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Research paper

Skin-infiltrating T cells and cytokine expression in Icelandic horses affected with insect bite hypersensitivity: A possible role for regulatory T cells

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ABSTRACT

Equine insect bite hypersensitivity (IBH) is a seasonally recurrent, pruritic skin disorder caused by an IgE-mediated reaction to salivary proteins of biting flies, predominantly of the genus *Culicoides*. The aim of this study was to define T cell subsets and cytokine profile in the skin of IBH-affected Icelandic horses with particular focus on the balance between T helper (Th) 1, Th2 and T regulatory (Treg) cells.

Distribution and number of CD4⁺, CD8⁺ and Forkhead box P3 (FoxP3)⁺ T cells were characterized by immunohistochemical staining in lesional and non-lesional skin of moderately and severely IBH-affected horses ($n = 14$) and in the skin of healthy control horses ($n = 10$). Using real-time quantitative reverse transcription-polymerase chain reaction, mRNA expression levels of Th2 cytokines (Interleukin (IL)-4, IL-5, IL-13), Th1 cytokines (Interferon- γ), regulatory cytokines (Transforming Growth Factor β 1, IL-10) and the Treg transcription factor FoxP3 were measured in skin and blood samples. Furthermore, *Culicoides nubeculosus* specific serum IgE levels were assessed.

Lesions of IBH-affected horses contained significantly higher numbers of CD4⁺ cells than skin of healthy control horses. Furthermore, the total number of T cells (CD4⁺ and CD8⁺) was significantly increased in lesional compared to non-lesional skin and there was a tendency ($p = 0.07$) for higher numbers of CD4⁺ cells in lesional compared to non-lesional skin. While the number of FoxP3⁺ T cells did not differ significantly between the groups, the ratio of Foxp3 to CD4⁺ cells was significantly lower in lesions of severely IBH-affected horses than in moderately affected or control horses. Interestingly, differences in FoxP3 expression were more striking at the mRNA level. FoxP3 mRNA levels were significantly reduced in lesional skin, compared both to non-lesional and to healthy skin and were also significantly lower in non-lesional compared to healthy skin. Expression levels of IL-13, but not IL-4 or IL-5, were significantly elevated in lesional and non-lesional skin of IBH-affected horses. IL-10 levels were lower in lesional compared to non-lesional skin ($p = 0.06$) and also lower ($p = 0.06$)

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in the blood of IBH-affected than of healthy horses. No significant changes were observed regarding blood expression levels of Th1 and Th2 cytokines or FoxP3. Finally, IBH-affected horses had significantly higher *Culicoides nubeculosus* specific serum IgE levels than control horses.

The presented data suggest that an imbalance between Th2 and Treg cells is a characteristic feature in IBH. Treatment strategies for IBH should thus aim at restoring the balance between Th2 and Treg cells.

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1. Introduction

Equine insect bite hypersensitivity (IBH) is a seasonally recurrent, intensely pruritic skin disorder of horses due to hypersensitivity to biting flies, mainly of the genus *Culicoides* (Baker and Quinn, 1978; Scott and Millier, 2003). Lesions are classically distributed along the dorsal midline and initially comprise papules, urticaria and edema, but are rapidly replaced by secondary lesions including crusts, dandruff, excoriations, alopecia and lichenification (Scott, 1990). The allergenic proteins are most probably located in the salivary glands of *Culicoides* spp., and several candidate allergens have been identified (Hellberg et al., 2006; Wilson et al., 2008; Langner et al., 2009; Schaffartzik et al., 2010).

IBH is the most common allergic skin disease of the horse and shows a close to worldwide distribution. A notable exception is Iceland, which is free of IBH as *Culicoides* spp. do not occur there (Hrafnisdottir, 2005). Horses that are born in Iceland and exported to continental Europe are highly susceptible to IBH. About 50% of Icelandic-born horses develop IBH within two years following export to *Culicoides*-infested areas (Bjornsdottir et al., 2006). In contrast, the prevalence of IBH in horses of Icelandic breed that are born abroad is much lower and resembles that found in other horse breeds (Brostrom et al., 1987; Halldorsdottir and Larsen, 1991; van Grevenhof et al., 2007).

Several lines of evidence suggest that IBH is a type I hypersensitivity reaction (Fadok and Greiner, 1990; Marti et al., 1999; van der Haegen et al., 2001; Wagner et al., 2006). Following skin tests, an additional delayed response indicative of type-IV hypersensitivity has been described (Baker and Quinn, 1978; Fadok and Greiner, 1990; McKelvie et al., 1999). The main mediators in type I allergies are T helper (Th) 2 cells. Th cells have been divided into different subgroups according to the cytokines they secrete and the resulting functional properties (Mosmann and Sad, 1996). Th2 cells secrete IL-4, IL-5 and IL-13 and thus induce B cells to switch to IgE production and promote eosinophil infiltration. In contrast, Th1 cells produce IL-2 and IFN- γ and mediate delayed-type hypersensitivity reactions, auto-immune disease and graft-rejection. In a healthy setting, Th2 cells play a major role in the defense against helminths and other extracellular parasites, whereas Th1 cells predominantly fight intracellular pathogens (Biedermann et al., 2004). The actions of both Th1 and Th2 cells are controlled by a group of T regulatory (Treg) cells, which have suppressive properties (Robinson et al., 2004). A lack in number or function of Treg cells is discussed as a crucial factor in the development of

allergy (Larche, 2007) and various autoimmune diseases (Anderton and Liblau, 2008; Horwitz, 2008).

Although differing in the route of antigen encounter, equine IBH shares several features with atopic dermatitis (AD). AD is a chronic relapsing skin disorder in response to environmental allergens which occurs in humans as well as in dogs and cats (Rees, 2001; Leung and Bieber, 2003). Similar to lesions observed in AD, IBH lesions are characterized by a mixed perivascular to diffuse cellular infiltrate consisting of T cells and mainly CD4⁺ T cells (McKelvie et al., 1999) and, especially in acute lesions, eosinophils (Scott, 1990; Kurotaki et al., 1994). While the cytokine microclimate in IBH has not been characterized yet, various studies suggest that acute AD lesions contain mainly Th2 cells, as demonstrated by elevated cytokine levels of IL-4, -5 and -13. In chronic lesions, on the other hand, Th1-type cytokines such as IFN- γ are predominant (Thepen et al., 1996; Leung et al., 2004).

