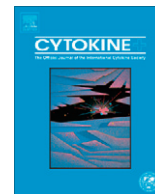




Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Expression analysis and specific blockade of the receptor for human thymic stromal lymphopoietin (TSLP) by novel antibodies to the human TSLPR α receptor chain

Andreas Borowski^a, Tina Vetter^a, Michael Kuepper^b, Andreas Wohlmann^a, Sebastian Krause^c, Thomas Lorenzen^d, Johann Christian Virchow^b, Werner Luttmann^e, Karlheinz Friedrich^{a,*}

^a Institute of Biochemistry II, Jena University Hospital, Jena, Germany

^b Department of Pneumology, University of Rostock, Rostock, Germany

^c INVIGATE GmbH, Jena, Germany

^d Institute of Transfusion Medicine, Jena University Hospital, Jena, Germany

^e ImmunoTools GmbH, Rostock, Germany

ARTICLE INFO

Article history:

Received 11 June 2012

Received in revised form 25 October 2012

Accepted 27 October 2012

Available online xxxx

Keywords:

Thymic stromal lymphopoietin

TSLP

Cytokine receptors

Inhibitory antibody

Asthma bronchiale

ABSTRACT

Thymic stromal lymphopoietin (TSLP) is an interleukin-7 (IL-7)-like cytokine with a pivotal role in development and maintenance of atopic diseases such as allergic asthma and atopic dermatitis. Moreover, recent studies show an involvement of TSLP in the progression of various cancers. TSLP signaling is mediated by the TSLP receptor (TSLPR), a heterodimeric type I cytokine receptor. It consists of the IL-7 receptor alpha chain (IL-7R α), which is shared with the IL-7 receptor, and the TSLPR α chain as a specific subunit. Blocking signal release by TSLP without affecting IL-7 function is a potentially interesting option for the treatment of atopic diseases or certain tumors. By employing the extracellular domain of human TSLPR α chain (hTSLPR α_{ex}) as an antigen, we generated a set of monoclonal antibodies. Several binders to native and/or denatured receptor protein were identified and characterized by cytometry and Western blot analysis. A screen based on a STAT3-driven reporter gene assay in murine pro-B cells expressing a functional hTSLPR yielded two hybridoma clones with specific antagonistic properties towards hTSLP, but not IL-7. Kinetic studies measuring blockade of hTSLP-dependent STAT phosphorylation in a TSLP-responsive cell line revealed an inhibitory constant in the nanomolar range.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The IL-7 like cytokine TSLP was first identified as a growth and differentiation factor for mouse B cells and T-cells in the culture supernatant of thymic stromal cells [1,2]. Human orthologs of both TSLP and the TSLP receptor (hTSLP and hTSLPR) were cloned in 2001, but sequence homology between human and mouse proteins is low [3–5]. The TSLP receptor is a heterodimeric cytokine type-I receptor [6,7] consisting of the IL-7 receptor alpha subunit (IL-7R α) and the TSLP receptor alpha chain 1 (TSLPR α). TSLPR α specifically binds TSLP, but also shows similarities to the common gamma cytokine receptor chain (γ_c). The TSLPR is mainly expressed in liver, brain, skeletal muscle, kidney, spleen and thymus [7]. Initial

reports pointed to differences between the murine and human TSLPR system with regard to intracellular signaling, holding that the hTSLPR triggers tyrosine phosphorylation of STAT5 without an involvement of Janus kinases [4–7]. We and others have shown recently, however, that human TSLP signals via tyrosine phosphorylation of STAT1, STAT3 and STAT5 and that JAK1 and JAK2, but not JAK3 and TYK2 are participating in this process [8–10]. hTSLP was identified a key mediator of atopic diseases such as allergic asthma and atopic dermatitis. Levels of hTSLP are elevated in the asthmatic lung and correlate with the severity of disease [11–14]. Experimental lung-specific expression of TSLP in mice leads to transgene-induced allergic airway inflammation characterized by a massive infiltration of leukocytes, goblet cell hyperplasia, and subepithelial fibrosis, as well as by increased serum IgE levels [11]. After temporary blockade of TSLP signaling, asthmatic symptoms could be reduced significantly in a mouse model [15]. In asthma, TSLP is highly expressed by epithelial cells and smooth muscle cells as a reaction to allergen challenge or treatment with NF- κ B activating cytokines such as IL-1 β and TNF- α [16]. TSLP promotes a TH2-driven immune response directly by action on T-cells [12], mast cells [17] and natural killer cells [18] and indirectly by

Abbreviations: hTSLP, human thymic stromal lymphopoietin; hTSLPR α , human thymic stromal lymphopoietin receptor α chain; hTSLPR α_{ex} , soluble extracellular domain of human thymic stromal lymphopoietin receptor α chain; γ_c , common gamma receptor chain; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

* Corresponding author. Address: Institute of Biochemistry II, Jena University Hospital, Nonnenplan 2–4, D-07743 Jena, Germany. Tel.: +49 3641 9 396405.

E-mail address: khf@mti.uni-jena.de (K. Friedrich).

activating CD11c⁺ dendritic cells (DCs) localized within the epithelium of lung or skin [11]. TSLP-primed DCs migrate to the lymph nodes where they activate CD4⁺ T-cells to differentiate to TH2 cells. Ultimately, TH2 cells infiltrate the lung where they produce large amounts of proinflammatory cytokines such as IL-4, IL-5, IL-13 and TNF- α , but, interestingly, no IL-10 or interferon- γ [13]. Within this sequence of events, TSLP represents a critical mediator in the development of asthmatic symptoms. Hence, a blockade of TSLP signaling is considered a promising therapeutic option for the treatment of atopic diseases [11,19].

In this work, we generated specific mouse monoclonal antibodies against the human TSLPR α chain. Two of the obtained antibodies efficiently block hTSLP-dependent activation of the hTSLPR without affecting IL-7 signaling. Another antibody clone allows for the characterization of hTSLPR surface expression on blood cells with unprecedented sensitivity.

2. Materials and methods

2.1. Cells and cell culture

All cells were maintained at 37 °C with 5% CO₂. HEK293T cells were obtained from the German Collection of Microorganisms and Cell Lines (DSMZ, Braunschweig, Germany) and cultured in DMEM with L-glutamine (Gibco, Darmstadt, Germany) supplemented with 10% heat inactivated fetal calf serum (GE Healthcare) and 1% Gentamycin (GE Healthcare, Munich, Germany). hTSLP responsive cell line Ba/F3-TSLPR stably expressing the bipartite human TSLP receptor has been described [10]. Ba/F3-TSLPR cells were grown in RPMI1640 supplemented with 10 ng/ml hTSLP (ImmunoTools, Friesoythe, Germany). Hybridoma clones were cultured in RPMI1640 with L-glutamine supplemented with 10% heat inactivated fetal calf serum, 1% Gentamycin and 20 ng/ml human IL-6 (ImmunoTools). X63AG8.653 myeloma cells were obtained from the German Collection of Microorganisms and Cell Lines (DSMZ). Human peripheral blood mononuclear cells (PBMCs) were isolated as described before [20].

2.2. DNA constructs and cell transfection

The coding sequence for the extracellular domain of hTSLPR α chain preceded by an amino-terminal signal sequence originating from the human IL-4 receptor α -chain and a P5D4 epitope tag was obtained by PCR using plasmid pcDNA-phTSLPR α [10] as template. Primers employed were STA12 (5'-CAGTGGATCCCTAAGAG ACCCTCATGTAT-3') and TSLPRex-rev (5'-CTGAAGTCGACTAT TAATGGTGATGATGGTGATGGCCTGACCCGAGCCATGGTGATGATG GTGATGGCC-3'). TSLPRex-rev encoded a double His₆ tag to be fused to the carboxyl terminus of the receptor domain for purification purposes. The PCR product was cleaved with Bam HI und Sal I and inserted into pcDNA3.1Neo (Invitrogen) to obtain expression plasmid pcDNA-phTSLPR α ex-His. Expression constructs pcDNA-pmTSLPR α , pcDNA-pmIL-7R α , and pcDNA-phIL-7R α encoding epitope-tagged murine TSLPR α chain, murine IL-7R α chain and the human IL-7R α , respectively, were described [10]. Expression plasmid pKCR-phyc encoding the human γ_c receptor chain has been published [21]. Reporter gene construct pGL3-SOCS3-luc has been described previously [10]. All constructs were verified by DNA sequencing.

