

Toll-like receptor-ligand induced thymic stromal lymphopoietin expression in primary equine keratinocytes

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ABSTRACT

Background – Thymic stromal lymphopoietin (TSLP) plays a key role in the development of allergic inflammation. Little is known about possible triggers of equine TSLP expression.

Hypothesis/objectives – To investigate TSLP expression in equine insect bite hypersensitivity (IBH) skin lesions. The capacity of TLR 1-8 ligands (L) and of atopic cytokine milieu as potential triggers of TSLP and of interleukin (IL)-6 as a downstream effector molecule of TLR signalling, were examined in primary equine keratinocyte cultures.

Animals – Lesional skin from IBH-affected and healthy skin from control-horses (n = 9 each group) was sampled.

Methods and materials – Keratinocyte cultures were established from six healthy horses and stimulated with TLR 1-8-L, and with IL-4 and tumor necrosis factor- α , to mimic an atopic inflammation cytokine milieu. TSLP and IL-6 gene expression was assessed by quantitative real-time PCR.

Results – Expression of TSLP was significantly greater in IBH lesions compared to healthy skin. TLR 1-8-L significantly upregulated TSLP expression in keratinocytes. The strongest upregulation was induced by TLR 1/2-L and TLR 3-L. Combination of atopic cytokine milieu and TLR 1/2-L or TLR 3-L further increased TSLP expression. TLR-L 1-5 stimulation significantly upregulated IL-6 expression.

Conclusions and clinical importance – The data herein suggest that the upregulation of TSLP expression in lesional skin of IBH-affected horses might play a role in IBH development. Moreover, TSLP expression is induced by TLR-L, in particular by TLR 1/2-L and TLR 3-L, and is further increased by atopic cytokine milieu, indicating a mechanism for TSLP-mediated exacerbation of IBH.

Introduction

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7 like cytokine that plays a central role in TH-2 cell differentiation and development of allergic inflammation.¹ Many cell types produce TSLP including epithelial, mast cells and dendritic cells (DCs), as well as cancer and cancer-associated cells.²

TSLP-treated DCs induce a strong proliferation of naïve T cells. In addition, under the influence of TSLP, DCs secrete TH2-recruiting chemokines, and induce naïve T-cell differentiation into “inflammatory” TH-2 cells, which in turn produce IL-4, IL-5 and IL-13, and tumor necrosis factor (TNF)- α through the OX40 ligand.³ Moreover, TSLP does not induce the expression of IL-12 or type I

interferons, which would drive the induction of TH-1 differentiation,⁴ thus further skewing the immune response towards a TH-2 phenotype. Furthermore, TSLP activates Langerhans cells.⁵ In combination with IL-1 and TNF- α , TSLP can act directly on human mast cells that in turn produce IL-5 and IL-13. It also can act on human eosinophils inducing their prolonged survival and secretion of pro-inflammatory molecules.⁶

TSLP is highly expressed in lesional keratinocytes of humans affected by atopic dermatitis (AD), where it plays a central role in triggering allergic diseases.⁴ Keratinocytes are the predominant cell type in the epidermis, forming the physical barrier to the external environment.⁷ Besides this role, keratinocytes also produce several cytokines and chemokines that ultimately shape the immune response. For example, in response to external stimuli, human keratinocytes produce cytokines such as IL-25, IL-33 and TSLP.⁷ The production of TSLP by keratinocytes, together with its functional ability to skew the immune response towards TH-2 subtype, makes TSLP a critical factor linking responses at the interface between

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the body and the external environment to TH-2 phenotype.⁸

Information on the environmental and endogenous triggers of TSLP expression, which is essential to elucidate the underlying mechanisms of allergic diseases, is still limited. Bogiatzi *et al.* have shown that pro-inflammatory and TH-2 cytokines act synergistically to induce TSLP expression in skin explants from healthy donors.⁹

In horses, keratinocytes and TSLP have not been studied extensively and little is known about their possible role in the development of allergic diseases such as insect bite hypersensitivity (IBH). IBH is an IgE-mediated, type I hypersensitivity caused by bites of insects in the genus *Culicoides*, and clinically is seen as a recurrent dermatitis in horses.¹⁰ Although the possible role of TSLP in IBH development is unknown, one study reported the generation of equine TSLP-specific antibodies and showed that TSLP production was induced in equine primary keratinocytes by stimulation with whole-body extract of *Culicoides* midges, whose salivary gland proteins are causative allergens of equine IBH.¹¹ However, keratinocytes from only one healthy horse was used in that study. Another study reported TSLP upregulation in bronchial alveolar lavage cells derived from horses affected by equine asthma (RAO) and in bronchial epithelial cells (BAC) after stimulation with hay dust extract *in vitro*.¹² Possible endogenous or exogenous triggers of equine TSLP expression in homeostasis or disease state remain to be elucidated.

Based on the demonstration that TSLP is the linking factor between the environmental stimuli and the type of immune response, it is hypothesized herein that the expression of equine TSLP is upregulated in primary equine keratinocytes by Toll-like receptor (TLR) ligands (TLR-L). TLRs are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) on various microbes and therefore play an essential function in innate immunity.¹³ Binding of ligands to TLRs leads to the activation of several signalling pathways, including NF- κ B and MAP (mitogen-activated protein) kinases that in turn induce the expression of various genes involved in host defence, such as IL-6 and IL-1, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules. In one study, equine keratinocytes responded to stimulation with LPS (TLR-4 ligand) and flagellin (TLR-5 ligand) by increased expression of inflammatory cytokine genes, suggesting that equine keratinocytes express functional TLR-4 and TLR-5.¹⁴

The primary aim of this study was to investigate whether equine TSLP is differentially expressed in lesional skin of IBH-affected horses compared to the skin of healthy horses. A further aim was to examine in primary equine keratinocytes the capacity of TLR 1-8-L to induce the expression of TSLP and the pro-inflammatory cytokine IL-6, as a downstream effector molecule of TLR signalling. For comparison, the TLR-L-induced TSLP and IL-6 expression in peripheral blood mononuclear cells were examined. Finally, it was investigated whether allergic inflammation cytokine milieu (IL-4 and TNF- α) induces TSLP expression in keratinocytes from nonallergic horses and acts synergistically with TLR-1/2 and TLR-3-induced TSLP expression.

Methods and materials

Sample collection

This study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (nos BE 2/17 and BE 69/18).

Nine skin biopsies from IBH-affected and nine skin samples from control horses were taken from the neck or midline area. Biopsies were collected from slaughtered horses or from IBH-affected horses. Subsequently, the biopsies were bisected and submerged in RNAlater (Thermo Fisher Scientific; Waltham, MA, USA) and stored at -80°C until further processing. Blood for isolation of peripheral blood mononuclear cells (PBMCs) was collected from three nonallergic horses.

Isolation and culture of primary equine keratinocytes

Primary equine keratinocytes were isolated from skin samples obtained from six slaughtered horses with no apparent skin disease. The cells were cultured using a modification of protocols described previously.^{11,15} Briefly, skin samples were incubated at 4°C for 24 h with 10 mg/mL Dispase II (Roche; Basel, Switzerland) in Williams E medium (Bioconcept; Allschwil, Switzerland). Subsequently, epidermis was separated from the dermis using sterile forceps and further digested for 20 min in accutase (CELLnTEC; Bern, Switzerland) in order to produce single-cell suspensions. Once acquired, keratinocytes were seeded at 12×10^3 cells per cm^2 of cell culture flask and grown in Williams E medium supplemented with 10% fetal bovine serum (Merck; Kenilworth, NJ, USA), 10 ng/mL human epidermal growth factor (Sigma Aldrich; St Louis, MO, USA), 2 mM glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin and amphotericin B (Bioconcept). Cells were grown in 75 cm^2 flasks at a density of 9×10^5 cells per flask and grown at 35°C , 5% CO_2 until they reached 90% confluence. Cells from passage three were used for stimulation experiments.

