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Author: Katrin Schaper Dr. Kristine Rossbach Brigitta K*ö*ther Holger Stark Manfred Kietzmann Thomas Werfel Ralf Gutzmer

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Stimulation of the histamine 4 receptor upregulates thymic stromal lymphopoietin (TSLP) in human and murine keratinocytes

Katrin Schaper[1], Kristine Rossbach[2], Brigitta Köther[1], Holger Stark[3] Manfred Kietzmann[2], Thomas Werfel[1], Ralf Gutzmer[1]

Authors Affiliations: [1] Division of Immunodermatology and Allergy Research, Department for Dermatology and Allergy, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany. [2]Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Buenteweg 17, Hannover, Germany. [3]Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University, Universitaetsstr. 1, 40225 Duesseldorf, Germany

Corresponding author: Dr. Katrin Schaper, Department for Dermatology and Allergy, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany

Email: schaper-katrin@mh-hannover.de

Phone: +495115325054

Fax: +495115328112

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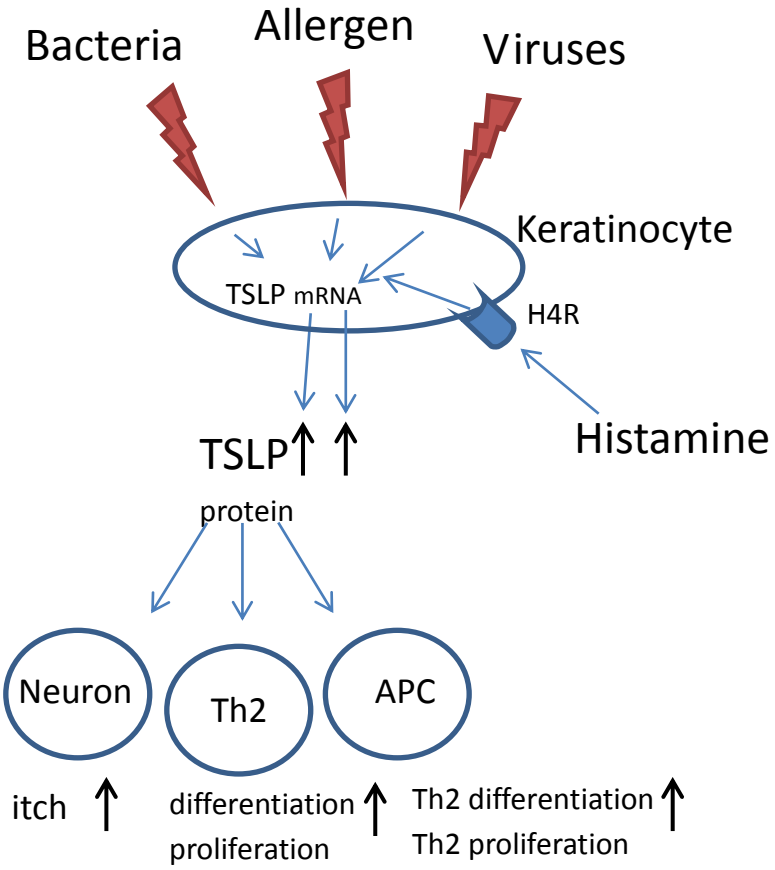
Graphical abstract



Abstract

The cytokine thymic stromal lymphopoietin (TSLP) is involved in the development and the progression of allergic diseases. It is mainly released by epithelial cells at barriers such as skin and gut in response to danger signals. Overexpression of TSLP in keratinocytes (KC) can

provoke the development of a type 2 inflammatory response. Additionally, TSLP directly acts on sensory neurons and thereby triggers itch. Since histamine is also increased in lesions of inflammatory skin diseases, the aim of this study was to investigate possible effects of histamine as well as different histamine receptor subtype agonists and antagonists on TSLP production in KC. We therefore stimulated human KC with histamine in the presence or absence of the known TSLP-inductor poly I:C and measured TSLP production at protein as well as mRNA level. Histamine alone did not induce TSLP production in human KC, but pre-incubation with histamine prior to challenge with poly I:C resulted in a significant increase of TSLP production compared to stimulation with poly I:C alone. Experiments with different histamine receptor agonists (H1R: 2-pyridylethylamine ; H2R: amthamine; H2R/H4R: 4-methylhistamine (4MH)) revealed a dominant role for the H4R receptor, as 4-MH in combination with poly I:C displayed a significant increase of TSLP secretion, while the other agonists did not show any effect. The increase in TSLP production by 4MH was blocked with the H4R antagonist JNJ7777120. This effect was reproducible also in the murine KC cell line MSC.