2.3. Purification of soluble hTSLPR α receptor protein

For protein expression, samples of 2×10^7 HEK293T cells were transfected with 5 μ g of expression plasmid pcDNA-phTSLPR α ex-His by electroporation using the Nucleofector[®] device and Trans-

fection Kit V (Lonza, Bergisch-Gladbach, Germany). After cultivation for 72 h in 100 ml medium, supernatants were collected and dialyzed against a buffer containing 300 mM NaCl and 20 mM Na₂HPO₄ $\times$ 12 H₂O pH 7.5. Receptor protein was purified from conditioned medium by Nickel affinity chromatography employing an ÄKTA purifier FPLC system and Nickel sepharose matrix (GE-Healthcare). Elution was achieved by application of an imidazole gradient. Fractions were analyzed by Western blot, protein containing fractions were combined, dialyzed against PBS and stored after sterile filtration at a concentration of 0.5 mg/ml at 4 °C.

2.4. Immunization of mice and generation of hybridoma clones

Protein-based immunization of mice was performed to generate monoclonal antibodies (mAbs) to hTSLPR α (hTSLPR α ex). BALB/c mice were injected with 20–90 μ g hTSLPR α ex four times within 12 weeks. Two days after the final immunization, splenectomy was performed and spleen cells were fused with X63AG8.653 myeloma cells by PEG (Sigma, Karlsruhe, Germany). Subsequently, hybridoma cells were selected in hypoxanthine–aminopterin–thymidine (HAT) medium (Biochrom, Berlin, Germany). Hybridoma clones were subcloned three times in hypoxanthine–thymidine (HT) medium (Biochrom). Culture supernatants of individual clones were screened and verified for antibodies specifically recognizing hTSLPR α by binding to TSLPR α expressed on Ba/F3-TSLPR cells or to immobilized TSLPR α ex. Inhibitory characteristics were identified and characterized by interference with hTSLP-dependent proliferation, STAT tyrosine phosphorylation and proliferation of Ba/F3-TSLPR cells.

2.5. Purification and FITC conjugation of antibodies

Monoclonal antibodies were purified from supernatants of individual hybridoma clones by affinity chromatography using HiTrap Protein A HP matrix and an ÄKTA FPLC system (GE Healthcare). After elution by a shift to pH 3.0, purified antibodies were dialyzed against PBS, sterile filtered and tested for binding and inhibitory activity by flow cytometric analysis and reporter gene assay. Purity of antibodies was >99% as determined by SDS-PAGE.

Purified antibody preparations were conjugated to FITC basically by the protocol of The and Feltkamp [22]. Briefly, 1 mg purified antibody dissolved in 2 ml 0.5 M Carbonate buffer pH 9.5 was combined with 160 μ g FITC dissolved in 20 μ l DMSO. Conjugation was achieved by agitation for 90 min at room temperature in the dark. Fluorescein-conjugated antibody was transferred to storage buffer (PBS/1% BSA/0.1% NaN₃). Absence of non-specific binding to Ba/F3 cells was verified by cytometry.

2.6. Flow cytometry

For flow cytometry experiments, samples of 5×10^5 Ba/F3-TSLPR cells Ba/F3-TSLPR were incubated with 1 ml hybridoma supernatant or 1 μ g purified antibody for 1 h, washed three times with PBS and incubated with a FITC-labeled secondary anti-mouse antibody (ImmunoTools) for 30 min in the dark. Cells were analyzed on a CyFlow[®] space instrument (Partec, Münster, Germany) and analyzed using the Cyflogic[®] software (freeware). Dead cells were excluded by gating out PI-positive cells during assessment of antibody binding. As controls, samples treated with either FITC-labeled secondary anti-mouse antibody alone or IgG1 control antibody were employed. Other antibodies used were obtained from eBioScience (Frankfurt, Germany) (monoclonal anti-human TSLPR), Biolegend (San Diego, CA, USA) (monoclonal anti-human TSLPR), R&D Systems (Wiesbaden, Germany) (polyclonal serum against hTSLPR), and ImmunoTools (APC-conjugated anti-CD14 and PE-conjugated anti-CD19).

2.7. Elisa

Antibody binding to soluble hTSLPR α protein or interaction of recombinant hTSLPR α ex with ligand TSLP was assayed by determining binding to recombinant protein immobilized on 96 well ELISA plates (Greiner, Frickenhausen, Germany). Plates were incubated overnight with protein at various concentrations at 4 °C, washed three times with 300 μ l PBS and blocked with 2% BSA for 2 h. After three additional washes with PBS, 100 μ l of hybridoma supernatant, PBS containing purified antibody, or recombinant soluble hTSLPR α ex protein (as applicable) was added and incubated under gentle agitation for 2 h at room temperature. In the latter case, an additional round of washing was performed, followed by incubation with 15 μ g/ml purified monoclonal anti-P5D4 antibody (directed to the aminoterminal P5D4 tag of the receptor protein). Antibody binding was detected using a HRP-coupled secondary anti-mouse antibody diluted 1:2000 and a HRP specific substrate (Glo Substrate Reagent Pack, R&D Systems). Binding was quantified by analyzing chemiluminescence at 495 nm using a plate reader (TECAN, Crailsheim, Germany).

2.8. SDS PAGE, Western blot

Samples of 2×10^6 Ba/F3-TSLPR cells were washed three times in RPMI1640, cytokine-starved for 12 h in growth factor free culture medium, optionally supplemented with antibody to TSLPR α and subsequently stimulated with 10 ng/ml human TSLP for 30 min. Cells were then washed with ice-cold PBS and cell lysates were prepared using NP-40 lysis buffer containing Complete Mini (Roche Applied Biosciences) and phosphatase inhibitors (Sigma-Aldrich, Taufkirchen, Germany) as described previously [10]. 40 μ g of protein were loaded per lane and separated by electrophoresis on 8.4% SDS-gels, transferred to nitrocellulose membrane and blocked with 5% dry milk for one hour. Proteins were probed with antibodies established in this study (hybridoma supernatant or purified immunoglobulin, compare 3.2) or antibodies to tyrosine phosphorylated STAT1 (p-STAT1: pTyr(701) from Santa Cruz Biotechnology, Biotechnology, Heidelberg, Germany), STAT3 (p-STAT3: pTyr(705) from Cell Signaling Technology, Frankfurt, Germany, and STAT5 (pTyr(694) from Cell Signaling Technology, Frankfurt, Germany) and then detected using an enhanced chemiluminescence kit (Roche, Mannheim, Germany). For loading control, blots were stripped and reprobed with antibodies to STAT1, 3, or 5 (Cell Signaling Technology).

Silver staining of PAA gels was performed according to a published protocol [23].

2.9. Reporter gene assay

For reporter gene assays, Ba/F3-TSLPR cells were washed three times in PBS and then cytokine-starved for 6 h in growth factor free culture medium. Transfection with 3 μ g of reporter gene plasmid pSOC3-luc by electroporation was performed as described [10] using the Nucleofector[®] device (Lonza, Bergisch-Gladbach, Germany) and Transfection Kit V following the manufacturer's instructions.

For inhibition experiments, antibody was added to cell samples at varying concentrations one hour before stimulation with 300 pg/ml hTSLP (ImmunoTools), 5 ng/ml mTSLP (R&D Systems), or 5 ng/ml and hIL-7 (ImmunoTools), respectively. 16 h after stimulation, cells were harvested and lysed with the Passive Lys Buffer (PLB, Promega) for 30 min. 20 μ l of cell lysate were mixed with 30 μ l Luciferase Assay Reagent (LAR, Promega) and luminescence was measured in a microplate luminometer (GloMax 96, Promega).