Isolation of peripheral blood mononuclear cells

Blood from three nonallergic horses was collected from the jugular vein using sodium heparin-containing vacutainers (Vacuette, Greiner; St Gallen, Switzerland). Blood was transferred immediately to the laboratory where PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque in accordance with Hamza *et al.*¹⁶

Immunofluorescence

In order to confirm the presence of primary equine keratinocytes in the cultures, cells were grown in chambered cell culture slides (Sarstedt; Nümbrecht, Germany) until they reached 70% confluence. Subsequently, cells were fixed using 4% paraformaldehyde (Thermo Fisher Scientific) for 20 min at room temperature. Cells then were permeabilized using 0.2% Triton (Biorad; Hercules, CA, USA) and blocked in 10% normal goat serum (AbCam; Cambridge, UK). They were incubated overnight at 4°C with the following primary antibodies: polyclonal rabbit anti-bovine cytokeratin (Agilent; Santa Clara, CA, USA) and mouse monoclonal anti-human vimentin (Agilent) at 4°C . Washing with 0.025% Tween 20 (Sigma Aldrich) and 45 min incubation at room temperature with the corresponding secondary antibodies, Alexa Flour 647-labelled goat anti-rabbit (Jackson ImmunoResearch; West Grove, PA, USA) and Alexa Flour 647-labelled goat-anti mouse (Invitrogen) followed. Nuclei were counterstained with Hoechst (Sigma Aldrich). Cells were imaged using Zeiss AxioImager Z1 (Carl Zeiss AG; Oberkochen, Germany). The staining was carried out for all keratinocyte cultures used in this study.

Stimulation of keratinocytes and PBMCs

Primary equine keratinocytes of passage three were seeded in six well plates at a density of 3×10^5 cells per well and grown at 35°C , 5% CO_2 until they reached 90% confluence. For stimulation experiments, cells were cultured in complete Williams E medium.

After isolation, equine PBMCs were cultured in 12 well plates at a density of 8×10^6 cells per well in RPMI medium.

Primary keratinocytes and PBMCs were cultured in medium only or with various TLR-L (as summarized in Table 1) for 4 h and 24 h.

Furthermore, a combination of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) and 1 µmol/mL ionomycin (Sigma Aldrich) or 5 µg/mL Concanavalin A (Sigma Aldrich) were used as positive controls. Additionally, a combination of 100 ng/mL recombinant equine TNF-α (R&D Systems; Minneapolis, MN, USA) and 100 ng/mL recombinant equine IL-4 (LubioScience; Zürich, Switzerland) was added to primary equine keratinocytes cultured in the presence or absence of Pam3CSK4 and Poly (I:C).

Isolation of RNA and cDNA synthesis

Total RNA was isolated from cultured keratinocytes using ReliaPrep RNA Cell miniprep system (Promega; Madison, WI, USA) according to the manufacturer's instructions. Total RNA was extracted from cultured PBMCs using the RNeasy Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. Before RNA extraction with the RNeasy Mini Kit, cell lysates were loaded onto a spin column (QIAshredder; Qiagen) and centrifuged at 16,000g for 2 min. Total RNA from lesional skin of IBH-affected (n = 9) and healthy horses (n = 9) was extracted using the RNeasy Fibrous Tissue Kit (Qiagen).¹⁷ Before RNA extraction, skin samples were lysed using MagNa tissue lyser (Roche) in which samples were homogenized by collision with ceramic beads (Roche) for 45 s at the shaking speed of 6,500, followed by 2 min cooling on ice. Homogenization was repeated for another 30 s at the shaking speed of 6,500 and supernatants were loaded onto a spin column (QIAshredder, Qiagen) and centrifuged at 16,000g for 2 min (Qiagen).

For all samples, contaminating genomic DNA was removed by on-column DNase treatment, and RNA was quantified spectrophotometrically at 260 nm (NanoDrop 2000c, Thermo Fischer Scientific; Reinach, Switzerland) and samples were stored at -80°C until use.

Reverse transcription (RT) of total RNA was performed using GoScript Reverse Transcription Mix (Promega) to generate complementary DNA (cDNA) that was used as a template for quantitative (q) RT-PCR. The cDNA synthesis reaction consisted of 500 ng RNA and 10 µL GoScript Reverse Transcription Mix, which contained 4 µL nuclease-free water, 4 µL GoScript Reaction Buffer and Random Primer, and 2 µL GoScript Enzyme Mix, in a total reaction volume of 20 µL. RNA samples and the RT mix were incubated at 25°C for 5 min. Reaction continued at 42°C for 1 h and was terminated by heating at 70°C for 15 min. The cDNA samples were stored at -20°C until further processing.

qRT-PCR of primary keratinocytes, PBMCs and skin

Expression of TSLP, IL-6 and the housekeeping gene *ubiquitin B* (*UBB*) was quantified using qRT-PCR. TSLP and UBB primer sequences were applied as previously published and IL-6 primers were designed using the PRIMER EXPRESS 3.0.1. program and synthesized by Microsynth (Balgach, Switzerland) (Sequences in Table 2).¹⁷ The qRT-PCR mixes consisted of: 1 µL cDNA per reaction, GoTaq Master Mix 2x (Promega), CXR reference dye 100x (Promega), and 100 µM primers. The qRT-PCR reactions were performed in a total volume of 10 µL and carried out in a 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). The UBB and TSLP qRT-PCR reaction consisted of initial 15 min at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C, elongation for 30 s at 72°C, and a melting curve program of 95°C for 15 s, followed by 30 s at 60°C and 15 s at 95°C.

The IL-6 qRT-PCR reactions consisted of an initial 2 min at 50°C and denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C, annealing at 60°C for 1 min, and the same melting curve program as for the UBB and TSLP qRT-PCR reaction. All reactions were performed in triplicate, and no template and RT-negative controls were included in each run. Efficiencies of qRT-PCRs were calculated using a standard curve derived from pool of cDNA (Table 2). TSLP and IL-6 gene expression in primary equine keratinocytes and PBMCs was quantified using the $\Delta\Delta C_t$ method. Expression of TSLP and IL-6 was normalized to the expression of *UBB* and to the nonstimulated control.

In six of nine skin samples from control horses, and two of nine samples from IBH-affected horses, TSLP expression was below the detection limit of the assay. A $C_t = 40$ was attributed to these samples to allow their inclusion in the statistical analysis. TSLP expression in skin samples was quantified using the $1/2^{\Delta C_t}$ method, as described in Hamza et al.; $1/2^{\Delta C_t}$ expresses the relative quantity of TSLP mRNA present in the sample compared to the amount of UBB in the sample.¹⁶

Statistical analyses

For statistical analysis, NCCS software [NCSS 12 Statistical Software (2018) NCCS, LLC; Kaysville, UT, USA, ncss.com/software/ncss] was used. Because the data were not normally distributed, descriptive statistics using median and ranges were used. Comparison of TSLP mRNA expression in lesional IBH skin to its expression in skin from healthy horses was performed using the Wilcoxon–Mann–Whitney U-test, as was comparison of cytokine expression between TLR-ligand stimulated and unstimulated keratinocytes. Bonferroni correction for multiple comparisons (cP) was performed manually (P -value \times number of comparisons = cP). On each occasion, $P \leq 0.05$ was regarded as significant.