Taken together, our study indicates a new role for the H4 receptor in the regulation of TSLP in keratinocytes. Therefore, blocking of the H4R receptor in allergic diseases might be promising to alleviate inflammation and pruritus via TSLP.

Keywords: TSLP, histamine, histamine 4 receptor, keratinocytes

Introduction

Histamine is a biogenic amine, which is mainly produced by mast cells and basophils but also from other cell types such as macrophages, dendritic cells, neutrophils and T-cells [1].

Histamine mediates its effects via four different G-protein coupled receptors (H1R-H4R), which are widely expressed on various immune cells, neurons and also on keratinocytes [2]. Thereby, histamine is involved in the immunomodulatory process of several allergic diseases, like asthma and atopic dermatitis (AD) and serves as an important mediator of pruritus. Recent studies in mice demonstrated, that blocking the H4R resulted on the one hand in a reduction of inflammation in an ovalbumin (OVA)-induced mouse model of asthma as well as AD [3, 4] and on the other hand in a decrease of scratching response [5-8]. Moreover, the H4R antagonist JNJ39758979 was effective in reducing histamine-induced pruritus in a randomized clinical study in healthy human subjects [9]. However, the mode of action of H4R antagonism for blocking pruritus is not clear yet. Possible mechanisms include a direct effect of histamine on sensory neurons [10] or an indirectly effect via modulating cytokines and chemokines implicated in mediating pruritus [11] or both. One of those mediators, which attracted attention in recent years, is thymic stromal lymphopoietin (TSLP). It is a cytokine, derived by epithelial cells, which is involved in the development and progression of allergic disease [12]. Thereby TSLP acts mainly at barrier functions such as skin and gut in response to danger signals [13], while it directly promotes T-cell proliferation and differentiation in mouse as well as human cells [14, 15] and enhances cytokine production from iNKT cells, innate lymphoid cells, eosinophils and mast cells [16-19]. Additionally, TSLP was found to promote itch directly via activation of cutaneous sensory neurons [20], which firmly underlines the role of TSLP as a pruritogenic molecule.

Since, both TSLP and histamine are increased in lesions of inflammatory skin diseases [21, 22], and have proven evidence for an important role in disease pathology, we decided to investigate possible effects of histamine and histamine receptor agonists and antagonists on TSLP production by human and murine keratinocytes.

Material and Methods

Reagents

Histamine receptor ligands: histamine (agonist for all histamine receptors; ALK-Scherax, Wedel, Germany), 4-methylhistamine (4MH; H2R/H4R agonist), 2-pyridylethylamine (H1R

agonist), amthamine (H2R agonist; all agonists from Tocris Bioscience, Bristol, United Kingdom), ST1006 (H4R agonist; Holger Stark, Düsseldorf, Germany), JNJ7777120 (JNJ, H4R antagonist, Tocris Bioscience). TLR3 ligand (poly I:C) was obtained from Sigma-Aldrich (Munich, Germany).

Keratinocyte cell culture

Adult human hair keratinocytes were isolated from the outer root sheath from plucked hair (HKC), as described previously [23]. Briefly, the hairs were placed in dishes with a feeder layer of 3T3 fibroblasts that had been treated with mitomycin C (Roche, Mannheim, Germany), the medium was changed every 2 to 3 days, and when sufficient HKC s had outgrown, they were selectively trypsinized and passaged further.

Neonatal human epidermal keratinocytes (NHEK) were obtained from Lonza (Cologne, Germany).

The murine keratinocyte cell line (MSC) was obtained from CLS Cell Lines Service (Eppelheim, Germany).

Stimulation of keratinocytes

From previous experiments regarding the effects of histamine on keratinocytes we already have established histamine and histamine receptor ligand concentrations [24]. Furthermore preliminary experiments revealed that pre-incubation with histamine agonists prior to poly I:C challenge obtained better effects, than simultaneous stimulation. Thus, human and murine KC were stimulated with histamine (10µM) and histamine receptor agonists (10µM) for 24h and the human KC subsequently with poly I:C at the indicated concentrations (1-10µg/ml) for another 24h. In some experiments the antagonist were added half an hour before the agonist.

