Structure and antagonism of the receptor complex mediated by human TSLP in allergy and asthma

Kenneth Verstraete1,2, Frank Peelman3, Harald Braun1,4, Juan Lopez5,6, Dries Van Rompaey7, Ann Dansercoer1,2, Isabel Vandenberghe2, Kris Pauwels8,9, Jan Tavernier3, Bart N. Lambrecht1,10, Hamida Hammad1,10, Hans De Winter7, Rudi Beyaert1,4, Guy Lippens5,11 & Savvas N. Savvides1,2

The pro-inflammatory cytokine thymic stromal lymphopoietin (TSLP) is pivotal to the pathophysiology of widespread allergic diseases mediated by type 2 helper T cell (Th2) responses, including asthma and atopic dermatitis. The emergence of human TSLP as a clinical target against asthma calls for maximally harnessing its therapeutic potential via structural and mechanistic considerations. Here we employ an integrative experimental approach focusing on productive and antagonized TSLP complexes and free cytokine. We reveal how cognate receptor TSLPR allosterically activates TSLP to potentiate the recruitment of the shared interleukin 7 receptor α-chain (IL-7Rα) by leveraging the flexibility, conformational heterogeneity and electrostatics of the cytokine. We further show that the monoclonal antibody Tezepelumab partly exploits these principles to neutralize TSLP activity. Finally, we introduce a fusion protein comprising a tandem of the TSLPR and IL-7Rα extracellular domains, which harnesses the mechanistic intricacies of the TSLP-driven receptor complex to manifest high antagonistic potency.

DOI: 10.1038/ncomms14937
The downside of aberrant signalling by TSLP has grave consequences for human health and imprints a massive healthcare and socioeconomic footprint. This is because type 2 helper T cell (Th2)-mediated inflammatory responses primed by activated DCs, are pivotal for the onset of widespread allergic diseases of the airways, skin and gut8. In fact, TSLP is now widely considered to underlie some of the most prevalent inflammatory allergies, disorders, such as the atopic dermatitis, asthma, atopic dermatitis, and atopic rhinitis, chronic obstructive pulmonary disease (COPD) and eosinophilic esophagitis9–12, and has been annotated as a genetic risk factor for the development of asthma3–15 and eosinophilic esophagitis16. Furthermore, a staggering 70% of atopic dermatitis clinical cases go on to develop asthma via the ‘atopic march’ (also known as ‘atopic march’)17, and adult asthmatics are strongly predisposed for acquiring COPD (ref. 18). Several recent developments have expanded the pathophysiological profile of TSLP. First, TSLP was shown to provide a signalling link between the skin epithelium and neuronal cells to trigger itch associated with atopic dermatitis19. Second, TSLP was shown to contribute to the development of psoriasis, a widespread autoimmune disease, by regulating IL-23 production by DCs20. Third, TSLP may drive tumour progression in breast- and pancreatic cancer21,22 but also manifest tumour protective effects23–26, while genetic rearrangements and mutations in the TSLPR gene (CRLF2) are found in paediatric acute lymphoblastic leukaemia (ALL)27. However, the role of TSLP in cancer is controversial8,28. Fourth, the human TSLP:TSLPR:IL-7Rα complex was shown to provide a signalling link between the skin epithelium and IL-7Rα-expressing immune cells41,42. Following enzymatic refolding from inclusion bodies produced in E. coli38, we were able to produce N-glycosylation variants of human TSLP and TSLPR (Supplementary Fig. 1C) and to adopt monodisperse assemblies obeying 1:1 stoichiometry as characterized by size-exclusion chromatography (SEC) (Fig. 1d,e), and were found to be highly homogeneous (Fig. 1f) and to produce non-glycosylated bioactive human TSLP lacking a basic cassette (127RRKRK131) (ref. 38) and the IL-7Rα ectodomain via in vitro refolding from inclusion bodies produced in E. coli37,40.

In parallel, we were able to produce N-glycosylation variants of TSLPR (TSLPRN47Q, TSLPRN47Q/110Q and TSLPRN47Q/N169Q) in HEK293S-TetR MGAT1−/− cells41,42. Following enzymatic trimming of residual TSLPR N-glycosylation, ternary TSLP:TSLPR:IL-7Rα complexes were assembled and isolated in a sequential manner by size-exclusion chromatography (SEC) (Fig. 1d,e), and were found to be highly homogeneous (Fig. 1f) and to adopt monodisperse assemblies obeying 1:1 stoichiometry as characterized by coupling SEC to multi-angle laser light scattering (MALLS) (Fig. 1g). Crystallization trials using purified TSLP N47Q/IL-7Rα (ref. 18) lead to optimized crystals that diffracted synchrotron X-rays to 2.55 Å resolution, and enabled determination of the crystal structure of the human TSLP:TSLPR:IL-7Rα complex by molecular replacement (Fig. 1h, Table 1).

Results
Reconstitution and cooperativity of the TSLP complex. Prior studies had suggested that the signalling complex mediated by human TSLP proceeds through an initial binary complex between TSLP and TSLPR to enable recruitment of IL-7Rα (refs 5,6,37). To determine the assembly order and kinetic profile underlying the TSLP:TSLPR:IL-7Rα complex we performed real time in vitro interaction studies via bio-layer interferometry (BLI) using mammalian-derived glycosylated TSLP, IL-7 and soluble TSLPR and IL-7Rα (Supplementary Fig. 1A). In accordance to prior observations human TSLP could only be produced in HEK293 cells upon abolishing its putative furin cleavage site38. Firstly, we determined that TSLP binds to TSLP with high-affinity (KD = 32 nM) and fast kinetics (kₐ = 1.7 × 10⁵ M⁻¹ s⁻¹ and k₋₋₋ = 5.2 × 10⁻³ s⁻¹) (Fig. 1a). In contrast, IL-7Rα, which was able to bind to cognate IL-7 (Supplementary Fig. 1B), showed no apparent binding to TSLP alone (Fig. 1b)39. However, IL-7Rα associated with preformed TSLP:TSLPR binary complex with high-affinity (KD = 29 nM; kₐ of 1.23 × 10⁵ M⁻¹ s⁻¹; k₋₋₋ of 3.6 × 10⁻³ s⁻¹) (Fig. 1c), thus, priming of human TSLP by its cognate receptor, TSLPR, is a mechanistic prerequisite for the recruitment of shared IL-7Rα to the extracellular ternary complex.

Such initial mechanistic insights formed the basis for a strategy to biochemically reconstitute the TSLP:TSLPR:IL-7Rα complex for structural studies. To facilitate the growth of well-diffracting crystals towards structural characterization of the complex at high-resolution by X-ray crystallography, we focused on the production of minimally glycosylated ternary complexes. We thus produced non-glycosylated bioactive human TSLP lacking a basic cassette (127RRKRK131) (ref. 38) and the IL-7Rα ectodomain via in vitro refolding from inclusion bodies produced in E. coli37,40.

