

# Proprotein convertases generate a highly functional heterodimeric form of thymic stromal lymphopoietin in humans

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**Rationale:** Thymic stromal lymphopoietin (TSLP) is known to be elevated and truncated in nasal polyps (NPs) of patients with chronic rhinosinusitis and might play a significant role in type 2 inflammation in this disease. However, neither the structure nor the role of the truncated products of TSLP has been studied. **Objective:** We sought to investigate the mechanisms of truncation of TSLP in NPs and the function of the truncated products.

**Methods:** We incubated recombinant human TSLP with NP extracts, and determined the protein sequence of the truncated forms of TSLP using Edman protein sequencing and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. We investigated the functional activity of truncated TSLP using a PBMC-based bioassay.

**Results:** Edman sequencing and mass spectrometry results indicated that NP extracts generated 2 major truncated products, TSLP (residues 29-124) and TSLP (131-159). Interestingly, these 2 products remained linked with disulfide bonds and presented as a dimerized form, TSLP (29-124 + 131-159). We identified that members of the proprotein convertase were rate-limiting enzymes in the truncation of TSLP between residues 130 and 131 and generated a heterodimeric unstable metabolite TSLP (29-130 + 131-159). Carboxypeptidase N immediately digested 6 amino acids from the C terminus of the longer subunit of TSLP to generate a stable dimerized form, TSLP (29-124 + 131-159), in NPs. These truncations were homeostatic but primate-specific events. A metabolite TSLP

(29-130 + 131-159) strongly activated myeloid dendritic cells and group 2 innate lymphoid cells compared with mature TSLP. **Conclusions:** Posttranslational modifications control the functional activity of TSLP in humans and overproduction of TSLP may be a key trigger for the amplification of type 2 inflammation in diseases. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

**Key words:** Carboxypeptidase, chronic rhinosinusitis, dendritic cells, group 2 innate lymphoid cells, nasal polyps, posttranslational modification, proprotein convertases, thymic stromal lymphopoietin

Chronic rhinosinusitis (CRS) is a heterogeneous disease characterized by local inflammation of the upper airways and sinuses that persists for more than 12 weeks. CRS affects nearly 30 million Americans, is responsible for more than 500,000 surgeries annually, and produces significant morbidity.<sup>1-3</sup> Although there is an ongoing debate about endotypes of disease, most investigators still accept a paradigm in which CRS is generally divided into 2 groups on the basis of the presence or absence of nasal polyps (NPs): chronic rhinosinusitis with nasal polyps (CRSwNP) and CRS without NPs.<sup>4-7</sup> Although CRSwNP is usually characterized by strong signs of type 2 inflammation, including pronounced eosinophilia and accumulation of T cells, dendritic cells, mast cells, and group 2 innate lymphoid cells (ILC2s),<sup>7-12</sup> the mechanisms underlying the amplification of type 2 inflammation in CRSwNP have not been clarified.

An epithelial cytokine, thymic stromal lymphopoietin (TSLP), is known to control type 2 inflammation via activation of dendritic cells, mast cells, and ILC2s.<sup>12-16</sup> TSLP induces the recruitment of T<sub>H</sub>2 cells and the induction of T<sub>H</sub>2 differentiation in myeloid dendritic cells via production of chemokines, CC chemokine ligand (CCL)17 and CCL22, and induction of OX40 ligand, respectively.<sup>17</sup> Although TSLP alone is not sufficient to produce type 2 cytokines in mast cells and ILC2s, it strongly synergizes with IL-33 to do so.<sup>12,18-21</sup> TSLP is known to be highly expressed in several type 2 inflammatory diseases including atopic dermatitis and bronchial asthma.<sup>12-14,22-24</sup> We have recently found that TSLP is also upregulated in CRSwNP and that mRNA expression of TSLP positively correlates with markers of eosinophils and type 2 cytokines in NPs.<sup>25</sup> Recently, a clinical trial showed that an antihuman TSLP mAb (AMG 157) reduced allergen-induced allergic responses in patients with mild allergic asthma.<sup>26</sup> This indicates that TSLP may be a key factor in allergic and type 2 inflammatory diseases including CRSwNP, and is an important therapeutic target in these diseases.

