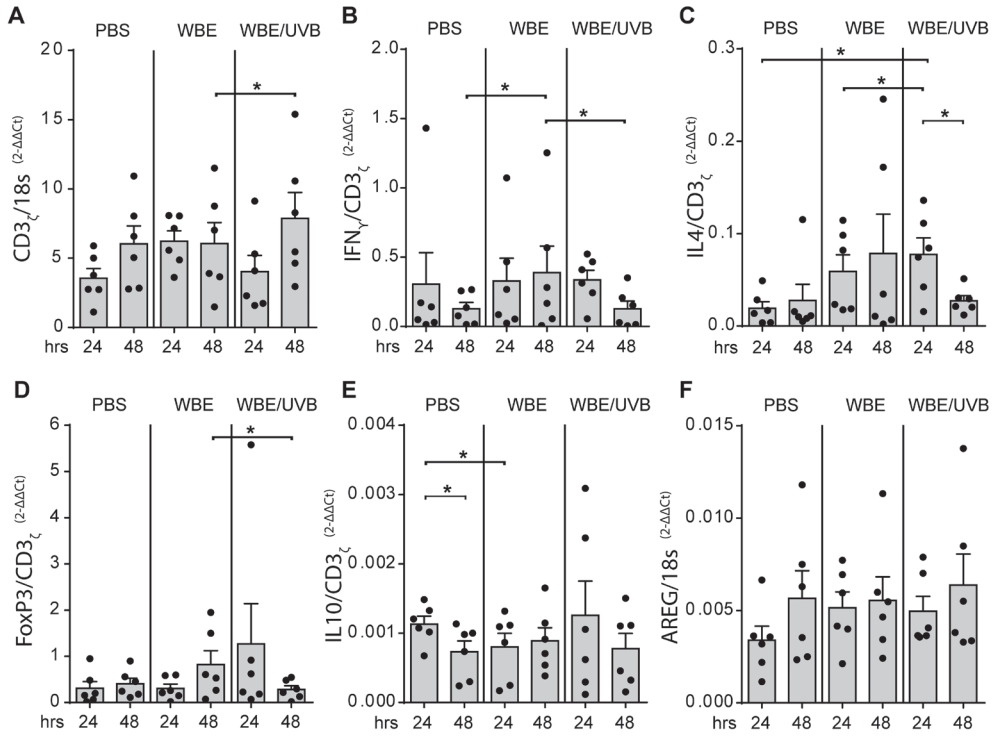

Figure 3: Expression of CD3ζ, IFNy, IL-4, FoxP3, IL-10, AREG mRNA. Non-irradiated skin was injected with WBE and PBS and biopsies were taken at 24h and 48h post challenge (PBS, WBE). In addition, UVB-irradiated skin in the same ponies was injected with WBE and biopsies were taken at 24h and 48h post challenge (WBE/UVB). The mRNA expression of A) CD3ζ, B) IFNy, C) IL-4, D) FoxP3, E) IL-10, F) AREG was determined with qRT-PCR and normalized against the housekeeping gene 18s using the Pfaffl method[22]. IFNy, IL-4, FoxP3, IL-10 mRNA values were divided by the corresponding CD3ζ value, to correct for the amount of T-cells. The bars represent the mean of all samples in the group.

Discussion

UVB-light therapy has been used as supportive treatment for various skin diseases in humans, in particularly AD.

In a previous study we showed that UVB treatment of naïve mice induced basophils to produce AREG that contributed to activation of antigen-specific Treg cells, suppressing contact hypersensitivity.[15] Furthermore, UVB treatment has shown to suppress several functions of antigen-presenting cells.[23] In the present study we wanted to investigate whether UBV-induced. WBE-specific immunosuppression could be accomplished in horses suffering from IBH. Therefore, we analyzed skin swelling, histology and mRNA expression of IFNy, IL-4, IL-10, FoxP3 and AREG.

The effect of priming of skin by UVB irradiation on immune reactivity was assessed by skin swelling subsequent to WBE challenge. A significantly lower RWD was found in the UVB-irradiated skin as compared to non-irradiated skin, 30 min after

WBE challenge but this was absent at 24h and 48h. Since wheal formation early on is dependent on mast cell degranulation and the release of inflammatory mediators (e.g. histamine) in the skin, these results may indicate that UVB irradiation has an inhibitory effect on mast cell degranulation after WBE challenge. In this study wheal diameter was used for evaluation of the effect of UVB irradiation. Although it is the most commonly used and standardized test, it may be that using skin thickness (in other words: wheal volume) would have given other results and another conclusion.

More lymphocytes and eosinophilic granulocytes were present at 24h in UVB-irradiated, WBE-challenged skin compared to only WBE-challenged skin. However, numbers of CD3ζ mRNA positive T-cells did not differ between these skin biopsies, suggesting infiltration of other lymphocytes such as B-cells and NK-cells. The only significant difference in CD3ζ mRNA found, was its higher expression in UVB-irradiated skin compared to non-irradiated skin 48h post WBE challenge, suggesting T-cell infiltration. The lack of CD3ζ mRNA upregulation at 24h post WBE challenge between UVB-irradiated and non-irradiated skin and a simultaneous increase in lymphocytes suggests infiltration from other lymphocytes e.g. B-cells and NK-cells.

UVB-irradiated skin 24h post WBE challenge had significantly more IL-4 mRNA compared to non-irradiated, whereas IFNy, IL-10, FoxP3 and AREG mRNA production did not differ. This suggests that UVB-irradiation did not downregulate IL-4 mRNA expression and thus did not suppress the immune responsiveness of IBH. The lack of increase in IL-10, FoxP3 and AREG mRNA in UVB-exposed skin indicates that there was no AREG-mediated Treg cells activation.

When comparing UVB-irradiated skin 48h post WBE challenge with non-irradiated skin, IFNy and FoxP3 mRNA were significantly downregulated whereas IL-4, IL-10 and AREG levels did not differ. Since IFNy, a Th1 cytokine, was upregulated upon challenge in ponies not suffering from IBH and is suggested to contribute to protection against IBH[16], we expected IFNy to be upregulated with UVB treatment. This suggests that the UVB treatment under the present conditions does not have a beneficial effect on IBH.

IL-4 did not differ significantly at 48h post WBE with UVB treatment however it did show a small decrease at 48h post WBE challenge compared to non-irradiated WBE challenged skin. In addition, IL-4 was significantly downregulated in UVB-irradiated skin 48h post WBE challenge compared to 24h. Since IL-4 is shown to be upregulated in IBH-affected ponies[16] when challenged with WBE and not in healthy ponies, our results suggests that UVB-irradiation may have some beneficial effect. Nonetheless, this has to be verified in additional studies. Only FoxP3 exhibited a significant lower expression in UVB-irradiated skin compared to non-irradiated skin 48h post WBE challenge, which is compatible with a lack of Treg induction. Together with the IL-10 and the AREG data, these results indicate that under the present conditions UVB treatment did not induce Treg cell activation mediated by AREG.

