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Modulation of Allergy Incidence in Icelandic Horses Is Associated with a Change in IL-4-Producing T Cells

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Key Words

Interferon-γ · Interleukin-10 · Interleukin-13 · Interleukin-4 · Interleukin-5 · Immediate-type hypersensitivity · T helper cells · Horse, Icelandic · Insect bite hypersensitivity

Abstract

Background: Equine insect bite hypersensitivity (IBH) is an immediate-type hypersensitivity reaction provoked by insect-derived allergens. Icelandic horses living in Iceland do not have IBH due to absence of relevant insects, but acquire it at high frequency after being imported to mainland Europe. In contrast, their offspring born in mainland Europe has reduced IBH incidence. T helper 1 (Th1) and Th2 cells and cytokines were determined in Icelandic horses born in Iceland and on the continent and which either have IBH or are healthy. ***Methods:*** Peripheral blood mononuclear cells (PBMC) from these horses were stimulated for 18 h during summer and winter with polyclonal T cell stimuli, IBH allergen(s) or irrelevant allergen(s). Cells were analysed by flow cytometry for interferon-γ (IFN-γ) and interleukin-4 (IL-4); RNA was analysed for IFN-γ, IL-4, IL-5 and IL-13 mRNA. ***Results:*** During summer, but not during winter, IBH PBMC stimulated polyclonally showed reduced IFN-γ mRNA and IFN-γ-producing cells when compared with those of healthy horses, regardless of origin. PBMC stimulated polyclonally or with IBH allergen showed increased IL-4 mRNA levels and higher numbers of IL-4-producing cells when born in Iceland or showing IBH symptoms. IL-5 and IL-13 mRNA were modulated neither by disease nor by origin. Abrogation of IL-4 production in healthy horses born in mainland Europe may be due, at least in part, to IL-10. There was an increased level of IL-10 in supernatants from PBMC of healthy horses born in mainland Europe and stimulated polyclonally or with IBH allergen. ***Conclusions:*** Modulation of IBH incidence is governed by altered Th1/Th2 ratio, which might be influenced by IL-10.

Introduction

For unknown reasons, the frequency of immediate-type allergic diseases is on the increase in most Western countries [1]. The role of immunoglobulin E (IgE) antibodies in immediate-type allergies is well established. However, the mechanism by which the immune response leads to an IgE response that in turn causes allergy is less clear. The original term atopy refers to the notion that the propensity towards immediate-type hypersensitivity against particular allergens is influenced in part by genetic factors [2, 3]. Recent studies suggest that factors independent of the genetic constitution and of allergen-mediated triggering, such as lifestyle and/or environmental factors, contribute to the incidence of immediate-type allergy, but the exact environmental factors governing allergy incidence are unclear [4, 5].

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Natural animal models of immediate-type hypersensitivity are rare. Here we discuss insect bite hypersensitivity (IBH) in Icelandic horses, a naturally occurring hypersensitivity whose incidence depends, in part, on the geographical origin. IBH is a recurrent allergic dermatosis affecting horses hypersensitive to bites of insects of the genus *Culicoides* (midges). Susceptible horses show clinical signs of IBH from spring to autumn, when the insects are active, and are in remission during winter [6–8]. Additional factors such as the birth place modulate the incidence of IBH. IBH does not occur in Iceland where insects conveying the IBH allergen(s) are absent. However, Icelandic horses exported to mainland Europe as adults (1st generation) have a $\geq$50% incidence of developing IBH; in contrast, their progeny (2nd generation) has a $<$10% incidence of contracting IBH [9, 10]. The offspring shares with their parents the genetic pool, and both groups are exposed to the same allergens in the environment for a number of years. Despite these similarities, there is a significant difference in IBH incidence, although the only apparent difference is that 2nd generation horses are born in an allergen-rich environment, whereas their parents are exposed to this environment only as adults.

T helper (Th) cells promote hypersensitivity reactions. For example, immediate-type hypersensitivity reactions are governed by Th2-type cells characterised by cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 [2]. Regulatory T cells, such as Tr1, control Th2 development, for example by IL-10 [11, 12]. The objective of this study was to determine (1) whether IBH is governed by a bias towards a Th2 reaction, (2) whether a difference is observed between 1st and 2nd generation horses and (3) whether it is observed in horses tested during exposure to allergens (summer) and during their absence (winter). Here we show that exposure to the specific antigen in vivo during summer is associated with an increase in IL-4 production in IBH-allergic horses (1st and 2nd generation), indicating a Th2 bias. This was not observed when allergen exposure was absent (winter). In 2nd generation horses, particularly in the healthy ones, there was a lower IL-4 production suggesting a Th2 cell suppression. This was consistent with the increased level of IL-10 found in supernatants from peripheral blood mononuclear cells (PBMC) of 2nd generation horses stimulated polyclonally, with IBH allergens or left unstimulated when compared with such supernatants of 1st generation horses. Thus, our results provide evidence that exposure to an allergen-rich environment early in life is associated with suppression of Th2 cell development which might be mediated, at least in part, by IL-10-producing cells.

Materials and Methods

Animals Enrolled in the Study

Horses were of Icelandic breed and lived in Switzerland for one to several years. They were assigned to 1 of 2 groups. One group was directly imported from Iceland at a mean age of 7 years [1st generation horses of both sexes, mean age of 16 years (range 8–38)]. The other group consisted of the progeny from the 1st generation horses which was born in mainland Europe [2nd generation horses of both sexes, mean age of 13 years (range 2–31)]. Each group encompassed 2 subgroups of healthy and IBH horses. The horses were from various stables in Switzerland and healthy control horses were selected randomly from the same stables where IBH-affected horses were living and thus were exposed to similar environmental conditions, including a similar exposure to insect bites. Furthermore, whenever available, 1st and 2nd generation horses from the same stable were tested at the same time. In the 1st generation horses, the mean time between import from Iceland and time when blood samples were taken was 8.7 years (range 1–32) for the healthy horses and 7.5 years (range 1–19) for the IBH-affected horses. All horses were dewormed and vaccinated regularly. The deworming and vaccination protocol of the horses that had lived in Iceland for some years is not known. The horses included in the study showed no clinical signs of other allergic or infectious diseases, and healthy horses had no previous history of skin problems. The diagnosis of IBH was based on clinical signs and history (recurrent seasonal pruritus and skin lesions along the typical anatomic sites, with remission in winter). In addition, a sulphidoleukotriene release assay (Cellular Antigen Stimulation Test (CAST); Bühlmann Laboratories, Schönenbuch, Switzerland) of high predictability (sensitivity 80%, specificity 97%) was used [13]. IBH-affected horses included in the study had shown clinical signs of IBH for at least 1 year and usually much longer, but in most cases the exact onset of disease had not been recorded by the owners. Almost all IBH-affected horses were treated against IBH in various ways, such as covering with blankets and/or local application of various lotions for insect and pruritus control. The numbers of horses tested in the various assays are given in the legends of the figures and tables. The number of subgroups of horses did not match the proportion found in nature; a sufficient number of horses was also tested for rare groups (for example IBH horses born in mainland Europe) to allow a meaningful statistical evaluation (at least 10 horses per subgroup).