In order to compare the effect of addition of inflammatory cytokine milieu on keratinocyte cultures within each culture conditions (i.e. within each TLR-ligand stimulation or without addition of TLR-ligand), a paired Wilcoxon signed rank test was performed.

Results

TSLP expression in lesional skin of IBH-affected horses

The mRNA expression of TSLP was significantly greater (median = 0.0114, range 0.0006–0.0580, $P < 0.05$) in lesional skin of IBH-affected horses compared to the skin of nonaffected controls (0.0009, 0.0005–0.0203) (Figure 1).

Establishment of primary equine keratinocyte cultures

Primary equine keratinocytes were morphologically distinctive in culture as cobble stone-shaped cells with ovoid nucleus and indistinct borders.¹⁵ Morphologically, all of the cultures herein corresponded to the description of keratinocytes. In addition, staining of the primary keratinocyte cultures with anti-cytokeratin confirmed that all of the cultures consisted of keratinocytes (Figure 2a).

Table 1. Toll like receptor ligands used in the study

TLR	Name	Origin	Concentration
TLR 1/2	Pam3CSK4	Synthetic triacylated lipoprotein ¹	200 ng/ml
TLR 3	Poly (I:C)	Synthetic analogue of dsRNA ¹	5 µg/ml
TLR 4	LPS	Lipopolysaccharide from <i>Escherichia coli</i> ¹	5 µg/ml
TLR 5	Flagellin	Flagellin from <i>Salmonella Typhimurium</i> ¹	200 ng/ml
TLR 7/TLR 8	Resiquimod	Imidazolquinolinamines ²	5 µg/ml
TLR 9	CpG-ODN (D-SL03)	Unmethylated CpG dinucleotides ¹	5 µg/ml

¹Invivogen, San Diego, CA, USA.

²Sigma Aldrich, St. Louis, MO, USA.

Table 2. Sequences of primers used for gene expression quantification by quantitative reverse transcription (qRT)-PCR

Gene	Primer	Sequence (5'-3')	R ² value
TSLP	Forward primer	CCACAGTAGACAGTGTGGTTGAA	0.9854
	Reverse primer	TAGGGCTGGTGTGCTGACCTAC	
IL-6	Forward primer	TTCACAGAATGAGTGGCTGAAGA	0.9994
	Reverse primer	GGCTGAACTGCAGGAAATCC	
UBB (ubiquitin B, housekeeping gene)	Forward primer	ATGTGAAGCCAAGATCCAG	0.9836
	Reverse primer	AAATCTGCATCCCACCTCTTG	

R² (coefficient of determination) values range from 0 to 1, where 1 indicates a total fit of the primer and 0 indicates the lowest fit.

Moreover, no staining was observed with the anti-vimentin antibody, indicating that the cultures were not contaminated with fibroblasts, which are common in keratinocyte cultures (Figure 2b). When anti-vimentin antibody was used on equine fibroblasts as control staining, positive staining was observed, confirming antibody reactivity on equine fibroblasts (Figure 2c,d).

TLR ligands and TSLP gene expression in primary equine keratinocyte cultures

It was then investigated whether TLR-1/2, 3, 4, 5, 7 and 8 ligands induce upregulation of TSLP gene expression in primary equine keratinocyte cultures and, for comparison, in PBMCs, after 4 h and 24 h stimulation. TLR-L 9 (CpG-ODN, D-SL03, InvivoGen; San Diego, CA, USA) was used in pilot experiments on both primary equine keratinocyte cultures and PBMCs, but as it did not elicit any TSLP response, it was not included in the main experiments.

A significant upregulation of TSLP gene expression was observed in primary equine keratinocytes after stimulation for 4 h with phorbol 12-myristate 13-acetate (PMA)-ionomycin as positive control, and all TLR-Ls used in the present study (Figure 3a). The strongest TSLP gene upregulation was induced by PMA-ionomycin (median = 1321.35) and Pam3CSK4 (231.72), followed by LPS (8.87), Poly (I:C) (4.98), resiquimod (5.61) and flagellin (3.03). Interestingly, Pam3CSK4, the ligand that induced the strongest upregulation of TSLP in keratinocytes (median = 231.72), only induced a moderate increase of TSLP expression in PBMC (34.9). Interestingly, PBMC displayed a stronger TSLP expression compared to keratinocytes after stimulation with LPS (median = 103.63) and flagellin (111.27) (see Figure S1a,b in Supporting Information).

After 24 h stimulation of keratinocytes (Figure 3b), the effect of some stimuli decreased and there was no significant difference for flagellin and resiquimod compared to unstimulated cells. TSLP was still significantly upregulated after 24 h by PMA-ionomycin, Pam3CSK4, Poly (I:C) and LPS. The strongest upregulation of TSLP after 24 h stimulation was induced by Poly (I:C) (median = 53.13). PMA-ionomycin (12.68) and Pam3CSK4 (4.25) induced a significant upregulation of TSLP as well, but to a lower extent than after 4 h, whereas TSLP expression induced by Poly (I:C) was 10-fold higher after 24 h compared to 4 h stimulation (Figure 3b). Medians of both TSLP and IL-6 expression values for all stimulation conditions are shown in Table S1.

TLR ligands and IL-6 gene expression in primary equine keratinocytes and PBMCs

In a similar way to TSLP expression, the expression of the pro-inflammatory cytokine IL-6 was significantly

upregulated in primary equine keratinocytes after 4 h stimulation with PMA-ionomycin, Pam3CSK4, Poly (I:C), LPS and flagellin. However, in contrast to TSLP expression, stimulation with resiquimod did not induce a significant IL-6 increased expression. After 4 h stimulation (Figure 3c), the strongest upregulation of IL-6 in equine keratinocytes was induced with Pam3CSK4 (median = 42.00), followed by LPS (13.27), PMA-ionomycin (7.84) and Poly (I:C) (7.06). After 24 h stimulation (Figure 3b), similar to TLR-L-induced TSLP expression, the effect of some TLR-L on IL-6 upregulation decreased. A significant upregulation of IL-6 after 24 h was induced only by Poly (I:C) and LPS stimulation. As for TSLP, the strongest IL-6 response was observed after stimulation with Poly (I:C) (median = 54.94) (Figure 3d).

In comparison to keratinocytes, IL-6 was upregulated in PBMCs after 4 h by PMA-ionomycin, Pam3CSK4, LPS, flagellin and resiquimod, and weakly by Poly (I:C) stimulation (Figure S1c,d). Effects of IL-4 and TNF α with Pam3CSK4 and Poly (I:C) on TSLP and IL-6 gene expression in primary equine keratinocytes.

The subtitle was removed from here, which in my opinion makes the part of cytokine milieu less visible. Since this is quite an interesting part, I would be very grateful if we could have a subtitle here. Treatment with recombinant equine IL-4 and TNF- α , which mimics an atopic inflammatory cytokine milieu, significantly upregulated TSLP gene expression after 4 h and 24 h in primary equine keratinocytes. Moreover, the combination of Pam3CSK4 and atopic cytokine milieu had a synergistic effect on upregulation of TSLP gene expression. Although Pam3CSK4 alone already induced a strong upregulation of TSLP, the combination with atopic cytokine milieu further increased TSLP expression. Moreover, although the effect of stimulation with Pam3CSK4 was only strong after 4 h and dampened after 24 h stimulation, the combination with atopic cytokine milieu significantly enhanced TSLP expression even after 24 h (Figure 4a,b).

The same effect of atopic cytokine milieu was observed in combination with Poly (I:C). The synergistic effect of the milieu and Poly (I:C) on TSLP gene expression was seen both after 4 h and 24 h stimulation, with TSLP gene expression significantly enhanced at both time points, when compared to stimulation with Poly (I:C) only (Figure 4a,b).