In summary, the results of this pilot study show no indications for a beneficial effect of UVB-irradiation on IBH skin by AREG-mediated immunosuppression. A possible reason for the latter can be that UVB-mediated immunosuppression of contact dermatitis, as observed in our mouse studies,[15] can only be induced in naïve individuals upon initial contact and not in individuals that are already sensitized, as is the case in IBH.[15] Why this occurs is not exactly known, but may be due to the fact that UVB-induced Treg are unable to migrate into the skin.[24] On the other hand we did

find a downregulation of IL-4, IFNy and (based on wheal reduction) mast cell mediators, suggesting that UVB treatment may work through other mechanisms. Since traditionally, UVB-light therapy is performed for several weeks before beneficial effects are observed, studies with more prolonged exposure times should be considered before definitive conclusions of (non-)effectivity are rendered.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Chapter 8

Summarizing Discussion

Insect bite hypersensitivity (IBH) is a seasonal allergic dermatitis primarily caused by *Culicoides* midges like *C. obsoletus*.[1] The welfare of IBH-affected horses is compromised due to severe itch with secondary dermatitis and skin infections.[2, 3] Similar to most allergies, IBH can only be controlled rather than permanently cured. The research described in his thesis aimed at better understanding of the immunopathogenesis as a basis to improve diagnosis and to explore potential immune modulatory strategies for IBH. In the following paragraphs, the results and conclusions presented in this thesis will be summarized and discussed.

T cells in Insect Bite Hypersensitivity

IBH is thought to be a Type-1 hypersensitivity with potential involvement of Th2 immune responsiveness. It is generally characterized by upregulation of IL-4 and IL-13[4-7] and at least partially mediated by *Culicoides* antigen-specific IgE. Surprisingly, we did not find any differences in IL-4 or IFNγ mRNA expression between skin of IBH-affected and healthy control animals at the end of the IBH season (**chapter 3**). Moreover, no differences were observed in IL-4 or IFNγ mRNA expression between lesional and non lesional skin from IBH-affected ponies, which is in line with a study done by Heiman *et al*.[5] However, contrary to Heiman *et al*.,[5] we found no differences in IL-13 mRNA expression.

To get more insight in T cell responsiveness in the skin in course of time, we compared (the same) healthy and IBH-affected ponies both during the IBH-season and in the off-season. A general increase of $CD3_{\zeta}^{+}$ T-cells expression was observed in skin collected during the IBH-season as compared to that in the off-season of IBH-affected as well as healthy ponies. This indicates that immune responsiveness in the skin of all Culicoides exposed animals was activated at the end of the IBH-season. Examination of whole body extract (WBE) of *C. obsoletus*-challenged skin in the off-season (**chapter 4**) showed a significant upregulation of IL-4 mRNA in IBH skin compared to non-challenged IBH-affected skin. In addition, significantly more IL-4 was present in the IBH skin compared to healthy skin. These results clearly suggest a Th2 skewing of the immune system due to earlier exposure, reflected in the skin of IBH-affected ponies upon challenge. On the other hand we again did not find significant differences in IL-13 mRNA expression healthy versus IBH-affected (data not shown). Since upregulation of IL-13 at earlier or later time points than 24h hence its potential importance in other stages of the pathogenesis cannot be excluded.

When examining IFNγ mRNA expression off-season, we observed a significant upregulation in healthy skin challenged with WBE of *C. obsoletus* compared to IBH-affected skin, hence clear Th1 immune reactivity (**chapter 4**). Moreover, upon *in vitro* stimulation of cultured T-cells with WBE of *C. obsoletus* we found significantly more IFNγ-producing allergen-specific CD4+ T-cells in cultures of healthy horses compared to those of IBH-affected horses (**chapter 5**). This strongly suggests that allergen-specific Th1 immune responsiveness protects against IBH development. Thus, in contrast to the general consensus, healthy animals are responsive to the allergen exposure, but in a non-pathogenic way.

It has been suggested that regulatory T-cells (Treg) are important players in avoidance of allergic diseases. In contrast to Heimann *et al*.,[5] we did not find a significant decrease in FoxP3 mRNA in lesional skin from IBH-affected horses compared to non-lesional skin and skin from healthy horses. We only observed a decrease in FoxP3 mRNA expression in healthy skin in the IBH-season suggesting a decrease in the number of Treg cells (**chapter 3**). In addition, even when assessing skin from IBH-affected and healthy ponies 48h after WBE challenge, we did not find differences in FoxP3 positive cells (**chapter 4**). Hence, Treg cells do not seem to be able to suppress the development of clinical IBH. The differences in results between the studies may be due to the different selection methods for IBH-affected horses included. We selected horses on the basis of severe and mild IBH symptoms in the course of several years, whereas Heimann *et al.* excluded horses with mild IBH symptoms. Furthermore, and similar to the observations of Heimann *et al*.,[5] we did not find a difference in IL-10 mRNA expression in skin challenged with WBE of *C. obsoletus* between healthy and IBH-affected animals , nor within the same animals between different seasons. This suggests that there is no IL-10 production by Treg cells.

Taken together our data cannot fully explain why some individuals are allergic and others are not, but they do suggest that IBH is the result of a Th2-biased immune response, while healthy animals show Th1 immune responsiveness that prevents the development of IBH. Consequently, it is tempting to claim that therapeutic interventions which aim at the induction of allergen-specific Th1 immune responses may diminish symptoms in allergic horses and may protect individuals from developing allergies.

We assessed whether two recently identified *C. obsoletus* recombinant allergen combinations P1 (Cul o 1, Cul o 2 and Cul o 5) and P2 (Cul o 3, Cul o 5 and Cul o 7)[(16)] were capable to stimulate antigen-specific T-cells after prior exposure of animals to *C. obsoletus* insects (**chapter 5**). We found significantly more cells expressing IFN-γ, mRNA as well as the protein, present in DC and specific antigen-stimulated T-cell cultures from both control and IBH-affected ponies, when using the recombinant allergen pools P1 and P2, as compared to WBE of *C. obsoletus* and unstimulated cultures. These observations are in contrast to those of Hamza *et al*. who could not detect IFN-γ expression after antigenic stimulation of PBMC with WBE of *C. nubeculosus*.[4] Our results suggest that these recombinant allergen pools stimulate antigen-specific T-cells even better than WBE, likely due to a difference in relevant antigen concentration.

