Reduced incidence of insect-bite hypersensitivity in Icelandic horses is associated with a down-regulation of interleukin-4 by interleukin-10 and transforming growth factor-β1

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Abstract

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by IgE-mediated reactions to bites of insects of the genus Culicoides. IBH does not occur in Iceland due to the absence of Culicoides. However, Icelandic horses exported to mainland Europe as adults (1st generation) have a ≥50% incidence of developing IBH. In contrast, their progeny (2nd generation) has a <10% incidence of IBH. Here we show that peripheral blood mononuclear cells (PBMC) from Icelandic horses born in mainland Europe and belonging either to the IBH or healthy subgroup produce less interleukin (IL)-4 after polyclonal or allergen-specific stimulation when compared with counterparts from horses born in Iceland. We examined a role of IL-10 and transforming growth factor (TGF)-β1 in down-regulation of IL-4 in healthy 2nd generation Icelandic horses. Supernatants of PBMC from 2nd generation healthy horses down-regulated the proportion of IL-4-producing cells and IL-4 production in stimulated cultures of PBMC from 1st generation IBH. This inhibition was mimicked by a combination of IL-10 and TGF-β1 but not by the single cytokines. Cultures of stimulated PBMC of healthy 2nd generation horses produced a low level of IL-4, but IL-4 production was increased by anti-equine IL-10 and anti-human TGF-β1. This shows for the first time that in horses, IL-10 and TGF-β1 combined regulate IL-4 production in vitro. It is suggested that in this naturally occurring IgE-mediated allergy, IL-10 and TGF-β1 have a role in the down-regulation of IL-4-induced allergen-specific Th2 cells, thereby reducing the incidence of IBH.

Keywords: Icelandic horses; Insect-bite hypersensitivity; Interleukin-4; Interleukin-10; Transforming growth factor-beta; Allergy; Immediate-type hypersensitivity

1. Introduction

Equine insect-bite hypersensitivity (IBH) is an immediate-type hypersensitivity reaction to bites from insects of the genus Culicoides (midges). Earlier studies suggested that it is mediated by IgE (Hellberg et al., 2006). Both genetic and environmental factors influence the development of IBH (Marti et al., 1992; Van Grevenhof et al., 2007). Icelandic horses living in Iceland do not have IBH due to the absence of relevant insects, but acquire it at high frequency (∼50%) after being imported to mainland Europe (1st generation); in contrast, their offspring born in mainland Europe (2nd
generation) has a reduced (<10%) IBH incidence (Brostrom et al., 1987; Halldorsdottir and Larsen, 1991). 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. However, 2nd generation horses are born in an environment where the allergen for IBH (Culicoides spp.) is present, whereas their parents are exposed to bites of these insects only as adults.

A previous study showed a higher frequency of IL-4-producing cells and higher interleukin (IL)-4 expression levels in horses imported from Iceland to continental Europe when compared with their offspring born on the continent. However, there was no significant difference in the levels of IL-5 and IL-13 mRNA (Hamza et al., 2007). This is consistent with a suppression of IL-4 production in horses exposed to the allergen(s) early in life (2nd generation healthy horses). In humans, it has been shown that the balance between allergen-specific T regulatory cells (Tr)1 and Th2 cells may be decisive in the development of allergy (Akdis et al., 2004). Tr1 cells control a Th2-biased immune response through their ability to produce high levels of IL-10 and TGF-β1, which neutralizes human TGF-β1, which neutralizes IL-10 (req IL-10) and affinity-purified goat anti-equine IL-10, were from R&D Systems, London, UK. Monoclonal anti-human TGF-β1, which neutralizes human TGF-β1, was also from R&D Systems. Dose response experiments were performed for reqIL-10 and rhuTGF-β1 individually and combined. The concentrations for these cytokines were found to be optimal at 100 ng/ml reqIL-10 and 1 ng/ml rhuTGF-β1. Dose response experiments were also performed for anti-equine IL-10 and anti-human TGF-β1, resulting in optimal concentrations of 1 μg/ml. Anti-equine CD3 was kindly provided by Dr. Jeff Stott, University of California, Davis, CA, USA. Monoclonal anti-equine IL-4 was previously described (Wagner et al., 2006).