The role of Treg cells in the development of allergic disease has recently received much attention. Two main groups of Treg cells are in focus because of their capacity to suppress Th2 responses: natural Treg cells, currently best defined by their lineage-specific transcription factor Forkhead box P3 (FoxP3) (Fontenot and Rudensky, 2005), and adaptive Treg cells, mostly identified by their secreted suppressive cytokines, TGF- β 1 and IL-10 (Roncarolo et al., 2001). Several independent studies indicate that in humans, allergic individuals are deficient in number or suppressive capacity of Treg cells (Akdis et al., 2004; Grindebacke et al., 2004; Ling et al., 2004). The authors propose that an inappropriate balance between Th2 cells and Treg cells may be responsible for the development of allergic disease.

Little is known about the relevance of these regulatory mechanisms in equine skin allergies. Knowledge of the presence of Treg cells in horses is only just emerging. Evidence for IL-10 producing T cells has been found in the circulation of horses (Wagner et al., 2008) and FoxP3⁺ cells were recently detected by immunohistochemistry in equine lymphoid tissue (Steinbach et al., 2009). To the best of our knowledge, no study investigating the Th1 and Th2 cytokine profiles in horse skin has been published to date. However, Hamza et al. have conducted a series of studies examining the cytokine profile of cultured equine PBMC derived from Icelandic horses with and without IBH (Hamza et al., 2007, 2008, 2010). Their results show that in IBH-affected horses, there is an increase in IL-4 production with a concomitant decrease in IFN- γ production during IBH season, indicating a Th2-biased immune response. They furthermore demonstrated that *in vitro*, IL-4 produc-

tion is down-regulated in healthy horses by the combined effect of the regulatory cytokines IL-10 and TGF- β 1, implying a possible role for Treg cells in the maintenance of a healthy immune response toward *Culicoides*.

To investigate whether IBH is characterized by an imbalance between Th1, Th2 and Treg cells, we have compared skin-infiltrating T cell subsets and cytokine expression in IBH-affected and non-affected Icelandic horses which were exposed to bites of *Culicoides*. Our results show a local decrease in expression of FoxP3 in IBH-affected horses in combination with an increase of the Th2-type cytokine IL-13.

2. Materials and methods

2.1. Animals

A total of 31 Icelandic horses located in Sweden were included in the study. Horses with IBH ($n=21$, age range 5–24 years) were selected based on clinical signs and history, i.e. recurrent seasonal pruritus and skin lesions along the typical anatomic sites with remission in winter. Control horses ($n=10$, age range 4–19 years) were chosen randomly wherever possible from the same stables as the IBH-affected horses and had no history or clinical signs of skin disease. All except two of the IBH-affected horses were born in Iceland and imported to Sweden at least two years prior to sampling, while all but two of the control horses were Swedish-born. The two Icelandic-born control horses had been imported 5 and 11 years ago, respectively. Almost all IBH-affected horses were protected against biting insects by stabling (part-time), blankets and/or insect repellents. Horses receiving corticosteroid treatment were excluded from the study. All horses were dewormed and vaccinated at regular intervals.

IBH-affected horses were graded prior to sampling according to the severity of clinical signs by one of the authors (HB). Grading was performed as follows: First, horse owners were asked to rate the overall degree of the horse's skin problems, i.e. pruritus and severity of lesions (0–3). Then, clinical signs were recorded separately for each of the following regions of the horse: Face, ears, mane, neck, chest, dorsal abdomen, ventral abdomen including midline, flank, thighs, front- and hindlimbs and tail. The clinical parameters assessed were edema, alopecia, crusts, excoriations, and lichenification on a scale from 0 (no signs) to 3 (severe). The average of the attained score was determined and formed the horse's IBH severity grade.

This study has been approved by the Animal Experimentation Ethics Committee in Uppsala (C118/8, Uppsala Djurförsöksetiska Nämnd).

2.2. Collection and processing of skin and blood samples

The sampling of all biopsies was performed in mid June 2008. All biopsies were taken from the dorsolateral neck of the horses under general sedation with detomidine hydrochloride (0.01 mg/kg iv; Domosedan, Orion Pharma Animal Health, <http://www.orionvet.se/>) combined with Butorphanol tartrate (0.025 mg/kg iv; Butador vet, Vetoquinol, <http://www.vetoquinol-scandinavia.com/>). From

each IBH-affected horse, one lesional and one non-lesional skin sites were sampled. Preferably, acute skin lesions, characterized by edema and small crusted papules, were chosen whereas chronic lesions as indicated by lichenification, excoriations or widespread alopecia were avoided as far as possible. Non-lesional skin was defined as having the same macroscopic aspect as skin from healthy control horses.

Two biopsies were taken per sampling site: First, one 8-mm punch biopsy, which was cut in halves perpendicularly to the skin surface and parallel to the orientation of the hairs. One half was subsequently fixed in 4% neutral buffered formaldehyde for 24–48 h, the other half embedded in O.C.T. compound (Tissue Tek, Sakura Finetek Europe, Zoeterwoude, Netherlands) and immediately snap-frozen in liquid nitrogen. Second, a 5-mm punch biopsy was taken and immersed in RNeasy lysis buffer (Qiagen, Crawley, UK), stored at 4 °C overnight and then frozen at –20 °C to be used for determination of cytokine RNA via real-time quantitative reverse transcription-PCR (qRT-PCR).

Blood samples were collected by jugular venipuncture. Serum was harvested and frozen at –80 °C until analyzed for *Culicoides* specific IgE. PAXgene tubes (Qiagen AG, Basel, Switzerland) were used according to the manufacturer's instructions for blood samples intended for RNA isolation.

2.3. Measurement of *Culicoides* specific serum IgE levels

Culicoides specific IgE levels were measured in the sera of all participating horses by ELISA. Microtiter plates (Immunolon-2HB, Thermo Scientific, Waltham, MA, USA) were coated with 100 μ l of 1 μ g/ml *Culicoides nubeculosus* whole body extract (Marti et al., 1999) diluted in 0.2 M carbonate–bicarbonate buffer pH 9.6 (Pierce Biotechnology, <http://www.piercenet.com>) and incubated for 2 h at 37 °C. Plates were washed in PBS containing 0.05% Tween 20 and then incubated with blocking buffer (PBS containing 5% dry milk powder and 5% Tween 20) for 1 h at 37 °C. Duplicate serum samples (100 μ l/well) were applied to the plates in twofold serial dilutions from 1:5 to 1:40 in blocking buffer and incubated overnight at 4 °C. After washing, 100 μ l of 0.5 μ g/ml monoclonal mouse anti-equine IgE (3H10, Wilson et al., 2006), was applied for 2 h at room temperature (RT) on a shaker. Plates were washed again and incubated with alkaline-phosphatase-conjugated goat anti-mouse IgG (Fc γ fragment, Jackson ImmunoResearch, Newmarket, UK), diluted 1:1500, for 1.5 h at RT on a shaker. Following a final wash, plates were developed with p-nitrophenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA) in 10% diethanolamine buffer, pH 9.8. Absorbance readings were measured at 405 nm with a Molecular Devices Reader (Molecular Devices, Sunnyvale, California, USA) and converted to relative ELISA units (REU). For this purpose, a horse serum with high *Culicoides* specific IgE was used as reference serum and assigned a value of 100 REU. Serial dilutions of the reference serum were used to generate a standard curve, and the test sample results were calculated from the curve using an ELISA software program (SOFTmax[®] 2.31 for Windows[™], Molecular Devices Co., Sunnyvale, CA, USA). The concentration of the original

serum samples was calculated as the average of the values from those dilutions where the concentration fell within the standard curve.