Next, the effect of the atopic cytokine milieu and Pam3CSK4 or Poly (I:C) on IL-6 gene expression was examined. After 4 h stimulation (Figure 4c), as expected, stimulation with Pam3CSK4 and Poly (I:C) alone induced IL-6 expression. However, by contrast to TSLP expression, addition of IL-4 and TNF- α to the culture medium did not induce any significant upregulation of IL-6 after 4 h

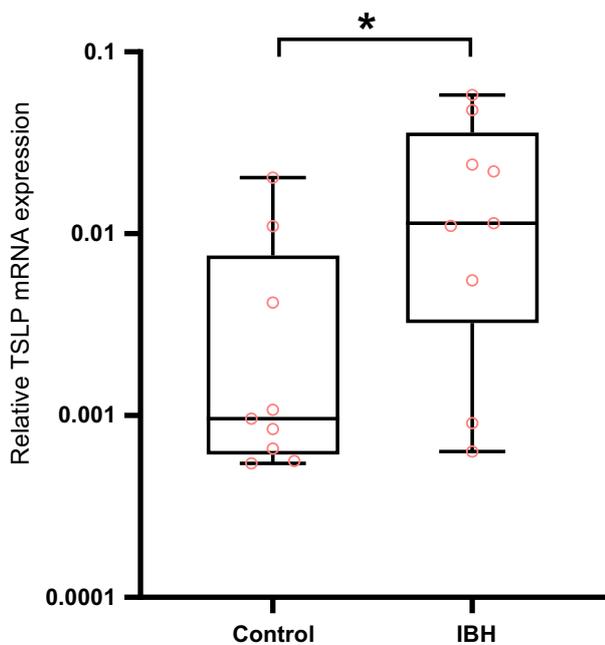


Figure 1. Thymic stromal lymphopoietin (TSLP) expression in lesional skin of Insect Bite Hypersensitivity affected ($n = 9$) and in skin of healthy horses ($n = 9$) assessed by qRT-PCR. Statistical analysis of differences between groups was performed using the Mann Whitney U-test. * $p \leq 0.05$

stimulation. Moreover, addition of cytokine milieu to Pam3CSK4 did not induce a significant upregulation of IL-6 after 4 h stimulation, either. Combination of Poly (I:C) and atopic cytokine milieu induced a significant upregulation of IL-6 after 4 h stimulation, when compared to stimulation induced by Poly (I:C) only, but the effect was much weaker for IL-6 compared to TSLP expression. Twenty-four hour stimulation with atopic cytokine milieu significantly upregulated IL-6 gene expression. The addition of atopic inflammation cytokine milieu to Pam3CSK4 or Poly (I:C) induced a statistically significant further upregulation of IL-6 (Figure 4d).

Discussion

The present study demonstrates that equine TSLP is significantly upregulated in lesional skin from IBH-affected horses when compared to healthy controls, thus indicating a potential role of this cytokine in the pathogenesis of IBH. A study has shown that TSLP protein is highly expressed in lesional skin of AD human patients, with no expression in patients without skin disease, which suggests that TSLP expression patterns are similar in equine and human counterparts.⁴

The data herein suggested that TSLP is involved in the pathogenesis of equine IBH, so the possible triggers of TSLP expression were investigated. Being on the interface between body and the environment, keratinocytes are in constant contact with a plethora of bacteria and viruses. Thus, it was hypothesized that TLR-L that correspond to certain bacterial- and viral PAMPs upregulate TSLP expression. First, primary equine keratinocytes cultures were established using modified protocols.^{11,15} Although fibroblast contaminations can be common in

keratinocyte cultures, the protocol used herein yielded fibroblast-free cultures, as demonstrated by immunofluorescence staining of cytokeratin and vimentin.

Even though all TLR-L except TLR-L 9, induced a significant TSLP upregulation, the strongest upregulation after 4 h was induced by Pam3CSK4, which is a synthetic triacylated lipoprotein found in both Gram-positive and -negative bacteria, and ligand for TLR 1/2. Interestingly, the strongest TSLP upregulation after 24 h stimulation was observed with Poly (I:C), and this upregulation was much stronger when compared to 4 h upregulation. Poly (I:C) is a synthetic analogue of double-stranded RNA (dsRNA), that can be recognized by TLR-3. Unlike TLR-1/2, -4 and -5, TLR-3 is an intracellular TLR, which may account for the prolonged time needed for the Poly (I:C) to induce TSLP and IL-6 upregulation, in comparison with ligands stimulating TLRs expressed on the cell membrane.¹⁸ In human keratinocytes, TSLP upregulation is induced via TLR-L 2/6, 3 and 5, after 18 h stimulation.^{19–22} Interestingly, Pam3CSK4 did not induce TSLP expression in human keratinocytes.¹⁹ A possible reason for this discrepancy between the effect of Pam3CSK4 on human and equine keratinocytes might be the origin of the reagent. Additionally, 200 ng/mL Pam3CSK4 was used, whereas human keratinocytes were stimulated with 5 $\mu\text{g/mL}$.¹⁹ When 5 $\mu\text{g/mL}$ Pam3CSK4 was used to stimulate equine keratinocytes, TSLP gene expression was upregulated as compared to nonstimulated cells, but the upregulation was not as strong as with a lower concentration of this TLR-L, suggesting that 5 $\mu\text{g/mL}$ of Pam3CSK4 might not be the optimal concentration. Human keratinocytes, however, upregulated TSLP when stimulated with FSL-1, a TLR 2/6-L.¹⁹ TLR-2 can form heterodimers with either TLR-1 or TLR-6, and it is possible that human keratinocytes form TLR-2/6 dimers whereas equine keratinocytes form TLR-1/2 dimers, and thus upregulate TSLP when stimulated with corresponding ligands.¹³ Interestingly, conversely to humans, flagellin did not induce a strong upregulation of TSLP in equine keratinocytes; however, this TLR-L induced a strong TSLP production in equine PBMCs. How these TLR-L actually impact skin inflammation remains to be elucidated.

In order to investigate whether TLR-L-induced TSLP expression is indeed mediated via TLR signalling, the expression of the pro-inflammatory cytokine IL-6 induced via NF- κ B, a downstream TLR signalling pathway effector, was examined.^{18,23} IL-6 was upregulated as expected by stimulation with TLR 1-5-L, but not with TLR 7-L and 8-L, suggesting that resiquimod induced TSLP expression may not be mediated via TLR 7 and 8. Moreover, it is known that some ligands, such as dsRNA (TLR-3 ligand) also can be recognized by molecules other than TLR 3, such as melanoma differentiation antigen 5 (MDA5), *retinoid-inducible gene 1* (*RIG-1*) and IFN-inducible double stranded RNA-activated protein kinase (PKR).^{24–26} Therefore, further studies will be required to decipher the exact mechanisms of TLR-L-induced TSLP expression in primary equine keratinocytes.