Blood Collection and Isolation of PBMC

Both during the presence of IBH allergens (summer) and during their absence (winter), horses belonging to the IBH and healthy subgroups from 1 or several stables were bled on the same days (approximately 10 horses). Horses from all subgroups (63 in total) were tested twice, once in summer and once in winter. Blood was collected aseptically from the jugular vein using sodium heparin-containing vacutainers (Vacuette®; Greiner, St. Gallen, Switzerland). Blood was transferred immediately ($<$4 h) to the laboratory. PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (d = 1.077 kg/l; Biochrom, Berlin, Germany).

Cell Culture

PBMC were cultured for 18 h in the presence or absence of T cell mitogens. These were 10^{-7} M phorbol 12-myristate 13-acetate

Table 1. Sequences of PCR primers and probes used

Cytokine	Primer	Sequence
IL-4 (73)	forward	AAA CGC TGA ACA ACC TCA CAG A
	reverse	GCA AAG GCA TCC GCT ACA GT
	probe	GCT CCA TGC ACG AAT TCT TGC CCT TT
IL-5 (78)	forward	CAC TGC TCT CCA CTC ATC GAA CT
	reverse	GGT GAT TTT TAT GTT CAG GAG TAG GAA
	probe	CAT CAG GTT CCC ATC GCC TAT CAG CA
IL-13 (75)	forward	CTG GAG TCC CTG AGC AAC GT
	reverse	AGG GCA GAG TTT AGT CAG CAT CTT
	probe	CCA CCT GCA GTG CCA TCC AAA ACA
IFN-γ (80)	forward	TCT TTA ACA GCA GCA CCA GCA A
	reverse	GCG CTG GAC CTT CAG ATC AT
	probe	AGA CTT CCA AAA GCT GAT TCA GAT TCC GGT

Figures in parentheses are amplicon lengths given as the number of nucleotides. Sequences are presented from the 5′ to the 3′ end. Accession numbers of the Genbank database are AF305617 for IL-4 and U91947 for IL-5. IL-13 was provided by F. Steinbach. IFN-γ was used according to Ainsworth et al. [29].

(PMA; Sigma-Aldrich, St. Louis, Mo., USA), combined with 1 μM calcium ionophor (A23187; Sigma-Aldrich) or 5 mg/l concanavalin A (Sigma-Adrich). PBMC were also cultured for 18 h with *Culicoides nubeculosus* whole-body extract (10 mg/l) or with irrelevant allergen(s) (*Parascaris equorum* extract, 30 mg/l). Both extracts were prepared as described [7]. Culture medium was RPMI 1640 supplemented by Glutamax (446 mg/l; Invitrogen, Paisley, UK), HEPES (25 mM), penicillin (10,000 IU/l), streptomycin (10,000 μg/l), 2-mercapoethanol (50×10^{-6} M) and 10% autologous serum. Preliminary studies showed that culturing PBMC for 4 and 18 h in the presence of T cell mitogens yielded the maximum number of T cells expressing interferon-γ (IFN-γ) and IL-4 protein, respectively. Culturing PBMC for longer than 18 h neither significantly increased the number of responding cells nor did it induce a shift in the proportion of IFN-γ- to IL-4-producing cells.

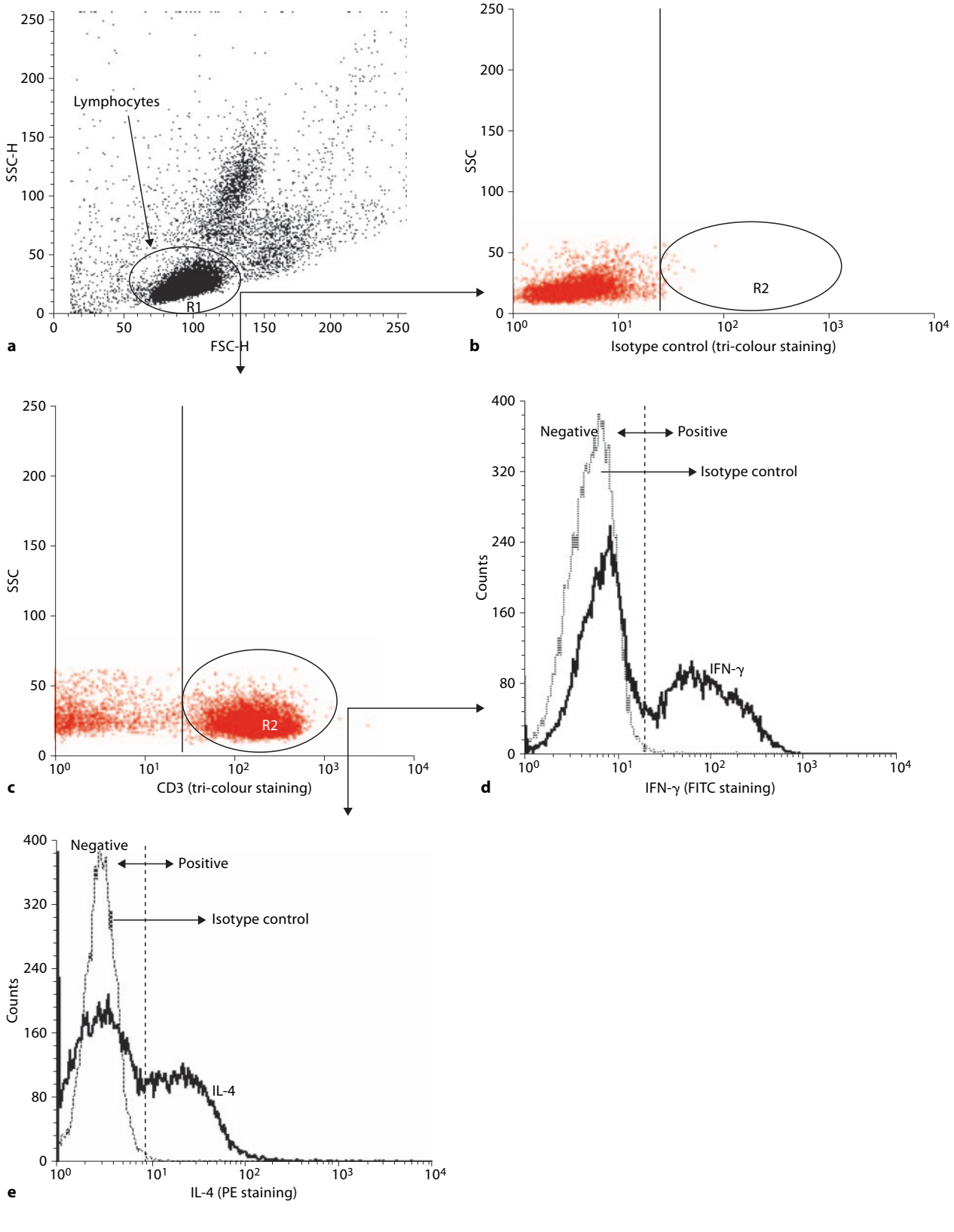
After PBMC culture, cells were harvested. One moiety of cultured cells was subjected to flow cytometry which was performed on the same day. The other was lysed and stored at –80°C until testing by quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR).