2.10. Cell proliferation assay

Samples of 10^4 growth factor-starved Ba/F3-TSLPR cells in 100 μ l culture medium were stimulated with 5 ng/ml hTSLP (ImmunoTools) for 72 h before proliferation was quantified using the "CellTiter 96 Aqueous One Solution Cell Proliferation Assay" Kit (Promega) following the manufacturer's instructions. Absorption was measured using a microplate reader (Sunrise; TECAN, Crailsheim, Germany) at a wavelength of 490 nm. In inhibition experiments, cell samples were incubated with antibodies at varying concentrations one hour before hTSLP stimulation.

3. Results

3.1. Generation and characterization of recombinant soluble hTSLPR α protein

To generate human TSLPR α protein suitable to serve as antigen for the immunization of mice, a cDNA sequence encoding the

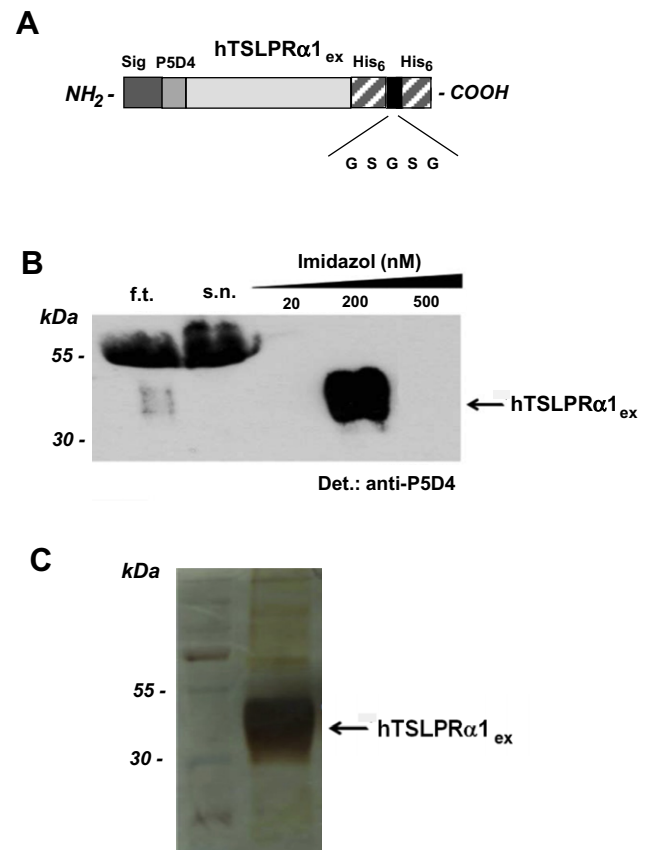


Fig. 1. Structure, expression and purification of recombinant soluble hTSLPR α protein. (A) Schematic representation of the engineered extracellular domain of hTSLPR α employed as antigen in this study: (Sig = signal peptide of the human IL-4R α chain; P5D4 = P5D4 epitope-tag derived from Vesicular Stomatitis Virus; hTSLPR α ex = extracellular domain of hTSLPR α chain; His₆ = His₆ tag; GSGSG = internal Glycine-Serine linker). (B) and (C) Purification of tagged hTSLPR α ex protein analyzed by Western-Blot and SDS-PAGE/silver stain analysis. Culture supernatant from transiently transfected HEK293T cells was subjected to nickel sepharose chromatography. Raw supernatant (s.n.), column flowthrough (f.t.) and fractions after elution with the indicated concentrations of imidazole (50 μ g protein per lane) were probed after separation through an 8.4% SDS-PAA gel and transfer to nitrocellulose with an antibody directed to the amino-terminal P5D4 tag. The fractions eluted by 200 nM imidazole were collected, dialyzed against PBS and stored at -20 °C. 1 μ g of hTSLPR α ex protein preparation was analyzed for purity by separation through an 8.4% SDS-PAA gel and subsequent silver staining (C).

extracellular domain of TSLPR α flanked N-terminally by a signal peptide sequence and a P5D4 epitope tag and C-terminally by a twin His₆ tag (see Fig. 1A) was inserted into expression vector pcDNA3.1Neo as detailed in the Materials and Method section to yield expression plasmid pcDNA-pTSLPR α ex-His. Transient transfection of HEK293T cells with the expression construct followed by 72 h of incubation resulted in accumulation of recombinant protein in the culture supernatant and allowed for its efficient preparation by nickel-sepharose based affinity chromatography. The soluble receptor domain could be specifically eluted with 200 nM imidazole. Routinely, yields of roughly 0.5 mg receptor exodomain per liter of conditioned culture medium were obtained with a typical purity >85% as analyzed by SDS-PAGE, immunoblotting and silver staining of SDS gels (Fig. 1B and C).

The receptor protein was tested for its ligand binding proficiency and, thus, correct folding by quantifying interaction of purified hTSLPR α ex with immobilized hTSLP. Specific ligand binding was observed, saturation occurred at a receptor concentration of 5 μ g/ml or roughly 1 nM (Fig. 2A). This value is consistent with the affinity of cell-bound hTSLP receptor [4,5] and, thus, strongly argues for native folding of the recombinant receptor protein. This notion is further backed up by the finding that recombinant hTSLPR α ex could be readily released by from solid-phase bound hTSLP by competition with soluble hTSLP (Fig. 2B).

3.2. Generation of monoclonal antibodies to the hTSLP receptor α chain and characterization of antigen binding properties

Purified, epitope and His-tagged recombinant hTSLPR α ex was employed for immunization of Balb-c mice and generation of hybridoma clones as described in the Material and Methods section. Culture supernatants of hybridomas were collected and combined to pools each containing conditioned medium from roughly ten individual clones. They were incubated with murine Ba/F3-TSLPR expressing heterologous hTSLPR and tested for the presence of mAbs with specific affinity for the receptor by flow cytometry (Fig. 3A). Sixteen pools positive for receptor binding were subjected to limiting dilution to yield individual clones. Supernatants from roughly 200 clones were subsequently screened by ELISA for binding to immobilized hTSLPR α ex. Five clones were identified which revealed intense binding to the antigen, but did not show significant interaction with amino- or carboxyterminal tags intrinsic to the recombinant receptor protein or with serum components, respectively (data not shown).

To obtain semi-quantitative information about the affinity for the native hTSLPR protein, we employed clone VGG12 (chosen as representative clone due to its particularly weak unspecific binding to serum) to cytometrically analyze its interaction with Ba/F3-TSLPR cells and to compare it with established monoclonal