Cytokine determination

TSLP was measured in the supernatants from stimulated cells by enzyme-linked immunosorbent-assays (ELISA) using commercially available kits according to the manufacturer's protocol (R&D System, Wiesbaden, Germany).

mRNA isolation, reverse transcription and quantitative RT-PCR

For real-time PCR cells were stimulated for 6h, harvested, lysed and mRNA was isolated according to the manufacturer's instructions with the Analytic Jena Kit (Jena, Germany). The cDNA was synthesized by reverse transcription the Quantitect reverse transcription kit (Qiagen, Hilden, Germany). Real-time quantitative LightCycler PCR (Roche Molecular Biochemicals, Mannheim, Germany) was performed with Quantitect primer assay for TSLP (QT00051464), TLR3 (QT00007714) and RPS (QT00003290) using SYBR Green according to the manufacturer's instructions (Roche).

Statistical analysis

For statistical analysis GraphPadPrism (Version 5.02) was applied. Statistical significance was assessed by t-test.

Results

Histamine enhances the production of TSLP in human NHEK and HKC

In preliminary experiments, human keratinocytes were stimulated with different cytokines (Interleukin 4 (IL-4), TNFα, IL-13 and the TLR3 agonist poly I:C) to induce TSLP production, like it is described in former publications [25, 26]. In our setting poly I:C led to the highest production of TSLP (data not shown), thus we proceeded using poly I:C for further experiments. Pre-incubation of NHEK with histamine for 24h increased the poly I:C induced TSLP secretion significantly (Figure 1A). Histamine alone had no impact on TSLP production (data not shown). To verify our findings regarding histamine and TSLP, we further used primary isolated HKC from adult patients. Again, histamine enhanced the TLR3-induced TSLP production (Figure 1B). Since it is reported that TSLP content is elevated in skin diseases like atopic dermatitis (AD) and psoriasis [12, 27], we performed experiments with HKC obtained from patients suffering from AD and psoriasis. Comparing the TSLP production upon poly I:C stimulation, keratinocytes derived from psoriatic patients produced more TSLP (mean: 189 pg/ml), than keratinocytes derived from AD patients (mean: 103 pg/ml) or healthy subjects (mean: 98 pg/ml). In the AD and psoriasis patients histamine also tended to enhance TSLP secretion, although the data were not significant.

Histamine modulates TSLP production via the H4R

Next, we investigated, which histamine receptor subtype is responsible for the TSLP-modulating effect. Therefore, we pre-incubated keratinocytes with specific histamine receptor agonists: 2-pyridylethylamine (H1R agonist), amthamine (H2R agonist) and 4-methylhistamine (4MH) (H4R agonist). While H1R and H2R had no impact on TSLP production, pre-incubation with the H4R-agonist increased TSLP production in NHEK as well as HKC (Figure 2A and B). Since it is described that the H4R agonist 4MH also binds to the H2R (although to a much lesser extend), we additionally incubated the cells with the selective H4R antagonist JNJ7777120 prior to 4MH- and poly I:C stimulation. In both cell

types, NHEK and HKC JNJ7777120 blocked the increase of 4MH-induced TSLP production (Figure 3). To exclude that stimulation of the H4R affects the expression of TLR3 and therefore causing an upregulation of TSLP production, we additionally investigated TLR3 expression at mRNA level. Both, poly I:C and the combination with 4MH slightly upregulated TLR3 expression, although no difference between H4R stimulation and poly I:C could be observed (Figure 3C).

Stimulation of the H4R induce TSLP production in the murine KC cell line MSC

MSC were incubated with the H4R agonists 4MH and ST-1006 (all 1-100µM). While 4MH did not display any effects (data not shown), ST-1006 showed a dose dependent increase in TSLP secretion, which could be blocked with JNJ7777120 (10µM) (Figure 4).

Discussion

In this study we investigated the effect of histamine on TSLP secretion, which displays an important molecule in the pathogenesis of allergic diseases like asthma or AD. We demonstrated that stimulating the H4R resulted in an increase of TSLP production in human and murine keratinocytes.

Keratinocytes represent more than 95% of cells in the skin and are therefore the predominant cell type. They have the potential to secrete a wide variety of cytokines, whereby the pattern of cytokine production plays a critical role in modulating tissue inflammation. It is already known that keratinocytes express three of four histamine receptor subtypes, while only for the H1R and H4R functional effects have been described on keratinocytes thus far [24, 28]. Stimulation of the H1R induced nerve growth factor, GM-CSF, matrix metalloproteinase-9 (MMP-9) and CCL5 secretion [28-30], while it suppressed keratinocyte differentiation [31].