Furthermore there tended to be more IL-4 mRNA expressing cells in cultures stimulated with P1, P2 and WBE (not shown) compared to unstimulated cultures; however, no increase in IL-4 protein could be detected. This is in contrast to Hamza *et al.* who stimulated PBMC with WBE of *C. nubeculosus* only. Our cultures did not reveal a clear difference in Th1/Th2 response between IBH and healthy horses. The reason for results differing from those of Hamza *et al.* may be the different horse population (Shetland ponies versus Icelandic horses) and/or the different culture conditions used in both studies.[4] The prolonged culture of the antigen-specific T-cells in our study may have resulted in selective expansion and activation of T-cells and thus, contrary to Hamza *et al.*[4], it does not reflect the actual *in vivo* Th1/Th2 response,

Since the two pools of recombinant *C. obsoletus* complex allergens stimulate antigen-specific Th1 cells in DC/T cell cultures of *C. obsoletus* complex-exposed ponies, they are promising candidates for immunotherapy of IBH.

IgE and IgG(-subtypes) in Insect Bite Hypersensitivity

IgE and IgG(-subtypes) have been suggested to play a role in the immunopathogenesis of IBH.[5, 8] Our study (**chapter 3)** showed that *C. obsoletus*-specific IgE titers were higher in IBH-affected ponies compared to control ponies in the off-season as well as in the IBH-season (April-September in The Netherlands), similar to what is found in flea allergy dermatitis in dogs.[9, 10] In addition, IgE titers did not differ between the IBH-season and the off-season, suggesting that they are stable throughout the year. This finding differs from what is known in humans, where circulating IgE has a short half-life of 2-3 days. However, IgE bound to the Fc receptors on basophils and mast cells, can survive up to several months.[11] Moreover, there is evidence that some IgE-secreting plasma cells in allergic individuals are long-lived and that these plasma cells can survive in the bone marrow, lymph nodes or spleen even up to years,[12-15] and are not affected by antigen challenge or immunosuppression.[16, 17] Hence, the persistent titers of allergen-specific IgE in ponies may be due to production by *Culicoides* allergen-specific long-lived plasma cells.

In addition to IgE, IgG-subtypes may also be involved in the immunopathogenesis of IBH.[8] To investigate the possible association between IgG isotypes and the IBH pathogenesis and to address their potential use in diagnostic tests, we examined specific binding of IgGa, IgGc, IgGb, and IgG(T) with WBE from *C. obsoletus* in an ELISA **(Fig. 1)**.

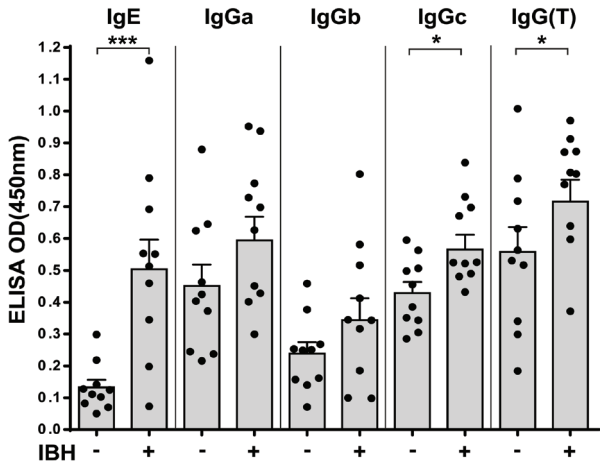

Figure 1: *C. obsoletus*-specific IgE, IgGa, IgGb, IgGc and IgG(T) serum levels in the IBH-season. IgE and IgG subtype serum levels of 10 IBH (+) and 10 non IBH-affected ponies (-) as determined by ELISA assay and expressed as OD minus OD of the negative control. The IBH-affected ponies showed significantly higher antigen-specific IgE (***P=0.0008), IgGc (*P=0.0255) and IgG(T) (*P=0.0489) serum levels compared to the non-IBH-affected ponies.

In line with previous studies [6, 8] IgG(T) levels were significantly higher in IBH-affected ponies compared to non IBH-affected ponies. Wagner *et al*.[6] performed intradermal injections in horses from different breeds with saline, histamine, WBE of *C. nubeculosus*, anti-IgE, anti-IgG(without IgG(T)), anti-IgGa, anti-IgGb, anti-IgGc and anti-IgG(T). They found that only anti-IgE and anti-IgG(T) showed skin reactions similar to histamine, suggesting that only IgE and IgG(T) can bind to skin mast cells with subsequent degranulation and skin swelling. In a previous study described in **chapter 3** with Shetland ponies, we also found that IgG(T) was significantly upregulated in IBH-affected ponies (p=0.0021) and control ponies (p=0.0001) in the IBH- season compared to the off-season **(Fig. 2)**. Moreover, we found that IgG(T) was significantly higher in IBH-affected ponies (p=0.0041) compared to control ponies in the IBH-season. Together, these results suggest that IgG(T) plays a role in the immunopathogenesis of IBH and that IgG(T) may be used as an additional diagnostic marker for IBH in the IBH season.

Considering IgGc, levels in plasma were variable throughout our experiments. Whereas a significant difference between healthy and IBH-affected could be shown in the experiment represented in **figure 1** this could not be confirmed in two additional experiments.

Until recently, there was no reliable and easy test available to diagnose IBH. Both the IgE based ELISA and the histamine release test (HRT) examined in **chapter 2** may be more reliable alternatives for the current diagnostic standard: clinical