2.4. Histological examination of skin biopsies

Formalin-fixed samples were paraffin-embedded, sectioned at 4 μ m and stained with haematoxylin–eosin (HE) for routine histopathology. A semi-quantitative grading system (0 = none, 1 = mild, 2 = moderate, 3 = severe) was used to assess the degree of epidermal changes (hyperplasia, hyperkeratosis), cellular infiltration (eosinophils, mononuclear cells), and edema. The grades in each category were summed up to form the histological score for each biopsy. All slides were analyzed by the same person (CvT), who was blind with regard to the identity of the samples.

2.5. Immunohistochemistry

Immunohistochemical staining was performed for CD4 and CD8 on acetone-fixed cryostat sections and for FoxP3 on formalin-fixed, paraffin-embedded sections using an avidin–biotin complex (ABC) technique.

The following primary antibodies were used in this study: Monoclonal mouse anti-equine CD4, clone HB61A (VMRD, Pullman, WA, USA), monoclonal mouse anti-equine CD8, clone HT14A (VMRD), both diluted 1:20,000, and monoclonal mouse anti-human FoxP3, clone 221D/D3 (Serotec, Oxford, UK), at a dilution of 1:500. Primary and secondary antibodies were diluted in 4% normal horse serum in PBS. All incubations were done at RT unless stated otherwise.

Briefly, frozen samples were sectioned at 5 μ m onto silane-coated slides (StarFrost, Knittel Glaeser, Braunschweig, Germany), fixed in acetone and transferred to PBS (pH 7.4). Endogenous peroxidase was inactivated by immersing the slides in 0.5% hydrogen peroxide containing 0.1% sodium azide in PBS for 30 min. After 20 min incubation in 4% normal horse serum to block non-specific protein-binding, the sections were incubated with primary antibody overnight at 4 °C. Biotinylated pan-specific secondary antibody (horse anti-mouse/anti-rabbit/anti-goat, Vector Laboratories, Linaris, Wertheim-Bettingen, Germany) was applied for 30 min in a dilution of 1:200. Sections were then incubated with peroxidase-conjugated ABC (Vectastain Elite ABC kit, Linaris) according to the manufacturer's instructions. The enzymatic reaction was developed by 5 min incubation with 3,3'-diaminobenzidine (DakoCytomation, Carpinteria, CA, USA). Finally, sections were lightly counterstained with haematoxylin, cleared in Tissue Clear (Sakura Finetek Europe), and mounted with permanent mounting medium (Mountex, Histolab Products, Gothenburg, Sweden).

Formalin-fixed, paraffin-embedded sections were cut at 2 μ m and collected on SuperFrost Plus slides (Menzel-Glaeser, Braunschweig, Germany). Sections were dewaxed, rehydrated, and endogenous peroxidase activity was quenched by immersing the slides for 15 min in 0.5% hydrogen peroxide in methanol. The staining procedure was carried out as described above, with the addition of a heat-induced epitope retrieval (HIER) step after the endogenous

peroxidase block. HIER was performed by boiling the sections for 15 min in Tris–EDTA buffer (pH 9.0) using a conventional microwave oven. Sections were allowed to cool down for 20 min at RT before the staining was continued with the protein blocking step.

A negative isotype control (mouse IgG1, Serotec) at the same protein concentration as the primary antibody was included in all staining runs. Sections of normal equine lymph nodes were used as positive control tissue.

2.6. Quantification of immunohistochemical staining

Positively stained cells were quantified under a light microscope at 400 $\times$ magnification using a square eyepiece reticule as described in [van der Haegen et al. \(2001\)](#) and the number of positive cells was calculated per square millimeter in the dermis and per mm in the epidermis. Intraepidermal cells were counted along the entire length of the epidermis. In the dermis, all positive cells located between the epidermal–dermal junction and the base of the hair follicles were counted. Cells in the wall of hair follicles were excluded. Due to the higher number of CD4+ and CD8+ cells, their counting was limited to 5 adjacent square reticules in the epidermis. Cells in the underlying dermis were counted from epidermal–dermal junction to the base of the hair follicles. Samples were numbered randomly to exclude a bias and analyzed by the same person (MH).

2.7. Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from blood with the Paxgene Blood RNA Kit (Qiagen AG) and from skin samples with the RNeasy Fibrous Tissue Mini kit (Qiagen AG) according to the manufacturer's instructions. Skin samples were homogenized for 2 $\times$ 6 min with a rotor–stator homogenizer (TissueLyser, Qiagen AG) prior to RNA extraction. Genomic DNA was eliminated by a DNase treatment supplied with the kit. Total RNA was quantified spectrophotometrically at 260 nm (NanoDrop 1000, Thermo Scientific) and samples were stored at –20 °C until use.

cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 1 μ g of total RNA was added to a reagent mix consisting of random hexamers, dNTPs and RNase-free water, incubated at 65 °C for 5 min and subsequently chilled on ice. Then, SuperScript Reverse Transcriptase was added in the presence of 5 $\times$ First-Strand buffer and dithiothreitol (DTT) and kept at 50 °C for 60 min followed by 15 min at 70 °C. cDNA was frozen and stored at –20 °C until use. For every RNA sample, an additional reaction was performed without reverse transcriptase (RT-negative control) to exclude contamination with genomic DNA.

A 5' nuclease assay using TaqMan probes was employed for qRT-PCR amplification. Expression of the following genes was assessed: IL-4, IL-5, IL-10, IL-13, TGF- β 1, IFN- γ and FoxP3 (for details see [Table 1](#)). cDNA (2 μ l), correspond-

Table 1
Probes and primers used in qRT-PCR.