The TLR-L-induced expression patterns of TSLP and IL-6 differed between equine keratinocytes and PBMCs. For example, the strongest TSLP upregulation in PBMCs was induced by flagellin, which induced only a moderate

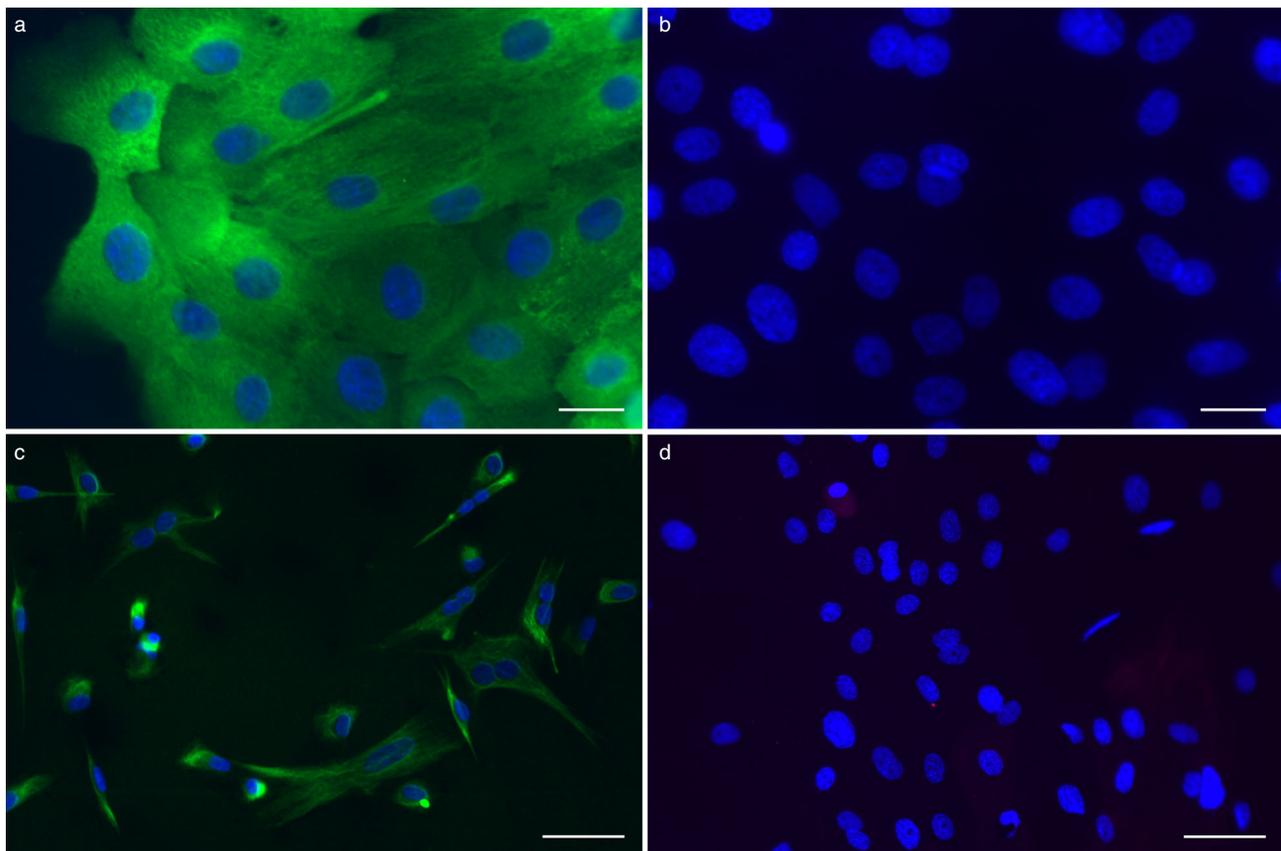


Figure 2. Immunofluorescence staining of keratinocytes and fibroblasts.

Primary equine keratinocytes and fibroblasts were stained with anti-cytokeratin and anti-vimentin antibody. (a) Staining of primary keratinocyte cultures with anti-cytokeratin: cytoplasmic cytokeratin is shown in green; nuclei were counterstained with Hoechst and are shown in blue. (b) Staining of keratinocytes with anti-vimentin: no staining was observed with this antibody. Nuclei are shown in blue. 40x magnification. (c) Equine fibroblasts stained with anti-vimentin: cytoplasmic vimentin is shown in green; nuclei were counterstained with Hoechst and are shown in blue. (d) Staining of fibroblasts with anti-cytokeratin: no staining was observed with this antibody. Nuclei are shown in blue. 20x magnification

expression of this cytokine in keratinocytes. This indicates that certain cell types in PBMCs express a different set of TLRs when compared to keratinocytes and are therefore stimulated to produce TSLP. However, it is not known which cell types are sources of equine TSLP in PBMCs, and this needs further investigation. Moreover, patterns of TLR expression in equine PBMCs remain to be elucidated. In addition, the expression profile and activity of different TLRs in equine keratinocytes are currently unknown.

In allergic humans, TH-2 cytokines (IL-4, IL-13) and TNF- α have been described as atopic cytokine milieu.^{19,21,22} Keratinocytes can come into contact with this milieu through interactions with TH-2 cells, basophils or mast cells that produce these cytokines. Therefore, to study the possible effect of atopic cytokine milieu alone or in combination with Pam3CSK4 or Poly (I:C) on TSLP expression, cytokines of atopic inflammatory milieu (recombinant equine IL-4 and TNF- α) were added to the culture medium. The milieu was combined only with Pam3CSK4 and Poly (I:C), the TLR-L that induced a clear TSLP upregulation in the present study. Addition of the atopic cytokine milieu alone already induced a significant TSLP upregulation, and when combined with Pam3CSK4 or Poly (I:C), it synergistically further upregulated TSLP expression. In skin allergic conditions including IBH,

clinical signs are exacerbated by secondary bacterial infections.^{10,27} As mentioned, Pam3CSK4 is a constituent of bacterial membranes recognized by TLR1/2 heterodimers.²⁸ The results herein showing a strong upregulation of TSLP expression by Pam3CSK4 and its synergistic effects with atopic cytokine milieu, suggest that secondary bacterial infections in an allergic microenvironment can amplify TH-2 inflammation via the induction of TSLP, thus forming a vicious circle and exacerbation of the disease. Likewise, combining Poly (I:C) with the atopic cytokine milieu significantly increased TSLP gene expression after both 4 h and 24 h stimulation, when compared to Poly (I:C)-induced TSLP expression. Interestingly, dsRNA and Poly (I:C) TLR-3 have been associated with the mechanism for detection of skin damage and early inflammation initiation independent of viral activation.²⁹ Because IBH lesions have evident skin damage that in chronic cases can occur as epidermal necrosis, it is speculated that dsRNA released from damaged cells can trigger TSLP production in keratinocytes and could thus result in exacerbation of the disease. These synergistic effects of the atopic cytokine milieu and TLR-L on TSLP expression also have been described in human: TLR-L Poly (I:C), FSL-1 and flagellin-induced TSLP expression, and, additionally, when combined with allergic inflammation cytokine milieu, these TLR-L synergistically further