### 2.2. Subjects

A total of 78 horses of the Icelandic breed was included in the study. They fell into four groups: healthy and IBH-affected Icelandic horses imported from Iceland as adults (1st generation), and their offspring born in mainland Europe (2nd generation). All horses lived in stables in Switzerland, which most often had horses belonging to all 4 groups. In each experiment, horses from several groups and from several stables were examined, thereby minimizing any influence of a given assay day on the results. In the 1st generation horses, the mean time between import from Iceland and time when blood samples were taken was 8.6 years (3–17 years) for the healthy horses and 9.8 years (4–15 years) for the IBH-affected horses; this difference was not statistically significant. The horses were all adult animals but the 1st generation horses were significantly older (mean age of 16.6 years, range: 4–29 years) than the 2nd generation horses (mean age of 12.7 years, range: 8–24 years). This may be explained by the fact that only adult horses were imported from Iceland to Switzerland (mean age of 7.4 years at time of import, range: 4–11 years) and that we did not include horses in our study that had been imported for less than 3 years, in order to exclude horses from the 1st generation healthy group that were still healthy but would develop IBH. Imported horses usually develop IBH in the second to third summer after importation.

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 (sLT) release

### 2. Materials and methods

#### 2.1. Reagents

An allergen preparation was made from extracts of *Culicoides nubeculosus* whole bodies as described (Marti et al., 1999). Concanavalin A (ConA) was purchased from Sigma–Aldrich, St. Louis, MO, USA. Recombinant human TGF-β1 (rhuTGF-β1) was from Peprotech EC, London, UK. Mature equine TGF-β1 protein is 99% identical with mature human TGF-β1 (Penha-Goncalves et al., 1997). Recombinant equine IL-10 (req IL-10) and affinity-purified goat anti-equine IL-10, were from R&D Systems, London, UK. Monoclonal anti-human TGF-β1, which neutralizes human TGF-β1, was also from R&D Systems. Dose response experiments were performed for reqIL-10 and rhuTGF-β1 individually and combined. The concentrations for these cytokines were found to be optimal at 100 ng/ml reqIL-10 and 1 ng/ml rhuTGF-β1. Dose response experiments were also performed for anti-equine IL-10 and anti-human TGF-β1, resulting in optimal concentrations of 1 μg/ml. Anti-equine CD3 was kindly provided by Dr. Jeff Stott, University of California, Davis, CA, USA. Monoclonal anti-equine IL-4 was previously described (Wagner et al., 2006).
assay (Cellular Antigen Stimulation Test (CAST®); Bühlmann Laboratories, Schönenbuch, Switzerland) was used (Hamza et al., 2007). Almost all IBH-affected horses were treated topically against IBH in various ways such as covering with blankets and/or local application of various lotions for insect and pruritus control. Horses under treatment with corticosteroids were excluded. 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. All horses were regularly dewormed and vaccinated.

2.3. Cell culture

PBMC were isolated from healthy 2nd generation Icelandic horses \( n = 21 \) as described (Hamza et al., 2007) using a Ficoll–Hypaque procedure. Immediately thereafter, they were cultured with a set of stimuli such as Culicoides extracts (specific allergen; 10 \( \mu \)g/ml), ConA (polyclonal stimulation; 5 \( \mu \)g/ml) or were left unstimulated. After 48 h, supernatants collected from several horses of this subgroup were pooled according to the stimulation of the cultures (ConA, Culicoides allergen or none) to overcome any individual variation, and stored at \(-20^\circ\text{C}\) to be used in the assay in which IL-4 production by cultured PBMC was tested (see below).