TSLP evokes receptor–receptor interactions. Our crystallographic analysis contributes structural insights at high-resolution of human TSLP and TSLPR (Supplementary Fig. 1C) and reveals how TSLP wedges between TSLPR and IL-7Rα to mediate a T-shaped extracellular assembly (Fig. 2a), as further supported by small-angle X-ray scattering (Supplementary Fig. 2; Supplementary Table 1). TSLP employs two opposing surface patches to interact with the elbow tips of the cytokine-binding homology regions (CHRs) of TSLPR (site I) and IL-7Rα (site II), which allow the membrane-proximal parts of the two receptors to engage in heterotypic receptor–receptor interactions (site III) (Fig. 2a). TSLP and TSLPR display pronounced electrostatic complementarity spanning the entire site I, with TSLP presenting a positively charged surface patch associating with the negatively charged interdomain elbow of TSLPR (Fig. 2b). This suggests that long-range electrostatic interactions may play an important role in attracting TSLP to TSLPR at the cell surface to establish the mechanistically critical binary complex. Interestingly, electrostatic
Figure 1 | The TSLP signalling complex assembles via a cooperative stepwise mechanism. (a–c) BLI data traces (black) and fitted 1:1 binding model (red) are plotted as the spectral nanometre shift in function of time for the interaction of TSLPR with TSLP (a), IL-7Rα with TSLP (b) and IL-7Rα with preformed TSLP:TSLPR complex (c). 4 nM of biotinylated TSLP was loaded for experiments (a–c). In experiment (c) TSLP-coated biosensors were incubated with 320 nM TSLPR and 320 nM of TSLPR was included in kinetics buffer and IL-7Rα samples. The reported $K_d$, $k_a$ and $k_d$-values represent average values and their s.d. from three technical replicate experiments. (d) SEC elution profiles on a Superdex 75 16/600 column of EndoH-treated TSLPRN47Q (dashed line) and TSLP$^{\Delta_{127-131}}$:TSLPRN47Q complex (solid line) plotted as the ultraviolet absorbance at 280 nm in function of elution volume. (e) SEC elution profile on a Superdex 200 16/600 column of TSLP$^{\Delta_{127-131}}$:TSLPRN47Q:IL-7Rα complex plotted as the ultraviolet absorbance at 280 nm in function of elution volume. (f) Coomassie-stained reducing SDS-PAGE gel of glycan-minimized ternary complex used for crystallization trials. The theoretical protein molecular weights for IL-7Rα, TSLPR and TSLP are 25.7, 24.0 and 14.6 kDa. (g) SEC elution profile on a Superdex-200 10/300 GL column of glycan-minimized ternary TSLP complex plotted as the ultraviolet absorbance at 280 nm (left Y-axis) as a function of elution volume. The molecular weight (right Y-axis) as determined by MALLS is reported as the number average molecular mass and its s.d. (h) Crystals of the TSLP$^{\Delta_{127-131}}$:TSLPRN47Q:IL-7Rα complex.

Potential calculations on the TSLP:TSLPR binary complex show that this molecular entity would project a negative electrostatic potential, which would render it compatible with the positive electrostatic potential of unbound IL-7Rα.

Consistent with annotations of human TSLP as a member of the IL-2 family of cytokines, its mature sequence (residues 29–159) adopts a four-helix bundle with ‘up-up-down-down’ topology stabilized by three disulfide bridges (Cys34-Cys110, Cys69-Cys75 and Cys90-Cys137), in which the four α-helices—designated αA, αB, αC, αD—are threaded via a BC loop and two long overhand AB and CD loop regions, with the latter largely invisible in the electron density maps (Fig. 2a; Supplementary Fig. 3A). The functional role of the flexible CD loop containing the seven residue basic cassette (residues 125–131) remains enigmatic (Supplementary Fig. 3A). It has been hypothesized that its embedded furin cleavage site is linked to a mechanism limiting the availability of proinflammatory TSLP in vivo. Moreover, it was recently shown that in nasal polyp tissues this loop region can be proteolytically processed to yield a biologically active nicked form of human TSLP (ref. 44). In addition, the positive charge density may mediate interactions with glycosoaminoglycans in the extracellular matrix, as proposed for IL-7 (ref. 45).

The structure of human TSLP is unique among helix bundle cytokines in three main ways. First, it adopts a rather open helix...
bundle core that is perforated by an elongated internal void volume (~120 Å³) running from the αA-αC face to the β6-αC face of the helical bundle (Fig. 2c). Second, it harbours a fully buried structural water at the heart of the helical bundle, coordinated by a conserved trio of amino acids (Trp148, Thr102, Thr83) (Fig. 2c; Supplementary Fig. 3A) suggesting that this central water molecule is an integral part of the protein fold. This notion is supported by molecular dynamics (MD) simulations of TSLP using an explicit solvent model, whereby TSLP devoid of water molecule inserted into the αA-αC face to the β6-αC face. Relative higher flexibility of these regions compared to the helical parts of the structure (Fig. 2f).

**The AB loop in TSLP relays IL-7Rα recruitment.** The atypical open helical bundle core of TSLP and the intriguing π-helical turn in helix αA of TSLP prompted us to hypothesize that the priming of TSLP by TSLPR for recruitment of IL-7Rα might be linked to the intrinsic plasticity and dynamics of TSLP. To this end, we performed a series of nuclear magnetic resonance (NMR) experiments on isotopically labelled TSLP and pursued complementary MD simulations. Assignment of the NMR spectra by triple resonance spectroscopy on isotopically labelled TSLP revealed that unbound TSLP comprises the four α-helices as delineated in the structure of TSLP bound to its receptors (Fig. 2e). Furthermore, 1H–15N heteronuclear NOE analysis showed decreased NOE values for the overhand AB and CD loops, as well as for the N- and C-termini, reflecting the relative higher flexibility of these regions compared to the helical parts of the structure (Fig. 2f).

Altogether with the structure of receptor-bound TSLP, these findings provide the rationale for tracing possible structural transitions in TSLP upon complex formation. In particular, TSLP employs the C-terminal half of αD (residues 142–152), the C-terminal short tail extending from αD (residues 153–158) and a continuous stretch of 10 residues located in the long overhand AB-loop region (residues 60–69) to interact with a complementary interaction epitope formed at the elbow tip of the CHR-module of TSLPR (Fig. 2a; Supplementary Table 2). On the basis of our NMR studies, the AB-loop and C-terminal tail would undergo significant conformational changes to achieve their observed bound state. This is additionally supported by extensive MD simulations for TSLP and TSLP:TSLPR (Fig. 2g,h), and might have profound mechanistic implications.

**Table 1 | X-ray data set and refinement statistics**

| Source        | Proxima 2A (SOLEIL, France) | Proxima 2A (SOLEIL, France) |
|---------------|----------------------------|----------------------------|
| Detector      | ADSC QUANTUM 315r           | EIGER 9M                   |
| Space group   | C 2                        | P 3 2 1                   |
| a, b, c (Å)   | 135.8, 66.6, 92.0           | 51.7, 51.7, 370.0          |
| α, β, γ (°)   | 90.0, 109.2, 90.0           | 90.0, 90.0, 120.0         |
| Resolution (Å) | 50.0–2.56 (2.72–2.56)      | 55.0–2.30 (2.44–2.30)     |
| Completeness (%) | 97.8 (94.2)               | 97.0 (83.8)               |
| Redundancy    | 3.2 (3.1)                  | 8.4 (4.2)                 |
| Mean I/σI    | 14.2 (1.7)                 | 14.23 (1.6)               |
| Rfactors (%)  | 6.2 (77.6)                 | 11.0 (74.7)               |
| Wilson B (%)  | 99.8 (78.2)                | 99.8 (64.2)               |
| Resolution (Å) | 45.76–2.56 (2.67–2.56)    | 44.80–2.30 (2.39–2.30)   |
| No. reflections | 24,638 (2,738)            | 25,275 (2,306)            |
| No. non-H atoms | 4,033                  | 4,120                     |
| Protein      | 3,966                      | 3,997                     |
| Ligands      | 38                         | 15                        |
| Water        | 29                         | 108                       |
| Average ADP (Å²) | 89.40 (51.90)            | 89.30 (52.00)             |
| Protein      | 89.40                      | 51.90                     |
| Ligands      | 118.30                     | 67.60                     |
| Water        | 69.70                      | 48.50                     |
| r.m.s.d.'s   | 1.71                       | 0.77                      |