RNA Extraction, Reverse Transcription of RNA and QRT-PCR

Total RNA was isolated from cultured PBMC using the ABI Prism™ procedure. Cultured cells were lysed by adding 400 μl of 1× Nucleic Acid Purification Lysis Solution (Applied Biosystems, Rotkreuz, Switzerland) per 2×10^6 cells. Lysates were stored at –80°C until they were processed for total RNA isolation. This was performed with a semi-automated system (ABI Prism 6100 Nucleic Acid PrepStation; Applied Biosystems) according to the manufacturer's protocol. Cell lysates were transferred to a purification plate with a maximum concentration of 1×10^6 cells/well. For each type of stimulation, RNA of 2×10^6 cells was collected in 200 μl elution buffer (Applied Biosystems). The RNA concentration was measured by a spectrophotometer (Nanodrop, Wilmington, Del., USA). Total RNA (500 ng) was incubated for 50 min at 42°C with the reverse transcription (RT) enzyme Superscript II (RNase H-Reverse Transcriptase; Invitrogen), generating the complementary DNA (cDNA) used for QRT-PCR. cDNA was stored at –20°C for subsequent quantification of mRNA coding for cytokines, using a TaqMan® procedure. Primers and probes used are listed in table 1. Primers spanned, in each case, an intron. Both test samples and negative control samples were incubated as described above, but in the absence of the RT enzyme they were tested with each set of primers and probes in order to assure that genomic DNA was not co-amplified. A volume of 5 μl cDNA was amplified in duplicate for 50 cycles (30 s at 90°C and 60 s at 60°C). Amplification was performed in an Mx4000 instrument (Stratagene, Amsterdam, The Netherlands), using Brilliant QPCR Master Mix (Stratagene). The assay had a detection limit corresponding to a C_t value of 40. The C_t value is defined as the point at which the fluorescent signal for a given amplification product is significantly above background [14]. The majority of unstimulated cells had cytokine mRNA contents below the detection limit. Cytokine gene expression was normalised to the 18s rRNA content (TaqMan ribosomal RNA control reagents; Applied Biosystems), and $1/2^{\Delta Ct}$ was calculated. The ΔC_T value reflects the difference in the number of molecules between the 18s rRNA and the cytokine of interest in the sample analysed. Assuming a 100% efficiency of the PCR reaction, the $1/2^{\Delta Ct}$ expresses the relative quantity of specific cytokine mRNA molecules present in the samples compared to the number of the 18s rRNA molecules in the sample.

Flow Cytometry

PBMC was cultured as mentioned above in the presence of the protein transport inhibitor monensin (2 μM; Becton-Dickinson, Mountain View, Calif., USA), which was added to the culture for the last 12 h. Cells were then harvested (1×10^6/ml) by centrifu-



gation (250 *g* for 8 min at room temperature) and stained with monoclonal anti-equine CD3 kindly provided by Dr. Jeff Stott, University of California, Davis, Calif., USA (20 min on ice), followed by staining with the secondary antibody, which was Tricolour-labelled goat anti-mouse IgG1 (Caltag, South San Francisco, Calif., USA). Stained cells were fixed in 4% paraformaldehyde for 10–15 min and then treated with Permeabilising Solution 2 (Becton-Dickinson). The intracytoplasmic cytokine was labelled with a monoclonal anti-bovine IFN-γ antibody (2 mg/l, clone CC302; Serotec, Düsseldorf, Germany) and a monoclonal anti-bovine IL-4 antibody (5 mg/l, clone CC303; Serotec). Staining was performed for 20 min at room temperature in the presence of the permeabilising solution. Isotype controls were mouse IgG1 and mouse IgG2a (DAKO, Glostrup, Denmark). They were used at the same concentrations as anti-IFN-γ and anti-IL-4, respectively. FITC-conjugated goat anti-mouse IgG1 and phycoerythrin-conjugated goat anti-mouse IgG2a (Southern Biotech, Birmingham, Ala., USA) were used for detection of anti-bovine IFN-γ and IL-4, respectively. Washed cells were resuspended in PBS containing 0.02% sodium azide and analysed by flow cytometry using a FACScan (Becton-Dickinson). Gates for CD3-positive cells were positioned according to CD3-FL3 and side scatter. Gated cells were analysed for IFN-γ or IL-4 production by displaying FL1 and FL2 histogram plots, respectively. The histogram plots were then overlaid with their respective isotype control antibodies to discriminate negative from positive events, and differences were calculated (fig. 1). Since the threshold of detection was estimated to be 1%, differences below this value were put to 1%. As predicted by an earlier study [15], the monoclonal antibody specific for bovine IFN-γ (CC302) labelled a large subset of equine T cells expressing IFN-γ. Moreover, preliminary experiments using a CHO cell line stably transfected with equine IL-4 and kindly provided by Dr. David Horohov (University of Kentucky, Lexington, Ky., USA) via Dr. Falko Steinbach (New Haw, Addlestone, UK) confirmed that the mouse anti-bovine IL-4 monoclonal antibody (CC303) cross-reacts with equine IL-4 (data not shown).

ELISA

A capture ELISA for detection of equine IL-10 [16] was adapted using monoclonal anti-bovine IL-10 (clones CC318 and CC320; Serotec) and carrier-free recombinant equine (req)IL-10 (R&D Systems, London, UK) as a positive control and for establishing a standard curve. No cross-reactivity was observed with reqIL-2 (R&D Systems). Microtitre plates (MaxiSorp; Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 100 μl coating antibody (CC318, 6 mg/l). Incubations of freshly frozen (–20°C) supernatants were done at 37°C for 60 min. After washing the plates, non-specific reactions were blocked using 100 μl PBS with 1% BSA and 0.05% Tween-20. Plates were washed and reqIL-10 or the undiluted supernatants to be assayed were added. Following washing, 100 μl of biotin-labelled detection antibody (CC320, 2 mg/l) was added to the wells and incubated. After further washing, 100 μl streptavidin-horseradish peroxidase (Zymed, Geneva, Switzerland) diluted 1:1,000 was added, and plates were incubated. This was followed by washing and addition of 100 μl 3,3′,5,5′ tetramethyl benzidine substrate (Sigma-Aldrich). The evolving colour reaction was stopped using 50 μl 2 N H_2SO_4, and the optical density was read at 450 nm. Optical density values were converted into micrograms per litre of IL-10, using a standard curve generated by serially diluting reqIL-10. The assay had a detection limit of 0.165 μg/l reqIL-10.