Activation of the H4R increased the production of IL-8 mRNA in HaCaT-cells [32], whereas it induced proliferation in keratinocytes obtained from AD patients [24]. Our results of histamine receptor stimulation in regard to TSLP secretion are consistent with these rather pro-inflammatory effects mediated by the H1R and H4R. TSLP is an important cytokine for the triad of atopic diseases. While under physiological conditions, TSLP modulates Th2-type homeostasis, dysregulated TSLP expression triggers a type 2 inflammatory response [12]. Since histamine is elevated in the skin of patients suffering from inflammatory skin diseases like AD or psoriasis [2], it is possible that histamine intensifies the Th2 response by augmenting TSLP production of keratinocytes.

Noteworthy, it has to be distinguished between two isoforms of TSLP: the long and the short form. Previous studies demonstrated that TLR ligands and a Th2 cytokine milieu (IL-4, IL-13, TNF-α) predominantly upregulated the gene expression of the long TSLP form, whereas the short form is expressed at steady state and not influenced by inflammatory stimuli [33, 34]. Additionally, Xie et al. showed that the short form has little or no effects on the synthesis of TSLP protein [34]. Thus, we assume that the effect of histamine on TSLP production is mediating via the long form of TSLP. If histamine also displays an impact on the short form still needs to be elucidated in further investigations.

Our results revealed that histamine mediates its effect via the H4R. We could exclude that stimulation of the H4R causes an increase of TLR3 expression, which could have been one reason for the elevated TSLP expression. It has been shown that TSLP production is depending on NF- kappa B activation [35]. Since previous studies demonstrated an impact of histamine and also of 4MH on NF-kappa B activation in human microglia [36] and in a mouse model of rheumatoid arthritis [37], it is tempting to speculate that also in keratinocytes the activation of NF-kappa B upon H4R stimulation plays a pivotal role in triggering TSLP

production. The pro-inflammatory role of the H4R in Th2 related diseases is in accord with previous investigation, where we showed that stimulation of the H4R blocked the production of the Th1 cytokine IL-12 in monocyte derived dendritic cells [11] and led to an upregulation of the Th2 cytokine IL-31 [38]. Histamine receptors are not only involved in inflammatory processes, but also in mediating pruritus. It is well known, that histamine-induced pruritus can be sufficiently blocked by H1R antagonist, also known as antihistamines. However, in AD, where pruritus is the most characteristic symptom [39], itch is not well controlled by H1R antagonists as reported in several mouse models and clinical trials [40]. In contrast, blocking the H4R showed to inhibit scratching response in mice induced by models of dermatitis or haptens and moreover in a phase 2 study in adults with moderate AD [8, 41, 42]. However, the side of action for inhibiting pruritus, whether it is mediating directly via sensory neurons or indirectly via itch-inducing cytokines is not clear yet. Decreasing TSLP production through blocking the H4R could be one explanation for inhibiting scratching symptoms in patients of AD, although it should be taken into account that pruritus in AD is the result of several factors.

Comparing keratinocytes derived from healthy subjects with keratinocytes from patients with AD or psoriasis, poly I:C induced TSLP expression was highest in psoriasis patients, which is in line with investigations from Volpe et al. [27]. In previous studies, we found that KC derived from patients with AD expressed more H4R at mRNA level compared to healthy subjects [24]. Thus, we expected that stimulation of the H4R in these patients would have shown a more distinct effect on TSLP production compared to health controls. However, the effect of histamine in AD and psoriasis patients on TSLP secretion was even weaker. Due to the lack of reliable H4R antibodies, we were not able to check the receptor expression at

protein level. Thus, it cannot be excluded, that in our cell culture setting the receptor expression at the surface of the cells is different compared to the mRNA data.