- Values in parentheses correspond to the highest-resolution shell.
- Final refinement was performed in autoBuster 2.10.2 for the TSLP:TSLPR:IL-7Rα complex and in PHENIX 1.9-1692 for the TSLP:Fab complex.
- Maximum likelihood estimate of the Wilson B-factor from phenix.xtriage.
Figure 2 | Structure of the TSLP:TSLPR:IL-7Rα complex and TSLP structural dynamics. (a) View of the determined X-ray structure for the TSLP:TSLPR:IL-7Rα complex. TSLP (blue) is shown in cartoon representation with its four helices labelled as αA to αD. TSLP loop regions are highlighted in red with the disordered CD loop region represented as a dashed line. The extracellular regions of TSLPR (yellow) and IL-7Rα (grey), each comprising twoFnIII-like domains, D1 and D2, are shown as cartoons overlaid on transparent surface representations. TSLPR and IL-7Rα strand and loop regions contributing to sites I, II and III are labelled. Disulfide bridges are shown as yellow spheres. The water molecule in the core of TSLP is shown as a red sphere. Modelled GlcNAc moieties on TSLPR are shown as grey sticks. The C-terminal loop extending from TSLPR strand G2 is coloured purple. BSA: buried surface area. (b) Bottom and side view cartoon representations of the TSLP complex with the electrostatic potential at the solvent accessible surfaces shown for TSLP (top) and TSLPR (bottom). (c) The coordinated water molecule in the bundle core (red sphere) is located adjacent to an elongated internal void volume (pink mesh). (d) Structural comparison of human TSLP (blue) as seen in the TSLP:TSLPR:IL-7Rα complex and human IL-7 (orange) as seen in the IL-7:IL-7Rα complex (pdb 3DI2, chain A). (e) 13C-consensus chemical shift index (CSI) analysis of TSLP D127–131. The location of crystallographically observed α-helices in the TSLP:TSLPR:IL-7Rα complex as annotated by DSSP is schematically shown on top with the π-helical turn coloured in red. (f) Backbone 1H–1H heteronuclear NOE values measured on TSLP plotted as a function of TSLP residue number. Data points for residues in the π-helical turn are coloured red. (g) Snapshot for one of five different 250 ns MD simulations runs for full-length TSLP is shown as a cartoon. Residues are coloured according to their average root mean square fluctuations (r.m.s.f.) of backbone positions over the five different MD runs. The width of the cartoon loop radius varies with the r.m.s.f. value. (h) Average backbone r.m.s.f. values and s.d. (shaded region) for unbound TSLP (black) and for TSLP in complex with TSLPR (red) plotted in function of TSLP residue number. Average r.m.s.f. values and s.d. were calculated from five 250 ns MD-runs, each with a different starting model for TSLP.
Our NMR analysis show that among all residues defining the four $\alpha$-helices of TSLP, Thr46 and Ile47 in the $\pi$-helical turn midway $\alpha$A exhibit the highest degree of flexibility in unbound TSLP (Fig. 2f), yet they become well-ordered at the TSLP:IL-7R$\alpha$ interface. We therefore wondered about the origin of the structural features of the atypical $\alpha$A of TSLP and about its possible role in IL-7R$\alpha$ recruitment. Indeed, analysis of the NOE strips through each of the resonances of residues in $\alpha$A showed that the relative intensity of the amide-amide proton cross peak compared to the diagonal peak is 15–25% for all amide proton pairs, except for the Thr46 H$_N$–Ile47 H$_N$ NOE where the normalized cross peak rises to 54% (Supplementary Fig. 3E). This discrepancy agrees with the distances between consecutive amide normalized cross peak rises to 54% (Supplementary Fig. 3E). This implies a 2.9 Å. Thus, unbound TSLP in solution also displays the kinked TSLPR and IL-7R$\alpha$ domains, D1 and D2 (Fig. 2a). The membrane-distal TSLPRD1 domain is characterized by a $AB$F/C $C\alpha$-helical bundle core in the context of somatic mutations in the juxtamembrane loop and N-terminal residues of the F strand of TSLPR D1 (Val78, Leu100, Ile102) and IL-7R$\alpha$ D1 (Tyr159, Tyr212, Phe213) to clamp onto the AC-face of the TSLP helical bundle as defined by residues protruding from $\alpha$A of TSLP (Ala41, Ala42, Leu44, Ser45, Thr46, Leu47, Lys49) and $\pi$C (Met97, Met100, Lys101, Ala104, Ala105, Ile108 and Trp109) (Figs 2a and 3e; Supplementary Table 2). With $\approx$1,120 Å$^2$ of buried solvent accessible surface area, the hydrophobic TSLP:IL-7R$\alpha$ interface (site II) is markedly more limited than the TSLP:IL-7R$\alpha$ interface (site I).

To interrogate the importance of TSLP residues at the TSLP:IL-7R$\alpha$ interface, we evaluated a set of TSLP variants carrying mutations at site II (Supplementary Table 3). While our selected set of single site TSLP mutants had no apparent effect, we found that a double TSLP mutant carrying Ser45Arg/Thr46Arg mutations at the $\pi$-helical turn of $\alpha$A (Fig. 3f) showed reduced capacity in inducing TSLPR:IL-7R$\alpha$-mediated STAT5-signalling (EC$_{50}$ = 5.3 pM versus for IC$_{50,WT}$ = 0.11 pM), while the affinity towards IL-7R$\alpha$ remained largely unaffected (IC$_{50}$ = 720 pM versus IC$_{50,WT}$ = 320 pM) (Fig. 3g). At site II, mutations in the hydrophobic EF loop region of IL-7R$\alpha$I1 (Leu100Ser/Ile102Ser, EC$_{50}$ = 470 pM) also led to a decreased signalling potential (EC$_{50}$ = 85 pM) (Fig. 3d).

Comparisons with the human IL-7:IL-7R$\alpha$ binary complex$^{51}$ show that IL-7$\alpha$ adopts a highly similar structure in the two complexes (r.m.s.d. = 0.66 Å for 195 C$\alpha$ atoms) and offers preformed binding sites to either cytokine as evidenced by the structure of IL-7R$\alpha$ in the absence of cytokine (Supplementary Fig. 6B). Although IL-7 and TSLP exhibit marginal sequence similarity (Supplementary Fig. 3C), IL-7R$\alpha$ employs a near identical set of residues to interact with IL-7 and TSLP, burying 740 Å$^2$ and 630 Å$^2$ of solvent-accessible surface, respectively (Supplementary Fig. 6C,D). Thus, the cytokine binding degeneracy of IL-7R$\alpha$ originates from a promiscuous hydrophobic platform at the IL-7R$\alpha$ elbow tip combined with unique structural features shared between the TSLP and IL-7 cytokines.
Receptor–receptor interactions potentiate TSLP signalling. One of the observed hallmarks of the receptor complex mediated by human TSLP concerns the compact network of interactions between the membrane-proximal regions of TSLPRD2 and IL-7RαD2 (Figs 2a and 4a; Supplementary Table 2). The ensuing heterotypic receptor interface buries ~780 Å² of solvent-accessible surface area contributed by the AB2, CC'2 and EF2 loops of TSLPRD2, and the ABE2-face, and AB2 and EF2 loops of IL-7RαD2. The interface displays several electrostatic interactions and close van der Waals contacts, such as the packing of TSLPR-
Figure 4 | Membrane-proximal receptor-receptor interactions potentiate TSLP signalling. (a) Detail of the IL-7Rα:TSLPR interface (site III) as viewed from the membrane-proximal side. Interface residues are shown as sticks. Hydrogen bonds and salt bridges are indicated with a dashed line. (b) TSLP-induced STAT5 activity assay in cells expressing WT or mutant forms of TSLPR. The EC50-values were 11 pM for the control and 85 pM for TSLPR-Asp157Ala/Glu159Ala, 400 pM for TSLPR-Phe156Ala/Asp157Ala/Glu159Ala and 49 pM TSLPR-Asp176Ala/Glu178Ala/Lys179Ala. Each experiment was carried out in triplicate; data shown are averages, and error bars were calculated as s.e.m. (c,d) BLI data traces (black) and the fitted 1:1 binding model (red) are plotted as the spectral nanometre shift in function of time for the interaction of soluble TSLPR with immobilized IL-7Rα (c) or TSLPR (d). The reported averaged K_D-, k_a- and k_d-values and their s.d. are derived from three technical replicate experiments. Biosensing surfaces were generated by loading 2.5 nm of biotinylated IL-7Rα for experiment (c) and 1.5 nm of biotinylated TSLPR for experiment (d). The concentration range of soluble TSLPR in d was identical as in c.