PBMC from 1st generation IBH horses \( n = 20 \), 2nd generation IBH horses \( n = 12 \) and 1st generation healthy Icelandic horses \( n = 15 \) were isolated as above. PBMC were stimulated by Culicoides allergen, ConA or were left unstimulated. Moreover, PBMC from 1st generation IBH horses \( n = 20 \) were cultured as above but under two types of condition. In the first, they were cultured with or without pooled supernatant from healthy 2nd generation Icelandic horse PBMC precultured with their corresponding stimulus for 48 h (see above; final concentration 50%). In the 2nd, they were cultured in the presence or absence of putatively inhibitory cytokines (reqIL-10 and rhuTGF-\( \beta1 \)). Four days later, supernatants were collected for determination of IL-4 by ELISA. Cells were harvested for determination of IL-4-producing cells by flow cytometry.

In another set of experiments, PBMC were isolated from healthy 2nd generation horses \( n = 13 \). They were cultured as described above but in the presence or absence of anti-equine IL-10 and anti-human TGF-\( \beta1 \) antibodies. After 4 days of culture, IL-4-producing cells and IL-4 concentrations were determined by flow cytometry and ELISA, respectively. In some experiments, respective isotype control antibodies were included. These were goat IgG isolated on a protein-G column (Zymed Laboratories, South San Francisco, CA, USA) and mouse IgG1 purchased from Dako, Glostrup, Denmark.

All cell cultures were performed in RPMI 1640 supplemented by Glutamax (446 \( \mu \)g/ml; Invitrogen, Paisley, UK), autologous serum (10%), Penicillin (100 IU/ml), Streptomycin (100 \( \mu \)g/ml) and 2-mercapto ethanol (50 \( \mu \)M).

2.4. Flow cytometry

PBMC cultured for a total of 4 days with a set of stimuli as described received the cytokine transport inhibitor monensin (Sigma; 2 \( \mu \)M) for the last 12 h. Cells were harvested after 4 days of culture by centrifugation (250 \( \times \) g, 8 min, room temperature). To measure T cells producing IL-4, cells were first stained for equine CD3 (final dilution 1:40) at 4 \( ^\circ\text{C}\) for 30 min, followed by washing, staining with goat-anti mouse IgG1-FITC (Southern Biotechnology Inc., Birmingham, AL, USA), fixing with paraformaldehyde (final concentration 4%) and permeabilizing, using Permeabiling Solution 2 (Becton-Dickinson; final dilution 1:10) according to the directions of the manufacturer. Then, cells were stained with anti-equine IL-4 (final dilution 1:10) or an isotype control (mouse IgG1; Dako) at room temperature for 30 min, and washed. They were then stained with phycoerythrin-conjugated goat-anti-mouse IgG1 (Southern) and washed again. Thereafter, cells were analysed in a FACScan analyser (Becton-Dickinson, San Jose, CA, USA). The percentage of IL-4-positive cells within CD3-positive cells was determined after positive–negative discrimination; which was done as described (Hamza et al., 2007), using an isotype control and leaving \( \leq 1\% \) cells positively staining with the IgG1 control antibody. The buffer system used in all labelling and washing steps consisted of PBS containing EDTA (13.4 mM), gelatine (1%) and sodium azide (0.02%).

2.5. ELISA

An antibody capture ELISA for detection of equine IL-4 was carried out according to Wagner et al. (2006) using affinity-purified monoclonal anti-equine IL-4 as coating antibody (5 \( \mu \)g/ml). Recombinant equine IL-4/ IgG fusion protein (Wagner et al., 2005) was used as a standard for quantification. The detection antibody purchased from Pierce Biotechnology (Endogen Brand, Rockford, IL, USA) was a rabbit anti-equine IL-4 antiserum and was used at a 1:500 dilution. The reaction
was made visible using a peroxidase-conjugated mouse anti-rabbit IgG (anti-heavy and light chain, Jackson ImmunoResearch Inc., West Grove, PA, USA) and a 3,3’,5,5’ tetramethyl benzidine (TMB)/H2O2 substrate system (Sigma–Aldrich). The buffer used was phosphate-buffered (10 mM) saline (pH 7.4) with 1% bovine serum albumin and 0.05% Tween-20. Results were expressed in units (U) per ml, which were obtained by a calibration curve consisting of a series of 2-fold dilutions of purified recombinant equine IL-4 supernatant ranging from 1000 to 15.63 IU/ml. The limit of detection has previously been determined to be 4.57 U/ml.