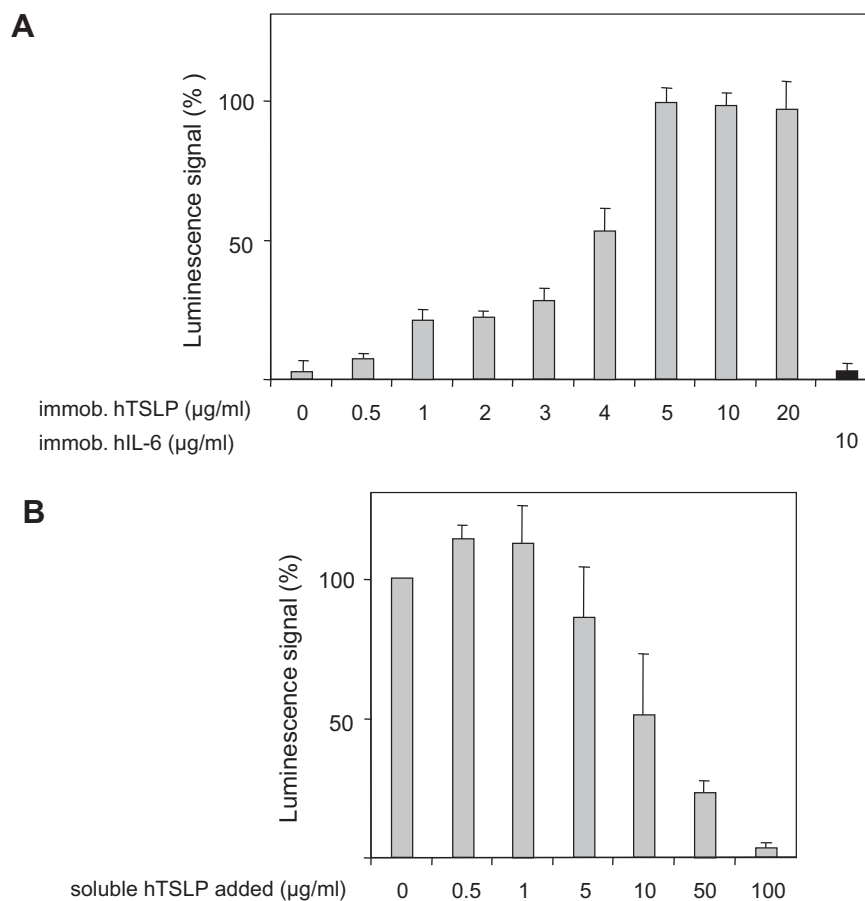


Fig. 2. Specific ligand binding properties of recombinant hTSLPR α ex. (A) ELISA plates were coated with hTSLP by overnight incubation with the indicated concentrations of cytokine and subsequently incubated with a saturating concentration of recombinant tagged hTSLPR α ex (15 μ g/ml). For detection and quantification of antibody binding, an anti-P5D4 antibody and a HRP-coupled secondary antibody were used to elicit luminescence signals as described in Section 2. The maximum signal achieved was set 100%. As specificity control, a parallel experiment employing plates coated with 10 μ g/ml human IL-6 was performed. Data is derived from three independent experiments. (B) Displacement of hTSLPR α ex from solid phase bound hTSLP by soluble hTSLP. ELISA plates were coated with 5 μ g/ml hTSLP and saturated with 15 μ g/ml hTSLPR α ex as in (A). Subsequently, increasing concentrations of soluble hTSLP were added. After washing with PBS, remaining solid phase bound hTSLPR α ex was quantified by means of anti-P5D4 as in (A). The signal obtained without addition of soluble TSLP was set 100%. IL-6-coated plates were used as control as in (A). Data is derived from three independent experiments.

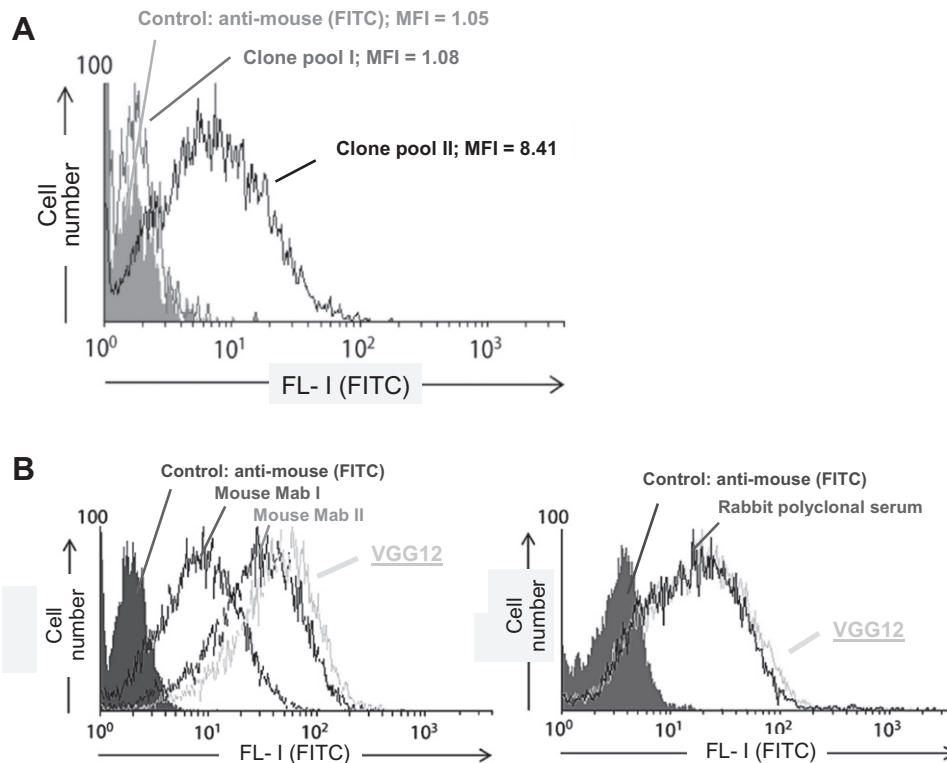


Fig. 3. Identification of monoclonal antibodies with specific affinity to hTSLPR α . (A) Cytometric analysis of two hybridoma clone pools for antibodies binding to hTSLPR α . Samples of murine Ba/F3-TSLPR expressing heterologous hTSLPR were incubated with conditioned medium from hybridoma pools, stained with FITC-labeled goat anti-mouse antibody and analyzed for binding by flow cytometry. Fluorescence intensity is plotted against cell number, a control sample (gray) was incubated with FITC-labeled secondary antibody only. A typical run from three experiments is shown. Mean fluorescence intensity (MFI) values were determined using the Cylogig[®] software and are given with the respective curves. (B) Comparative evaluation of surface antigen recognition by antibody VGG12. Samples of 10^4 Ba/F3-TSLPR cells were incubated with 1 μ g of VGG12 for 1 h. A FITC-conjugated secondary goat anti-mouse antibody was employed to monitor binding by flow cytometry. For comparison, cell samples were stained with two established commercial mouse monoclonal antibodies (Mabs I and II, left hand side) and with a rabbit polyclonal serum to hTSLPR α (right hand side). Typical runs from three experiments are shown.

antibodies (Fig. 3B) and a polyclonal antiserum (Fig. 3C) to the hTSLPR. In these tests, VGG12 showed a binding strength exceeding that of all as yet available monoclonal antibodies to the hTSLPR. Notably, the signal elicited by VGG12 is comparable in intensity with that of a polyclonal antiserum.

To evaluate the applicability of VGG12 to sensitively detect TSLPR α expression on relatively rare cell populations in human blood, we double-stained human PBMCs with FITC-conjugated VGG12 and either APC-conjugated anti-CD14 or PE-conjugated anti-CD19 specific for monocytes or B cells, respectively. As shown in Fig. 4, TSLPR α surface expression could be readily detected on both monocytes and B cells within PBMCs without prior enrichment.

Finally, we were interested in the ability of the novel antibodies to discriminate between native and denatured hTSLPR α protein. Hence, antibodies VGG12, D9C10, D9E9, 1E10 and 6F4 were employed to probe recombinant, P5D4-tagged hTSLPR α ex protein in Western blot experiments (Fig. 5A). In addition, 1E10 was also tested for recognition of full-length hTSLPR α in lysates of Ba/F3-TSLPR cells (Fig. 5B). VGG12, 1E10 and 6F4 can detect denatured protein and are likely to recognize continuous epitopes within the protein. D910 and D9E9 require native receptor protein to bind. They are, thus, probably conformation specific antibodies.

3.3. Identification and characterization of antibodies to hTSLPR α with antagonistic properties

All antibodies to hTSLPR α which had been shown to specifically bind the receptor protein were screened for inhibitory properties

with regard to hTSLP-induced hTSLPR activation. To this aim, Ba/F3-TSLPR cells were subjected to hTSLP induced reporter gene activation assays in the presence of hybridoma clone culture supernatants. In this test, two hTSLPR α binding antibodies reduced the cells' response to hTSLP by 60–70%. (Fig. 6A). The two antibodies 1E10 and 6F4 were purified and characterized for dose dependency and specificity of their inhibitory properties in comparison to a non-inhibitory hTSLPR α binder by reporter gene and proliferation assays. As shown in Fig. 6B and C, hTSLP-induced signal transduction could be reduced by more than 90% by antibodies 1E10 and 6F4, half maximal inhibition was reached at concentrations around 15 μ g/ml (100 nM). To further evaluate the inhibitory performance of 1E10 and 6F4, we tested comparable concentrations of a commercial goat polyclonal antiserum (R&D Systems) for potential neutralizing activity towards TSLP-induced reporter gene activation and did not observe significant effects (data not shown).