Figure 3 | Structure-based dissection of TSLP cytokine-receptor interfaces. (a) Detail of the TSLP:TSLPR interface (site I). Interface residues are shown as sticks. Water molecules are shown as red spheres. Hydrogen bonds and salt bridges are indicated with a dashed line. (b) Competitive binding assay measuring displacement by either wild type (WT) or mutated TSLP of bound TSLP-SEAP fusion protein from HEK293T cells expressing TSLPR. (c) STAT5 activity induced by WT or mutated TSLP, as measured using a luciferase-based reporter system in HEK293T cells expressing WT TSLPR and IL-7Rα. The EC50-values were 0.15 pM for TSLP-WT, 2.8 pM for TSLP-Arg149Ser, 9.5 pM for TSLP-Arg150Ser, 100 pM for TSLP-Arg149Ser/Arg150Ser and 19 pM for TSLP-Arg153Ser. (d) TSLP-induced STAT5 activity assay in cells expressing WT or mutant forms of either TSLPR or IL-7Rα. The EC50-values were 85 pM for the control, 87 nM for TSLPR-Asp92Ala and 470 pM for IL-7Rα-Leu100Ser/Ile102Ser. For mutants TSLPR-Trp112Ala and TSLPR-Trp112Arg the EC50-value could not be accurately determined. (e) Detail of the TSLP:IL-7Rα interface (site II). Interface residues are shown as sticks. Water molecules are shown as red spheres. Hydrogen bonds and salt bridges are indicated with a dashed line. (f) STAT5 activity induced by WT or TSLP-Ser45Arg/Thr46Arg in cells expressing wild-type TSLPR and IL-7Rα. The EC50-values were 0.11 pM for TSLP-WT and 5.3 pM for TSLP-Ser45Arg/Thr46Arg. (g) Competitive binding assay measuring displacement by either WT (IC50 = 320 pM) or TSLP-Ser45Arg/Thr46Arg (EC50 = 720 pM) from bound TSLP-SEAP fusion protein from HEK293T cells expressing TSLPR. Each experiment was carried out in triplicate; data shown are averages, and error bars were calculated as s.e.m.
Receptor fusion proteins are potent TSLP antagonists. TSLP is increasingly gaining a central role in the pathophysiology of allergic diseases. To identify novel TSLP antagonists, we designed TSLP cytokine traps by fusing the TSLPR and IL-7Rα extracellular regions in both orientations with a flexible (Gly-Gly-Ser)10 linker, hereafter termed TSLP-trap1 and TSLP-trap2 (Fig. 5a), and produced them in stably transfected T-Rex-293 cells (Fig. 5b; Supplementary Fig. 7A). We found that TSLP-trap1 binds 250-fold stronger to TSLP (Kd = 120 pM) (Fig. 5d) than the unlinked receptor ectodomains do (Fig. 1c). Importantly, the corresponding kinetic profile shows that such high-affinity can be traced to a drastically reduced dissociation rate constant (koff = 2 × 10⁻⁵ s⁻¹) as compared to the dissociation rate of the unfused ectodomains (Fig. 1c). A similar binding profile was observed for TSLP-trap2 (data not shown).

To compare the binding properties of our TSLP-traps to Tezepelumab (AMG-157/MEDI9929) (ref. 54), to our knowledge the most potent anti-TSLP antagonist to date, we produced AMG-157 and its Fab fragment in HEK293T cells (Supplementary Fig. 8A,B). AMG-157 is a fully human neutralizing immunoglobulin G subclass 2 (IgG2) anti-TSLP monoclonal antibody (mAb) and was recently shown to alleviate most measures of both early and late asthmatic responses in patients with mild allergic asthma. AMG-157 Fab displays comparable affinity and binding kinetics to TSLP when compared to TSLP-trap1 (Fig. 5e). However, cellular activity assays in HEK293T cells at 10 pM TSLP (EC50 = 9 pM) demonstrated that TSLP-trap1 and TSLP-trap2 are 20–30 fold more potent in inhibiting TSLP-induced STAT5 signalling (IC50 = 67 and 44 pM, respectively) (Fig. 5f, Supplementary Fig. 7B) compared to AMG-157 mAb and its Fab fragment (IC50 = 1.4 and 1.7 nM, respectively) (Fig. 5f). Remarkably, the TSLP-traps show about 1,000-fold higher inhibitory potency over equimolar mixtures of unlinked TSLPR and IL-7Rα (IC50 = 49 nM) (Fig. 5g), suggesting that fusion of the two receptor ectodomains harnesses certain key mechanistic features underlying the TSLP-receptor complex. Parallel assays at 100 pM TSLP resulted in analogous observations but with overall higher IC50-values (Supplementary Fig. 7C). Finally, neither TSLP-trap1 nor TSLP-trap2 inhibited STAT5 signalling in HEK293T cells transfected with IL-7Rα and the common gamma-chain (γc) mediated by IL-7 at 10 and 100 pM (EC50 = 41 pM) (Fig. 5h; Supplementary Fig. 7D,E).

Plasticity and functional role of the α-helix turn in TSLP. The TSLP:AMG-157 complex provides a unique view of the IL-7Rα binding site on TSLP in the absence of the shared receptor, thereby fuelling insights into the possible structural transitions associated with the cooperative recruitment of IL-7Rα to the TSLP-mediated signalling complex. Perhaps the most intriguing feature of TSLP as bound to AMG-157 concerns an ordered water molecule, the AB-loop and C-terminal tail extending from zD adopt different conformations, with the rest of the TSLP main chain being very similar (r.m.s.d. of 0.56 Å for 89 aligned Cα atoms). In fact the AB-loop and the C-terminal tail of zD in the TSLP:AMG-157 complex are only partly resolved in the electron density maps, indicating that these regions are flexible in the absence of TSLPR consistent with our NMR and MD studies of TSLP (Figs 2f and 7d).

Structure and mechanism of TSLP antagonism by Tezepelumab. We seized the opportunity to obtain structural and mechanistic insights into how Tezepelumab (AMG-157) might exert its antagonistic effects on TSLP and to characterize TSLP in a binary complex with a non-signalling binding partner, by pursuing the crystal structure of TSLP in complex with AMG-157Fab. We were able to biochemically reconstitute and crystalize the TSLP:AMG-157Fab complex and to determine its crystal structure to 2.3 Å resolution (Fig. 7a; Table 1, Supplementary Fig. 8D). The structure reveals that the complementarity determining regions (CDRs) of the variable heavy chain domain (VH) of AMG-157 target TSLP at the AB-loop region and C-terminal region of helix D, while the variable light chain fragment does not interact with TSLP at all (Fig. 7a). The TSLP:AMG-157 interface buries a total of 1,200 Å² of accessible surface area, with all three VH-CDR loops contributing to a polar footprint (Fig. 7b; Supplementary Table 4). Most notably, Glu110 in the CDR-3 loop makes a bifurcated salt-bridge with TSLP:Arg150 and TSLP:Arg153, and Trp105 packs against TSLP:Glu75 in a surface pocket formed between TSLP zD and the overhand AB-loop (Fig. 7b). Importantly, we now show that AMG-157 competes against a critical part of the TSLP binding site on TSLP and remains completely clear of the IL-7Rα binding site on the other side of the TSLP helical bundle (Fig. 7c). Furthermore, structural superposition of TSLP in its two complexes shows that the AB-loop and C-terminal tail extending from zD adopt different conformations, with the rest of the TSLP main chain being very similar (r.m.s.d. of 0.56 Å for 89 aligned Cα atoms). In fact the AB-loop and the C-terminal tail of zD in the TSLP:AMG-157 complex are only partly resolved in the electron density maps, indicating that these regions are flexible in the absence of TSLPR consistent with our NMR and MD studies of TSLP (Figs 2f and 7d).
complex, IL-7Rα cannot be recruited to the TSLP:AMG-157 complex (Supplementary Fig. 8C).

