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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

Mareike Heimann[a,1], Jozef Janda[b,c,1], Olöf G. Sigurdardottir[a], Vilhjalmur Svansson[a], Jolanta Klukowska[b], Claudia von Tscharner[c,d], Marcus Doherr[b], Hans Broström[e], Lisa S. Andersson[f], Sigurjón Einarsson[e], Eliane Marti[b,c,*], Sigurbjörg Torsteinsdottir[a]

[a] Institute for Experimental Pathology, University of Iceland, Keldur, v/Vesturlandsveg, 112 Reykjavík, Iceland
[b] Department of Clinical Research and Veterinary Public Health, University of Berne, 3001 Berne, Switzerland
[c] Dermfocus Vetsuisse Faculty, University of Berne, 3001 Berne, Switzerland
[d] Institute of Animal Pathology, Vetsuisse Faculty, University of Berne, 3001 Berne, Switzerland
[e] Department of Clinical Sciences, Swedish University of Agricultural Sciences, 75007 Uppsala, Sweden
[f] Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, 75124 Uppsala, Sweden

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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 β1, 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 ($p = 0.06$)

* Corresponding author at: Department for Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Länggass-str 124, 3001 Bern, Switzerland. Tel.: +41 31 631 23 30; fax: +41 31 631 26 30.
E-mail address: eliane.marti@itz.unibe.ch (E. Marti).
[1] Equal contribution.

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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.

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 (Hrafnsdottir, 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 micromilieu 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-β1 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-