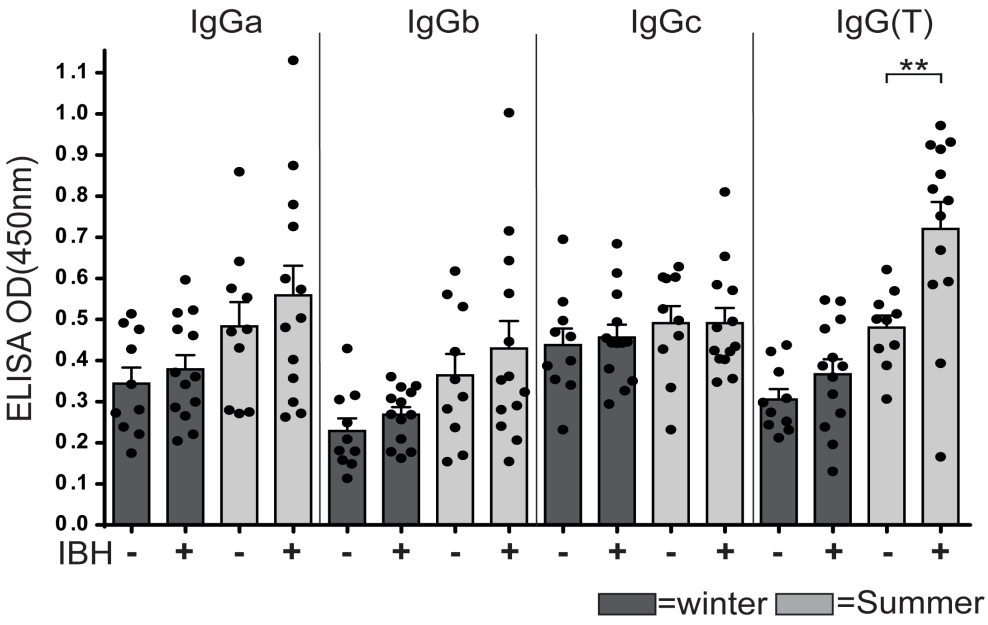

Figure 2: *C. obsoletus*-specific IgG subtype serum levels in the off-season and in the IBH-season. IgG serum levels of 14 control and 16 IBH ponies (off-season) and 11 control and 15 IBH ponies (IBH-season) were examined.by ELISA assay and expressed as OD minus OD of the negative control. The IBH-affected ponies showed significantly higher antigen-specific IgG(T) (**P=0.0041) serum levels compared to the non-IBH-affected ponies.

diagnosis together with intradermal allergy testing, which has low sensitivity and reproducibility.[18-21] Our results show that all (n=10) IBH-affected horses were found positive for IBH using the HRT, and only 60% was found positive with ELISA, whereas all healthy horses had negative scores for both assays. This suggests that the HRT may be more reliable than the ELISA. However, this experiment was performed with a relatively small number of horses, besides, HRT requires fresh blood samples and test results have been reported to differ between months of testing.[18, 22, 23] ELISA on the other hand can be performed with previously frozen samples, is better suitable for high throughput screening, and plasma *C. obsoletus-specific* IgE titers can be measured throughout the year (**chapter 3**). For these reasons the ELISA was chosen to be further evaluated with a large number (n=203) of horses, which demonstrated a sensitivity of 93% and a specificity of 90%.

When comparing different *Culicoides spp*. as antigens in assays, some studies report that there is no difference in the immune response that is measured in the IgE ELISA, HRT or in the intradermal test,[24, 25] while others report strong differences between the different tests.[19, 26] Despite the presence of various *Culicoides spp.* in the horses' habitat[27] we found that nearly all *Culicoides* insects collected from horses belong to the *C. obsoletus* species (**chapter 2**), which is in line with other studies in the Netherlands.[28, 29] In addition, the use of WBE of *C. obsoletus* resulted in higher skin test reactivity compared to WBE from other non-native *Culicoides spp*.[19] These results suggest that our horses were sensitized against *C. obsoletus,* but not against *C. nubeculosus*. Furthermore, in line with other studies,[19, 26] it suggests that there is low cross-reactivity between the different *Culicoides* species used for testing. We therefore like to recommend that when testing horses for IBH, protein from *Culicoides spp*. that are present in the same habitat and that have been shown to feed on horses, should be used.

Serological tests and HRT using WBE for *in vitro* diagnosis, so far showed low sensitivity and/or specificity.[22, 24, 30] Van der Meide *et al.* identified and characterized 7 *C. obsoletus* allergens (Cul o 1 – Cul o 7),[31] which bound significantly more IgE in an IgE ELISA with serum from IBH-affected horses compared to healthy controls. Combining Cul o 2, Cul o 5 and Cul o 7 resulted in a higher sensitivity (89%) and specificity (89%) of the ELISA compared to the 86% found with WBE,[32] suggesting that these recombinant allergens may be a good alternative for WBE in the diagnosis of IBH.

Can the immune response in IBH be modulated?

Immunotherapy has been used for centuries as a desensitizing therapy for allergic diseases and represents the only curative and specific method of treatment.[33] In humans allergic to insect bites, immunotherapy has shown to shift cytokine production to a more Th1 cytokine profile, [34, 35] to increased IL-10 production,[36] to reduced histamine release by basophils,[37] and to reduced allergen-specific IgE production.[38]

To date, only a few immunotherapy trials have been performed for IBH using WBE of *Culicoides spp*., their the results have been contradictive so far.[39-41] The exact mechanisms underlying the effect of immunotherapy are still unclear. It has been shown in humans that clinical improvement and the induction of peripheral tolerance in individuals with allergies to cow's milk,[42] bee venom,[43] birch pollen allergens,[44] and

mosquito bites [45, 46] is associated with an increase in allergen-specific IgG4 and a decrease in allergen-specific IgE. IgG4 antibodies prevent mast cell degranulation by competing against antigen-specific IgE for allergens.[47] It is suggested that this change in isotype production is mediated by IL-10-producing regulatory T-cells.[47] In addition, it has been reported that immunotherapy in patients with atopic dermatitis resulted in less basophil activation and higher allergen-specific IgG4 levels, however without changes in specific or total IgE levels.[48]

Recently, Hamza *et al*. showed that PBMC of healthy horses stimulated with WBE of *C. nubeculosus*, contained more $CD4^{+}CD25^{+hi}FoxP3^{+}$ T-cells able to suppress effector T-cells as compared to PBMC of IBH-affected horses.[49] For immunotherapy the use of recombinant allergens is preferred over WBE. The latter contains non IBH-related proteins which may cause side effects,[50-52] may act as new sensitizing allergens,[53, 54] may contain too low concentrations of IBH-relevant allergens, or may exhibit poor immunogenicity, all potential reasons for a less successful immunotherapy.[55]

In contrast, recombinant antigen cocktails only contain IBH-relevant allergens and can be produced in large amounts, with standard quality. Finally, using recombinant antigens allows for tailor made immunotherapy for each individual.[56]

Stimulation of T-cells with the two recombinant antigen pools *in vitro*, as described in **chapter 5**, did not result in increased FoxP3 or IL-10 mRNA in healthy horses compared to IBH-affected horses. This supports our findings in **Chapter 3 and 4** in which no differences were found in naturally and experimentally challenged IBH-affected skin compared to heathy skin. However, in contrast to our data concerning mRNA expression we found *in vitro* an increase in FoxP3-positive, IL-10-producing $CD3^{+}CD4^{+}CD25^{high}$ T-cells in T-cell cultures from both healthy and IBH-affected ponies stimulated with WBE of *C. obsoletus* (data not shown), or the recombinant *C. obsoletus* allergen pools P1 and P2 (**chapter 5**). These data suggest that, *in vitro,* WBE of *C. obsoletus* as well as the two antigen pools are able to stimulate the development of antigen-specific Treg cells and their production of the immune suppressive cytokine IL-10. Although Hamza *et al*.[49] showed that *in vitro* Treg cells from IBH-affected horses are less proficient in suppressing effector T-cells, it would still be interesting to see if the antigen-specific Treg cells can be induced *in vivo* and are able to suppress the IBH immune response.