Additional insights into the possible structural states of TSLP are provided by our NMR analysis. Close inspection of the 1H,15N HSQC TSLP spectrum at 900 MHz uncovered conformational heterogeneity on the second timescale, which is much slower than can be sampled by MD-simulations. Specifically, we identified two populations for the Tyr43-Leu44-Ser45-Thr46 amide resonances located in the α-helical turn in αA of TSLP, as well as for the side-chain resonance of Trp148, which stacks right above the α-helical turn of TSLP (Fig. 7f; Supplementary Fig. 9A–E). The minor conformations observed for Ser45 and Trp148 are populated to 20±2% based on deconvoluted integrals of their respective signals, suggesting that they represent the same structural heterogeneity that connects the core of TSLP to αA. Although our NMR analysis does not provide structural details for the two TSLP conformations, together with the distinct conformational states of active versus antagonized TSLP, it provides independent support for the structural heterogeneity of TSLP.
Discussion

The emergence of TSLP as a central orchestrator of Th2 responses that initiate allergy and inflammation has placed therapeutic targeting of TSLP-mediated signalling against major chronic diseases such as allergic asthma and atopic dermatitis at center stage. However, in order to maximally harness the therapeutic potential of TSLP-mediated signalling, it will be essential to dissect the structural and mechanistic basis for its bioactivity. Recent efforts that have leveraged mechanistic and structure-based considerations of cytokine-mediated receptor activation, have led to the development of engineered IL-2, IL-4 and IL-13 variants with drastically improved kine-mediated receptor activation, have led to the development of leveraged mechanistic and structure-based considerations of cytokine receptors.

The intrinsic cooperativity of the TSLP:TSLPR:IL-7R complex is also the mechanistic pillar for the high in vitro potency of the TSLP-traps we have developed by linking the TSLPR and IL-7Rz ectodomains to create a single protein. Our TSLP-trap fusion proteins neutralize TSLP via a very drastic improvement in the $K_D$ compared to the unlinked counterparts by nearly three orders of magnitude ($K_D = 120$ pM) manifested primarily by very slow off-rate kinetics ($t_{1/2} \sim 10$ h). This strong increase in binding affinity is functionally reflected by the potent antagonistic activity and specificity of the TSLP-traps against TSLP signalling in our cellular inhibition assays with IC$50$-values below 100 pM (Fig. 5). Such binding properties gain important biological context in light of the ability of both TSLP-trap1 and TSLP-trap2 to effectively antagonize TSLP-mediated molecular responses relevant for Th2 immunity in human primary cells (Fig. 6). Remarkably, the IC$50$-values obtained for the TSLP-traps are 20–40-fold lower than those obtained for the AMG-157 mAb and its derived Fab fragment, which we propose is inextricably linked to the mechanistic modalities of the TSLP-mediated receptor complex (Fig. 8). Fusion proteins comprising receptor ectodomains and decoy receptors foster attractive binding properties to serve as effective therapeutics, as exemplified in the targeting of IL-1 (Rilonacept) or TNF$\zeta$ (Etanercept) for the treatment of CAPS-syndrome and rheumatoid arthritis, respectively. Indeed, we are currently performing in vivo studies to assess the antagonistic potency of such fusion proteins. Furthermore, we anticipate that the current version of the TSLP-traps can be additionally improved in a number of ways, including optimizing linker length and introducing mutations to enhance the affinity and/or cross-linkage of the TSLPR and IL-7Rz ectodomains to each other. Indeed, the therapeutic potential of targeting TSLP to treat allergic diseases mediated by Th2 responses is large, in particular in the context of combined approaches co-targeting the bioactivity of IL-25 and IL-33 (ref. 35).

Finally, our work on human TSLP provides opportunities to further investigate recent intriguing findings describing a second
isoform of TSLP, termed short form TSLP (sfTSLP)\(^{63–65}\). Evidence for sfTSLP mainly pertains to the transcriptional levels of sfTSLP, and led to proposals that sfTSLP might be the constitutively expressed isoform of TSLP. sfTSLP is 63 residues in length and approximately covers the C-terminal half of human TSLP (residues 97–159). On our structure of human TSLP, sfTSLP would encompass \(\alpha C\), the long CD loop and \(\alpha D\) (Supplementary Fig. 3A). It is presently unclear if sfTSLP can adopt any helical structure in the absence of \(\alpha A\) and \(\alpha B\). However, a propensity to form amphipathic helices combined with a high isoelectric point (pI) of 11.1 would support its presumed function as antimicrobial peptide\(^{63,66}\). sfTSLP may also

---

**Figure 7 | Structure of the TSLP:AMG-157Fab complex and insights into TSLP antagonism and plasticity.** (a) Cartoon representation of the determined X-ray structure for AMG-157Fab-fragment in complex with TSLP. TSLP helices (\(\alpha A\)-\(\alpha D\)) are coloured in blue and TSLP loop regions in white, except the AB loop (red). The red dashed line represents two disordered residues in the TSLP AB loop. N- and C-termini are indicated. The VH, CDR-loops and VL, C\(_L\) fragments of AMG-157Fab are coloured in maroon and green, respectively. V\(_H\), CDR-loops are labelled and shown as yellow. BSA: buried surface area. (b) Detail of the TSLP:AMG-157Fab interface. Selected interface residues are labelled and shown as sticks. Dashed lines represent salt bridges and hydrogen bonds. (c) Superposition of the TSLP-TSLPR:IL-7R\(\alpha\) and TSLP:AMG-157Fab complexes based on the structural alignment of the two TSLP structures. TSLPR (yellow) and the light chain (green) and heavy chain (maroon) of the AMG-157Fab fragment are displayed in surface mode. (d) Aligned human TSLP X-ray structures are shown as cartoons and are coloured according to a rainbow scheme with the N-terminus in blue and the C-terminus in red. (e) Structural comparison of the main chain conformations of the \(\pi\)-helical turn in TSLP helix A in both determined TSLP structures. The water molecule in the \(\pi\)-helical turn of helix \(\alpha A\) of TSLP complex by AMG-157Fab (blue) is shown as a red sphere. (f) Zoomed regions of the \(^{1}H,^{15}N\) HSQC-spectrum for TSLP\(^{127–131}\) at 900 MHz show the Nc-Hc Trp148 side chain (left) and Ser45 main chain amide (right) resonances. The projection of these regions on top of each panel shows that both minor forms adopt an identical population.
TSLP pool secreted by keratinocytes, lung and gut epithelial cells

TSLP traps IC50 = 50 pM

AMG-157 mAb IC50 = 1 nM

Figure 8 | Mechanistic recapitulation for the assembly and antagonism of the pro-inflammatory TSLP mediated complex. Following capture and rearrangement of TSLP by TSLPR at the cell surface, shared IL-7Rα is recruited to initiate intracellular pro-inflammatory JAK-STAT pathways. TSLP cytokine traps and anti-TSLP mAbs may represent effective strategies for therapeutic targeting of TSLP. Positive and negative signs represent the surface electrostatics of TSLP and TSLPR, respectively. Stars indicate site I, II and III. Left right arrows represent protein interactions.

Methods

Protein expression in mammalian cells and purification. HEK293T (ATCC CRL-3216), HEK293S-TetR MGAT1+/− (ref. 41) and T-REx-293 (Thermo Fisher Scientific) cells were grown in high-glucose DMEM medium supplemented with 10% fetal calf serum, 105 units per l penicillin G and 1 g l−1 streptomycin in a 5% CO2 atmosphere at 37 °C. The medium of T-REx-293 cells was supplemented with 5 μg ml−1 blasticidin. Mammalian expression constructs for secreted proteins carrying a C-terminal hexahistidine-tag were generated in the pHLsec (ref. 68) and/or pcDNA4/TO vector (Thermo Fisher Scientific). For transient expression experiments 25 KDa branched PEI was used as transfection agent68. Before addition and/or pcDNA4/TO vector (Thermo Fisher Scientific) cells were grown in high-glucose DMEM medium supplemented with 100 μg ml−1 G418 and/or small-scale IMAC analysis using an HRP-coupled antibody directed against the C-terminal His tag at 1:5,000 dilution ratio (Invitrogen, catalogue no. R931-25) and/or small-scale IMAC purifications in batch mode using 2 ml of conditioned medium.

Stable, tetracycline-inducible polyclonal cell lines for pcDNA4/TO expression constructs were generated in HEK293S-TetR MGAT1−/+ cells or T-REx-293 cells by selection with 200 μg ml−1 zeocin62. To induce expression the growth medium was changed to serum-free medium. Post transfection of 3–4 days, secretion of recombinant protein into the conditioned medium was confirmed by western blot analysis using an HRP-coupled antibody directed against the C-terminal His tag at 1:5,000 dilution ratio (Invitrogen, catalogue no. R931-25) and/or small-scale IMAC purifications in batch mode using 2 ml of conditioned medium.