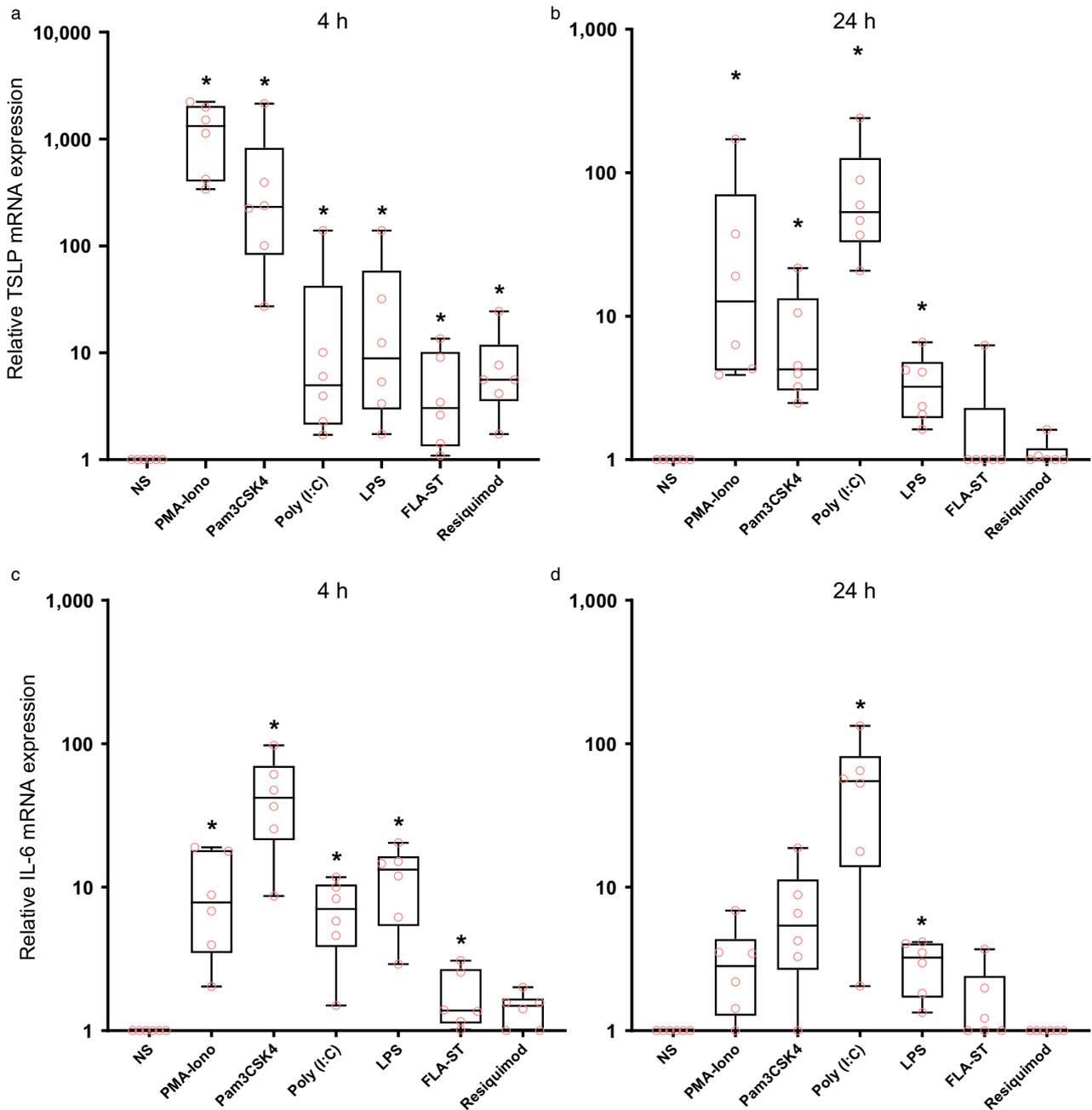


Figure 3. Thymic stromal lymphopoietin (TSLP) and IL-6 gene expression in primary equine keratinocytes derived from six horses assessed by qRT-PCR.

Cells were cultured with medium alone, PMA - phorbol 12-myristate 13-acetate (50 ng/ml) + ionomycin (1 μ mol/mL), Pam3CSK4 (200 ng/ml), Poly (I:C) (5 μ g/ml), Lipopolysaccharide (LPS) (5 μ g/ml), Flagellin from *Salmonella Typhimurium* (FLA-ST) (200 ng/ml) and resiquimod (5 μ g/ml) for 4 h and 24 h. (a) and (b) TSLP expression after 4 h and 24 h of stimulation. (c) and (d) IL-6 expression after 4 h and 24 h of stimulation. Asterisk (*) indicates statistically significant TSLP and IL-6 upregulation compared to non-stimulated control in the Mann Whitney U-test, after correction for multiple comparisons (* $cP \leq 0.05$). NS = non-stimulated control.

upregulate TSLP expression.^{19,21,22} Based on the data herein, it is therefore hypothesized that stimulation of TLR on keratinocytes by bacterial or viral PAMPs from the environment may lead to an increased release of TSLP in horses suffering from IgE-mediated skin allergies. This effect may be due to the allergic cytokine microenvironment in the skin of these animals, as demonstrated for IBH and recurrent urticaria.^{17,30} Further studies to investigate TSLP expression at the protein level are now required to confirm the role of TSLP in equine skin allergy.

In conclusion, the present study strongly suggests an important role for TSLP in the pathogenesis of IBH, as demonstrated by increased mRNA expression in the skin of IBH-affected horses as well as by the strong ability of equine keratinocytes to produce TSLP in response to TLR-L, present in the skin microenvironment and possibly also in the saliva of the causative insects. Further studies will be needed to elucidate whether the response of keratinocytes differs solely between allergic and nonallergic horses, or whether the observed