Gene	Primer	Sequence
IL-4	Forward	CGT GCA TGG AGC TGA CTG TAG
	Reverse	CAG CCC TGC AGA TTT CCT TT
	Probe	FAM- ATG CCT TTG GCC CGA AGA ACA CAG A -TAMRA
IL-5	Forward	TGC TTC TGC ATT TGA GTG TGC TA
	Reverse	TGT TCA TGGG GAC TTT CTA CAG CAA
	Probe	FAM- CTC TTG GAG CTG CCT ACG TCT GTG CC -TAMRA
IL-10	Forward	GAC ATC AAG GAG CAC GTG AAC TC
	Reverse	CAG GGC AGA AAT CGA TGA CA
	Probe	FAM- AGC CTC ACT CGG AGG GTC TTC AGC TT -TAMRA
IL-13	Forward	CTG GAG TCC CTG AGC AAC GT
	Reverse	AGG GCA GAG TTT AGT CAG CAT CTT
	Probe	FAM- CCA CCT GCA GTG CCA TCC AAA ACA -TAMRA
IFN- γ	Forward	AAG TGA ACT CAT CAA AGT GAT GAA TGA
	Reverse	CGA AAT GGA TTC TGA CTC CTC TTC
	Probe	FAM- TCG CCC AAA GCT AAC CTG AGG AAG C -TAMRA
TGF- β 1	Forward	CCC TGC CCC TAC ATT TGG A
	Reverse	TGT ACA GGG CCA GGA CCT T
	Probe	FAM- CCT GGA CAC GCA GTA CAG -TAMRA
FoxP3	Forward	AGG GAG GTG GTG CAG TCT
	Reverse	CGC CCA GCT TCT CCT TCT C
	Probe	FAM- CAC CAG CTG CTG CTC C -TAMRA
18S rRNA		TaqMan ribosomal RNA control reagents; Applied Biosystems

ing primers and fluorescence-labeled probe were mixed with TaqMan Universal PCR Mastermix (Applied Biosystems, Austin, TX, USA). PCR reactions were performed in a total volume of 25 μ l and carried out in a 7300 Real-Time PCR System (Applied Biosystems). Amplification conditions were the same for all primer pairs: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Each reaction was run in duplicate and a no-template control was included to exclude contamination of reagents with cDNA on every plate. In case the standard deviation of the duplicate samples exceeded 0.5, the measurement was repeated until reliable values were attained. PCR efficiencies were calculated using a relative standard curve derived from a cDNA pool of all samples taken. Gene expression was quantified using the $\Delta\Delta$ Ct method. Expression of each gene of interest was normalized to the expression of the housekeeping gene 18S ribosomal RNA (18S rRNA).

2.8. Statistical analyses

Statistical analyses were carried out with the software package NCSS 2001 (NCSS, Kaysville, UT, USA). As the data were not normally distributed, the Mann–Whitney *U* test was used to compare values from IBH-affected horses 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). Differences between different severity groups were evaluated with the Kruskal–Wallis one-way ANOVA on Ranks. For parameters with only a low number of distinct values (histological scoring), the *p*-value of the test statistic after correction for ties was used. The Kruskal–Wallis-Multiple comparison-Z value test with Bonferoni correction was used to determine significant differences between the severity groups. Results were considered significant at $p \leq 0.05$.

3. Results

3.1. *Culicoides* specific IgE levels

Culicoides nubeculosus specific serum IgE levels were significantly higher in IBH-affected than in non-allergic control horses (median: 47.6 and 5.5 REU, respectively, $p < 0.0001$), as illustrated in Fig. 1.

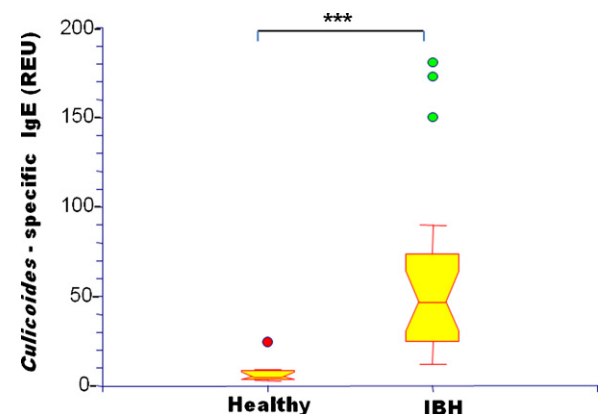


Fig. 1. *Culicoides nubeculosus* specific IgE levels in sera from 10 healthy and 21 IBH-affected horses: data are presented as relative ELISA units (REU). The centre horizontal line of the box plot marks the median of the sample. The edges of the box mark the first and third quartiles. The whiskers define the upper adjacent value which is the largest observation that is less than or equal to the 75th percentile plus 1.5 times the interquartile range (IQR) and the lower adjacent value which is the smallest observation that is greater than or equal to the 25th percentile minus 1.5 times IQR. The circles represent the outside values. ***Significant difference ($p \leq 0.0001$).

Table 2

Results (median and (range)) of histopathological evaluation of HE-stained slides in skin biopsies from 10 healthy control horses and in lesional and non-lesional skin of 14 moderately or severely IBH-affected horses.

	Healthy (n = 10)	Non-lesional (n = 14)	Lesional (n = 14)
Epidermis			
Hyperkeratosis	1 ¹ (0–2)	1 (0–2)	1 (0–3)
Hyperplasia	1 (0–1)	1 (0–3)	1 ^{a*} (1–2)
Dermis			
Eosinophils sup. dermis	0 (0–0)	0 ^b (0–2)	0 ^a (0–2)
Eosinophils deep dermis	0 (0–1)	0 (0–3)	1 ^{a*} (0–3)
Mononuc. cells sup. dermis	1 (1–2)	1 (1–2)	2 ^{a,†} (0–3)
Mononuc. cells deep dermis	0 (0–1)	0 (0–2)	1 ^(a) (0–2)
Edema	0 (0–1)	0 (0–2)	1 (0–2)
Score	4 (1–6)	4 (1–14)	6 ^{a*,†} (2–13)

¹ Results of semi-quantitative grading system (0–3).

^(a) $0.05 < p \leq 0.07$ in Mann–Whitney *U* test compared to healthy skin sample.

^a $p \leq 0.05$ in Mann–Whitney *U* test compared to healthy skin sample.

^b $p \leq 0.05$ in Mann–Whitney *U* test compared to healthy skin sample.

^{a*} $p \leq 0.01$ in Mann–Whitney *U* test compared to healthy skin sample.

^(†) $p = 0.06$ in Wilcoxon Signed-Rank paired test compared to non-lesional skin sample.

[†] $p \leq 0.05$ in Wilcoxon Signed-Rank paired test compared to non-lesional skin sample.