Although some studies have shown that recombinant allergens used in immunotherapy can be successful,[57, 58] these allergens still have immunological features similar to their natural counterparts and bear the risk of inducing adverse allergic events as a consequence of IgE or T cell reactivity.[59] To overcome this problem, the recombinant antigens can be modified for instance by binding to an adjuvant like unmethylated cytosine-phosphate-guanosine-oligodeoxynucleotides (CpG-ODN). Inhalation of gelatin nanoparticles (GNP)-bound CpG-ODN resulted in remarkable IL-10 expression in bronchoalveolar lavage fluid in a small group of horses with recurrent airway obstruction (RAO).[60] In addition, administration of GNP-CpG-ODN resulted in significant clinical improvement compared to placebo in RAO-affected horses.[61] When the recombinant *C. obsoletus* antigens alone are not able to induce immune tolerance, CpG-ODN may be useful for the optimization of immunotherapy against IBH.

A different therapeutic approach for IBH may be UVB irradiation, which has been

moderately successful in psoriasis and atopic dermatitis,[62, 63] and which induced effective suppression of allergic contact hypersensitivity reactions in mice models.[64, 65] In **chapter 6** we report that UVB-mediated immunosuppression in mice was facilitated by basophil-derived amphiregulin (AREG)-induced Treg cells, and not by mast cell-derived AREG. Thus, results reveal a novel function of basophils in the regulation of immune responses in the skin and are in line with findings that basophils contribute to inducing Th2 immune responses, suppress the immune system and repair tissue damage.[66, 67] This adds to earlier findings that basophils are important in regulating skin homeostasis by mediating the differentiation of inflammatory monocytes into so-called M2 macrophages at the site of inflammation.[68] These M2 macrophages within allergic skin lesions significantly contribute to the alleviation of allergic skin inflammation.[68]

In a pilot study we investigated in horses affected by IBH whether UVB-mediated immunosuppression could be induced and if similar mechanisms were involved (**chapter 7**). We found that FoxP3, IL-10 and AREG in UVB-irradiated, IBH-affected skin were not upregulated upon challenge with WBE of *C. obsoletus*. This may indicate that UVB-mediated immunosuppression can only be induced in naïve individuals (as in the mouse model)[69] and not in individuals that are already sensitized i.e. IBH affected horses.[70] Taken together, there are no indications for AREG-mediated immunosuppression in IBH after UVB irradiation under the current experimental conditions. Although it was a pilot experiment, the results indicated a downregulation of IL-4, IFNy and a reduction in skin test reactivity which suggests a downregulation of both Th2 and Th1 responsiveness in the skin, possibly representing an alternative regulatory mechanism.

Concluding remarks

Horses with IBH, living in a *C. obsoletus*-rich environment, appear to have much more IgE antibodies against *C. obsoletus* proteins compared to *C. sonorensis* and *C. nubeculosus* proteins. In these environments a *C. obsoletus*-specific IgE ELISA can be routinely used to diagnose IBH throughout the year. In addition, IgG(T) may be considered as an additional diagnostic marker for IBH in the IBH-season.

It was shown that IBH-affected animals have a Th2-skewed immune response against *Culicoides*-specific antigens. Moreover, animals without IBH, in contrast to the so far established dogma, are not ignorant of *Culicoides*-specific antigens, but have a Th1-skewed immune response. Such response may protect them against an allergic reaction and hence IBH-associated symptoms.

Two *C. obsoletus* complex recombinant allergens pools, P1 (Cul o 1, Cul o 2 and Cul o 5) and P2 (Cul o 3, Cul o 5 and Cul o 7) were able to stimulate antigen-specific Th1 and IL-10 producing Treg cells in prolonged T cell cultures from IBH-affected animals *in vitro*. Therefore, it would be interesting to investigate these recombinant allergens *in vivo* for immunotherapy in combination with adjuvants.

Finally, we discovered that basophils can enhance the suppressive capacity of local Treg cell populations by producing and releasing amphiregulin after UVB irradiation in mouse skin. However, our pilot study in horses showed no indications for a beneficial effect of UVB-irradiation on IBH skin by amphiregulin-mediated immunosuppression under the present experimental conditions. More research

needs to be done to find out whether or not UVB irradiation may be a complimentary treatment for horses with IBH.

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Nederlandse samenvatting

Samenvatting

Staart- en maneneczeem (SME) is een veel voorkomende allergische huidaandoening bij paarden en pony's en wordt veroorzaakt door de beten van kleine insecten behorende tot de *Culicoides* familie, ook wel knutten genoemd. Deze komen in Nederland alleen voor van april tot en met september, waardoor SME een zogenaamde seizoensgebonden aandoening is. Beten van *Culicoides* soorten kunnen hevige jeuk veroorzaken en door krabben, bijten en schuren ontstaan huidveranderingen (laesies) zoals, roodverkleuring, schilfering, korstvorming, verdikking van de huid en haarverlies. De klinische verschijnselen worden vaak gezien bij de staartwortel en de manenkam, vandaar de naam staart- en maneneczeem.

SME is een multifactoriële aandoening, waarbij de omgevingsfactoren en de genetische aanleg van het paard bepalend zijn voor de ontwikkeling van klinische symptomen. Tot op heden zijn er nog geen effectieve preventie- of behandelmethoden beschikbaar. Daarnaast is er grote behoefte aan een betrouwbare diagnostische test voor SME, dat momenteel uitsluitend op basis van de klinische verschijnselen wordt vastgesteld.

Immuunpathogenese van SME

De immuunpathogenese van SME berust op een combinatie van zogenaamde type-1 (IgE-gemedieerd) en type-4 (Th2-gemedieerd) overgevoeligheidsreacties en kent een sensibilisatiefase en een effectorfase.