Production of recombinant TSLP and IL-7Rα. To produce biotinylated TSLP, TSLPR and IL-7Rα cDNA fragments for TSLP (NP_002176.2; residue 1–239), and the TSLPR and IL-7Rα ectodomains were cloned into the pDisplay-BirA-vector (Novagen). Biotinylated thrombin was removed by incubation with streptavidin agarose beads. Refolded proteins were further purified by size-exclusion chromatography using a Superdex 75 column with HBS pH 7.4 as running buffer.

Preparation of TSLP:TSLPR:IL-7Rα complex for crystallization. Following purification of TSLPRN47Q from stable HEK293S-TetR MGAT1−/+ cells, N-linked glycosylation was trimmed by overnight incubation at room temperature with EndoH (New England Biolabs) using 7.5 kU of EndoH per mg of complex. Biotinylated thrombin was removed by incubation with streptavidin agarose beads. Refolded proteins were further purified by size-exclusion chromatography using a Superdex 75 column with HBS pH 7.4 as running buffer.

Production of TSLP and IL-7 and soluble TSLPR and IL-7Rα. Complementary DNA (cDNA) fragments (Genscript) encoding full-length human TSLP(NP_002176.2; residue 1–239) and the extracellular fragments of human TSLPR (NP_071431.2; residue 1–221) and human IL-7Rα (NP_002176.2; residue 1–239) were cloned into the pHL-AVITAG vector68. Before transfection in E. coli BL21(DE3) strain as inclusion bodies and refolded in vitro60 using 6 M Guanidine-HCl as denaturing agent. Following refolding, the N-terminal His tag was removed using biotinylated thrombin (Novagen). Biotinylated thrombin was removed by incubation with streptavidin agarose beads. Refolded proteins were further purified by size-exclusion chromatography using a Superdex 75 column with HBS pH 7.4 as running buffer.

Preparation of TSLP:TSLPR:IL-7Rα complex for crystallization. Following purification of TSLPRN47Q from stable HEK293S-TetR MGAT1−/+ cells, N-linked glycosylation was trimmed by overnight incubation at room temperature with EndoH (New England Biolabs) using 7.5 kU of EndoH per mg of complex. The binary TSLP:TSLPRN47Q complex was formed by adding a molar excess of refolded TSLP127–131 to EndoH-treated TSLPRN47Q. The binary complex TSLP127–131:TSLPRN47Q was isolated and separated from the excess of TSLP127–131 by SEC using a Superdex 75 column with HBS pH 7.4 as running buffer. The ternary complex was then formed by adding a molar excess of the refolded IL-7Rα ectodomain...
to the binary TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup> complex. The ternary complex was isolated and separated from the excess of IL-7Rα by SEC using a Superdex 200 column with HBS pH 7.4 as running buffer. Fractions corresponding to the ternary TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup>:IL-7Rα complex were pooled and concentrated by centrifugal ultrafiltration to a concentration of 6 mg ml<sup>−1</sup>. The protein sample was then aliquoted and flash frozen in liquid nitrogen.

**Production of anti-TSLP mAb and Fab fragment and Fab:TSLP.** cDNA fragments (Gen9) encoding the Tezepelumab (AMG-157) lambda light chain, the IgG2 heavy chain, and the PCR-derived V<sub>H</sub>-V<sub>κ</sub> chain fragment were produced (Supplementary Table 5) and cloned between the AgeI and KpnI sites of the pHSec vector, in frame with the vector’s signal peptide. At the C-terminus, the heavy chain and the V<sub>κ</sub>-C<sub>κ</sub> fragment were purified from the conditioned medium by IMAC (Roche cOmplete column) and SEC (Superdex 200) using HBS pH 7.4 as running buffer. The Fab:TSLP complex was formed by incubating the Fab-fragment with a molar excess of refolded TSLP<sup>127–131</sup> produced in E. coli as described above. The complex was then isolated from the molar excess of TSLP<sup>127–131</sup> by SEC and concentrated to 10 mg ml<sup>−1</sup>. The protein sample was then aliquoted and flash frozen in liquid nitrogen.

**Protein crystallization.** Nanoliter-scale vapour diffusion crystallization experiments were set up to 293 K using a Mosquito crystallization robot (TTP Labtech) and commercially available sparse-matrix screens (Molecular Dimensions, Hampton research). The TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup>:IL-7Rα ternary complex crystals were produced and cryo-cooled by direct plunging into liquid nitrogen.

**Crystallographic structure determination.** X-ray diffraction measurements were conducted at the Proxima2B beam line (Synchrotron SOLEIL, Gif-sur-Yvette, France). All data were integrated and scaled using the XDS suite<sup>76</sup>. The structures for the TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup>:IL-7Rα complex and the TSLP<sup>127–131</sup>:AMG-157<sub>αβ</sub> complex were determined by maximum-likelihood molecular replacement (MR) as implemented in the program suite Phaser (ref. 71). Human TSLP and TSLPR search models were derived from an X-ray structure for human IL-7:IL-7R<sub>α</sub> complex (PDB entry 4NN5) 37. The search model for human IL-7Rα was obtained from the human IL-7:IL-7R<sub>α</sub> complex (PDB entry 5J12, to be published).

**Constructs for cellular activity assays and binding studies.** The pMET7-Flag-TSLPR was created by ligating the same linker fragment together with the expression cassette containing different analyte concentrations. IL-7, TSLPR and IL-7Rα assay, non-functionalized biosensors were used as a control. To measure the interaction between IL-7Rα and the TSLP:TSLPR complex, TSLP-loaded sensor tips were incubated with 320 nM of TSLPR which was also included in the assay buffer. The TSLP-TSLPR samples. The sensor traces from zero concentration samples were subtracted from the raw data traces before data analysis. To correct for bulk effects during the measurements the interaction between IL-7Rα and TSLPR a column of non-functionalized sensors was used to enable double reference subtraction. All data were fitted with the ForteBio Data Analysis 9.0.4 software using a 1:1 ligand model.

**Small-angle X-ray scattering data collection and analysis.** SAXS data were measured on the SWING beam line at the SOLEIL Synchrotron (Gif-sur-Yvette, France). Around 50 µl of glycan-minimized ternary TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup>:IL-7Rα complex (6 mg ml<sup>−1</sup>), as prepared for crystallographic studies, was injected onto an Agilent 1.4 × 300 mm Bio SEC-3 column with 300 Å pore size and HBS pH 7.4 as running buffer at a flow speed of 0.2 ml min<sup>−1</sup> at 288 K. X-ray scattering data were collected in continuous flow mode with 1 s exposure time per frame and an acquisition rate of 1 frame every 2 s. Data were recorded within a momentum transfer range of 0.1 Å<sup>−1</sup> < q < 0.6 Å<sup>−1</sup>, with q = 4πsinθ/λ. Raw data were radially averaged and subtracted using Fovx v3.27 (developed at Synchrotron SOLEIL and provided by Xenocs (Saenonce, France)). The quality of the data set was checked with Fovx, including checking the stability of the intensity over the length of the elution peak and by scaling all curves to the most intense scattering profile. The final scattering curve was obtained by averaging the unscaled, buffer-subtracted scattered profiles from frames 119–128, which correspond to the top of the elution peak. Structural parameters were determined with the ATOMS suite<sup>76</sup>.