To verify the specificity of the antibodies' inhibitory properties towards the human TSLP receptor, related receptors were expressed transiently in Ba/F3 cells, stimulated by their respective ligands and analyzed for reporter gene activation in the presence of 1E10 or 6F4. Fig. 6D shows that both antibodies efficiently blocked signal transduction by the hTSLPR, neither the human IL-7 receptor nor the murine TSLP receptor was affected. Lastly, we performed a saturation binding experiment to quantify the affinity of inhibitory antibody 1E10 for immobilized hTSLPR α ex protein. As evident from Fig. 6E, half maximal binding is reached at an antibody concentration of roughly 2 nM. This result places 1E10 clearly among high affinity antibodies.

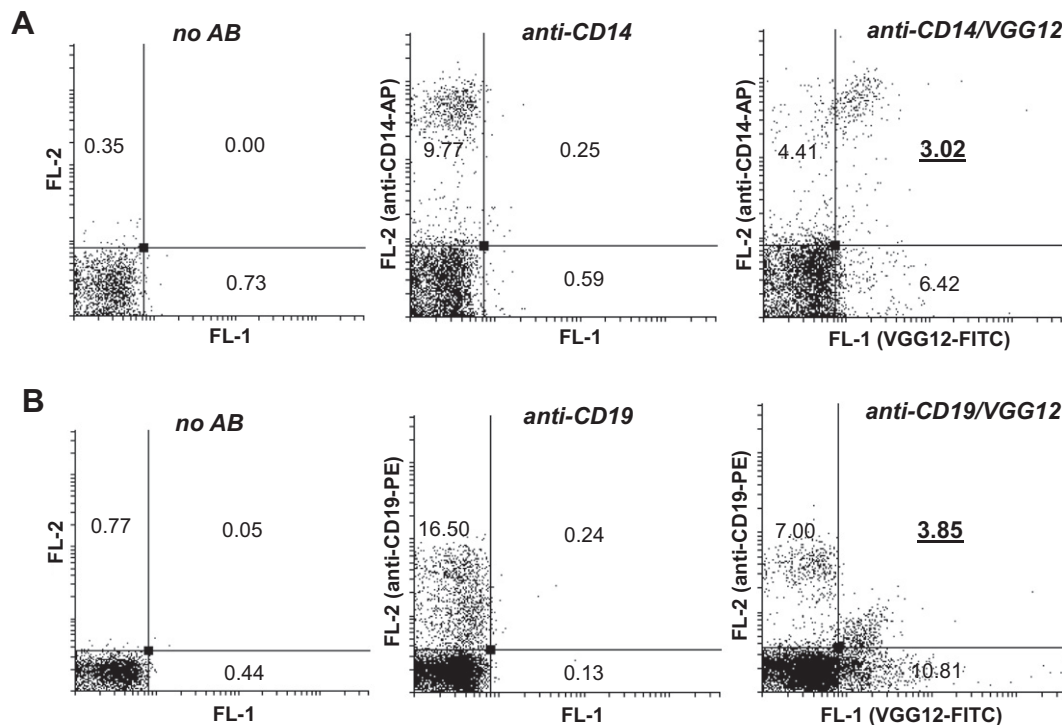


Fig. 4. Cell-type specific cytometric analysis of hTSLP α surface expression in human blood. Samples of roughly 10^5 human peripheral blood mononuclear cells (PBMCs) were kept for 12 h in RFG medium and were then simultaneously incubated with 1.5 μ g of each FITC-conjugated anti-hTSLP α 1 antibody VGG12 and either (A) anti-CD14-APC or (B) anti-CD19-PE specific for monocytes or B cells, respectively in a volume of 50 μ l for 1 h at room temperature in the dark. Vital cells were gated by forward/sideward scatter detection and analyzed by dot-plot representation. Control measurements were performed with unstained PBMCs and PBMCs stained only with cell type specific antibodies anti-CD14 or anti-CD19 as indicated. Percentages of single and double positive cells for FITC- and APC/PE-specific fluorescence channels FL-1 and FL-2 are given in the respective quadrants. Data from a typical out of four independent determinations is shown.

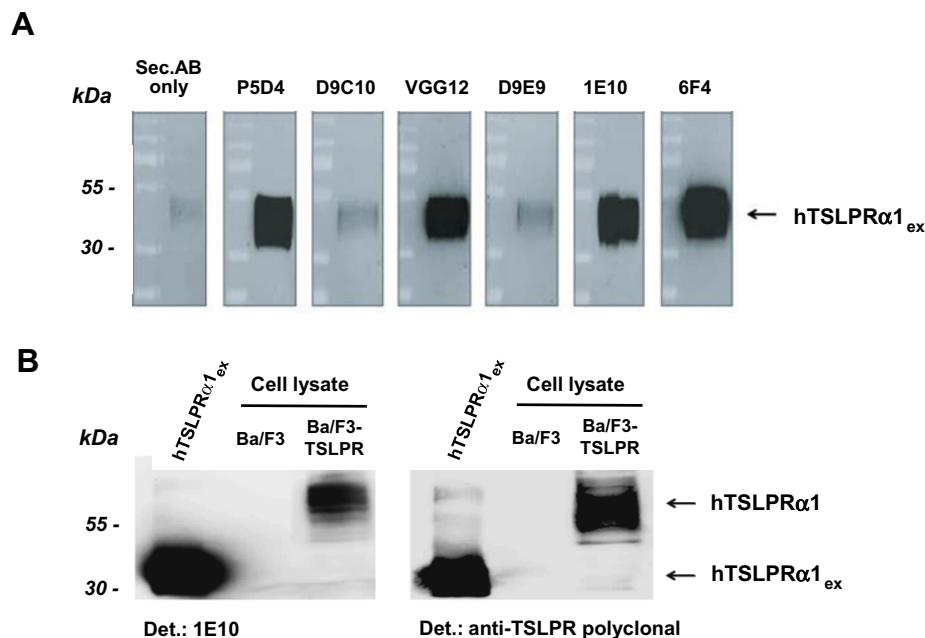


Fig. 5. Analysis of antibodies to TSLP α _{ex} protein for recognition of denatured antigen. (A) Employment of Mabs as probes to detect recombinant TSLP α _{ex} in Western blot analysis. Samples of 500 ng of purified recombinant receptor domain (compare Fig. 1) were separated by PAGE, blotted onto nylon membrane and incubated overnight with solutions containing 1 μ g/ml of the indicated antibodies. Antibody binding was subsequently visualized using enhanced chemiluminescence. (B) Test of antibody 1E10 for Western blot detection of full length hTSLP α in cell lysates. Samples of 10^6 Ba/F3 cells or Ba/F3-TSLPR cells stably expressing hTSLP α were lysed, and 40 μ g of protein extracts were subjected to PAGE and Western blotting. 500 ng recombinant TSLP α _{ex} served as positive control. For comparison, an equivalent blot was probed with a polyclonal antiserum to TSLP α (right hand side).