Uit humaan onderzoek is bekend dat wanneer een individu voor het eerst in contact komt met allergenen (eiwitten, die een allergische reactie kunnen veroorzaken), de sensibilisatiefase, deze door antigeen-presenterende cellen (Langerhans cellen; LC) in de huid worden opgenomen en afgebroken tot kleine fragmenten (peptiden). Na migratie van de LC naar de lymfeklieren worden de allergene peptiden via MHC II moleculen (Major Histocompatibility Complex) op de LC aangeboden aan T cel receptoren (TCR) van naïeve T cellen. In homeostase is er in het immuun systeem een evenwicht tussen zogeheten T helper 1 (Th1) en T helper 2 (Th2) reacties, echter, de consensus is, dat in SME de naïeve T cellen zullen prolifereren en differentiëren tot met name Th2 cellen. Ook B cellen nemen allergenen op, verwerken ze en presenteren deze via hun MHC II moleculen aan de TCR op de Th2 cellen. De Th2 cytokines, de interleukines IL-4, IL-5 en IL-13, zijn verantwoordelijk voor proliferatie en differentiatie van de allergeen-specifieke B cellen tot IgE producerende plasmacellen. Tenslotte bindt het antigeen-specifieke IgE aan receptoren (FcεR1) op mestcellen en eosinofiele granulocyten in de huid en basofiele granulocyten in het bloed, waarmee de sensibilisatie fase is afgerond. In dit proefschrift hebben we onderzocht of het immunologisch milieu in de huid bij paarden en pony's met SME ook daadwerkelijk meer in de richting vanTh2 verschoven is (hoofdstuk 3 en 4).

Als een gesensibiliseerd individu voor een tweede keer met het allergeen in aanraking komt, de effector fase, wordt het allergeen gebonden door meerdere allergeen-specifieke IgE antilichamen aan het oppervlak van mestcellen, eosinofiele en basofiele granulocyten (cross-linking). Deze cross-linking zorgt voor degranulatie: blaasjes in de cel gevuld met proinflammatoire componenten (componenten die ontsteking veroorzaken) zoals histamine, geven hun inhoud af aan de omgeving. De jeuk en zwelling die daardoor ontstaan, resulteren uiteindelijk in de klinische symp-

tomen.

Naast B cellen en effector T cellen kunnen ook regulatoire T cellen (Treg) een rol spelen in allergische reacties, de mate waarin zij het effectief functioneren van B cellen, Th2 cellen en LC remmen, o.a. via IL-10 en TGFβ productie, bepaald uiteindelijk het ontstaan van klinische symptomen. In hoofdstuk 3, 4 en 5 van dit proefschrift hebben we onderzocht of Treg cellen een rol spelen bij de regulatie van immuunresponsen in SME.

Allergenen en diagnose

Diverse allergeen preparaten worden gebruikt om aan te tonen of een individu allergisch is. In het geval van SME zijn dat met name "whole body extracts" van *Culicoides*. Deze extracten bevatten meerdere allergene eiwitten die kunnen binden aan IgE in het serum en aan IgE op cellen in bloed en huid van pony's met SME. De specificiteit van het allergeenbindende IgE voor die eiwitten kan variëren, waardoor het lastig is om aan te geven welk eiwit het belangrijkste is bij het ontstaan van SME. Uit een aantal studies met *C. nubeculosus* en *C. sonorensis* extracten waarin antigeen-specifieke IgE niveaus bepaald werden, bleek dat er weinig homologie is tussen de allergenen afkomstig van verschillende *Culicoides* soorten. In Nederland is *C. obsoletus* de meest voorkomende *Culicoides* soort, die ook op paarden gevonden wordt. Om die reden hebben wij onderzocht of IgE afkomstig van pony's en paarden met SME sterker reageert met *C. obsoletus* extract dan met extracten van twee andere *Culicoides* soorten, *C. nubeculosus* en *C. sonorensis*.

Het gebruik van "whole body extracts" van knutten heeft nadelen omdat ze ook eiwitten bevatten die niets met SME te maken hebben, een mogelijke complicatie voor diagnostische testen. Individuele allergene eiwitten hebben dit probleem niet en zouden dus beter geschikt zijn voor diagnose en eventuele immunotherapie. Een aantal dominante *C. obsoletus* allergenen werden geïdentificeerd en als recombinante eiwitten tot expressie gebracht (Cul o 1 – Cul o 7). In hoofdstuk 5 van dit proefschrift hebben we onderzocht of pools van deze recombinante eiwitten antigeen-specifieke T cellen *in vitro* kunnen stimuleren.

Tot nu toe werd de diagnose SME gesteld door in opeenvolgende jaren, in het SME-seizoen, specifieke klinische symptomen vast te stellen. Huidtesten uitgevoerd met allergeen extracten van *Culicoides* insecten, bleken niet specifiek genoeg en slecht reproduceerbaar. In verschillende onderzoeken naar SME wordt gebruik gemaakt van *in vitro* testen, maar deze zijn nog niet geoptimaliseerd en gevalideerd voor routinematig gebruik in de diagnostiek. Voorbeelden van *in vitro* testen zijn de Enzyme-Linked Immuno Sorbent Assay (ELISA) gebaseerd op binding van allergeen-specifiek IgE aan Culicoides extracten, en cellulaire testen zoals de basofielen degranulatietest, gebaseerd op degranulatie van cellen na cross-linking van IgE. In dit proefschrift hebben we onderzocht welke van deze twee testen het best gebruikt kan worden als diagnostische test voor SME (hoofdstuk 2).

UVB bestraling en allergie

In muizen kan allergische contact dermatitis (ACD) worden geïnduceerd door middel van het aanbrengen van DNFB (dinitrofluorbenzeen) op de huid. Net als *Culicoides* allergenen wordt DNFB opgenomen door LC en stimuleert het antigeen-specifieke degranulatie van mestcellen en basofiele granulocyten door middel van IgE

cross-linking. In dit muizenmodel kan de effector fase van ACD onderdrukt worden door voorafgaand aan sensibilisatie fase te bestralen met UVB (ultraviolet B), waardoor IL-10 producerende, antigeen-specifieke Treg cellen worden geïnduceerd, en daarmee tolerantie voor DNFB. In dit proefschrift hebben we onderzocht hoe deze antigeen-specifieke Treg worden geactiveerd tot het onderdrukken van ACD (hoofdstuk 6).

In de humane kliniek wordt UVB bestraling veelvuldig toegepast ter behandeling van huidaandoeningen zoals atopische dermatitis en psoriasis. In dit proefschrift hebben we dan ook in een pilot studie het potentiële effect van UVB bestraling bij staart- en maneneczeem onderzocht (hoofdstuk 7).

In dit proefschrift

Het onderzoek beschreven in dit proefschrift had tot doel meer inzicht te krijgen in de immunopathogenese van staart- en maneneczeem (SME) en daarmee aanknopingspunten voor verbetering van de diagnostiek. Daarnaast werd het potentieel immunomodulerende effect van UVB bestraling op de huid van muizen met ACD en van paarden met SME onderzocht.