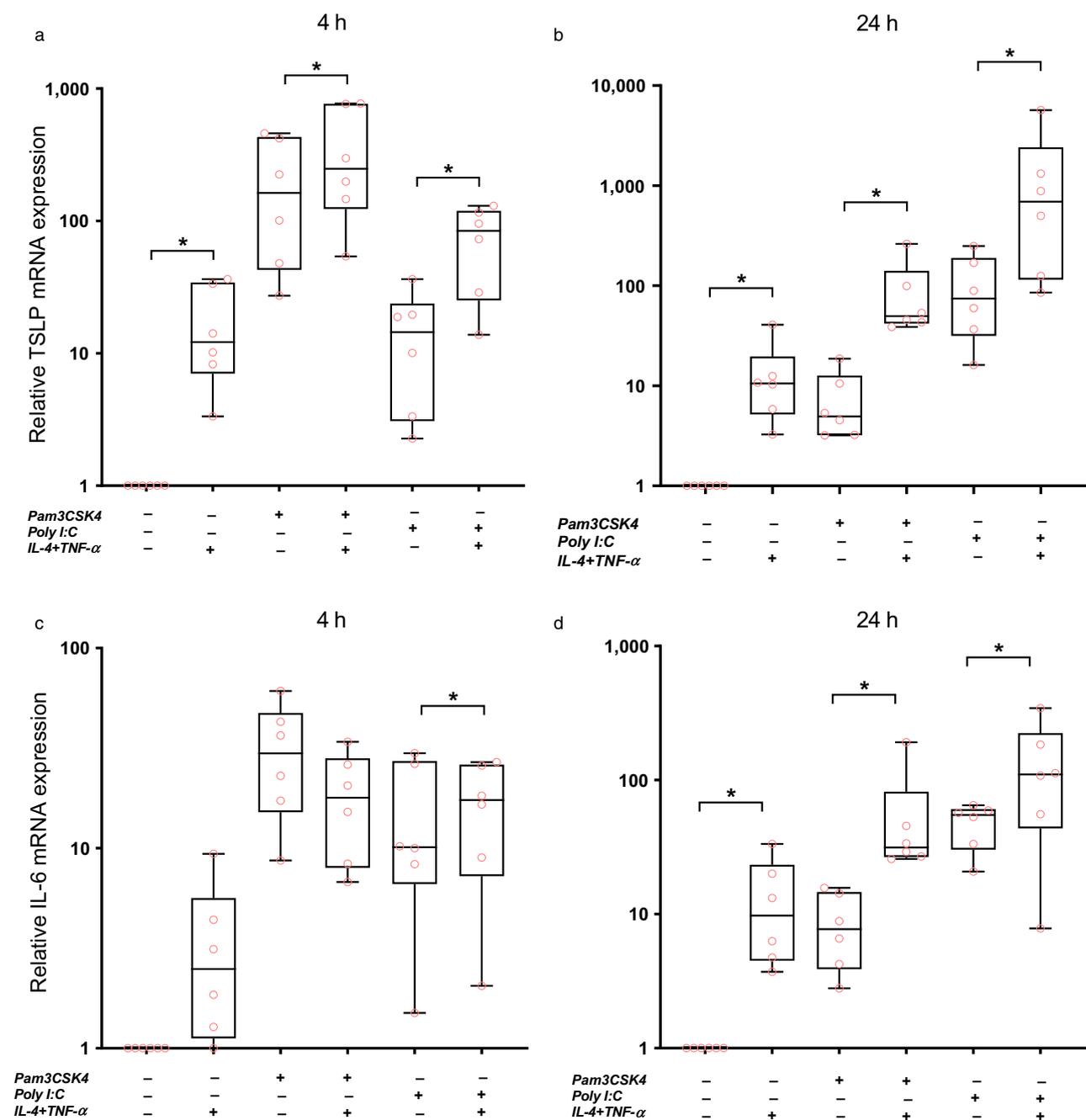


Figure 4. Thymic stromal lymphopoietin (TSLP) and IL-6 gene expression in primary equine keratinocytes ($n = 6$) assessed by qRT-PCR.

Cells were stimulated with medium alone, allergic inflammation cytokine milieu consisting of IL-4 (100 ng/ml) and TNF- α (100 ng/ml), Pam3CSK4 (200 ng/ml) and Poly (I:C) (5 μ g/ml) alone or in combination with the cytokine milieu for 4 h and 24 h of stimulation. (a) and (b) TSLP expression after 4 h and 24 h of stimulation. (c) and (d) IL-6 expression after 4 h and 24 h of stimulation. Asterisk (*) indicates statistically significant TSLP and IL-6 upregulation in the Wilcoxon Signed-Rank test (* $p < 0.05$)

increased TSLP expression is only associated with the allergic inflammation micro-milieu present in the skin of IBH-affected horses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Thymic stromal lymphopoietin (TSLP) (a-b) and IL-6 (c-d) gene expression in peripheral blood mononuclear cells (PBMCs) isolated from three horses, assessed by qRT-PCR.

Table S1a. Median Thymic stromal lymphopoietin (TSLP) mRNA expression (presented as Log₂ ΔΔCT) in keratinocytes stimulated with PMA-Ionomycin as positive control and various Toll like receptor-Ligands in comparison to non-stimulated cells for 4 and 24 hours.

Table S1b. Median IL-6 mRNA expression (presented as Log₂ ΔΔCT) in keratinocytes stimulated with PMA-Ionomycin as positive control and various Toll like receptor - Ligands in comparison to non-stimulated cells for 4 and 24 hours.

Table S1c. Effect of a combination of IL-4 and TNF-α on TSLP expression.

Table S1d. Effect of a combination of IL-4 and TNF-α on IL-6 expression.

Résumé

Contexte – Le TSLP (Thymic stromal lymphopoietin) joue un rôle clé dans le développement de l'inflammation allergique. On en sait peu sur les facteurs possibles d'expression de TSLP chez le cheval.

Hypothèses/objectifs – Étudier l'expression de TSLP dans les lésions cutanées de l'hypersensibilité aux piqures d'insectes (IBH) chez le cheval. La capacité des ligands TLR 1-8 (L) et des cytokines atopiques comme déclencheurs potentiels de TSLP et d'interleukine (IL)-6 comme molécule effectrice de régulation du signal TLR, ont été examinés dans des cultures de kératinocytes équins primaires.

Sujets – La peau lésionnelle d'IBH atteints et de peau saine de chevaux contrôles (n=9 pour chaque groupe) a été prélevée.

Matériel et méthodes – Les cultures de kératinocytes ont été réalisées à partir de six chevaux sains et stimulés par TLR 1-8-L, et avec IL-4 et TNF- α , pour mimer une inflammation atopique. L'expression des gènes de TSLP et IL-6 a été déterminée par PCR quantitative en temps réel.

Résultats – L'expression de TSLP est significativement plus élevée dans les lésions d'IBH comparé à la peau saine. Les TLR 1-8-L ont significativement augmenté l'expression de TSLP dans les kératinocytes. L'augmentation de régulation la plus marquée était induite par TLR 1/2-L et TLR 3-L. La combinaison de milieu de cytokine atopique et TLR 1/2-L ou TLR 3-L augmente davantage l'expression de TSLP. La stimulation de TLR 1-5 a significativement augmenté l'expression d'IL-6.

Conclusion et importance clinique – Les données ci dessus suggèrent que l'augmentation de régulation de l'expression de TSLP dans les lésions cutanées de chevaux atteints d'IBH pourrait jouer un rôle dans le développement d'IBH. En outre, l'expression de TSLP est induite par TLR-L, en particulier par TLR 1/2-L et TLR 3-L, et est d'autant plus augmenté que le milieu de cytokine atopique, indiquant un mécanisme d'exacerbation d'IBH médié par TSLP.

Resumen

Introducción – la linfopoyetina del estroma tímico (TSLP) desempeña un papel clave en el desarrollo de la inflamación alérgica. Poco se sabe sobre posibles desencadenantes de expresión de TSLP equina.

Hipótesis/objetivos – investigar la expresión de TSLP en lesiones cutáneas de hipersensibilidad por picadura de insecto en equinos (IBH). Se examinaron la capacidad de los ligandos TLR 1-8 (L) y del medio de citoquinas en atopia como posibles desencadenantes de TSLP y de interleuquina (IL) -6 como una molécula efectora posterior a la señal estimulante de TLR en cultivos primarios de queratinocitos equinos.

Animales – se tomaron muestras de piel lesionada de caballos con IBH y de piel sana de caballos control (n = 9 cada grupo).

Métodos y materiales – se establecieron cultivos de queratinocitos a partir de seis caballos sanos y se estimularon con TLR 1-8-L, y con IL-4 y factor de necrosis tumoral- α , para imitar un medio de citoquinas en inflamación en atopia. La expresión del gen TSLP e IL-6 se evaluaron por PCR cuantitativa en tiempo real.

Resultados – la expresión de TSLP fue significativamente mayor en los queratinocitos de lesiones de IBH en comparación con los de la piel sana. TLR 1-8-L aumentó significativamente la expresión de TSLP en queratinocitos. La regulación positiva más fuerte fue inducida por TLR 1/2-L y TLR 3-L. La combinación del medio de citoquinas en atopia y TLR 1/2-L o TLR 3-L aumentó aún más la expresión de TSLP. La estimulación de TLRL 1-5 aumentó significativamente la expresión de IL-6.

Conclusiones e importancia clínica – los datos en este documento sugieren que la regulación positiva de la expresión de TSLP en la piel lesionada de los caballos afectados por IBH podría desempeñar un papel en el desarrollo de IBH. Además, la expresión de TSLP es inducida por TLR-L, en particular por TLR 1/2-L y TLR 3-L, y aumenta aún más por medio de citoquinas en atopia, lo que indica un mecanismo para la exacerbación de signos clínicos de IBH mediada por TSLP.

Zusammenfassung

Hintergrund – Das Thymic Stromal Lymphopoietin (TSLP) spielt eine Schlüsselrolle bei der Entwicklung allergischer Entzündung. Es ist wenig bekannt über die möglichen Auslöser der Expressierung des equinen TSLP.

Hypothese/Ziele – Das Ziel war eine Untersuchung der TSLP Expressierung in Hautveränderungen bei der Insektenstich Hypersensibilität des Pferdes (IBH). Die Fähigkeit von TLR 1-8 Liganden (L) und des atopischen Zytokinmilieus als mögliche Auslöser von TSLP und von Interleukin (IL)-6 als Downstream Effektor Molekül für die TLR Signalgebung wurden in primären Keratinozytenkulturen von Pferden untersucht.