2.6. Statistical analysis

Values were assessed as follows: Values below the limit of detection in either assay (flow cytometry and ELISA) were put to the limit of detection. These data were log-transformed in order to obtain a normal distribution. Values shown in Fig. 1 were analyzed by one-way ANOVA with Bonferoni correction for multiple comparisons to compare the groups. Multiple variable analysis ANOVA was 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), ‘gender’ (male or female) and ‘age’ on the IL-4 production. Data from cultures with and without supernatant, with and without cytokines and with and without antibodies were subjected to a paired t-test. P values of ≤ 0.05 were regarded as significant.

3. Results

3.1. IL-4 levels in stimulated PBMC cultures from various groups of Icelandic horses

The level of IL-4 and the proportion of IL-4-producing cells were assessed in stimulated 4 day PBMC cultures from various groups of Icelandic horses. These included both healthy and IBH horses that were born either in Iceland and imported to mainland Europe (1st generation), or that were the offspring of the latter and born in Contintental Europe (2nd generation) (Fig. 1). Both disease state and origin influenced significantly the proportion of IL-4-producing cells, as assessed by flow cytometry, thus confirming earlier observations (Hamza et al., 2007). In the case of the IL-4 level, as assessed by ELISA, the health state and the place of origin were only significant in ConA-stimulated cultures, but not Culicoides-stimulated cultures, possibly due to lack of sensitivity of the assay. In contrast, neither sex nor age influenced the proportion of IL-4-producing cells nor the IL-4 levels (data not shown).

3.2. Supernatants from healthy 2nd generation horses down-regulate IL-4 production

To investigate the mechanism of IL-4 impairment we assayed the effect of adding pooled supernatant from allergen- or ConA-stimulated PBMC from 2nd generation healthy horses to cultures of PBMC from 1st generation IBH horses. PBMC isolated from 1st generation

Fig. 1. The influence of disease status and generation on the proportion of IL-4-producing cells. PBMC from various groups of horses were stimulated either with Culicoides antigen (specific allergen; panel A) or were stimulated by ConA (polyclonal stimulation; panel B), cultured for 4 days, and cells were collected and assessed by flow cytometry for the proportion of IL-4-producing T cells by flow cytometry. Each data point (triangles) represents one PBMC culture. Horizontal bars denote geometric means. Groups of horses tested are 1st generation IBH (n = 20), 1st generation healthy (n = 15), 2nd generation IBH (n = 12), and 2nd generation healthy (n = 13). Asterisks (*) denote significant differences between the groups (p < 0.05).
IBH horses were cultured with various stimuli, and it was tested whether the above supernatants inhibit the production of IL-4 by PBMC stimulated in the same manner and cultured for 4 days in the presence or absence of added supernatants (Fig. 2). As expected, 4 day PBMC cultures of allergen-stimulated cells and ConA-stimulated cells contained more IL-4-producing cells than unstimulated cultures (Fig. 2A). Using ELISA, IL-4 could be detected only in cultures of cells stimulated by ConA and Culicoides extracts (Fig. 2B). Importantly, in each case in which a positive signal was obtained, there was a significant reduction in the proportion of IL-4-producing cells and the IL-4 level when PBMC were cultured in the presence of 2nd generation healthy PBMC culture supernatant (Fig. 2).