Since pre-clinical research is always based on mouse models, we were interested if our results obtained from human cells could be transferred to mouse cells. We therefore stimulated the murine cell line MSC with the H4R agonists 4MH and ST-1006. While 4MH did not show any effect, ST-1006 displayed an increase of TSLP secretion even without an additionally stimuli, like it is required for human cells. We can only speculate about the reasons for the discrepancy between the two agonists. It could be shown that compared to the human H4R a trend towards decreased potency was detected at the murine receptor for 4MH [43], thus it is feasible that ST-1006 has a higher affinity towards the H4R in MSC cell line than 4MH. Also possible is a difference in the functional selectivity of the two agonists in humans and mice. Another reason could be that MSC cells display a different receptor expression pattern. As a result 4MH could bind to a greater extend to the H2R than in human cells and thereby mediates possibly opposite effects on TSLP production. However, the increase of TSLP protein in MSC cells with ST-1006 could be blocked with the specific H4R antagonist JNJ777710.

Conclusion

Taken together, our study indicates a possible role for the H4R in the regulation of TSLP in human and murine keratinocytes. In allergic diseases blocking the H4R could therefore lead to a decrease of TSLP production and subsequently result in an anti-inflammatory and anti-pruritic effect.

Conflict of interest

The authors declare no conflict of interest.

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Figure 1:

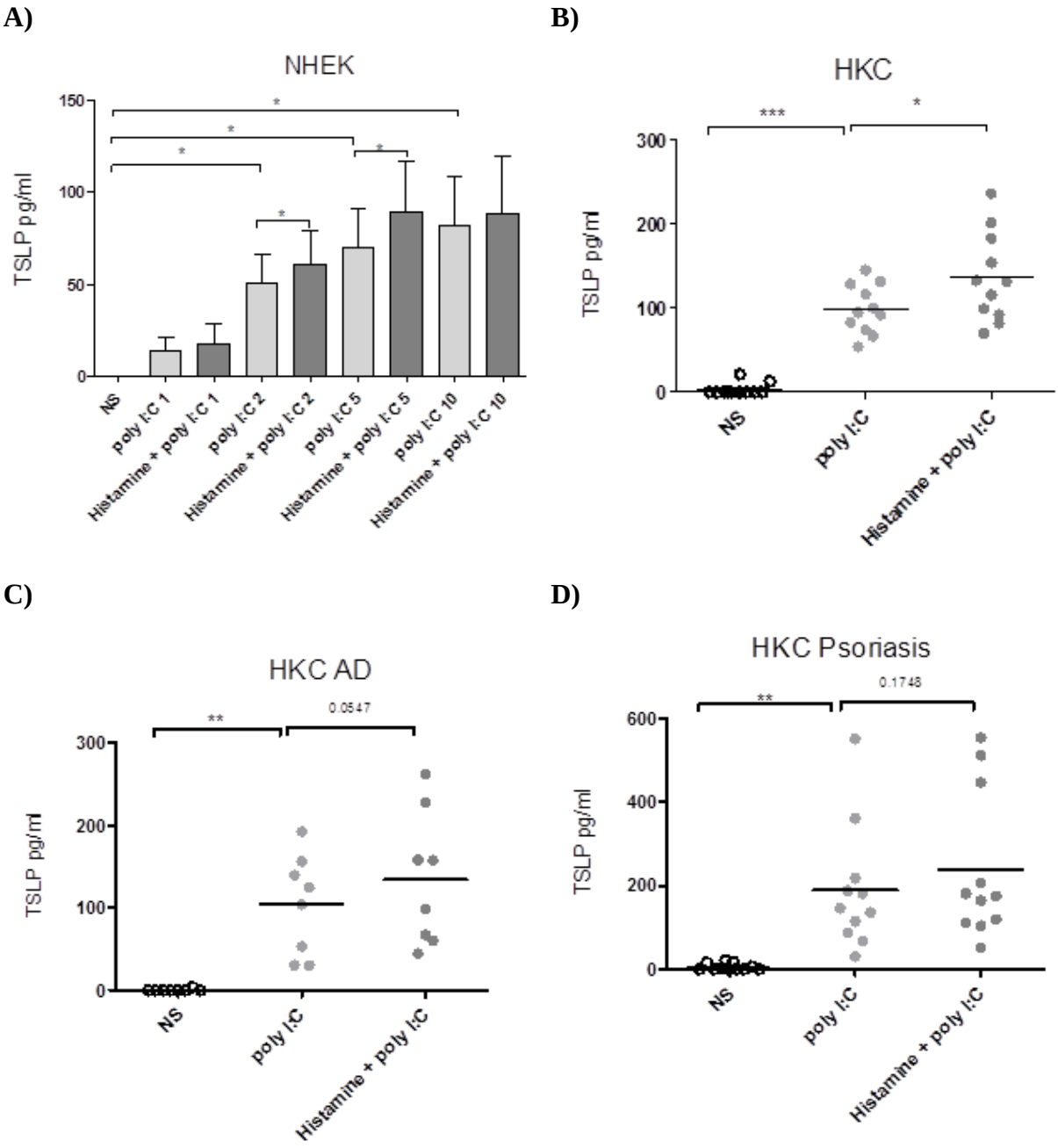


Figure 1. Poly I:C-induced TSLP-Production in NHEK. Histamine enhance the poly I:C-induced TSLP- production in both NHEK (A) as well as HKC from healthy donors (B). Keratinocytes from patients with AD (C) and psoriasis also produce more TSLP following incubation with histamine (D), also the data do not reach significance. Comparing the keratinocytes in regard to poly I:C-induced TSLP production, HKC from psoriatic patients show the highest amount (D). (NHEK: n=3-10; HKC NL n=11, AD n=8, Pso n=11)

Figure 2:

A) **B)**

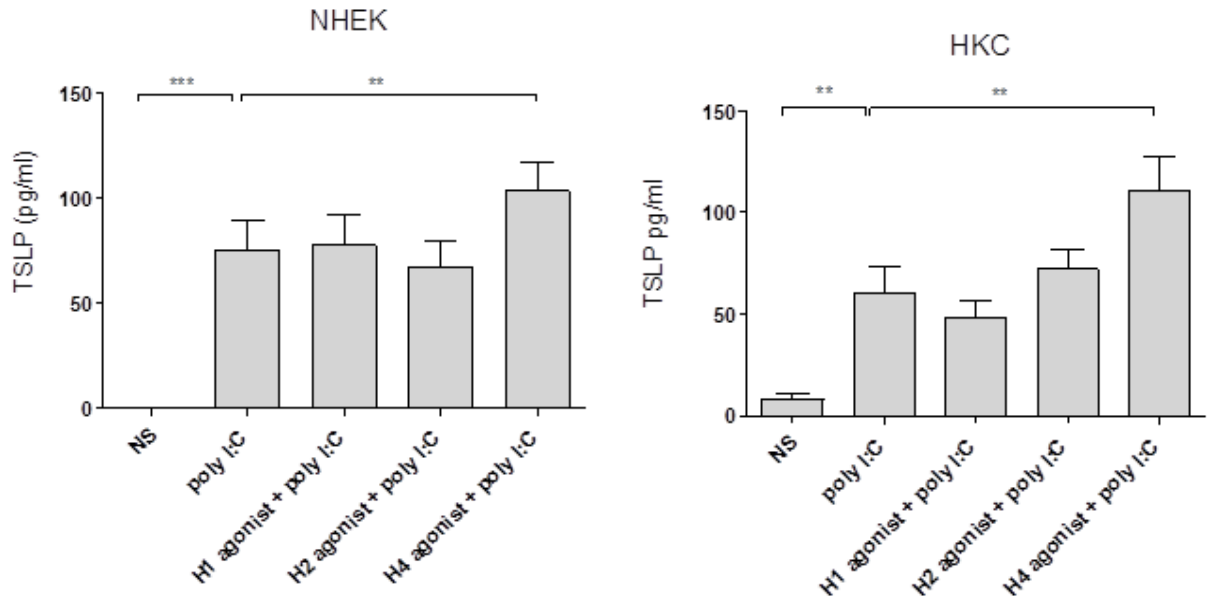
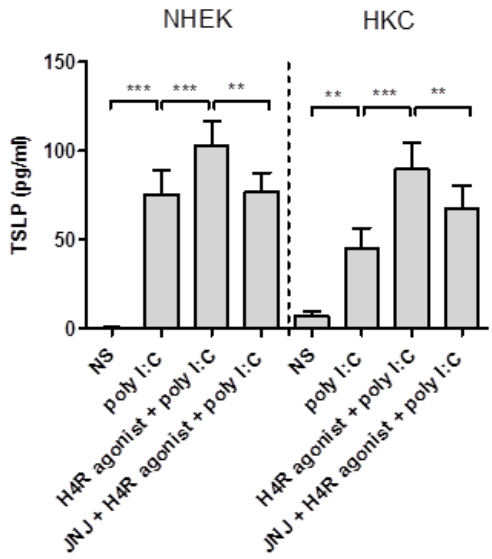


Figure 2. The H4R agonist 4-methylhistamine (4MH) in combination with poly I:C lead to an increase of TSLP production in NHEK (A) as well as HKC (B) compared to treatment with poly I:C alone, while we do not see an effect of other histamine receptor agonists. Keratinocytes were pre-treated with (H1R: 2-pyridylethylamine (10µM); H2R: amthamine (10µM); H4R: 4MH (10µM)) 24 h prior to poly I:C challenge for another 24 h. Mean and SEM of 12 (NHEK) or 9 (HKC) experiments are shown. ** P-Value < 0.01; *** P-Value < 0.001.