3.2. Clinical grading and histopathology

The 21 IBH-affected horses participating in this study were graded into three categories with respect to the severity of the clinical signs. Based on the grading system described previously, seven horses were classified as mildly, 10 horses as moderately and four horses as severely affected with IBH.

Histopathologic changes were evaluated by light microscopic examination of HE-stained sections according to a semi-quantitative grading system. First, we analyzed the data separately for each of the three IBH severity groups. As illustrated in Fig. 2, while lesional skin from moderately or severely IBH-affected horses had a significantly higher histological score than biopsies from mildly affected or

healthy horses, lesional biopsies from mildly IBH-affected horses did not differ significantly from the skin of healthy horses. For this reason, the seven mildly affected horses were discarded from the subsequent analyses. Compared to healthy skin, lesions from IBH affected horses showed a significantly higher degree of hyperplasia and a significantly increased eosinophil infiltration in superficial and deep dermis. Furthermore, infiltration of mononuclear cells in the superficial dermis was significantly higher in skin from IBH-affected horses than in skin from healthy controls (Table 2).

3.3. Distribution and number of CD4+, CD8+ and FoxP3+ cells in lesional and non-lesional skin of IBH-affected horses and in skin of healthy control horses

3.3.1. CD4+ and CD8+ T cells

CD4+ and CD8+ T cells were labeled immunohistochemically on acetone-fixed cryostat sections. Both in healthy and in IBH skin, CD4+ and CD8+ T cells were found in proximity to small blood vessels in the superficial and deep dermis as well as in the basal layer of the epidermis (Fig. 3). In healthy horse skin, the perivascular infiltrate was sparse and CD8+ cells were more frequent in the epidermis than CD4+ cells (Table 3). No positive staining was observed when the primary antibodies were replaced by the isotype control reagent.

There was a significantly increased number of CD4+ cells in lesional dermis of IBH-affected horses compared to skin from healthy controls (Table 3). The number of CD4+ cells in lesional skin was also slightly higher than in non-lesional skin but this difference did not reach statistical significance ($p = 0.07$). However, if the total number of CD4+ and CD8+ cells was considered, there was a significant increase in lesional compared to non-lesional dermis (94.8 and 72.5 cells/mm², respectively, $p \leq 0.05$, Table 3) and a tendency for increased total numbers of dermal CD4+ and CD8+ cells in IBH lesions compared to healthy skin biopsies ($p = 0.06$; Table 3).

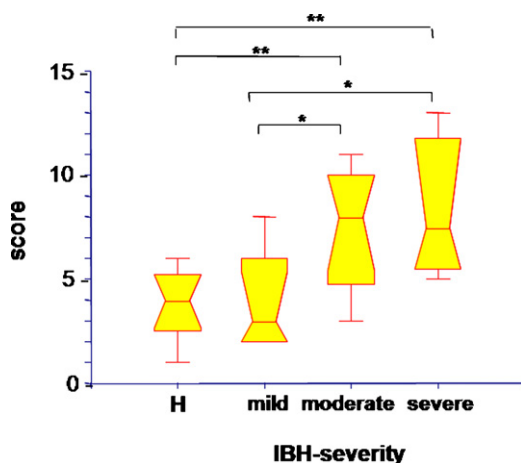


Fig. 2. Histologic score in biopsies from skin from healthy horses (H) and in lesional skin from IBH-affected horses graded according to clinical severity (mild, moderate and severe IBH). A semi-quantitative grading system (0 = none, 1 = mild, 2 = moderate, 3 = severe) was used to assess the degree of cellular infiltration (eosinophils, mononuclear cells), epidermal changes (hyperplasia, hyperkeratosis) and edema. The grades in each category were summed up to form the histologic score for each biopsy. For definition of the box plots and whiskers see legend of Fig. 1. ** $p \leq 0.01$; * $p \leq 0.05$.

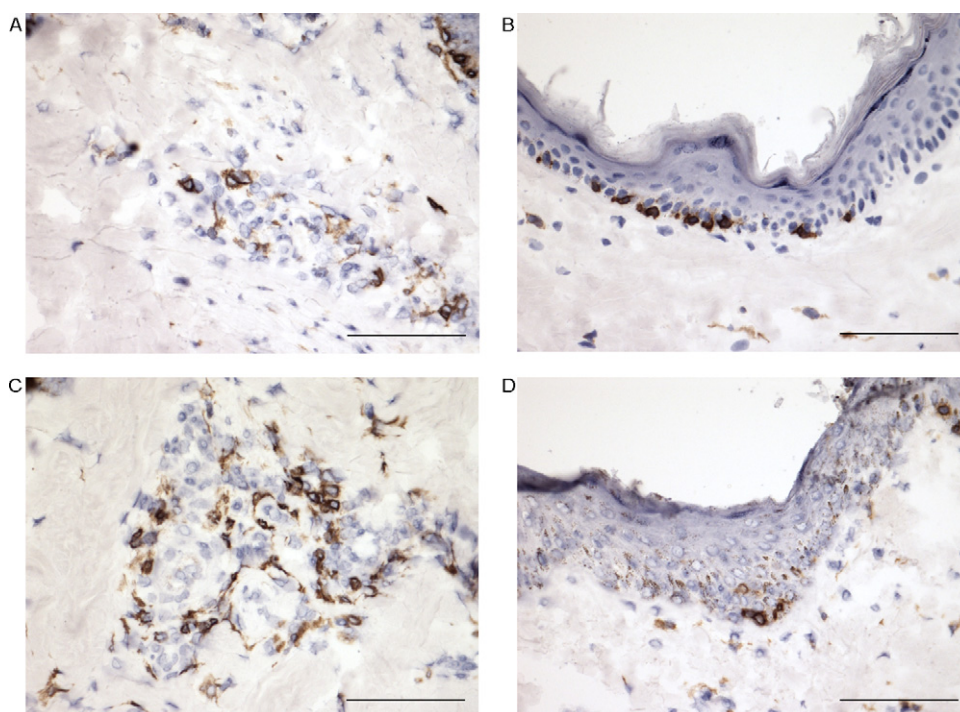


Fig. 3. Immunohistochemical staining of CD8 (A and B) and CD4 (C and D) positive cells on acetone-fixed cryostat sections of IBH skin. A and C: dermis (lesional); B and D: epidermis (non-lesional). Original magnification 400 $\times$. The bar corresponds to 100 μ m.

No significant differences in the number of CD8+ cells were observed between skin of healthy and IBH-affected horses. The CD4/CD8 ratio did not differ between the groups in the dermis, but it was slightly higher in the epidermis of lesional IBH skin than in the epidermis of healthy horse skin ($p=0.06$, Table 3).