**Biolayer interferometry.** BLI experiments were performed in PBS-buffer supplemented with 0.01% (w/v) BSA and 0.002% (w/v) Tween 20, with an Octet RED6 instrument (ForteBio), at operating at 25°C. Streptavidin-coated biosensors were functionalized with biotinylated TSLP<sup>127–131</sup>:TSLPR<sup>N47Q</sup>:IL-7Rα and quenched with a 10 µg ml<sup>−1</sup> biotin solution and then dipped into solutions containing different analyte concentrations. IL-7, TSLPR and IL-7Rα ectodomains produced from stable transfectant HEK293-Tef-TGRα<sup>−/−</sup> cells were used as analyte. To verify that no non-specific binding was present during the interaction assay, non-functionalized biosensors were used as a control. To measure the interaction between IL-7Rα and the TSLP:TSLPR complex, TSLP-loaded sensor tips were incubated with 320 nM of TSLPR which was also included in the assay buffer. The TSLP-TSLPR samples. The sensor traces from zero concentration samples were subtracted from the raw data traces before data analysis. To correct for bulk effects during the measurements the interaction between IL-7Rα and TSLPR a column of non-functionalized sensors was used to enable double reference subtraction. All data were fitted with the ForteBio Data Analysis 9.0.4 software using a 1:1 ligand model.
a codon optimized hTSLP cDNA-fragment into the Clal/XbaI opened pMef7-flag-mTSLP vector. pMef7-HA-IL-7R was created by ligating a codon optimized hIL-7R cDNA-fragment into the BglII-SalI opened pMef7-Flag. Site-directed mutations in these vectors were introduced via the Quikchange protocol (Stratagen). Site-directed mutations of pHl-hTSLP127A/R130S were first introduced in the pUC57-hTSLP vector via the Quikchange protocol, followed by ligation of the EcoRI/KpnI mutant hTSLP DNA fragment into the EcoRI/KpnI opened pHl-hTSLP127A/R130S vector. All constructs were used for site-directed mutagenesis of human TSLP, TSLPR and IL-7R.

Competitive TSLP-SEAP cell binding assay.

HEK293T cells were co-transfected with pMef7-TSLP, a codon optimized hTSLPR cDNA-fragment into the ClaI/XbaI opened pMet7-flag-Nature Communications | DOI: 10.1038/ncomms14937 | www.nature.com/naturecommunications

site-directed mutagenesis of human TSLP, TSLPR and IL-7R receptors, HEK293T cells were co-transfected with 150 ng pMET7-Flag-hTSLPR, reporter plasmid per well of a 6-well plate. When comparing wild type and mutant TSLP in FACS buffer. The concentration of wild type and mutant TSLP was determined by ELISA (Human TSLP Duoset ELISA, R&D Systems). The cells were washed three times with FACS buffer, and were used to quantify the amount of bound alkaline phosphatase activity using the PhospoLight kit (Tropix) in an Envision chemiluminescence counter (Perkin-Elmer). The data were plotted and fitted to a log inhibitor versus response curve as implemented in GraphPad Prism.

TSLP induced STAT5 reporter assay.

Competitive TSLP-SEAP cell binding assay.

For comparing wild type and mutant TSLP, HEK293T cells were co-transfected with 15 ng pMef7-Flag-TSLPR, 15 ng pMef7-HA-IL-7R, 900 ng empty pMef7 vector and 100 ng pGCL-β-casein-luci reporter plasmid per well of a 6-well plate. When comparing wild type and mutant receptors, HEK293T cells were co-transfected with 150 ng pMef7-Flag-hTSLP, 150 ng pMef7-HA-IL-7R, 600 ng empty pMef7 vector and 100 ng pGCL-β-casein-luci reporter plasmid per well of a 6-well plate. The pGCL-β-casein-luci luciferase reporter carries a set of five repeated STAT5 responsive motifs of the β-casein promoter. One day after transfection, the cells were detached with cell dissociation buffer (Life Technologies), and resuspended in DMEM + 10% fetal bovine serum. Following counting, 50% of the cells were seeded in a new six-well plate for FACS analysis, and 2% of the cells were seeded per well in 96 well plates and stimulated with increasing concentrations of hTSLP. The luciferase activity was determined on day two after transfection using an Envision chemiluminescence counter. The fold induction of luciferase activity was calculated by the ratio of the luminescence signal (cps) from cells stimulated with hTSLP to the signal from the unstimulated cells. The data were plotted and fitted to a log inhibitor versus response curve as implemented in GraphPad Prism.

The expression of Flag-tagged hTSLP at the cell surface was determined using a mouse monoclonal anti-Flag M2 antibody (Sigma) and AlexaFlour488 labelled goat anti-mouse antibody (Molecular Probes) on a FACSCalibur (BD Biosciences). The cells were then detached with 0.05% EDTA in phosphate buffered saline. Subsequently, 130,000 cells were incubated for 2h at 6°C with TSLP-SEAP containing conditioned medium (diluted 15-fold) and different concentrations of unlabelled wild type or mutant TSLP in FACS buffer. The concentration of wild type and mutant TSLP was determined by ELISA (Human TSLP Duoset ELISA, R&D Systems). The cells were washed three times with FACS buffer, and were used to quantify the amount of bound alkaline phosphatase activity using the PhospoLight kit (Tropix) in an Envision chemiluminescence counter (Perkin-Elmer). The data were plotted and fitted to a log inhibitor versus response curve as implemented in GraphPad Prism.

Nuclear magnetic resonance.

Isotopically labelled 15N-TSLP127–131 and 13C/15N-TSLP127–131 were produced in E. coli BL21(DE3) cells transformed with the PET15b-TSLP127-131 expression construct (see above). Cells were grown in minimal medium at 37°C supplemented with a 1 × MEM vitamin solution (Sigma Aldrich), 1 mg/l −1,N-acetyl-DL-Lysine (Sigma Aldrich), 100 μg/ml kanamycin, 10% fetal calf serum (Gibco) and glucose (EURISO-TOPL, CLM-1396) and induced with 1 mM IPTG. Isotopically labelled TSLP127–131 was refolded from inclusion bodies and its N-terminal His-tag was removed as described above. Protein samples for NMR measurements at concentrations of 582 μM (8.5 mg/ml) for 15N-TSLP127–131 and 628 μM (9 mg/ml) for 13C/15N-TSLP127–131 were generated for this region using Modeller 9.14 (ref. 79). To account for the structural heterogeneity of this loop, five diverse loop models were selected for molecular dynamics simulations. The apo-TSLP structures and TSLP:TLR complex structures were prepared separately. Five 250 ns molecular dynamics simulations, each with a different TSLP starting model, were performed for TSLP and TSLP:TLR (10 runs in total). All simulations were performed using Gromacs 5.1.1 (ref. 80) with the Amber99SB-ILDN force field and TIP3P explicit solvent. The crystal structure was placed in a rhombohedral dodecahedron extending 1.2 nm beyond the diameter of the system. An integration time step of 2 fs and the Verlet scheme of second order was used for all simulations. Van der Waals and electrostatic forces were truncated to 10 Å. Long-range Coulomb forces were treated with the particle mesh Ewald method and bonds involving hydrogen atoms were constrained. During equilibration, protein heavy atoms were harmonically restrained with a force constant of 1,000 kJ mol⁻¹ nm⁻². The crystal structure was relaxed using a steepest descent algorithm until the maximum force exerted on any atom was lower than 1,000 kJ mol⁻¹. A 300 ps NVT equilibration was then performed, starting at 30 K and increasing the temperature to 300 K over the course of 200 ps. Temperature control was achieved through two Bussi–Parinello thermostats coupled to protein and non-protein groups, each with a coupling time of 0.1 ps. Following equilibration, the system was coupled to a Berendsen barostat with a reference pressure of 1 bar and a coupling time of 0.5 ps for 500 ps of NPT equilibration. The 250 ns long production runs were performed using two Nose–Hoover thermostats with coupling times of 1 ps and reference temperatures of 300 K. Pressure control was achieved through a Parrinello–Rahman barostat with a reference pressure of 1 bar and a coupling time of 2 ps. Intraparticle snapshots were saved every 100 ps. Root-mean-square fluctuations around the average structure were calculated for the final 100 ns of simulation time.
Water-striped TSLP. An interesting feature of the TSLP crystal structure is the presence of a buried water molecule in the core. During the MD simulations described above, the buried water molecule remained bound in the protein core. To assess its structural role further, molecular dynamics simulations were performed in which the central water molecule was deleted from the starting model. Three 250 ns all-atom molecular dynamics simulations were completed. Spontaneous rehydration of this cavity through a channel located between the B and C helices by bulk water molecules was observed within 15–125 ns in each of three independent simulations. Inserted water molecule at the α-helical turn of TSLP helix A. A stabilizing water molecule can be observed in the A helix of the TSLP:AMG-157Fab crystal structure. We sought to investigate if water molecules were present at a similar position in our simulations by identifying water molecules for which the distance between the water oxygen, and the Tyr43 carbonyl oxygen and the Lys49 amide nitrogen was equal to or less than 3.5 Å. Such water molecules were identified in 19% of frames over the entire TSLP simulations.