Tiere – Es wurden Läsionen der Haut von Pferden mit IBH und gesunder Haut von Kontrollpferden (n = 9 in jeder Gruppe) untersucht.

Methoden und Materialien – Es wurden Keratinozytenkulturen von sechs gesunden Pferden hergestellt und mit TLR 1-8-L, mit IL-4 und Tumor Nekrose Faktor- α stimuliert, um das Zytokinmilieu einer atopischen Entzündung zu imitieren. Die TSLP und IL-6 Genexpressierung wurde mittels quantitativem Real-Time PCR untersucht.

Ergebnisse – Die Expressierung von TSLP war in IBH Läsionen im Vergleich zur gesunden Haut signifikant höher. TLR 1-8-L erhöhte die TSLP Expressierung in den Keratinozyten signifikant. Die stärkste Erhöhung wurde von TLR 1/2-L und TLR 3-L induziert. Eine Kombination des atopischen Zytokinmilieus und TLR 1/2-L oder TLR 3-L erhöhte die TSLP Expressierung noch deutlicher. Die TLRL 1-5 Stimulierung erhöhte die IL-6 Expressierung signifikant.

Schlussfolgerungen und klinische Bedeutung – Diese Ergebnisse weisen darauf hin, dass die Erhöhung einer TSLP Expressierung in läSIONaler Haut bei IBH-betroffenen Pferden eine Rolle bei der Entwicklung der IBH spielen könnte. Darüber hinaus wird die TSLP Expressierung durch TLR-L induziert, vor allem durch TLR 1/2-L und TLR 3-L und wird durch ein atopisches Zytokinmilieu weiter erhöht, was auf den Mechanismus einer TSLP-vermittelten Verschlimmerung der IBH hinweist.

要約

背景 – 胸腺間質性リンパ球新生因子(TSLP)は、アレルギー性炎症の発症に重要な役割を果たす。馬のTSLP発現のトリガーの可能性についてはほとんど知られていない。

仮説/目的 – 本研究の目的は、馬の昆虫刺咬性過敏症(IBH)の皮膚病変におけるTSLP発現を調査することである。TLRシグナル伝達の下流エフェクター分子としてのTSLPおよびインターロイキン(IL)-6の潜在的トリガーとしてのTLR 1-8リガンド(L)およびアトピー性サイトカイン環境の能力を、一次馬ケラチノサイト培養で調査された。

被験動物 – IBHに罹患した馬の病変皮膚および対照馬の健常皮膚(n = 9各グループ)をサンプリングした。

材料と方法 – ケラチン生成細胞培養を、6頭の健常馬によって確立し、TLR 1-8-L、IL-4および腫瘍壊死因子- α で刺激し、アトピー性炎症サイトカイン環境を模倣した。TSLPおよびIL-6遺伝子発現を、定量リアルタイムPCRにより評価した。

結果 – TSLP発現は、健常皮膚と比較してIBH病変において有意に多かった。TLR 1-8-Lは、ケラチン生成細胞におけるTSLP発現を有意に上方制御した。最も強いアップレギュレーションは、TLR 1 / 2-LおよびTLR 3-Lによって誘導された。アトピー性サイトカイン環境およびTLR 1 / 2-LまたはTLR 3-Lの組み合わせは、TSLP発現をさらに増加させた。TLRL 1-5刺激は、IL-6発現を有意にアップレギュレートした。

結論と臨床的重要性 – 本データは、IBHに罹患した馬の病変皮膚におけるTSLP発現の上方制御がIBHの発生に役割を果たす可能性があることを示唆している。さらに、TSLP発現は、TLR-L、特にTLR 1 / 2-LおよびTLR 3-Lによって誘導され、アトピー性サイトカイン環境によってさらに増加し、TSLPを介したIBHの増悪メカニズムを示している。

摘要

背景 – 胸腺基质淋巴生成素(TSLP)在過敏性炎症发生过程中起关键作用。目前对马的TSLP表达的可能触发因素知之甚少。

假设/目的 – 研究TSLP在马昆虫叮咬超敏反应(IBH)皮肤病变中的表达。在初代马角质细胞培养基中,研究TLR1-8配体(L)和异位性细胞因子环境作为TSLP的潜在触发因子,以及白介素(IL)-6作为TLR信号下游效应分子的能力。

动物 – 采集IBH的病变皮肤和对照组马的健康皮肤(每组9例)作为样本。

方法和材料 – 从六匹健康马身上制备角质细胞培养基,给予TLR1-8-L、IL-4和肿瘤坏死因子- α 刺激,模仿异位性炎症细胞因子环境。实时荧光定量PCR检测TSLP和IL-6基因表达。

结果 – 与健康皮肤相比,TSLP在IBH病变中的表达明显增加。TLR1-8-L显著上调TSLP在角质细胞中的表达。其中TLR1/2-L和TLR3-L诱导上调最强。异位性细胞因子环境与TLR1/2-L或TLR3-L联合进一步增加了TSLP的表达。TLRL1-5刺激显著上调IL-6的表达。

结论和临床意义 – 本文数据表明,IBH患马病变皮肤中的TSLP表达上调,可能在IBH的发生过程中发挥作用。此外,TSLP的表达由TLR-L诱导,尤其是TLR1/2-L和TLR 3-L,并在异位性细胞因子环境中进一步增加,提示TSLP介导IBH加重的机制。

Resumo

Contexto – A linfopoiétina estromal tímica (TSLP) desempenha um papel fundamental no desenvolvimento da inflamação alérgica. Pouco se sabe sobre possíveis gatilhos da expressão de TSLP em equinos.

Hipótese/objetivos – Investigar a expressão de TSLP em lesões cutâneas de hipersensibilidade à picada de inseto (IBH) em equinos. A capacidade dos ligantes TLR 1-8 (L) e do meio de citocinas atópicas como potenciais gatilhos da TSLP e da interleucina (IL) -6 como uma molécula efetora a jusante da sinalização de TLR, foram examinados em culturas primárias de queratinócitos equinos.

Animais – Coletou-se a amostras de pele lesional de equinos afetados por IBH e a pele saudável de cavalos controle (n = 9 cada grupo).

Métodos e materiais – Culturas de queratinócitos foram iniciadas a partir de material de seis equinos saudáveis e estimuladas com TLR 1-8-L, IL-4 e com fator de necrose tumoral- α , para imitar um meio de citocina de inflamação atópica. A expressão do gene TSLP e IL-6 foi avaliada por PCR quantitativo em tempo real.

Resultados – A expressão de TSLP foi significativamente maior nas lesões de IBH em comparação com a pele saudável. TLR 1-8-L aumentou positivamente a expressão de TSLP nos queratinócitos. Maior regulação positiva foi induzida por TLR 1/2-L e TLR 3-L. A combinação do meio de citocinas atópicas e TLR 1/2-L ou TLR 3-L aumentou ainda mais a expressão de TSLP. A estimulação com TLRL 1-5 aumentou significativamente a expressão de IL-6.

Conclusões e importância clínica – Os dados do presente estudo sugerem que a regulação positiva da expressão de TSLP na pele lesional de cavalos afetados por IBH pode desempenhar um papel no desenvolvimento da IBH. Além disso, a expressão de TSLP é induzida por TLR-L, em particular por TLR 1/2-L e TLR 3-L, e é ainda aumentada pelo meio de citocinas atópicas, indicando um mecanismo para a exacerbação da IBH mediada por TSLP.