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**Abbreviations used**

|              |                                             |
|--------------|---------------------------------------------|
| CCL:         | CC chemokine ligand                         |
| CMK:         | Decanoyl-Arg-Val-Lys-Arg-chloromethylketone |
| CPN:         | Carboxypeptidase N                          |
| CRS:         | Chronic rhinosinusitis                      |
| CRSwNP:      | Chronic rhinosinusitis with nasal polyps    |
| DTT:         | Dithiothreitol                              |
| ILC2:        | Group 2 innate lymphoid cells               |
| MS:          | Mass spectrometry                           |
| <i>m/z</i> : | Mass-to-charge ratio                        |
| NP:          | Nasal polyp                                 |
| PCSK:        | Proprotein convertase subtilisin/kexin      |
| PTM:         | Posttranslational modification              |
| TSLP:        | Thymic stromal lymphopoietin                |

It is now recognized that posttranslational modifications (PTMs) of cytokines and chemokines by endogenous proteases are important events in the fine-tuning of biological activity.<sup>27,28</sup> However, it is not known whether TSLP can be controlled by PTM after secretion from producing cells including epithelial cells. The human TSLP gene encodes a protein of 159 amino acids and contains a signal peptide (residues 1-28) in the N terminus.<sup>29,30</sup> After removal of the signal peptide, cells release 14.9 kDa TSLP (residues 29-159) as a mature protein, referred to as TSLP (29-159) or mature TSLP. Interestingly, we have recently found that mature TSLP is unstable in NP tissues and we have detected truncated TSLP with a molecular weight of approximately 10 to 11 kDa, when we incubate NP extracts with recombinant human mature 15.1 kDa TSLP (Met (start codon) + residues 29-159, referred to as TSLP (M29-159)).<sup>25</sup> Because a protease inhibitor cocktail completely blocks NP extract-mediated truncation and degradation of TSLP, this event is controlled by endogenous proteases in NPs. Importantly, NP extract-treated TSLP, which is a mixture of mature and truncated products, has a higher activity than does mature TSLP in mast cells.<sup>25</sup> These results indicate that TSLP may be posttranslationally modified by endogenous proteases in NPs and suggest that truncated TSLP may have enhanced activity when compared with mature TSLP. Although this was the first evidence that TSLP may be controlled by PTM, the truncation enzymes, the structure of truncated TSLP, and the function of the truncated products have not been identified.

In this study, we hypothesized that TSLP is posttranslationally modified by endogenous proteases and that cleaved active TSLP metabolites are involved in the amplification of type 2 inflammation in NPs. We therefore set out to define the TSLP products, their activities, and the enzymes that generate them in NP tissue.

**METHODS****Patients and biopsies**

Patients with CRS were recruited from the Otolaryngology Clinic and the Northwestern Sinus Center of Northwestern Medicine. NP tissue was obtained during routine endoscopic sinus surgery performed on patients with CRS. All patients met the criteria for CRS as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 and the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force.<sup>5,6</sup> Patients with an established immunodeficiency, pregnancy, coagulation disorder, or diagnosis of classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study.

Details can be found in this article's [Methods](#) section in the Online Repository at [www.jacionline.org](http://www.jacionline.org).

**TSLP cleavage assay**

Recombinant mature TSLP (M29-159) was preincubated with 1 mg/mL tissue extracts for 6 or 24 hours. In some experiments, TSLP was incubated with NP extract in the presence of 1% dimethyl sulfoxide (vehicle control) or protease inhibitors for 6 hours. Details can be found in the [Methods](#) section in this article's Online Repository.