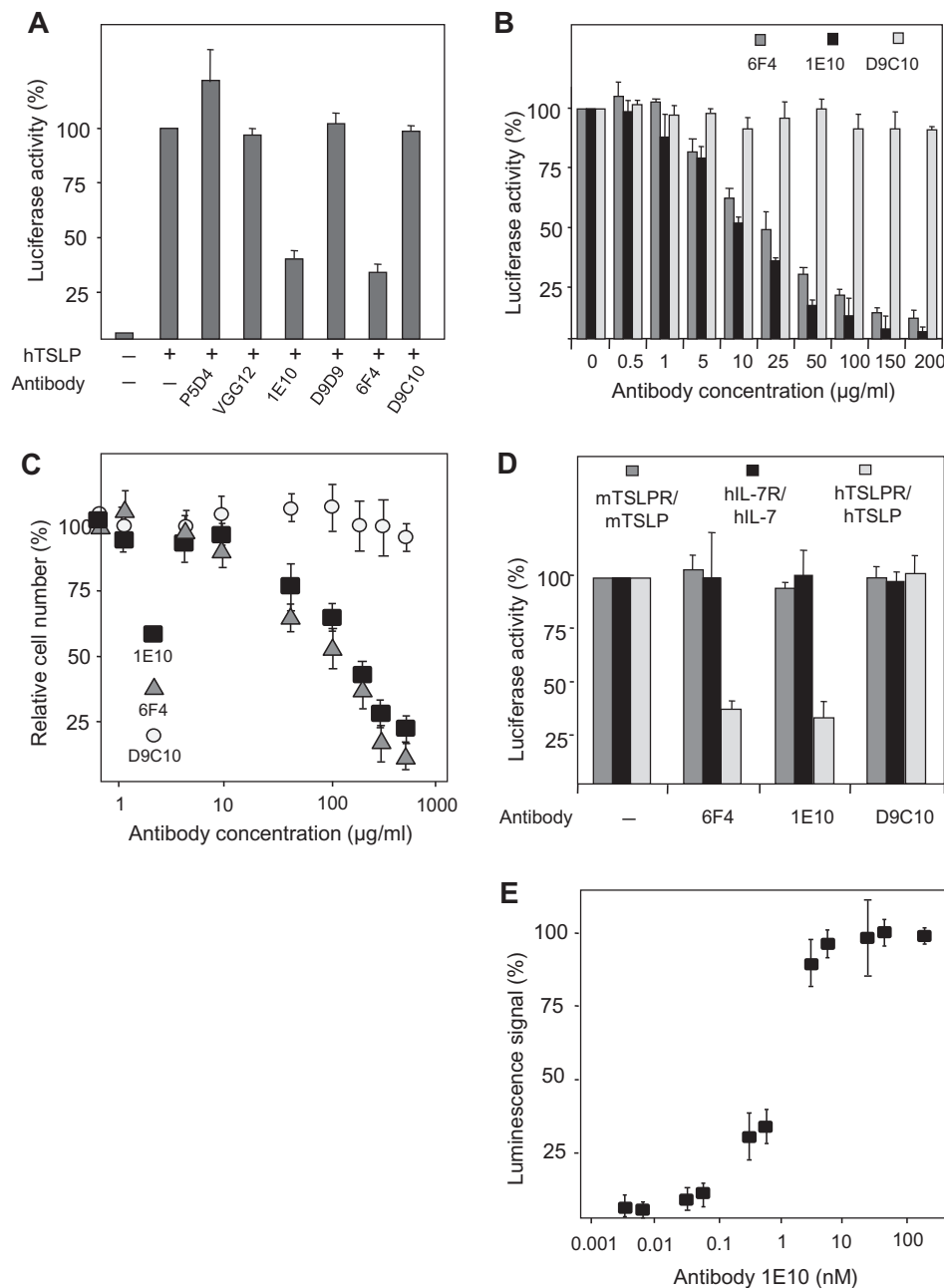


Fig. 6. Identification and characterization of hTSLPR α 1 binding antibodies with inhibitory properties. (A) Influence of antibodies to hTSLPR α on hTSLP dependent reporter gene activation in Ba/F3-TSLPR cells. Antibodies from 1.5 ml culture supernatant of the indicated hybridoma clones were collected by incubation with 10 μ l Protein A Sepharose (Sigma) for 12 h at room temperature. Immunoglobulins were released from the beads by incubation with 20 μ l 100 mM Glycine pH 3.0 followed by neutralization by addition of 2 μ l 500 mM Na₂PO₄ pH 7.5. Antibodies were incubated for 1 h with samples of 5×10^4 starved Ba/F3-TSLPR cells previously transfected with STAT3-inducible reporter gene construct pGL-SOCS3-luc before cells were stimulated with hTSLP and analyzed luminometrically for luciferase activity as described in Material and Methods. Luciferase activity elicited by hTSLP without prior addition of antibody was set 100%. Shown is one representative out of two independent experiments. All tests were performed in triplicate. (B) Dose-dependent inhibition of hTSLP-induced reporter gene activity in Ba/F3-TSLPR cells by purified antibodies. Samples of 5×10^4 of Ba/F3 TSLPR cells previously transfected with pGL-SOCS3-luc were incubated for 1 h with the indicated concentrations of purified antibodies 6F4 (dark gray bars) or 1E10 (black bars). Cells were then stimulated with hTSLP and luciferase activity was determined as in (A). As negative control, non-inhibitory antibody D9C10 (light gray bars) was employed. (C) Dose-dependent inhibition of hTSLP-induced proliferation of Ba/F3 TSLPR cells by purified antibodies. Samples of 5×10^4 transfected Ba/F3 TSLPR cells were hTSLP-starved for 6 h and incubated with the indicated concentrations of antibodies 1E10 (black squares) or 6F4 (gray triangles) for 1 h. Non-inhibitory antibody D9C10 (light gray circles) was employed as controls. Cells were then stimulated with 5 ng/ml hTSLP for 72 h and proliferation was determined luminometrically using the CellTiter 96 Kit (Promega). Shown is one of three independent experiments, data points were measured in triplicate. (D) Analysis of inhibitory specificity of antibodies for the human TSLP receptor. Samples of 4×10^5 Ba/F3 cells were transfected transiently with combinations of expression plasmids encoding cytokine receptor chains to yield functional receptors for murine TSLP (pcDNA-pmTSLPR α and pcDNA-pmIL-7R α , dark gray bars), human IL-7 (pcDNA-phIL-7R α and pKCR-phyc, black bars), or human TSLP (pcDNA-phTSLPR α and pcDNA-phIL-7R α , light gray bars), respectively, along with reporter gene construct pGL-SOCS3-luc. Cells were incubated with 200 μ g/ml inhibitory antibodies (1E10 or 6F4) or non-inhibitory control antibody D9C10 as indicated for 1 h before stimulation with receptor specific cytokines (5 ng/ml). After 16 h, cells were lysed and luciferase expression was quantified luminometrically as described in Material and Methods. Shown is a typical out of three independent experiments, data points were measured in triplicate. (E) Determination of specific affinity for hTSLPR α of inhibitory antibody 1E10: ELISA plates were coated with 15 μ g/ml hTSLP α and subsequently incubated with the indicated concentrations of 1E10. Detection and quantification of antibody binding was performed using a HRP-coupled secondary antibody as described in Section 2 and in Fig. 1. The maximum signal achieved was set 100%. All measurements were performed in triplicate, a typical out of three independent experiments is shown.

3.4. Specific inhibition of TSLP-induced intracellular signal transduction by an inhibitory antibody to TSLPR α

To further demonstrate the applicability of the novel inhibitory antibodies to block hTSLP-dependent cellular responses, we studied their effects on transcription factor activation. Ba/F3-TSLPR cells were analyzed for TSLP-dependent tyrosine phosphorylation of STAT1, 3, and 5 upon stimulation with hTSLP both in the absence and presence of varied concentrations of antibody 6F4. Fig. 7 shows that hTSLP-induced tyrosine phosphorylation of all three STAT factors was blocked by 6F4 in a dose-dependent manner with a half-maximal effect in the range of 10 $\mu\text{g/ml}$. The specificity of this effect is demonstrated by the failure of D9C10, a high affinity hTSLPR α binder (compare Fig. 3) to interfere with hTSLP-dependent tyrosine phosphorylation of STATs in Ba/F3-TSLPR cells even at extremely high concentrations. A comparable effect was observed with antibody 1E10.