Data Transformation and Statistical Analyses

A preliminary analysis had shown that the data were not normally distributed. Therefore, all values were log transformed. Values below the detection limit of the assay were replaced by a value at or below the limit of detection as follows: 1% in flow-cytometric analysis, 10^{-5} in QRT-PCR and 0.1 μg/l in the IL-10 ELISA.

Statistical analyses were carried out using the statistical software package NCSS 2004 (NCSS, Kaysville, Utah, USA). Analysis of variance (ANOVA) was performed in 3 ways: (1) When the same horses were analysed twice (that is during summer and winter), uni- and multivariable repeated measures ANOVA routines were used to examine the influence of the factors 'health state' (IBH or healthy), 'origin' (1st generation: born in Iceland; 2nd generation: born in mainland Europe), 'season' (summer/winter) and 'gender' (male or female). (2) An ANOVA routine was used to assess the influence of health state, origin and gender (IL-10 determinations). (3) For significant factors in any of the multivariable ANOVA routines, a one-way ANOVA with Bonferroni correction for multiple comparisons was used to identify significant group differences.

Fig. 1. Gating strategy used for determination of IL-4+ CD3+ and IFN-γ+ CD3+ lymphocytes in PBMC from Icelandic horses stimulated in various ways. **a** A gate was positioned around lymphocyte (R1). **b**, **c** Cells within this lymphocyte gate were used for gating CD3-positive cells (R2). **d**, **e** Cells within the R2 gate were then analysed for IFN-γ or IL-4 production. FL1 (IFN-γ) and FL2 (IL-4) histogram plots were displayed and overlaid with their respective isotype control antibodies to discriminate negative from positive populations.

Results

The Influence of Health State, Origin, Gender and Season on Th1 and Th2 Cytokine Production

PBMC isolated from the various subgroups of Icelandic horses during summer were cultured in the presence or absence of T cell mitogens (PMA + A23187), IBH allergen and irrelevant allergen. Cytokine (IFN-γ, IL-4, IL-5 and IL-13) mRNA expression was determined by QRT-PCR, and cytokine-producing T cells (CD3+ cells) were assessed by flow cytometry. Table 2 summarises the overall results of the repeated measures ANOVA. Following polyclonal stimulation, both the percentage of IFN-γ-producing T cells, as determined by flow cytometry, and expression levels of IFN-γ mRNA (QRT-PCR) were significantly influenced by health state (IBH or healthy) and season (summer or winter). Moreover, health state, origin of the horses and season had significant effects on the frequency of IL-4-producing T cells and levels of IL-4

mRNA expression when PBMC were polyclonally stimulated or stimulated with an IBH allergen, but not when they were stimulated by an irrelevant allergen. For IL-13 mRNA, an effect of origin and gender did not reach significance regardless of how cells had been stimulated, and showed significant differences for health state and season in 1 of 3 stimulation protocols only. For IL-5, the same results as for IL-13 were obtained except that winter and summer were always significantly different. When stimulation was done with the irrelevant antigen, the factors health state and origin had no significant influence on all parameters determined. Gender provided no significant differences for any of the parameters assessed.

For all factors examined in the repeated measures ANOVA (table 2), a one-way ANOVA was then used to determine the differences between the predetermined groups. For factors for which significance was achieved, differences between the subgroups are shown below.

Table 2. Influence of health state, origin, gender and season on Th1 and Th2 cytokine production by stimulated PBMC isolated from Icelandic horses

Parameters measured	Health state			Origin		
	healthy	IBH	p value	1st gen.	2nd gen.	p value
IFN-γ mRNA/polyclonal, $1/2^{\Delta CT}$	0.13 ×/: 0.04	0.0025 ×/: 0.04	<0.001*	0.0063 ×/: 0.04	0.05 ×/:0.04	0.06
IFN-γ-producing T cells/polyclonal, %	16 ×/:1.1	8 ×/:1.1	<0.001*	10 ×/:1.1	13 ×/:1.1	0.23
IL-4 mRNA/polyclonal, $1/2^{\Delta CT}$	0.001 ×/: 0.01	0.016 ×/: 0.03	0.001*	0.02 ×/: 0.027	0.0005 ×/: 0.018	<0.001*
IL-4 mRNA/IBH allergen, $1/2^{\Delta CT}$	0.000032 ×/: 0.0014	0.001 ×/: 0.01	<0.001*	0.00063 ×/: 0.009	0.00004 ×/: 0.0056	<0.001*
IL-4 mRNA/irrelevant allergen, $1/2^{\Delta CT}$	0.00007 ×/: 0.005	0.01 ×/: 0.004	0.37	0.000063 ×/: 0.005	0.000013 ×/: 0.004	0.16
IL-4-producing T cells/polyclonal, %	2 ×/:1.1	6 ×/:1.1	<0.001*	6 ×/:1.1	1.6 ×/:1.1	<0.001*
IL-4-producing T cells/IBH allergen, %	1.0 ×/:1.0	2.5 ×/:1.0	<0.001*	2.1 ×/:1.0	1.1 ×/:1.1	<0.001*
IL-4-producing T cells/irrelevant allergen, %	1.2 ×/:1.1	1.3 ×/:1.1	0.3	1.3 ×/:1.1	1.2 ×/:1.1	0.2
IL-5 mRNA/polyclonal, $1/2^{\Delta CT}$	0.0016 ×/: 0.012	0.0013 ×/: 0.015	0.58	0.002 ×/: 0.015	0.0013 ×/: 0.01	0.35
IL-5 mRNA/IBH allergen, $1/2^{\Delta CT}$	0.000025 ×/: 0.0005	0.00013 ×/: 0.02	0.02*	0.00010 ×/: 0.02	0.000032 ×/: 0.0003	0.06
IL-5 mRNA/irrelevant allergen, $1/2^{\Delta CT}$	0.0001 ×/: 0.0007	0.00016 ×/: 0.008	0.5	0.00013 ×/: 0.0075	0.0001 ×/: 0.001	0.75
IL-13 mRNA/polyclonal, $1/2^{\Delta CT}$	0.032 ×/: 0.06	0.11 ×/:0.08	0.035*	0.063 ×/: 0.07	0.032 ×/: 0.066	0.12
IL-13 mRNA/IBH allergen, $1/2^{\Delta CT}$	0.002 ×/: 0.015	0.013 ×/: 0.087	0.07	0.013 ×/: 0.079	0.002 ×/: 0.017	0.07
IL-13 mRNA/irrelevant allergen, $1/2^{\Delta CT}$	0.005 ×/: 0.037	0.0079 ×/: 0.02	0.80	0.0079 ×/: 0.03	0.0063 ×/: 0.026	0.86

PBMC were isolated from different groups of Icelandic horses during summer (n = 73) and winter (n = 63) and stimulated either polyclonally (PMA + A23187), with IBH allergen or with irrelevant allergen. Statistical analysis was carried out on log-transformed data using repeated measures ANOVA to examine the influence of health state (IBH or healthy), origin (born in Iceland, i.e. 1st generation, or born in mainland Europe, i.e. 2nd generation), gender (male or female) or season (summer or winter) on the parameters listed in the first column of the table. mRNA refers to QRT-PCR determinations; cells producing a given cytokine refer to flow-cytometric measurements. Values shown represent geometric means. ×/: = Standard error of the means. * $p \leq 0.05$.