Figure 3:

A)



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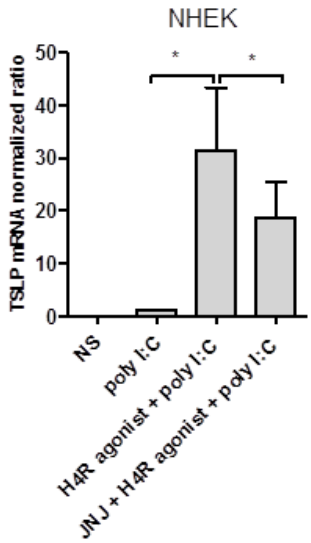

C)

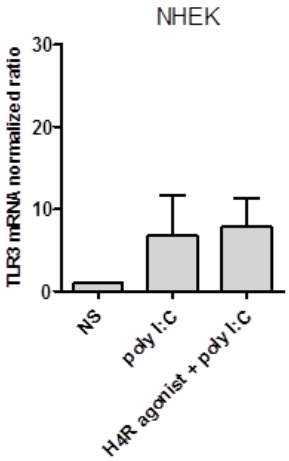

Figure 3. The increase of poly I:C (5µg/ml)-induced TSLP production by 4MH is blocked with the selective H4R antagonist JNJ7777120 (10µM) in both, NHEK and primary HKC (A). Using NHEK for PCR experiments, we also measure an upregulation at TSLP mRNA level upon stimulation with H4R agonist, which can be blocked with JNJ7777120 (B). Incubation with H4R has no influence on TLR3 expression compared to poly I:C treatment alone (C). Mean and SEM of 12 (NHEK), 14 (HKC) (A) or 6 (B/C) experiments are shown. * P-Value < 0.05; ** P-Value < 0.01; *** P-Value < 0.001.

Figure 4:

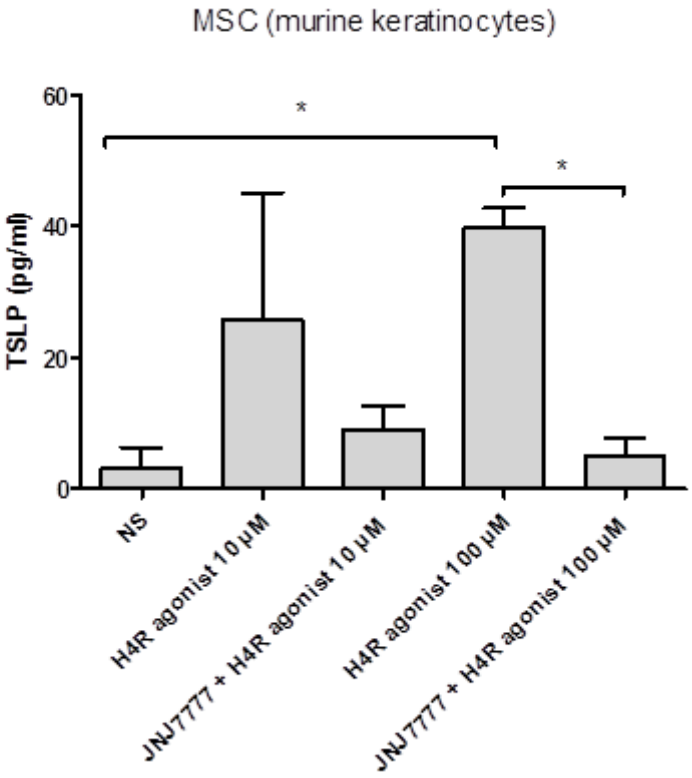

Figure 4: Murine keratinocytes (MSC) were incubated with the H4R agonists ST1006. In the indicated settings cells were pre-incubated with JNJ7777120 half an hour prior to H4R agonist. Mean and SEM of 6 experiments are shown. * P-Value < 0.05