Data availability. Protein Data Bank: Coordinates and structure factors for the crystal structure of the TSLPΔ17–131:TLSPN55Q1–72ζ complex and TSLPΔ17–131, AMG-157:Fab complex have been deposited with accession codes 5J11 and 5J13, respectively. Other PDB codes used in this study: 5J12, 4NNS, 3D12, 4HIE, 4HK0 and 3UP1.

Small Angle Scattering Biological Data Bank: SAXS data and coordinates of the best TLS model generated by the AllosMod-FoXS server have been deposited with accession code SASD899.

Protein sequences used in this study: Thymic stromal lymphopoietin (TSLP): NCBI NP_1490241.1; Thymic stromal lymphopoietin receptor (TSLPR): NCBI NP_071413.2 and Uniprot ID Q9H7C3; Interleukin-7 (IL-7): NCBI NP_000871.1; Interleukin-7 receptor α (IL-7Rα): NCBI NP_001278.2 and Uniprot ID P16871; Secreted alkaline phosphatase (SEAP): Uniprot P05157; Bovine Serum Albumine (BSA): Uniprot ID P00769; Bifunctional ligase/repressor BirA (BirA) UniprotID: L3K9G4. All other data are available from the corresponding author on reasonable request.

References
1. Quintemier, H. et al. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. Leukemia 15, 1286–1292 (2001).
2. Reche, P. A. et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. J. Immunol. 167, 336–343 (2001).
3. Soumelis, V. et al. Human epithelial cell triggered dendritic cell mediated allergic inflammation by producing TSLP. Nat. Immunol. 3, 673–680 (2002).
4. Bell, R. D. et al. The transcription factor STAT5 is critical in dendritic cells for the development of TH2 but not TH1 responses. Nat. Immunol. 14, 364–371 (2013).
5. Pandey, A. et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. Nat. Immunol. 1, 59–64 (2000).
6. Park, L. S. et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. J. Exp. Med. 192, 659–670 (2000).
7. Mackall, C. L., Fry, T. J. & Gress, R. E. Harnessing the biology of IL-7 for therapeutic application. Rev. Neuroimmunol. 11, 330–342 (2012).
8. Ghirardi, C. et al. No evidence for TSLP pathway activity in human breast cancer. J. Clin. Invest. 126, 458–470 (2016).
9. Noti, M. et al. Thymic stromal lymphopoietin-elicted basophil responses promote eosinophilic esophagitis. Nat. Med. 19, 1005–1013 (2013).
10. Redhu, N. S. & Gounni, A. S. Function and mechanisms of TSLP/TLSPR complex in asthma and COPD. Clin. Exp. Allergy 42, 994–1005 (2012).
11. Siracusa, M. C. et al. Thymic stromal lymphopoietin-mediated extramedullary hematopoiesis promotes allergic inflammation. Immunity 39, 1158–1170 (2013).
12. Ziegler, S. F. et al. The biology of thymic stromal lymphopoietin (TSLP). Adv. Pharmacol. 66, 129–155 (2011).
13. Hunninghake, G. M. et al. TSLP polymorphisms are associated with asthma in a large-scale, family-based study. Allergy 65, 1566–1575 (2010).
14. Liu, W. et al. Two single nucleotide polymorphisms in TSLP gene are associated with asthma susceptibility in Chinese Han population. Exp. Lung Res. 38, 375–382 (2012).
15. Torgerson, D. G. et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. Nat. Genet. 43, 807–889 (2011).
16. Rothenberg, M. E. et al. Common variants at 5q22 associate with pediatric eosinophilic esophagitis. Nat. Genet. 42, 289–291 (2010).
17. Spergel, J. M. From atopic dermatitis to asthma: the atopic march. Ann. Allergy Asthma Immunol. Nat. Immunol. 105, 99–106 quiz 107–9, 117 (2010).
18. Guerra, S. The biology of thymic stromal lymphopoietin (TSLP): Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation. Nature Commun. 4, 2675 (2013).
19. Vannella, K. M. et al. Combinatorial targeting of TSLP, IL-25, and IL-33 in 2 cytokine-driven inflammation and fibrosis. Sci. Transl. Med. 8, 337ra65 (2016).
20. Gauvreau, G. M. et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. N. Engl. J. Med. 370, 2102–2110 (2014).
21. Verstraete, K. et al. Structural basis of the proinflammatory signaling complex meditated by TSLP. Nat. Struct. Mol. Biol. 21, 375–382 (2014).
22. Lyman, S. D., Van Ness, K. P. & Paxton, R. J. Modified human thymic stromal lymphopoietin. US Patent 7709217 (2010).
23. Lundstrom, W. et al. Soluble IL7Rα potentiates IL-7 bioactivity and promotes autoimmune. Proc. Natl Acad. Sci. USA 110, E1761–E1770 (2013).
24. Verstraete, K. et al. Efficient production of bioactive recombinant human Flt3 ligand in E. coli. Protein J. 28, 57–65 (2009).
25. Lee, J. Y., Lin, S. C., Lee, J. Y., Lee, Y. C., Lee, H. G. & Lee, T. H. The expression and characterization of recombinant human Flt3 ligand. Acta Crystallogr. Sect. F 67, 325–331 (2011).
26. Comeau, M. R. & Ziegler, S. F. The influence of TSLP on the allergic response. Mucosal Immunol. 3, 138–147 (2010).
27. Poposki, J. A. et al. Proprotein convertases generate a highly functional heterodimeric form of thymic stromal lymphopoietin in humans. J. Allergy Clin. Immunol. doi:10.1016/j.jaci.2016.08.040 (2016).
28. Zhang, F., Li, A., Qian, N., Cai, J. & Qian, X. Biophysical characterization of glycosaminoglycan-IL-7 interactions using SPR. Biochimie 94, 242–249 (2012).
29. Cartailleau, J. P. & Luecke, H. Structural and functional characterization of TSLP ligand-receptor complexes. Acta Crystallogr. Sect. F 67, 325–331 (2011).
30. Peng, J. W. & Wagner, G. Investigation of protein motions via relaxation measurements. Methods Enzymol. 239, 563–596 (1994).
Acknowledgements

We thank the staff of Proxima 2A and SWING beam lines at SOLEIL synchrotron for excellent technical support; K.V. and K.P. are postdoctoral fellows of Research Foundation Flanders, Belgium (FWO). D.V.R. is a PhD fellow of the Research Foundation Flanders, Belgium (FWO). This work was supported by grants from the FWO (no G0C2214N to S.N.S. and F.P.), the Hercules Foundation (no AUGE-11-029 to S.N.S.), the Belgo-University 'Concerted Research Actions' (no BOF13-GOA-005 to R.B.), the Ghent University 'Group-ID Multidisciplinary research partnership' (to R.B.). The 900 MHz NMR facility was supported by the CNRS (TIGR RMN THC, FR-3050, France), University of Lille 1, the European community (EDRF), and the Région Nord-Pas-de-Calais (France). Computational resources and services for MD calculations were provided by the VSC (Flemish Supercomputer Center), funded by the FWO and the Flemish Government—department EWI. We thank Koen Verschueren for carefully reading the manuscript.

Author contributions

K.V. and A.D. cloned expression constructs, performed protein expression and purification and crystalization experiments. K.V. performed MALDIs and SAXS experiments. K.V. and S.N.S. determined crystallographic structures and carried out structural analyses. J.L. and G.L. performed NMR studies. J.L. and G.L. determined crystallographic structures and carried out structural analysis. J.L. and G.L. performed NMR studies. J.L. and G.L. performed NMR studies. K.V. and S.N.S. conceived and supervised the project. S.N.S. performed all the biophysical experiments.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: K.V., F.P., H.B., R.B. and S.N.S. have filed a patent application with the European Patent Office (EP 16163883.8) pertaining to the development of the TSLP-traps. The remaining authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/ reprintsandpermissions/

How to cite this article: Verstraete, K. et al. Structure and antagonism of the receptor complex mediated by human TSLP in allergy and asthma. Nat. Commun. 8, 14937 doi: 10.1038/ncomms14937 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.