IFN-γ mRNA Expression and Protein Production by Stimulated PBMC

During summer, that is when IBH allergens were present, the proportion of IFN-γ-producing T cells and the level of IFN-γ mRNA were significantly lower in the IBH subgroup compared to healthy animals of the same generation ($p \leq 0.05$). This was true for polyclonally stimulated cells (fig. 2). In most cases, no IFN-γ-producing T cells were detected after stimulation with IBH or the irrelevant allergen. Similarly, IFN-γ mRNA expression was also below the detection limit of the assay (data not shown).

IL-4 mRNA Expression and Protein Production by Stimulated PBMC

During the IBH season, stimulation of the PBMC induced a high level of IL-4 in 1st generation IBH horses compared to the other groups (fig. 3). This effect was seen

Gender			Season		
female	male	p value	summer	winter	p value
0.016 × /: 0.03	0.016 × /: 0.045	0.9	0.005 × /: 0.038	0.05 × /: 0.048	<0.001*
10 × /:1.1	13 × /:1.1	0.8	8 × /:1.1	16 × /:1.1	<0.001*
0.005 × /: 0.025	0.0032 × /: 0.025	1	0.008 × /: 0.026	0.0016 × /: 0.02	0.03*
0.0002 × /: 0.0077	0.00016 × /: 0.008	0.56	0.0005 × /: 0.01	0.00004 × /: 0.002	<0.001*
0.000013 × /: 0.0049	0.000063 × /: 0.004	0.9	0.000063 × /: 0.0006	0.000013 × /: 0.00046	0.47
3.2 × /:1.1	3.6 × /:1.1	0.27	8 × /:1.1	1.6 × /:1.1	<0.001*
1.6 × /:1.1	1.6 × /:1.0	0.20	2.5 × /:1.1	1 × /:1.0	<0.001*
1.3 × /:1.1	1.3 × /:1.1	1	1.6 × /:1.1	1.1 × /:1.1	0.002*
0.0025 × /: 0.015	0.0013 × /: 0.012	0.43	0.0008 × /: 0.014	0.0032 × /: 0.012	0.036*
0.00005 × /: 0.00068	0.000063 × /: 0.02	0.77	0.000025 × /: 0.0027	0.00016 × /: 0.027	0.001*
0.0001 × /: 0.007	0.00016 × /: 0.0068	0.11	0.00005 × /: 0.0001	0.0004 × /: 0.009	<0.001*
0.05 × /: 0.056	0.063 × /: 0.075	0.45	0.05 × /:0.055	0.063 × /: 0.09	0.72
0.0079 × /: 0.029	0.004 × /: 0.07	0.5	0.0050 × /: 0.027	0.0063 × /: 0.09	0.6
0.0079 × /: 0.029	0.0063 × /: 0.03	0.7	0.0032 × /: 0.036	0.016 × /: 0.02	0.05*

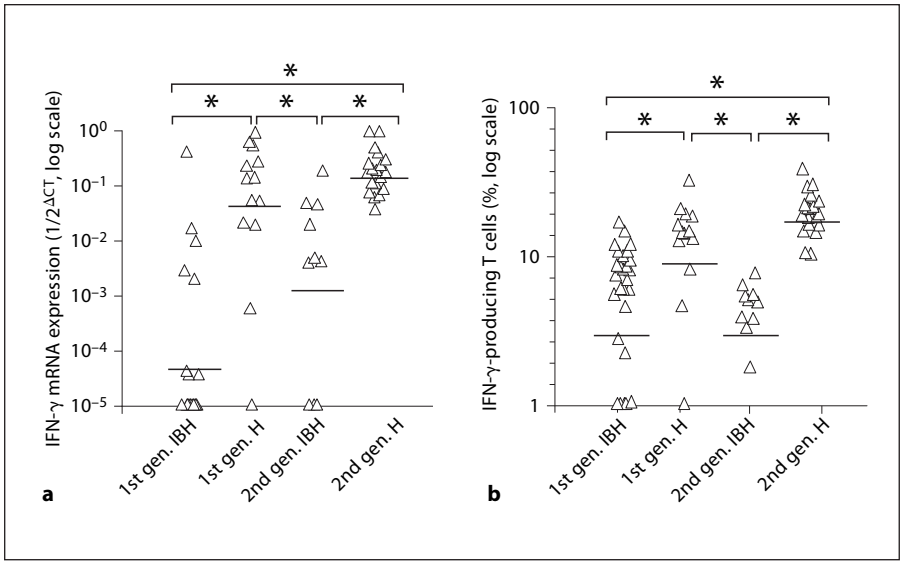


Fig. 2. Determination of IFN-γ mRNA (**a**) and protein (**b**) production in PBMC isolated from various groups of horses during the IBH season and stimulated polyclonally for 24 h. Groups of horses included 1st generation Icelandic horses suffering from IBH (1st gen. IBH; n = 27) or being healthy (1st gen. H; n = 15) and 2nd generation Icelandic horses suffering from IBH (2nd gen. IBH; n = 10) or being healthy (2nd gen. H; n = 21). **a** IFN-γ mRNA was quantified using QRT-PCR and the results are shown in relative terms ($1/2^{\Delta CT}$). **b** Intracellular IFN-γ production was determined by flow cytometry. Each data point represents 1 horse. The results are shown as percentage of IFN-γ+ CD3+ cells. Horizontal bars within values denote geometric means. One-way ANOVA was used to determine the significances of group differences. * $p \leq 0.05$.

regardless of how the cells had been stimulated (polyclonally or with IBH allergen) and which parameter was determined (IL-4 mRNA expression or IL-4-producing T cells). There was also a difference between the 1st and 2nd generation which reached significance in most instances, regardless of how the cells had been stimulated (polyclonally or with IBH allergen) and which parameter was determined (IL-4 mRNA expression or IL-4-producing T cells). No difference was seen if PBMC were stimulated with an irrelevant allergen (fig. 3e, f).

IL-5 and IL-13 mRNA Expression by Stimulated PBMC

The significance seen for 1 of 3 stimulation modes in table 2 (health state) was not paralleled by a significance between healthy and IBH subgroups (data not shown). No origin-related significance was obtained, regardless of the mode of stimulation of PBMC in vitro (data not shown).

Cytokine Production in the Presence or Absence of Allergen(s) in the Environment

Since modulation of IL-4 and IFN-γ production was observed (fig. 2, 3), it was of interest to compare PBMC collected from horses while allergen(s) were present in their environment (summer) with those collected during the absence of IBH-related allergen(s) in the environment (winter; fig. 4). Modulation of cytokine production (that is increase in IL-4 mRNA protein and decrease in IFN-γ mRNA and protein) was seen only during the time the horses showed IBH (summer) and not during the disease-free interval (winter). Increases and decreases were significant ($p \leq 0.05$). Such season-related alterations were not observed for IL-5 and IL-13 (data not shown). Moreover, season-related differences were not observed in healthy horses, regardless of which parameter was measured and how PBMC were stimulated in vitro (data not shown).