3.3.2. FoxP3+ T cells

We first confirmed reactivity of the anti-FoxP3 antibody 221D/D3 on formalin-fixed paraffin-embedded equine lymph nodes. 221D/D3 reacted with the nuclei of lymphocytes randomly scattered predominantly throughout the cortical areas of the lymph node. No positive staining was obtained with

Table 3

Results (median and (range)) of immunohistochemical quantification: stained cells in skin biopsies from 10 healthy control horses and in lesional and non-lesional skin of 14 moderately or severely IBH-affected horses.

	Healthy (n = 10)	Non-lesional (n = 14)	Lesional (n = 14)
CD4			
Epidermis	3.0 ¹ (0.0–10.0)	5.2 (0.0–13.2)	3.2 (0.4–9.2)
Dermis	50.5 (30.9–70.4)	42.0 (15.8–136.1)	60.6 ^{(a),(†)} (22.5–117.7)
CD8			
Epidermis	5.8 (2.4–12.4)	3.6 (0.8–10.4)	3.8 (1.2–10.8)
Dermis	27.0 (20.1–30.4)	22.7 (8.1–51.1)	24.9 (15.9–55.3)
CD4+CD8 total			
Epidermis	9.0 (2.4–19.6)	9.6 (1.6–21.2)	7.0 (2.0–18.4)
Dermis	76.3 (58.6–100.4)	72.5 (23.9–187.2)	94.8 ^{(a),(†)} (45.9–173.0)
CD4/CD8 ratio			
Epidermis	0.52 (0.0–1.9)	1.28 (0.0–7.5)	0.87 ^(a) (0.1–5.5)
Dermis	2.03 (1.1–2.9)	1.89 (0.9–4.0)	1.97 (1.0–6.1)
FoxP3			
Epidermis	0.1 (0.0–0.5)	0.1 (0.0–0.4)	0.1 (0.0–0.3)
Dermis	5.1 (2.3–8.0)	2.8 (1.3–6.3)	4.2 (2.2–13.3)
Ratio FoxP3 to CD4			
Epidermis	0.031 (0.0–0.056)	0.015 (0.0–0.25)	0.011 (0.0–0.50)
Dermis	0.104 (0.046–0.163)	0.062 (0.037–0.177)	0.072 (0.032–0.207)

¹ Epidermal cells expressed in cells/mm, dermal cells in cells/mm².

^(a) 0.05 < p $\leq$ 0.07 in Mann–Whitney U test compared to healthy skin sample.

^(†) p $\leq$ 0.05 in Mann–Whitney U test compared to healthy skin sample.

^(‡) 0.05 < p $\leq$ 0.07 in Wilcoxon Signed-Rank paired test compared to non-lesional skin sample.

[†] p $\leq$ 0.05 in Wilcoxon Signed-Rank paired test compared to non-lesional skin sample.

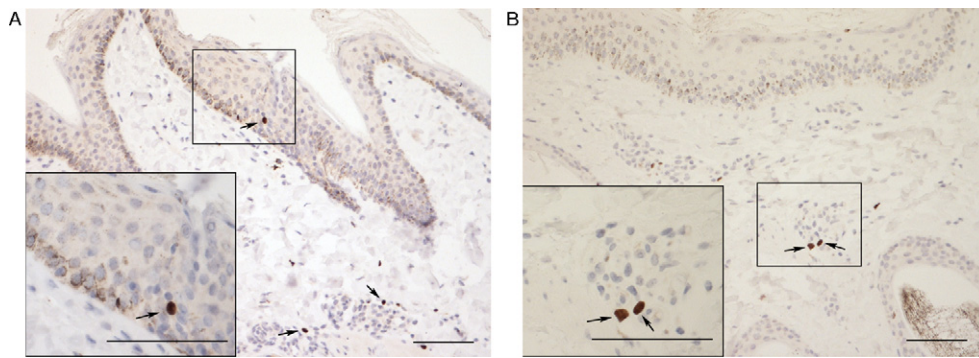


Fig. 4. Immunohistochemical staining of FoxP3+ cells in formalin-fixed paraffin-embedded equine tissue. FoxP3+ cells are present in the perivascular cell infiltrate and occasionally in the basal cell line of the epidermis in normal equine skin (A) and lesional IBH skin (B). Arrows indicate positive cells. Original magnification 200 $\times$ and 400 $\times$, respectively. The bar corresponds to 100 μ m.

the negative isotype control reagent (Suppl. Fig. 1).

Next, we used the antibody to stain skin sections from healthy horses and non-lesional and lesional skin from IBH-affected animals. In normal equine skin, a small number of FoxP3+ cells were distributed randomly in the dermis, most often in proximity to blood vessels. FoxP3+ cells were also present in the wall of hair follicles and occasionally within the basal cell line of the epidermis (Fig. 4A). In skin from IBH-affected horses, FoxP3+ cells were similarly distributed (Fig. 4B).

Neither absolute numbers nor the ratio of Foxp3+ to CD4+ cells differed significantly between lesional and non-lesional skin (Table 3). However, in the one-way ANOVA, horses with severe IBH lesions had a significantly lower ratio of Foxp3+ to CD4+ cells in the dermis than moderately affected or healthy horses (0.04 versus 0.092 or 0.104, respectively, $p < 0.05$). IBH lesions of these four severely affected horses contained more CD4+ cells (74.5 versus 60.6 and 50.5 respectively) and fewer FoxP3+ cells (2.9 versus 4.6 and 5.1, respectively) than the skin from moderately affected or healthy horses, but these differences did