3.3. IL-10 and TGF-β1 combined down-regulate IL-4 production

The previous results suggested that factors down-regulating IL-4 production are present in 2 day culture supernatants from 2nd generation healthy horses and raised the question as to the factor(s) in supernatants from 2nd generation healthy horses which down-regulate IL-4 production. IL-10 was considered as a down-regulative factor, since IL-10 was shown to down-regulate IL-4 production in vitro (Chung, 2001; Oida et al., 2006). However, IL-10 given as a single agent did not down-regulate the proportion of IL-4-producing cells (Figs. 3A, 4). Another candidate for down-regulation is TGF-β1 (Bridoux et al., 1997; Heath et al., 2000; Kunzmann et al., 2003). However, TGF-β1 had a non-significant effect on the proportion of IL-4-producing cells (Figs. 3B, 4). Pilot experiments suggested that even by increasing the concentration of either IL-10 or TGF-β1, IL-4 production was not decreased (Fig. 3). However, a combination of IL-10 and TGF-β1 showed a significant down-regulation of IL-4 (Figs. 3C, 4). This was further substantiated in a larger number of horses. PBMC from 1st generation IBH horses were cultured under various conditions in the presence or absence of reIL-10 and rhuTGF-β1 combined (Fig. 5 and supplementary Fig. S1 displayed online). The proportion of IL-4-producing cells was found highest or lowest in PBMC that had been ConA-stimulated or were left unstimulated, respectively. The PBMC stimulated with Culicoides-derived allergen took an intermediate position (Fig. 5A). Using ELISA, IL-4 production was only obtained if PBMC were stimulated by ConA, or by Culicoides-derived allergen (Fig. 5B). Importantly, in each case in which a strong positive signal for IL-4-producing cells or IL-4 levels was obtained, it could be significantly reduced or even totally abrogated by reIL-10 and rhuTGF-β1 combined (Fig. 5).

3.4. Anti-IL-10 and anti-TGF-β1 combined abrogate the IL-4 down-regulation in PBMC cultures from 2nd generation healthy horses

The combined role of IL-10 and TGF-β1 in down-regulating IL-4 was confirmed in experiments with IL-
10-specific and TGF-β1-specific antibodies added to stimulated PBMC cultures of healthy 2nd generation horses (Fig. 6). As expected, no IL-4 producing cells were observed when these cells were stimulated with Culicoides-derived allergen, and a low proportion of IL-4-producing cells was seen when PBMC were stimulated with ConA (Fig. 6A). Using ELISA, only a low level of IL-4 was measured when PBMC had been stimulated by ConA (Fig. 6B). However, the addition of antibodies significantly raised the proportion of IL-4-producing cells when PBMC were stimulated by Culicoides-derived allergen or by ConA, and a significant rise in IL-4 levels was noted in supernatants of these PBMC stimulated by ConA (Fig. 6).

To verify that it is the specific antibodies that increase IL-4 production, PBMC from 2nd generation healthy horses were stimulated by ConA in the presence or absence of anti-IL-10 and anti-TGF-β1 combined or of isotype control antibodies, and cells were analysed 4 days later by flow cytometry for IL-4-producing cells. Whereas the anti-IL-10 and anti-TGF-β1 antisera increased the proportion of IL-4-producing cells, isotype control preparations had no effect whatsoever (supplementary figure S2 shown online). This confirms that IL-10 and TGF-β1 specifically control the level of IL-4 in 2nd generation healthy horses.

4. Discussion

It is now established that genetic factors and environmental allergen influence the incidence of allergies. It also became clear that the incidence of
allergy is influenced by non-allergen-derived environmental factors (Piccinni et al., 2000; Von Ehrenstein et al., 2000). These, however, are unknown. IBH in Icelandic horses represents a natural allergic disease in which the incidence can be manipulated. For example, horses born in Iceland and imported from Iceland to mainland Europe have a high disease incidence (≥50%). However, their offspring born in mainland Europe has a significantly lower (<10%) disease incidence although derived from the same genetic pool. We report here and in a previous paper (Hamza et al., 2007) that this low disease incidence is associated with a reduced level of IL-4-producing PBMC upon polyclonal or allergen-specific stimulation. In the present study we showed that 48 h culture supernatants from 2nd generation healthy horses down-regulate the IL-4 production by 1st generation IBH horse PBMC stimulated either with allergen or mitogen. A similar effect was also observed in 1st generation healthy and 2nd generation IBH horses (Hamza et al., unpublished).
The mechanism of IL-4 down-regulation was further investigated. As studies performed in other species and other disease models suggested an involvement of IL-10 and TGF-β1 as potentially down-regulative factors (Taylor et al., 2006), the effect of these cytokines on IL-4 down-regulation was examined. Our experiments showed that culture of PBMC from 1st generation IBH horses with both reqIL-10 and rhuTGF-β1 reduce the level of IL-4 production induced by IBH allergen and ConA. In ConA-stimulated cultures of PBMC of 1st generation healthy and 2nd generation IBH-affected horses, the addition of reqIL-10 and rhuTGF-β1 resulted in a significant reduction of IL-4 production (Hamza et al., unpublished). This is the first report showing that the production of IL-4 by stimulated equine PBMC is significantly down-regulated by IL-10 and TGF-β1 combined but not by the single agents.