IL-10 Production by Stimulated PBMC

Results shown in figure 3 suggest that there is a suppression of IL-4 in 2nd generation IBH horses and even more so in 2nd generation healthy horses. IL-4 production by T cells could be influenced in several ways, for example by IL-10 [17, 18]. IL-10 levels were determined by ELISA in supernatants of PBMC stimulated for 24 h in various ways (polyclonal, IBH allergen) or left unstimulated.

Table 3 shows that using an ANOVA, there was a significant influence of the origin of the Icelandic horses (1st or 2nd generation) on IL-10 production following PBMC stimulation polyclonally, with the IBH allergen or leaving them unstimulated. Health state had a significant effect on IL-10 production upon polyclonal stimulation.

The individual group comparisons showed that following polyclonal stimulation, significantly higher concentrations of IL-10 were observed in PBMC cultures of 2nd generation IBH horses as opposed to 1st generation IBH horses (fig. 5a). Furthermore, significantly higher levels of IL-10 were determined in PBMC of 2nd generation healthy horses when compared with 1st generation IBH counterparts, regardless of stimulation (fig. 5). In-

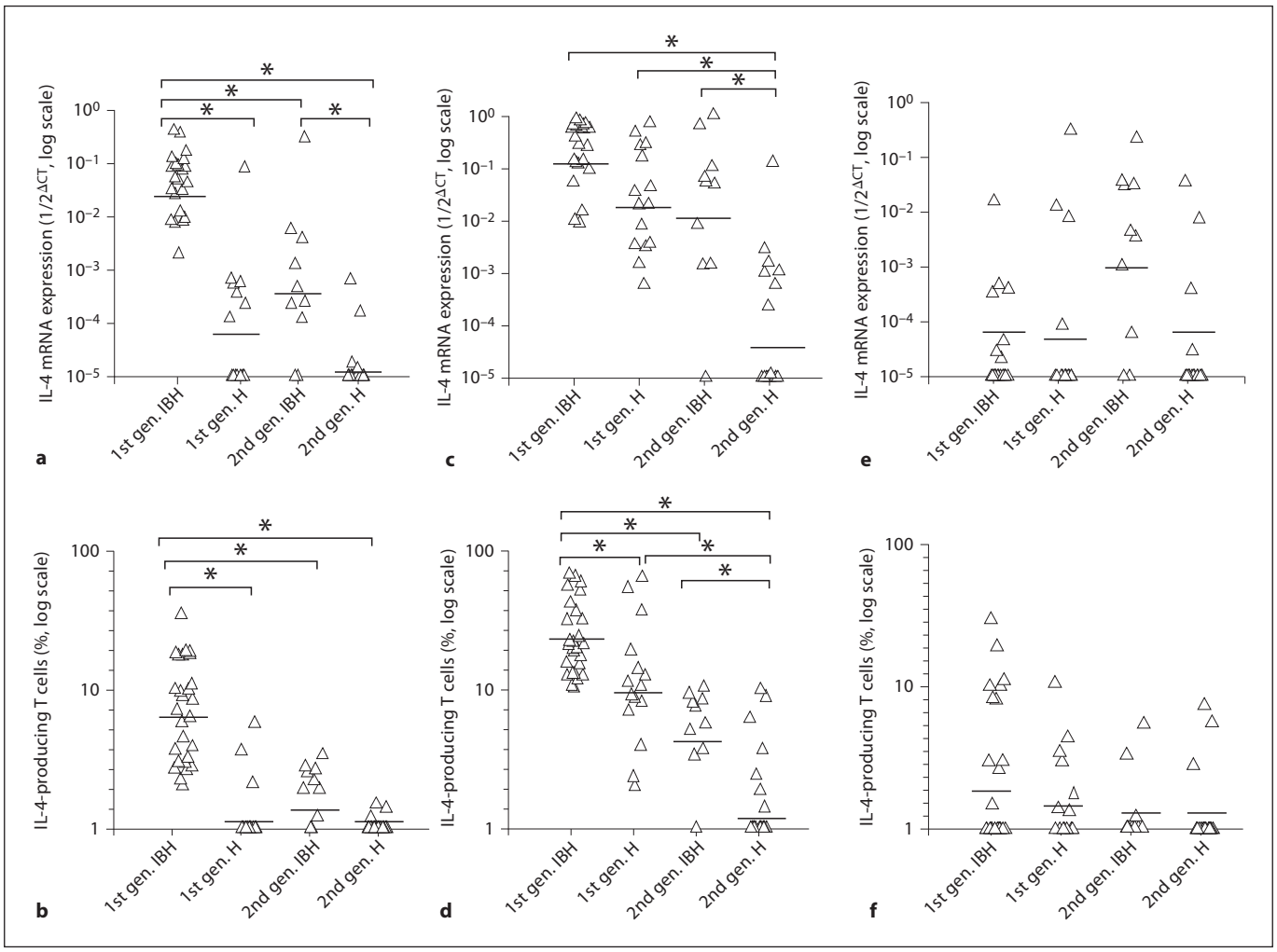


Fig. 3. Determination of IL-4 mRNA (**a**, **c**, **e**) and protein (**b**, **d**, **f**) production in PBMC isolated from various groups of horses during the IBH season and stimulated by the IBH allergen (**a**, **b**), polyclonally (**c**, **d**) or with irrelevant antigen (**e**, **f**). Groups and numbers of horses included are indicated in the legend of figure 2. Horizontal bars within values denote geometric means. One-way ANOVA was used to determine the significances of group differences. * $p \leq 0.05$.

Table 3. Influence of health state and origin on IL-10 production by PBMC isolated from Icelandic horses

Parameters	Health state			Origin		
	healthy	IBH	p value	1st gen.	2nd gen.	p value
IL-10/polyclonally stimulated PBMC, μg/l	6.0 ×/:1.3	2.1 ×/:1.3	0.03*	1.6 ×/:1.3	7.8 ×/:1.3	0.003*
IL-10/IBH allergen-stimulated PBMC, μg/l	2.8 ×/:1.5	0.9 ×/:1.5	0.06	0.8 ×/:1.5	3.2 ×/:1.5	0.03*
IL-10/unstimulated PBMC, μg/l	1.4 ×/:1.4	0.7 ×/:1.5	0.42	0.46 ×/:1.5	2.3 ×/:1.4	0.007*

PBMC isolated from different groups of Icelandic horses during summer (n= 66) were left either unstimulated or were stimulated polyclonally, with concanavalin A or with the IBH allergen. After 24 h, IL-10 was measured in the supernatants by ELISA. Statistical analysis was carried out on log-transformed data using ANOVA to examine the influence of health state (IBH or healthy), or origin (born in Iceland, i.e. 1st generation, or born in mainland Europe, i.e. 2nd generation) on the IL-10 production. Values shown represent geometric means. ×/: = Standard error of the means. * $p \leq 0.05$.