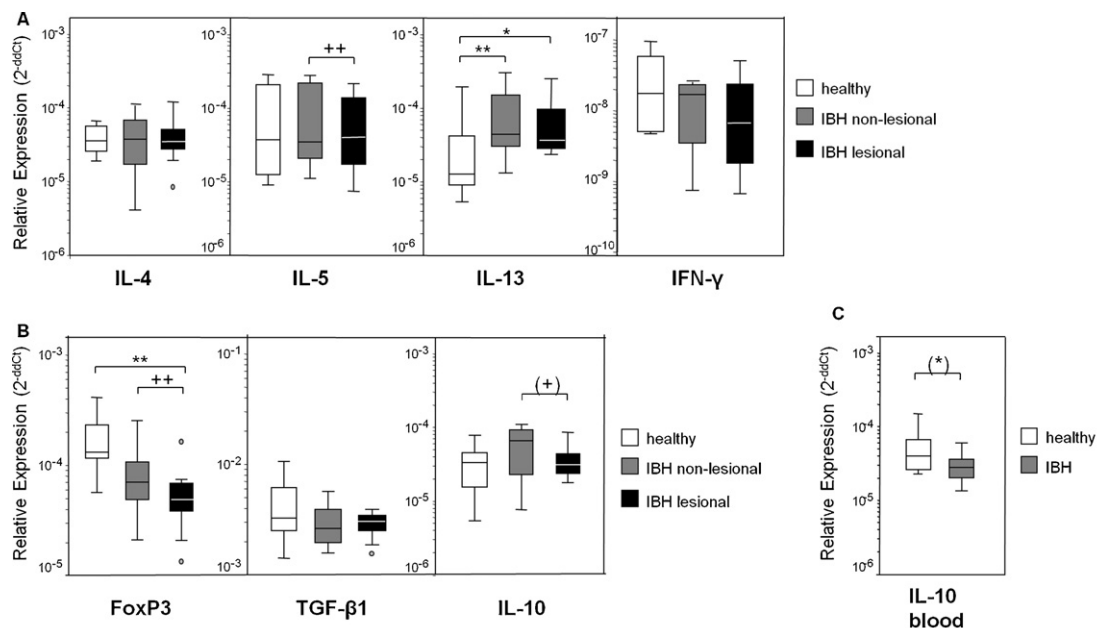


Fig. 5. Expression of Th1 and Th2 cytokines (A), regulatory cytokines and FoxP3 (B) in the skin and expression of IL-10 in blood (C) of 14 moderately or severely IBH-affected and 10 healthy horses. Expression levels were assessed by quantitative RT-PCR and normalized to 18S rRNA using the ddCt method. For definition of the box plots and whiskers see legend of Fig. 1. Comparison of the results of the skin from healthy horses with those of lesional or non-lesional skin from IBH affected horses was performed using the Mann–Whitney U test (* $p \leq 0.05$; ** $p \leq 0.01$). Comparison of the results of lesional and non-lesional skin of individual horses was performed with the paired Wilcoxon Signed-Rank Test (++) $p \leq 0.01$; + $p = 0.06$.

not reach statistical significance for these single parameters.

3.4. Expression levels of cytokine and FoxP3 mRNA in skin and blood of IBH-affected and healthy horses

3.4.1. Th1 and Th2 cytokines

Expression levels of the Th2-type cytokines IL-4, IL-5 and IL-13 and the Th1-type cytokine IFN- γ were measured in skin and blood samples of IBH-affected and healthy horses. Expression of IL-13 was significantly higher in lesional ($p < 0.05$) and non-lesional skin ($p \leq 0.01$) of IBH-affected horses compared to the skin of healthy horses (Fig. 5A), but no significant differences were observed regarding the expression levels of IL-4 and IFN- γ (Fig. 5A). Intriguingly, IL-5 levels were relatively low in lesional skin, leading to a significant difference when compared to non-lesional skin ($p < 0.01$, paired WSR test).

There were no differences in peripheral blood expression levels of IL-4, IL-5, IL-13 and IFN- γ between healthy and IBH-affected horses (data not shown).

3.4.2. Regulatory cytokines and FoxP3

To investigate the possible role of Treg cells in the pathogenesis of IBH, we assessed the expression of IL-10, TGF- β 1 and FoxP3 in the skin of IBH-affected and healthy horses. Expression levels of FoxP3 were significantly lower in lesional skin compared to non-lesional ($p < 0.01$, paired WSR test) and healthy skin ($p < 0.01$) and were also lower in non-lesional compared to healthy skin ($p < 0.05$; Fig. 5B). IL-10 expression was lower in lesional than in non-lesional skin; the difference nearly reached statistical significance (paired WSR test $p = 0.06$). Expression levels of TGF- β 1 did not differ significantly between skin of healthy and IBH-affected horses.

There was a tendency for reduced IL-10 expression levels in peripheral blood of IBH-affected horses compared to healthy horses ($p = 0.06$; Fig. 5C). There were no differences in peripheral blood expression of FoxP3 and TGF- β 1 between healthy and IBH-affected horses (data not shown).

4. Discussion

Equine IBH is a chronic recurrent dermatitis caused by IgE-mediated reactions to biting flies. In the past decade, a surge of interest has focused on the role of Treg cells in allergic diseases, and accumulating evidence indicates a connection between atopy and an increased ratio of Th2 to Treg cells (Taylor et al., 2006). We hypothesized that a similar shift of balance plays a role in the pathogenesis of IBH in the horse. In the present study, we examined the skin-infiltrating T cell subsets and the cytokine expression in lesional and non-lesional skin of IBH-affected horses and compared the results to the state in normal equine skin. Additionally, expression of cytokines and the Treg transcription factor FoxP3 was measured in peripheral blood.

All horses included in this study were of Icelandic breed. All except two of the healthy control horses were born in Sweden while all except two of the IBH-affected horses were born in Iceland and imported to Sweden

as adults. This difference reflects the high prevalence of IBH in horses born in Iceland and imported to Europe as adults. Although there may be some differences in the immune response toward *Culicoides* spp. in Icelandic-born or Sweden born IBH-affected Icelandic horses, the clinical signs and histopathological changes were the same in both groups and they were thus all included in our study. IBH-affected horses had a well-documented history of a seasonally recurrent dermatitis which would improve through insect control, such as stabling or blankets. Sensitization to midge antigens in these horses was confirmed by a highly significant increase in *Culicoides* specific serum IgE levels compared to the healthy control horses (Fig. 1). Due to thorough insect control, clinical signs at the time of sampling were only mild to moderate in most cases, even though the majority of horses had a history of clinically severe IBH.

Distinct histopathological changes were observed in biopsies from moderately and severely affected horses only: Horses which were clinically graded as mild did not differ in their histological score from healthy horses. These horses were not considered as suitable for our study and were removed from further analysis. A preliminary analysis had also shown that they did not differ from the healthy control horses for the other parameters investigated. In contrast, horses clinically graded as moderately or severely affected reached significantly higher histological scores than healthy or mildly affected horses (Fig. 2).

Skin-infiltrating mononuclear cells were further characterized by immunohistochemical staining for CD4, CD8 and FoxP3. We found a significant increase in total T cell numbers (CD4+ and CD8+) in the dermis of lesional compared to non-lesional IBH skin and lesional IBH skin contained significantly more CD4+ cells than the skin from healthy horses (Table 3). A T cell infiltrate due to an amplified recruitment of CD4+ Th cells is also a typical finding in AD lesions from humans and dogs (Roosje et al., 1998; Rho et al., 2004).