4. Discussion

The TSLP receptor system is of major medical interest due to its emerging importance in chronic inflammatory processes, e.g. allergic asthma as well as in certain cancers. We have generated a set of novel monoclonal antibodies to the hTSLPR α subunit which improve diagnostic sensitivity for TSLPR expression and, for the first

time, allow for specific blockade of hTSLP-dependent activation of the hTSLPR. The latter option obviously has potential therapeutic implications.

Inhibition of hTSLP-driven reactions proved efficient to alleviate asthmatic symptoms in mouse disease models. In one published approach this was achieved by a fusion of the extracellular domain of murine TSLPR α and an IgG2a Fc fragment. Intratracheal administration of this fusion protein prior to sensitization of mice significantly reduced symptoms such as allergen-specific serum IgE, airway tissue inflammation, inflammatory cell infiltration and bronchoalveolar TH2 cytokine levels [24]. In an alternative approach, asthmatic mice were treated intratracheally with a monoclonal antibody to the murine TSLPR which was able to interfere with mTSLP-dependent receptor activation [15]. Notably, this reagent significantly reduced eosinophilic airway inflammation, goblet cell hyperplasia and TH2 cytokines production. The authors showed that these effects were due to blockade of airway dendritic cell function in priming CD4⁺T cells in response to mTSLP stimulation.

These findings strongly suggest to develop reagents that can efficiently inhibit ligand-dependent activation of the human TSLP receptor. Various strategies are being pursued to achieve a blockade of cytokine signaling such as the employment of scavenging soluble receptor molecules, antagonistic cytokines acting as competitors or receptor blocking antibodies [25,26]. The receptor

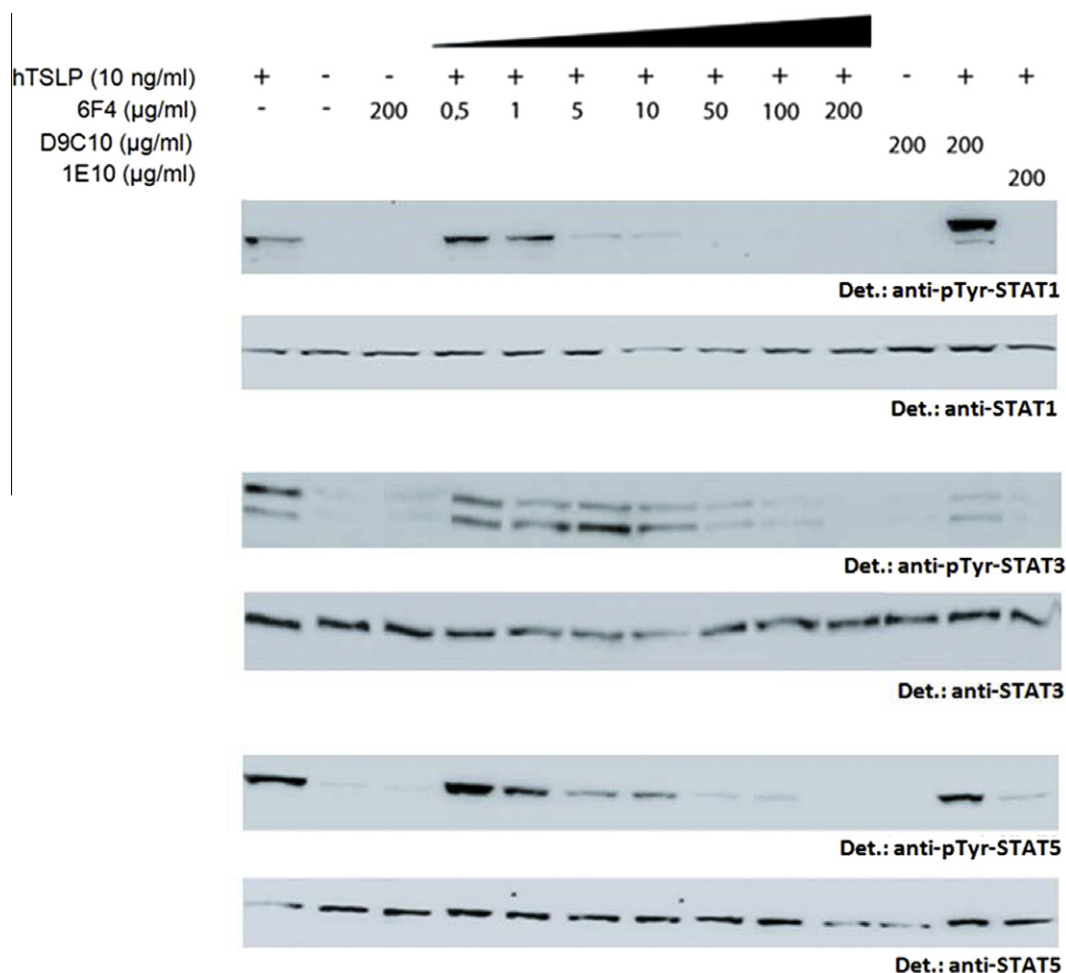


Fig. 7. Blockade of hTSLP-induced intracellular signal transduction by inhibitory antibodies 6F4 and 1E10. Samples of 1×10^6 Ba/F3 TSLPR cells were starved for 12 h in the absence or presence of the indicated concentrations of 6F4 or 1E10. As control, a 200 $\mu\text{g/ml}$ concentration of non-inhibitory antibody D9C10 was employed. Cells were then optionally stimulated with hTSLP (10 ng/ml) for 30 min, lysed and subjected to Western blot analysis. Blots were probed with the indicated specific antibodies to tyrosine phosphorylated STATs and, to verify equal loading, reprobed with antibodies recognizing the respective STATs in both phosphorylated and non-phosphorylated form.

antibody approach offers the advantage of inhibiting cytokine-induced signal transduction immediately and specifically on reactive cells. Furthermore, a relatively low number of receptor molecules can principally be saturated more easily than a higher concentration. Targeting membrane receptors by antibodies should be superior over targeting cytokine since antibodies to cytokines have the potential to stabilize circulating cytokine molecules and, thus, may ultimately increase the equilibrium cytokine concentration [27].

Although the receptor complex for hTSLP utilizes comprises as one component the hIL-7 receptor α -chain, the inhibitory antibodies developed in this study are highly specific for hTSLP signaling, and do not affect activities of IL-7. This is important for potential applications, since interference with IL-7 effects would disturb critical functions of IL-7 in the immune system such as control of T and B cell development [28–30]. Moreover, antibodies 6F4 and 1E10 antigen show inhibitory constants of roughly 100 nM, a figure well in the range of IC_{50} values measured for established inhibitory antibodies used in therapy such as Herceptin, Cetuximab or Bevacizunab [31–33]. It is conceivable to assume that the antigen affinity of the antibodies hTSLP α can be further increased by humanization as has been shown throughout the development of inhibitory antibodies to the Her2 receptor [34].

Asthmatic symptoms are critically influenced by both TSLP and IL-13. These two factors are believed to play combined roles in disease development and progression, a notion supported by the finding that TSLP obviously functions both upstream and downstream of IL-13 in murine airway tissue [35]. Consequently, after having generated inhibitory antibodies to the human IL-13 receptor [20], we now targeted the hTSLPR. The fact that in a mouse models, antagonistic reagents with the property to efficiently block asthma-related mTSLP signaling were developed [24,15] perspectively opens up the possibility to be applied in a combinatorial manner together with anti-IL-13R inhibitors. Such a strategy might offer unprecedented efficiency and specificity in the control of allergic inflammation.

Recently, growing evidence is emerging for important roles of hTSLP in cancer. For instance, 15% of patients with Acute Lymphatic Leukemia (ALL) have mutations in the hTSLP α 1 receptor, which in turn are associated with an unfavorable prognosis [36–38]. TSLP is also a determinant of tumor progression in breast cancer [39] as well as in pancreatic and lung cancer. The function of TSLP in these settings is that it promotes the development of regulatory T cells, TH2 cells and dendritic cells. This was shown to result in a decreased immune response and in enhanced metastasis [40–43]. These findings suggest that sensitive detection of hTSLPR expression and specific blockade of hTSLP-dependent signaling may be of considerable pharmacological interest also for the diagnosis and treatment of certain cancer entities.