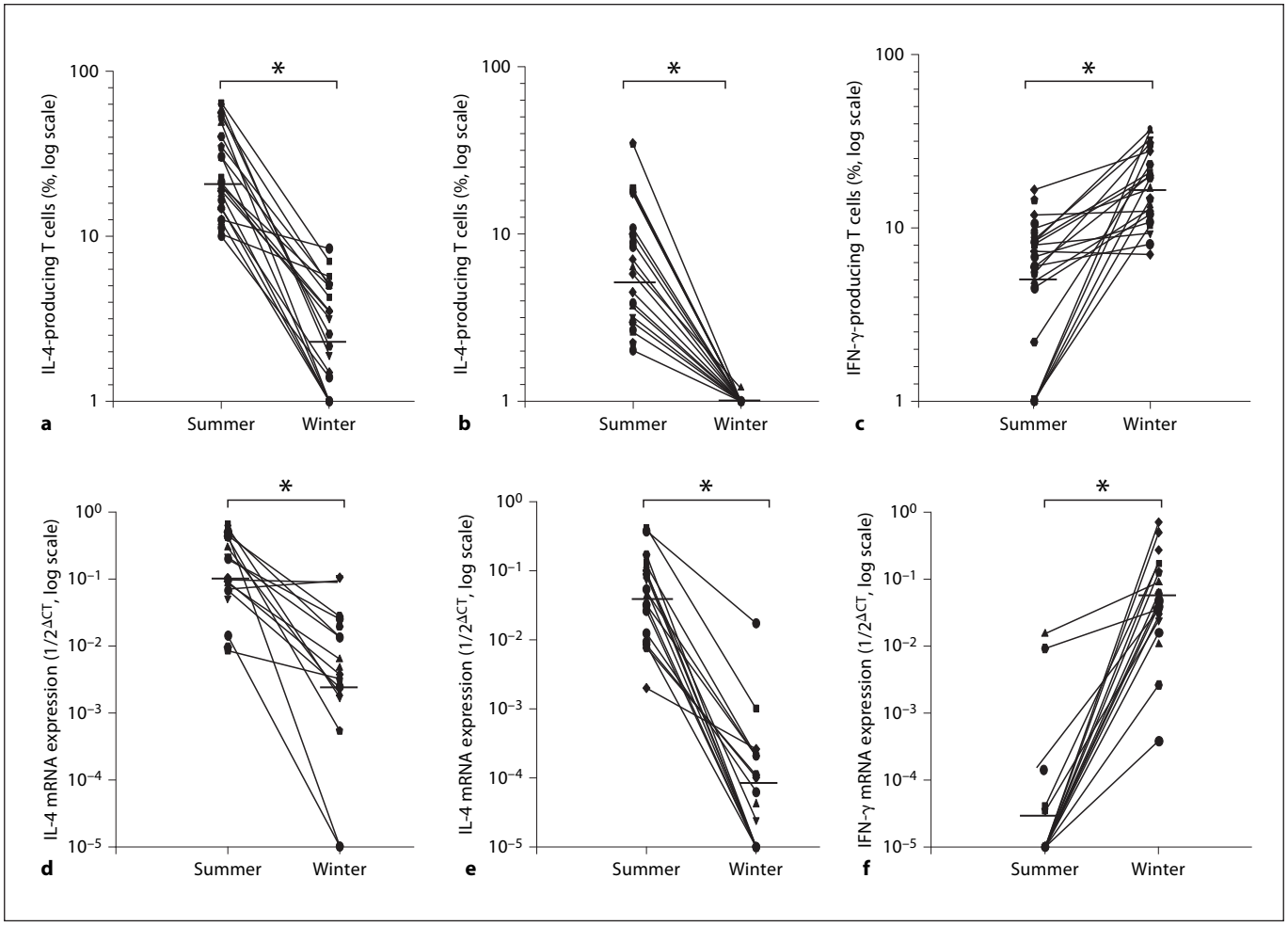


Fig. 4. Comparisons of the percentage of IL-4-producing T cells (**a**, stimulated polyclonally; **b**, stimulated by the IBH allergen) and IFN-γ-producing T cells (**c**, stimulated polyclonally) and the level of IL-4 mRNA expression (**d**, stimulated polyclonally; **e**, stimulated by the IBH allergen) and IFN-γ mRNA expression (**f**, stimulated polyclonally) between summer (i.e. during the IBH season) and winter (i.e. outside the IBH season) within the group of IBH 1st generation horses (n = 21). Horizontal bars within values denote geometric means. Statistical analysis was carried out using repeated measures ANOVA. * $p \leq 0.05$.

terestingly, there was a spontaneous production of IL-10 in supernatants of unstimulated PBMC, which was significantly higher in 2nd generation healthy animals compared to 1st generation IBH horses (fig. 5c). This supports the notion that in horses, IL-10 is involved in IL-4 suppression, although it does not prove it.

Discussion

In this study, Icelandic horses living in mainland Europe were tested for cytokines indicating either Th1 or Th2 cells. These horses differ in their disease incidence for IBH, a prototypic immediate-type allergy of the horse skin mediated by IgE antibodies [19]. Allergens are derived from bites of *Culicoides* species that are prevalent in mainland Europe, but are not found in Iceland. Horses exported to continental Europe from Iceland have a high incidence (≥50%) of contracting IBH, whereas their offspring born in mainland Europe have a significantly lower disease incidence (<10%), that is similar to that of other horse breeds. This is a naturally occurring model disease in which unknown environmental factors modulate the disease incidence. For the first time the number of Th1 and Th2 cells in healthy and IBH horses from the imported generation (1st generation), or born in mainland Europe (2nd generation), was determined. The expression of Th1 cytokines (IFN-γ) and Th2 cytokines