**Cell culture**

Human PBMCs were stimulated with TSLP (M29-159), TSLP (M29-124), TSLP (131-159), proprotein convertase subtilisin/kexin (PCSK)-treated TSLP, and 10 ng/mL IL-33 for 48 (CCL17) or 72 (IL-5) hours. The CCL17 and IL-5 concentrations in the supernatants were measured with DuoSet ELISA kits (R&D Systems, Minneapolis, Minn). Details can be found in the [Methods](#) section.

**Reagents, Western blot analysis, PCR, protein sequencing, and mass spectrometry**

Details can be found in the [Methods](#) section in this article's Online Repository.

**RESULTS****Determination of cleavage products of TSLP by NP extracts**

To determine the cleavage products of TSLP, we incubated TSLP (M29-159) with NP extracts, and assessed N-terminal protein sequences using an Edman-based sequencer. We found that the N-terminal sequence of the 10-kDa protein was identical to mature TSLP, indicating that the cleavage of TSLP by NP extracts was at a C-terminal site ([Fig 1, A](#)). Because there is no system to detect the protein sequence of the C terminus, we also used matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to assess the molecular weight of each product. The combination of these 2 methods allowed us to identify the structure of the truncation products. We initially sought 10 kDa truncated TSLP and found a spectral peak having mass-to-charge ratio (*m/z*) value of 10,713 (peak D) representing TSLP (M29-124) ([Fig 1, B](#)). We also detected 2 peaks (*m/z* 3517 [peak E] and 3131 [peak F]) representing TSLP (131-159) and TSLP (131-156), indicating that a major truncation site might be between residues 124 and 125 or between 130 and 131. In addition, proteases from NPs induced additional truncation between residues 156 and 157. Indeed, we also found a peak having an *m/z* value of 14,674 (peak C) representing TSLP (M29-156) after incubation with NP extracts ([Fig 1, B](#)).

We generated a PBMC-based TSLP bioassay (see this article's [Results](#) section and [Fig E1](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org)), and then examined the activity of synthesized recombinant TSLP (M29-124) and TSLP (131-159) peptide (see [Fig E2](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) and data not shown). Surprisingly, TSLP (M29-124), TSLP (131-159), and their mixture did not induce production of CCL17 in PBMCs ([Fig 2, A](#)). Because about 80% of synthesized recombinant TSLP (M29-124) was conjugated to glutathione ([Fig E2, B](#)), we also dissociated glutathione using dithiothreitol (DTT) and assayed activity using PBMCs. However, DTT-treated TSLP (M29-124) still did not stimulate PBMCs ([Fig E2, C](#)).