Acknowledgements

This work was supported by Grants FR 854/5-1 and VI 193/6-1 from the Deutsche Forschungsgemeinschaft (DFG), by the Thüringer Aufbaubank (European Regional Development Fund Grant FE 9034), by the Ministry of Economy, Work and Tourism of Mecklenburg-Vorpommern (Grant V220-630-08-TFMV-F-050), and by the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) of the Jena University Hospital. We thank Meike Hannstein for excellent laboratory assistance.

References

- [1] Friend SL, Hosier S, Nelson A, Foxworthe D, Williams DE, Farr A. A thymic stromal cell line supports in vitro development of surface IgM⁺ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol* 1994;22:321–8.
- [2] Ray RJ, Furlonger C, Williams DE, Paige CJ. Characterization of thymic stromal derived lymphopoietin (TSLP) in murine B cell development in vitro. *Eur J Immunol* 1996;26:10–6.
- [3] Tonoizuka Y, Fujio K, Sugiyama T, Nosaka T, Hirai M, Kitamura T. Molecular cloning of a human novel type I cytokine receptor related to delta1/TSLPR. *Cytogenet Cell Genet* 2001;93:23–5.
- [4] Quentmeier H, Drexler HG, Fleckenstein D, Zaborski M, Armstrong A, Sims JE, et al. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. *Leukemia* 2001;15:1286–92.
- [5] Reche PA, Soumelis V, Gorman DM, Clifford T, Liu MR, Travis M, et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 2001;167:336–43.
- [6] Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nature Immunol* 2000;1:59–64.
- [7] Park LS, Martin U, Garka K, Gliniak B, Di Santo JP, Muller W, et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med* 2000;192:659–70.
- [8] Rochman I, Watanabe N, Arima K, Liu Y-J, Leonard WJ. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4⁺ T cells. *J Immunol* 2007;178:6720–4.
- [9] Sebastian K, Borowski A, Kuepper M, Friedrich K. Signal transduction around thymic stromal lymphopoietin (TSLP) in atopic asthma. *Cell Commun Signal* 2008;25:5.
- [10] Wohlmann A, Sebastian K, Borowski A, Krause S, Friedrich K. Signal transduction by the atopy-associated human thymic stromal lymphopoietin (TSLP) receptor depends on Janus kinase function. *Biol Chem* 2010;391:181–6.
- [11] Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nature Immunol* 2005;6:1047–53.
- [12] Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med* 2005;202:829–39.
- [13] Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183–90.
- [14] Ying S, O'Connor B, Ratoff J, Meng Q, Fang C, Cousins D, et al. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;181:2790–8.
- [15] Shi L, Leu SW, Xu F, Zhou X, Yin H, Cai L, et al. Local blockade of TSLP receptor alleviated allergic disease by regulating airway dendritic cells. *Clin Immunol* 2008;129:202–10.
- [16] Lee H-C, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. *Proc Natl Acad Sci USA* 2007;104:914–9.
- [17] Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 2007;204:253–8.
- [18] Nagata Y, Kamijuku H, Taniguchi M, Ziegler S, Seino K. Differential role of thymic stromal lymphopoietin in the induction of airway hyperreactivity and Th2 immune response in antigen-induced asthma with respect to natural killer T cell function. *Int Arch Allergy Immunol* 2007;144:305–14.
- [19] Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP. *Nature Immunol* 2002;3:673–80.
- [20] Krause S, Behrendts J, Borowski A, Lohrmann J, Lang S, Myrtek D, et al. Blockade of interleukin-13-mediated cell activation by a novel inhibitory antibody to human IL-13 receptor alpha1. *Mol Immunol* 2006;43:1799–807.
- [21] Kammer W, Lischke A, Moriggl R, Groner R, Ziemiecki A, Gurniak CB, et al. Homodimerization of interleukin-4 receptor α chain can induce intracellular signalling. *J Biol Chem* 1996;271:23634–7.
- [22] The TH, Feltkamp TE. Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. *Immunology* 1970;18:875–81.
- [23] Winkler C, Denker K, Wortelkamp S, Sickmann A. Silver- and Coomassie-staining protocols: detection limits and compatibility with ESI MS. *Electrophoresis* 2007;28:2095–9.
- [24] Zhang F, Huang G, Hu B, Song Y, Shi Y. A soluble thymic stromal lymphopoietin (TSLP) antagonist, TSLPR-immunoglobulin, reduces the severity of allergic disease by regulating pulmonary dendritic cells. *Clin Exp Immunol* 2011;164:256–64.
- [25] Elliott MJ, Maini RN. Anti-cytokine therapy in rheumatoid arthritis. *Baillière's Clinical Rheumatology* 1995;9:633–52.
- [26] Jelusic M, Lukic IK, Batinic D. Biological agents targeting interleukin-18. *Drug News Perspect* 2007;20:485–94.
- [27] Mueller TD, Zhang JL, Sebald W, Duschl A. Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim Biophys Acta* 2002;1592:237–50.
- [28] Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T(–)B(+)NK(+) severe combined immunodeficiency. *Nature Genet* 1998;20:394–7.
- [29] Kikuchi K, Lai AY, Hsu C-L, Kondo M. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med* 2005;201:1197–203.

- [30] Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nature Rev Immunol* 2009;9:195–205.
- [31] Nakamura H, Takamori S, Fujii T, Ono M, Yamana H, Kuwano M, et al. Cooperative cell-growth inhibition by combination treatment with ZD1839 (Iressa) and trastuzumab (Herceptin) in non-small-cell lung cancer. *Cancer Lett* 2005;230:33–46.
- [32] Bull Phelps SL, Schorge JO, Peyton MJ, Shigematsu H, Xiang LL, Miller DS, et al. Implications of EGFR inhibition in ovarian cancer cell proliferation. *Gynecol Oncol* 2008;109:411–7.
- [33] Yang H, Jager MJ, Grossniklaus HE. Bevacizumab suppression of establishment of micrometastases in experimental ocular melanoma. *Invest Ophthalmol Vis Sci* 2010;51:2835–42.
- [34] Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992;89:4285–9.
- [35] Miyata M, Nakamura Y, Shimokawa N, Ohnuma Y, Katoh R, Matsuoka S, et al. Thymic stromal lymphopoietin is a critical mediator of IL-13-driven allergic inflammation. *Eur J Immunol* 2009;39:3078–83.
- [36] Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. *Blood* 2010;115:5312–21.
- [37] Yoda A, Yoda Y, Chiaretti S, Bar-Natan M, Mani K, Rodig SJ, et al. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 2010;107:252–7.
- [38] Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, et al. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. *Blood* 2011;117:2129–36.
- [39] Olkhanud PB, Rochman Y, Bodogai M, Malchinkhuu E, Wejksza K, Xu M, et al. Thymic stromal lymphopoietin is a key mediator of breast cancer progression. *J Immunol* 2011;186:5656–62.
- [40] Muerkoster S, Wegehenkel K, Arlt A, Witt M, Sipos B, Kruse ML, et al. Tumor stroma interactions induce chemoresistance in pancreatic ductal carcinoma cells involving increased secretion and paracrine effects of nitric oxide and interleukin-1 β . *Cancer Res* 2004;64:1331–7.
- [41] Ohara M, Yamaguchi Y, Matsuura K, Murakami S, Arihiro K, Okada M. Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer. *Cancer Immunol Immunother* 2009;58:441–7.
- [42] De Monte L, Reni M, Tassi E, Clavenna D, Papa I, Recalde H, et al. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J Exp Med* 2011;208:469–78.
- [43] Li H, Zhao H, Yu J, Su Y, Cao S, An X, et al. Increased prevalence of regulatory T cells in the lung cancer microenvironment: a role of thymic stromal lymphopoietin. *Cancer Immunol Immunother* 2011;60:1587–96.