In **hoofdstuk 2** zijn in eerste instantie in een kleine groep paarden (10 met SME en 10 gezonde controles) *C. obsoletus* en de commercieel verkrijgbare *C. nubeculosus* en *C. sonorensis* extracten met elkaar vergeleken voor hun toepasbaarheid voor SME diagnostiek. De drie extracten zijn getest op hun vermogen om paardenserum IgE te kunnen binden in een ELISA en in Western blots, en op hun vermogen om basofielen degranulatie op te wekken. Het *C. obsoletus* extract gaf de beste resultaten in alle testen. Om praktische redenen is er voor gekozen in verder onderzoek de ELISA te testen in een groter aantal paarden. Dit heeft geresulteerd in een test met hoge sensitiviteit en specificiteit, die daarmee ook geschikt is als diagnostische test voor SME.

Hoofdstuk 3 beschrijft *Culicoides*-specifieke immuunreactiviteit in SME pony's en gezonde controle dieren uit dezelfde leefomgeving, en zowel in het SME-seizoen als buiten deze periode (winter). Het gemiddelde *C. obsoletus*-specifieke IgE niveau van pony's met SME was significant hoger dan dat van de controle pony's en de niveaus in het SME seizoen bleken gelijk aan die in de winter. Dit geeft aan dat (a) de pony's waarbij de diagnose SME was gesteld op basis van klinische verschijnselen, ook daadwerkelijk SME hebben, en (b) dat SME zowel in het SME-seizoen als in de winter kan worden gediagnosticeerd m.b.v. een IgE ELISA.

In het SME-seizoen werden er in de lesionale huid van de pony's met SME histologische veranderingen gezien passend bij een Th2 immuunreactie, terwijl dit niet het geval was in niet-lesionale huid van deze dieren en in de huid van gezonde pony's. Er werden geen verschillen gevonden tussen IFNγ, IL-4, IL-13, FoxP3 en IL-10 mRNA expressie tussen lesionale en niet-lesionale huid van pony's met SME en ook niet tussen niet-lesionale huid en gezonde controle huid. Wel waren de IFNγ, CD3ζ (TCR marker) en IL-13 mRNA niveaus significant hoger in het SME-seizoen in vergelijking met het winter seizoen. Dit kan betekenen dat de cellen in de huid van zowel pony's met SME als in de huid van gezonde controle pony's geactiveerd zijn. Op basis van deze bevinding is het dan ook aan te bevelen om in het winterseizoen te meten, zodat de invloed van factoren anders dan van *Culicoides,* geminimaliseerd wordt. Verder zijn er geen verschillen gevonden in FoxP3 en IL-10 mRNA expressie,

wat suggereert dat Treg cellen niet geactiveerd worden in de huid van pony's met SME, noch in die van gezonde controle pony's in het SME-seizoen.

In de experimenten beschreven in **hoofdstuk 4** is onderzocht welke immunologische reacties er plaats vinden in de huid van pony's met en zonder SME, na een locale injectie met *C. obsoletus* extract (stimulatie). De cellen in de huid van zowel gezonde pony's als pony's met SME werden pas 24 uur na stimulatie geactiveerd. De IL-4 mRNA expressie niveaus, maar niet de IL-4 eiwit productie, waren significant hoger in de huid van pony's met SME ten opzichte van de gezonde controles. Dit geeft aan, dat het immuunreactiviteit in de huid meer in de richting vanTh2 verschoven is. In tegenstelling tot de heersende gedachte dat het immuunsysteem van gezonde (niet allergische) individuen niet zou reageren op allergenen, werd na stimulatie met allergeen in de huid van gezonde pony's significant meer expressie van IFNγ mRNA en eiwit waargenomen, passend bij een immuunreactiviteit van het Th1 type. Gezonde pony's lijken op deze wijze beschermd tegen de ontwikkeling van SME.

In **Hoofdstuk 5** wordt verslag gedaan van onze experimenten die tot doel hadden te onderzoeken of pools (pool 1= cul o 1, cul o 2, cul o 5, en pool 2= cul o 3, cul o 5, cul o 7) van recombinant *C. obsoletus* allergenen *in vitro* in staat zijn om antigeen-specifieke T cellen te stimuleren. Beide pools induceerden IL-4 mRNA expressie door T cellen afkomstig van zowel pony's met als zonder SME, maar er was geen verschil in productie van het cytokine zelf. Daarnaast was er alleen met pool 1 meer IL-4 mRNA waarneembaar in cellen afkomstig van pony's met SME dan van gezonde pony's. Pool 1 en 2 waren beiden in staat om IFNγ mRNA expressie en cytokine productie te induceren in gekweekte antigeen-specifieke T cellen afkomstig van zowel gezonde pony's als pony's met SME. Ook waren deze pools in staat om antigeen-specifieke Treg cellen afkomstig van beide diergroepen aan te zetten tot IL-10 productie. Daarom zijn deze recombinante antigenen interessante kandidaten voor nader onderzoek naar immunotherapie gericht op IFNγ en Treg activatie.

In **hoofdstuk 6** is in een muizenmodel onderzocht of het onderdrukken van allergische contact dermatitis (ACD) met UVB bestraling afhankelijk is van de aanwezigheid van de EGF-like groeifactor amphiregulin (AREG). In een colitis (ontsteking van de dikke darm) muismodel was aangetoond, dat AREG via de receptor voor epidermale groeifactor (EGF) Treg cellen kan stimuleren tot onderdrukking van immuunreacties. In ons DNFB muizenmodel hebben we gezien dat, onder invloed van UVB bestraling, basofiele cellen en niet mestcellen de voornaamste producent zijn van AREG. Verder suggereren onze resultaten dat ook in dit model AREG bindt aan de EGF-receptor op antigeen-specifieke Treg cellen die verantwoordelijk zijn voor de onderdrukking van de ACD reactie. Hiermee is een nieuwe rol voor basofiele granulocyten in immuunregulatie aangetoond.

In een pilot experiment beschreven in **hoofdstuk 7** hebben we onderzocht of UVB bestraling ook een gunstig effect heeft op SME en of dit AREG afhankelijk is. Na UVB bestraling van de huid van pony's met SME en daaropvolgend een lokale stimulatie met het *C. obsoletus* extract, trad significant minder zwelling op in de bestraalde huid dan in niet-bestraalde huid. Dit suggereert dat UVB een gunstig effect kan hebben op de ontstekingsreactie in de huid van pony's met SME.