We measured mRNA expression of IL-4, IL-5, IL-13 and IFN- γ to characterize the cytokine microclimate in the skin of IBH-affected and control horses. IL-13 mRNA levels were significantly elevated both in lesional and in non-lesional IBH skin compared to healthy skin, whereas there was no significant difference in IL-4 expression. To our knowledge, no data on Th2 cytokine expression in equine IBH skin have been published to date, but various studies have focused on the cytokine profile observed in human and canine AD. Elevated levels of IL-13 have repeatedly been described in AD skin (Hamid et al., 1996; Akdis et al., 1999; Obara et al., 2002; Tazawa et al., 2004), and it has been proposed that IL-13, not IL-4, is responsible for IgE production in human AD patients (Akdis et al., 1999). Data on IL-4 expression in human AD skin are controversial. While some authors describe significantly elevated levels (Hamid et al., 1994), others detected no difference in IL-4 expression between AD and healthy skin (Akdis et al., 1999; Tazawa et al., 2004). One possible reason for this discrepancy is the stage of lesions used for the study. In humans, only acute lesions are characterized by a predominant Th2-type cytokine profile, while Th1-type cytokines become prevailing in chronic

lesions (reviewed by Leung et al., 2004). Furthermore, IL-4 and IL-13 dominate at different stages of the Th2 reaction: While IL-4 has a primary role in the initiation of the allergic response, IL-13 is critically involved in its effector functions (Corry, 1999). IL-4 may thus be expressed far more transiently, as underlined in an atopy patch test experiment by Pfeffer et al. (2007) using lambs with allergic dermatitis against louse-antigens.

Conversely to our expectation, we detected significantly lower IL-5 expression in lesional than in non-lesional skin. We can presently not explain this finding. We have found no significant difference in IFN- γ expression between IBH and healthy skin. IFN- γ is a Th1-associated cytokine that has been described to be elevated in chronic AD lesions (Grewe et al., 1995). In our study, we have tried to avoid chronic lesions as biopsy sites. It is thus not surprising that the difference between the groups remained insignificant.

FoxP3 is presently used as a standard marker for detection of natural Treg cells (Hori et al., 2003; Fontenot and Rudensky, 2005; Solomon and Magro, 2008). Treg cells are able to suppress allergen-driven T cell proliferation and Th2 cytokine production *in vitro*, and it has been proposed that atopic disease could result from failure of such suppression (Bellinghausen et al., 2003; Ling et al., 2004). Possible reasons for an insufficient inhibitory activity of Treg cells include a numerical or a functional deficiency. In our study, immunohistochemical staining revealed no difference in absolute numbers of FoxP3+ cells between IBH and healthy skin. However, when the IBH samples were grouped according to clinical severity, it became apparent that the ratio of FoxP3+ cells to CD4+ cells decreased with increasing IBH severity grade and was significantly lower in the IBH lesions from severely affected horses. As the number of horses was small in the severe IBH group ($n = 4$), these results will need to be confirmed.

In our study, differences in FoxP3 expression were more striking at the mRNA level: FoxP3 mRNA expression was significantly reduced in lesional IBH skin compared to non-lesional or healthy skin (Fig. 5B). Combined with the results of the immunohistochemical staining described above, this finding suggests that expression levels of FoxP3 are reduced per individual cell. While it is well-established that presence of FoxP3 is required for development and function of natural Treg cells (Hori et al., 2003; Fontenot and Rudensky, 2005), it has only recently been shown that the level of expression is also critical: Wan and Flavell (2007) provide compelling evidence that a decreased expression of FoxP3 leads to defective suppressive function of natural Treg cells. Interestingly, they also show that Treg cells with reduced expression levels of FoxP3 instruct Th2 differentiation of conventional T cells, thus contributing to rather than inhibiting Th2-mediated immune diseases. Decreased FoxP3 protein expression in Treg cells has since been described in asthma patients (Provoost et al., 2009) as well as in patients with new-onset systemic lupus erythematosus (Zhang et al., 2008). As allergies have repeatedly been associated with a functional deficiency of Treg cells (Grindebacke et al., 2004; Ling et al., 2004), it is reasonable to assume that this deficiency may be based on reduced expression levels of FoxP3 within the Treg cell population.

It is furthermore of interest that in contrast to FoxP3 expression in the skin, FoxP3 mRNA levels in the blood of IBH affected horses were similar to those of healthy control horses. These results underline the importance to study Treg cell number and function at the site of inflammation. While most studies investigate peripheral blood Treg cells as those are easily accessible, the derived results do not necessarily reflect the situation in the affected organ (Cao et al., 2003; Belkaid, 2007; Franz et al., 2007).

In contrast to thymic-derived FoxP3+ natural Treg cells, adaptive Treg cells can be induced under certain conditions upon antigen encounter in the periphery and are characterized by their secretion of high levels of IL-10 and/or TGF- β (Roncarolo et al., 2001). IL-10 is a potent immunosuppressive cytokine that modulates many cells and effector functions associated with allergic disease, such as Th2 cell activation, mast cell and eosinophil function and IgG to IgE ratios (Hawrylowicz and O'Garra, 2005). In humans, it has been shown that IL-10 producing allergen-specific T cells are reduced in allergic individuals (Akdis et al., 2004) and that IL-10 levels in peripheral blood increase while patients undergo allergen-specific immunotherapy (Akdis et al., 1998; Till et al., 2004). In horses, like humans, IL-4 secretion *in vitro* is down-regulated by the combined effect of IL-10 and TGF- β (Hamza et al., 2008) and IL-10 producing T cells have been identified in peripheral blood (Wagner et al., 2008). In our study, there was a tendency for reduced IL-10 expression in the blood of IBH-affected horses compared to control horses and a tendency for decreased IL-10 expression in lesional compared to non-lesional skin ($p = 0.06$). These findings suggest that IL-10 producing Treg cells may play a role in prevention of allergy to *Culicoides* bites. In contrast, we did not find any significant differences for TGF- β 1 expression. This may be due to the fact that TGF- β 1 is secreted in an inactive form, and measurement of the total expression neglects the fact that it may be the ratio of active to latent TGF- β 1 that is important. In a study by Khalil et al. (2001), the authors show that while the total expression of TGF- β 1 is comparable between a patient and a control group, the percentage of active TGF- β 1 may still differ significantly.

In summary, we found reduced FoxP3 and increased IL-13 mRNA expression in lesional and non-lesional skin of IBH-affected horses, indicating a local imbalance between Th2 and Treg cells. Our findings suggest that treatment strategies for IBH should aim at restoring the balance between Th2 and Treg cells, for example by allergen specific immunotherapy, as demonstrated in the field of human allergy (Akdis et al., 1998).

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2010.11.016.

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