Evidence that IL-10 and TGF-β1 combined are indeed down-regulating IL-4 production in cultures of 2nd generation healthy horses was obtained from antibody inhibition experiments. Neutralization of both IL-10 and TGF-β1 substantially reduced the regulatory effect in these cultures. It may be speculated that by this mechanism, the generation of Th2 cells is reduced, and thus the cells promoting the development of B cells into IgE-producing plasma cells is impaired.

It is established that IL-4 levels are regulated by IFN-γ. It is, however, unlikely that IFN-γ is down-regulating IL-4 production in PBMC from 2nd generation healthy horses. IFN-γ-producing cells could not be detected in PBMC from this subgroup stimulated with Culicoides allergen, whereas IL-10 could be readily detected in supernatants from these cultures (Hamza et al., 2007). Neutralization of IL-10 and TGF-β1 in polyclonally stimulated cultures increased IL-4 levels significantly but this treatment did not significantly influence the proportion of IFN-γ-producing cells (Hamza, unpublished observation).

Previous experiments (Hamza et al., 2007) and this report show that in 2nd generation horses significantly less IL-4-producing cells are observed than in 1st generation counterparts. This raises the question as to whether there is down-regulation of IL-4 production or a lack of response of PBMC from 2nd generation horses. The data reported here is the first evidence of a suppression of IL-4 production and IL-4-producing cells rather than a lack of response, since supernatants from PBMC of 2nd generation healthy horses down-regulated IL-4 production, and this down-regulation could be abrogated by treatment with IL-10-specific and TGF-β1-specific antibodies.

We have determined in parallel IL-4-producing cells by flow cytometry and IL-4 production by antibody capture ELISA. Taken together, these two assays show the same picture although in ELISA, IL-4 levels were more often below the detection limit of the assay than in flow cytometric determinations. As the same IL-4-specific antibody was used in both assays, this suggests
that the determination of IL-4-producing cells by flow cytometry is more sensitive than the IL-4 ELISA. This is consistent with a recent report (Burns et al., 2000).

The results shown here suggest that an unusually high proportion of T cells produce IL-4. This observation was characteristic for IBH Icelandic horses, and even for healthy 1st generation horses. It was not observed in 2nd generation healthy horses, for which we suggest suppression of IL-4 upon allergen-specific or polyclonal stimulation of their PBMC. Notably, 2nd generation horses have a disease incidence not differing from that of other breeds (Wilson et al., 2006). Genetic factors or environmental factors related to their birth place may predispose Icelandic horses to high-levels of IL-4 production when imported to the continent later in life.