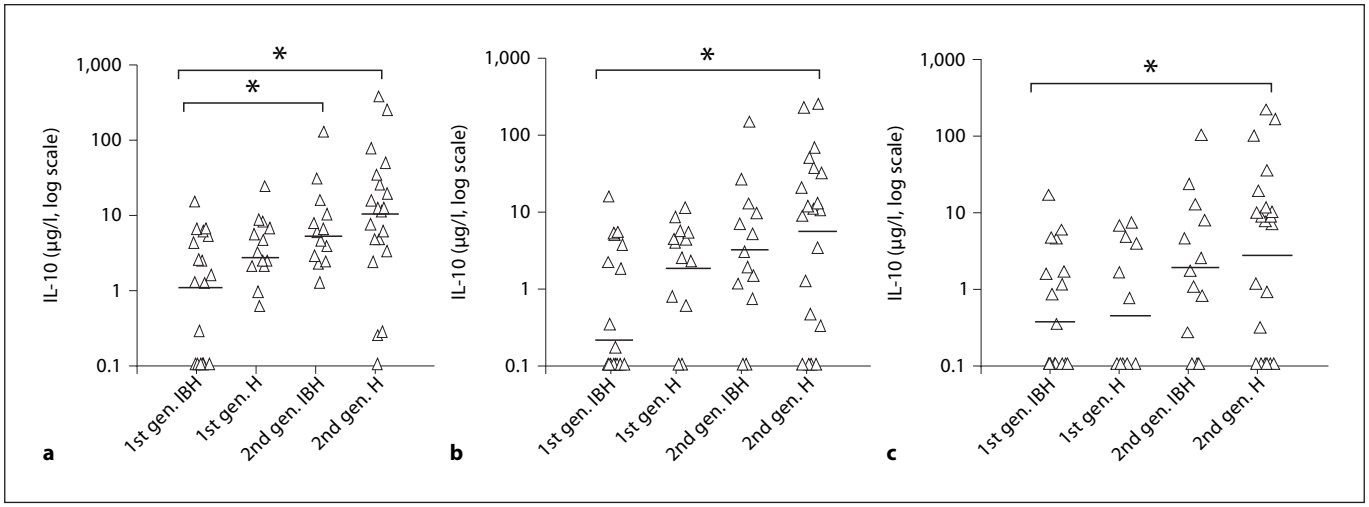


Fig. 5. Concentration of IL-10 protein measured in PBMC supernatants isolated from various groups of horses during the IBH season and cultured for 24 h in the presence of concanavalin A (**a**, polyclonal), IBH allergen (**b**) or cultured without stimulation (**c**, unstimulated). Groups of horses included 1st generation Icelandic horses suffering from IBH (1st gen. IBH; n = 19) or being healthy (1st gen. H; n = 13) and 2nd generation Icelandic horses suffering from IBH (2nd gen. IBH; n = 13) or being healthy (2nd gen. H; n = 21). Horizontal bars within values denote geometric means. One-way ANOVA was used to determine the significances of group differences. * $p \leq 0.05$.

(IL-4, IL-5 and IL-13) was assessed by QRT-PCR and/or flow cytometry. Both polyclonal and allergen-specific stimulation revealed whether there was a Th1 or a Th2 bias. Results suggest that there is a disease-associated reduction in IFN-γ and an increase in IL-4 expression in IBH horses during the IBH season, that is when the antigen was prevalent in the horses' environment. More importantly, there is an impaired IL-4 production in 2nd generation horses as opposed to 1st generation animals, regardless of the health state.

Using polyclonal stimulation does not only stimulate IBH allergen-specific CD4 T cells but is assumed to stimulate CD4 cells of any specificity. Therefore, it was expected that stimulating PBMC polyclonally would not show a bias of Th2 versus Th1 cells specifically sensitised to the IBH allergen. However, this was not the case, as polyclonal stimulation of PBMC clearly revealed disease-associated differences in both IL-4 and IFN-γ mRNA expression and protein production. One explanation is that during the IBH season, a greater number of IBH-specific precursors contributing to an IFN-γ or IL-4 response are present in the circulation. Indeed, upon stimulation with IBH allergen, an IL-4-dominated response was observed in IBH horses. Both polyclonal and antigen-specific Th2-biased responses were not observed with PBMC collected in winter. Another, although less likely, explanation is that Th1 and Th2 cells from the circulation respond by IL-4 and IFN-γ synthesis regardless of their immunological specificity. It is nevertheless remarkable that a local skin disease triggers the generation of a sufficient number of either Th1 or Th2 cells that enter the circulation.

It is noteworthy that there was both a disease-associated and origin-associated effect on T cell-derived IL-4, but no effect on IL-5 and IL-13. This result, together with others, suggests that in the model described there was no strict correlation in the production of the 3 Th2 cytokines (IL-4, IL-5 and IL-13). For example, QRT-PCR results suggest that there was a low level of constitutive IL-13, but neither IL-4 nor IL-5 mRNA expression. It is possible that disease- and origin-associated effects would also have become manifested for IL-5 and IL-13 when cells had been cultured for more than 18 h, since IL-4 is a cytokine inducing Th2 cells, whereas IL-5 and IL-13 are cytokines produced by Th2 cells once these have been formed.

The striking observation that there was an almost complete absence of IL-4 mRNA and protein expression in PBMC from healthy 2nd generation horses is open to several interpretations. The first hypothesis suggests that IL-4-generating (Th2 type) cells are absent from the circulation during the season because they are diverted from their normal circulation. The second, more likely, hypothesis suggests that IL-4 mRNA and protein synthesis is actively suppressed in polyclonally stimulated cul-

tures from these horses. What governs IL-4 production by cultured cells and what putatively suppresses IL-4 synthesis in 2nd generation horses is unknown. It is likely that a cytokine drives suppression, and the most probable candidates are IL-10 and TGF-β [20–23]. This hypothesis is compatible with our observation that IL-10 levels in PBMC cultures of 2nd generation horses were significantly higher than those of PBMC cultures of 1st generation horses. In keeping with this notion, it was reported that an increase in propensity of Th2 responses to aeroallergens in children with allergic dermatitis is associated with early impaired production of IL-10 as a regulatory cytokine [24, 25] and that IL-10 production at ≤12 months of age was observed in non-atopic children [26]. It has recently been suggested that Tr1 cells inhibit the generation of allergen-specific Th2 cells via multiple mechanisms, including the synthesis of IL-10 [11, 12]. It will be of interest to determine the source of IL-10 production in our system.

Earlier data on IBH of Icelandic horses demonstrated that 2nd generation horses have a far lower incidence of contracting IBH than 1st generation horses [9, 10], but the mechanism remains unclear. We showed that IBH is associated with reduced numbers of IFN-γ-producing cells and increased numbers of IL-4-producing cells in the circulation. Moreover, we showed that the low incidence for contracting IBH is associated with a decreased level of IL-4 mRNA expression and decreased numbers of IL-4-producing cells. Our study provides evidence that this is due to a suppression of IL-4, possibly via IL-10.

Many factors including time, route and level of exposure to an allergen may determine whether sensitisation or tolerance will ensue. It was also suggested that maternal antibodies play an important role [27]. Interestingly, the opposite of this study was observed in immediate-type hypersensitivity of dogs. Dogs sensitised intravenously early in their life developed significantly higher ovalbumin-specific serum IgE and lower IgG levels than dogs experiencing the sensitising allergen late in life [28]. That report was an experimental study of dogs, whereas we report on a naturally occurring disease in horses.

In humans, the variable incidence of immediate-type hypersensitivity is striking, but not understood. Despite the paucity of reagents characterising cytokines, cell subsets and signalling molecules in horses, this is one of the few models in which differences in incidence of immediate-type hypersensitivity can be tested in a breed that is principally atopic, displaying with high incidence a naturally occurring skin allergy. It has the advantage over human studies that the model is amenable to experimentation. Revealing the pathogenetic mechanism leading either to disease (1st generation) or to suppression/absence of disease (2nd generation) might provide novel therapeutic avenues by which to address allergy incidence.

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