Uit mRNA expressie analyse bleek verder, dat in de UVB behandelde huid zowel de Th1 als Th2 immuun reacties waren afgenomen. Er werd geen verschil gevonden

in Treg cel activatie en AREG mRNA expressie tussen wel en niet bestraalde huid. Dit suggereert dat UVB bestraling geen Treg cellen kan activeren via AREG, waarmee het werkingsmechanisme van UVB bestraling op de ontstekingsreactie bij SME onduidelijk blijft. Het feit dat er minder significant zwelling optrad met UVB geeft aanleiding voor meer onderzoek naar de effecten van UVB behandeling bij SME.

Samengevat, is door de resultaten van het onderzoek meer inzicht verkregen in de immunopathogenese van SME. Daarmee is ook een bijdrage geleverd aan het identificeren van mogelijke therapeutische aangrijpingspunten en nieuwe mogelijkheden voor diagnostiek. We laten zien dat ELISA een geschikte diagnostische methode is voor het vaststellen van SME, zowel in winter als in zomer, en dat het gebruik van allergenen afkomstig van *Culicoides* soorten uit de leefomgeving van het paard, van groot belang is voor een juiste diagnose. Daarnaast dragen de resultaten ook bij aan het vinden van geschikte kandidaat (recombinante) allergene eiwitten voor immunotherapie als alternatief voor de bestaande behandelingsmogelijkheden van SME. Hoewel dit proefschrift bijdroeg aan nadere opheldering van het mechanisme van onderdrukking van allergisch contact dermatitis door UVB bestraling in een muizenmodel, kon het resultaat van een pilot experiment m.b.t. UVB bestraling in SME paarden nog geen uitsluitsel geven over de mogelijkheden van deze therapie in SME paarden.

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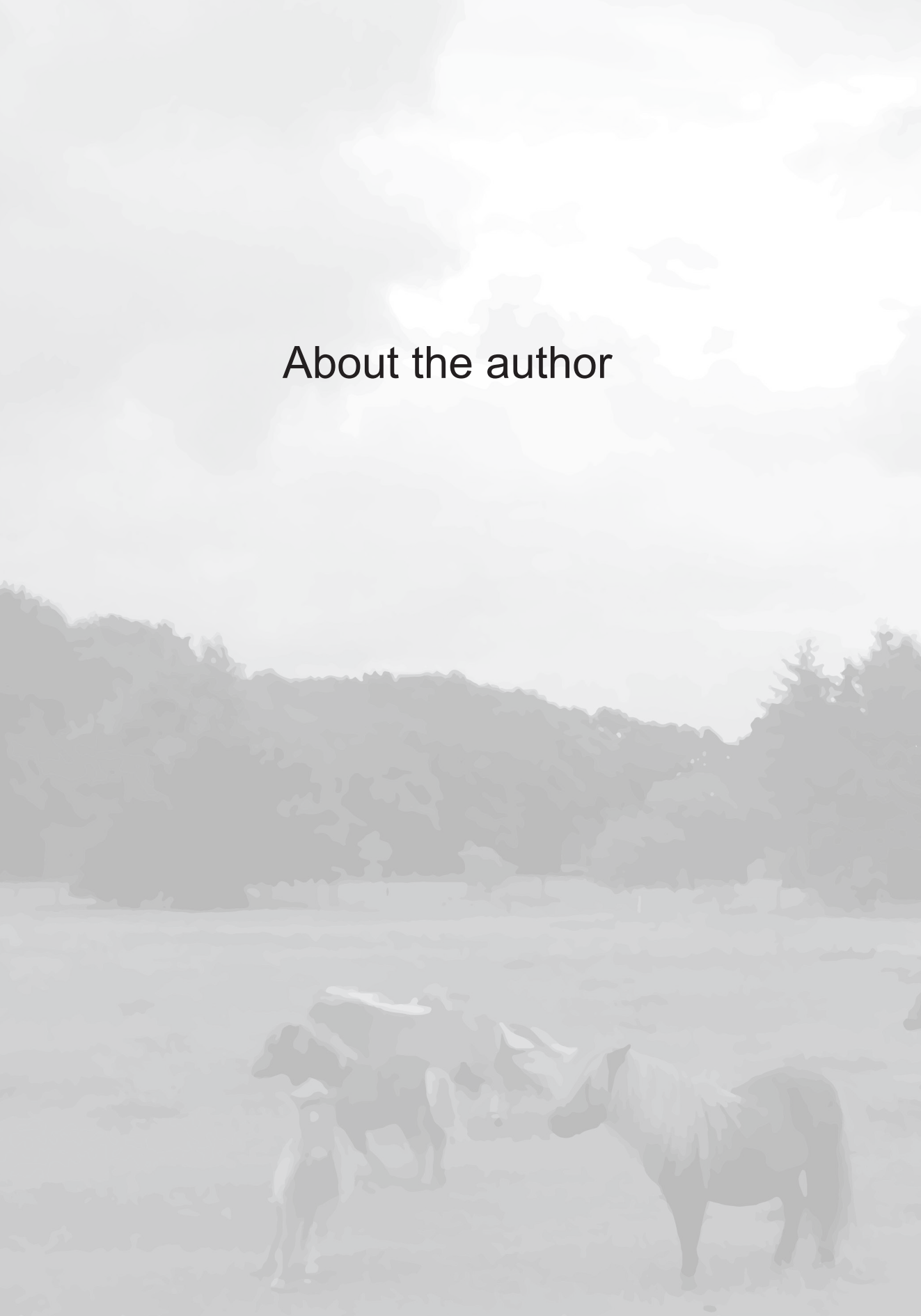
About the author



Curriculum vitae

Chantal Meulenbroeks was born on May 18th 1981 in Hapert, The Netherlands. In 2004 she obtained her Bachelor degree of Applied Science in Biology and Medical laboratory research at the Fontys institute, Eindhoven, The Netherlands, during which she did two research internships at the Department of Marine Biology at the University of Groningen on the silica frustule formation in *Navicula pelliculosa* and on developing a 2D-electrophoresis assay for *N. pelliculosa* and *N. Salinarum*. After working for 2 years as a research technician at TNO in Rijswijk, she started the Master of Animal Biology at Leiden University in 2006. She did a 9 month internship at the Molecular and cellular department on the function of reverse-induced LIM protein during embryonic development in zebrafish under the supervision of Dr. C. P. Bagowski. After receiving her master's degree in 2008 she worked on the Prediction of pharmacokinetic interactions between complementary and alternative medicines and (new) anticancer drugs at the Department of Biomedical analysis at Utrecht University. In November 2009 she started her PhD project on "Equine insect bite hypersensitivity: Pathogenesis, diagnosis and immunomodulation" led by prof. dr. Ton Willemse, prof. dr. Victor Rutten and dr. Dietmar Zaiss at the department of Infectious Diseases and Immunology at Utrecht University. The research results of this project are described in this thesis.

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