Our results suggest that both agents IL-10 and TGF-β1 are required to significantly down-regulate IL-4. Cooperation of IL-10 and TGF-β1 has been shown to induce T-cell down-regulation during allergen-specific immunotherapy (SIT) and in the control of healthy immune response to allergen (Akdís and Blaser, 2003; Akdis et al., 2006; Verhagen et al., 2006). For example, in the mucosa of normal donors both IL-10 and TGF-β1 are assumed to contribute to the down-regulation of Th2-mediated allergy to common allergens as neutralization of both IL-10 and TGF-β1 was required to reach a statistically significant increase in Th2 cytokine production (Jutel et al., 2003). These cytokines were reported to have different roles in the down-regulation of IL-4 (Joss et al., 2000; Oida et al., 2006). TGF-β1 has been shown to play a pivotal role in the differentiation of Foxp3-expressing regulatory T cells (Chen et al., 2004; Fantini et al., 2004; Heath et al., 2000) which then produce both IL-10 and TGF-β (Cottrez and Groux, 2001; Kitani et al., 2003) which down-regulate IgE production: these cytokines also induce the production of the non-inflammatory immunoglobulin isotypes IgG4 and IgA, respectively (Akdís et al., 1998; Punnonen et al., 1993; van Vlasselaer et al., 1992). Furthermore, they directly down-regulate allergic inflammation induced by effector cells such as mast cells, basophils and eosinophils (Taylor et al., 2006). Although this has not been addressed in this study, this is consistent with the hypothesis that these cytokines also operate in vivo, thereby reducing the Th2-inducing power of IL-4 which in turn leads to control of an allergic reaction.

Although both cytokines appear to be necessary, the exact mechanism how they influence IL-4 production has not been addressed. It is noteworthy that TGF-β1 exists in two stages, inactive and active, and that the culture of cells in autologous serum precludes exact determination of the levels of active TGF-β1, due to the contribution of platelet-derived TGF-β1 to the level of inactive TGF-β1 in horse serum (Kropf et al., 1997). Interestingly, IL-10 levels were significantly higher in the 2nd generation healthy group than in the 1st generation IBH group, a result consistent with our suggestion. Even unstimulated cells from 2nd generation healthy horses contributed to IL-10 levels (Hamza et al., 2007) which was in line with a regulatory effect of these supernatants (Fig. 2).

Our findings propose that in culture, a polyclonal stimulation of PBMC yields similar results as does allergen-specific stimulation of PBMC from IBH horses. This suggests that within PBMC, allergenspecific cells are also expanded. It may be noted that these experiments were performed during summer, i.e. when the disease was manifest. It may be speculated that during this period, a larger proportion of allergen-specific cells are in the circulation of IBH horses than in healthy 1st generation horses.

In a previous study we reported that IBH is associated with a significantly higher IL-4 level in response to IBH allergen in IBH 1st as compared to IBH 2nd and healthy 1st generations horses (Hamza et al., 2007). The present study shows that IL-10 and TGF-β1 reduce this difference between IBH and healthy horses, suggesting that IL-10- and TGF-β1 regulation are also responsible for the differences in the levels of IL-4 between IBH and healthy horses.

Anti-IL-10 and anti-TGF-β1 increase IL-4 production, i.e. they abrogate the putatively down-regulative effect seen in 2nd generation healthy horses. These inhibition experiments did not include a specificity control, e.g. equal isotypes from non-immune donors in all experiments. However, two lines of evidence support the hypothesis that the specific antibodies rather than non-specific factors in these preparations are responsible for abrogation of IL-4 down-regulation. (i) Antibodies against a single cytokine had no effect and (ii) in ConA-stimulated cultures, the combination of anti-IL-10 and anti-TGF-β1 significantly increased the proportion of IL-4-producing cells, whereas isotype control antibodies did not.

In this study, the source of IL-10 and TGF-β1 has not been addressed. Studies aimed at investigating whether regulatory T cells are present in cultures from PBMC of the 4 groups, e.g. by the study of appropriate markers such as CD25, FoxP3, CTLA-4, PD-1 and GITR are in progress.

This report also leaves open whether in addition to IL-10 and TGF-β1 other factors present in supernatants
may contribute to the down-regulation of IL-4 levels. Nevertheless, we suggest that the combination of IL-10 and TGF-β1 contribute to controlling IL-4 levels. Thereby they control the generation of Th2 cells leading to manifest disease. This natural IgE-mediated disease might be a model to better understand modulation of incidence of allergies unrelated to genetics or environment-derived allergen.

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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.2007.10.018.

References