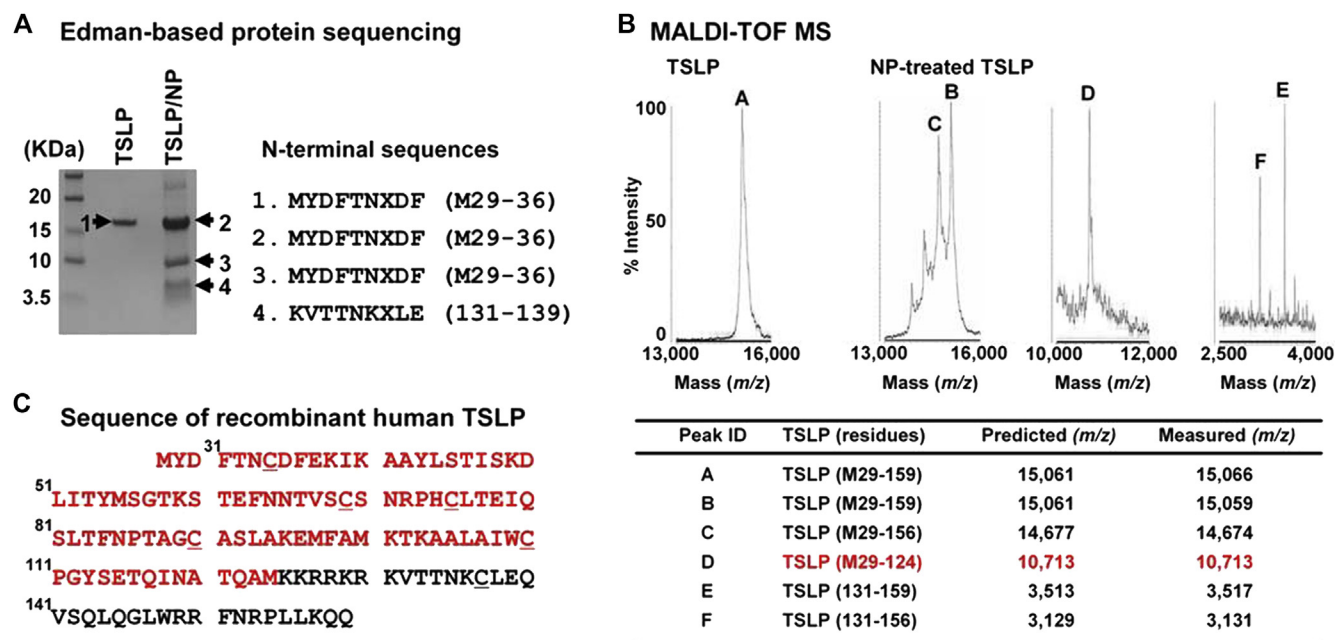


**FIG 1.** Identification of cleaved TSLP generated by NP extracts. Recombinant mature TSLP was incubated with 1 mg/mL NP extracts for 6 hours and TSLP proteins were separated by SDS-PAGE. N-terminal protein sequences of each product were detected using an Edman-based sequencer (**A**). TSLP was incubated with 1 mg/mL NP extracts for 24 hours and truncated products were detected by MALDI-TOF MS. The x-axis of the mass spectra represents  $m/z$  (**B**). Summary table of identified truncated products assigned by comparison of measured with predicted  $m/z$  by MALDI-TOF MS and N-terminal sequencing (Fig 1, **B**). Protein sequence of recombinant human TSLP can be found in **C** and red color indicates the sequence of a potential major active metabolite, TSLP (M29-124), produced by NP extracts. Underlined letters indicate the cysteine residues (Fig 1, **C**). The results are representative of 3 separate experiments with separate donors (Fig 1, **A** and **B**). MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight.

Because glutathione binds cysteine residues, we searched within the sequence of human TSLP protein and found 6 cysteine residues. Five were present in TSLP (M29-124) and 1 was in TSLP (131-159) (Fig 1, **C**). We therefore hypothesized that TSLP (M29-124) and TSLP (131-159) might still be dimerized via a disulfide bond after truncation. To test this hypothesis, we performed Western blots in reducing and nonreducing conditions. We found that the 10-KDa protein was not detected in nonreducing conditions (Fig 2, **B**). MALDI-TOF MS showed that the level of peak E (TSLP [M29-124]) was strongly enhanced and peak C (14,216  $m/z$ ) and peak D (13,830  $m/z$ ) were largely reduced in the presence of DTT (Fig 2, **C**). These results suggest that the 2 cleaved products are dimerized with a disulfide bond and the dimerized form of TSLP (referred to as TSLP [M29-124 + 131-159]) is a major active metabolite whereas TSLP (M29-124 + 131-156) is a minor dimerized metabolite in NPs (Fig 2, **D**).

### Proprotein convertases are rate-limiting enzymes in the truncation of TSLP

NPs are characterized by accumulation of inflammatory cells, including eosinophils, neutrophils, mast cells, and macrophages, all of which contain several proteases. To examine the potential role of proteases released from inflammatory cells on the truncation of TSLP in NPs, we incubated TSLP (M29-159) with supernatants from activated cultures of these cells. However, we could not detect the same 10-KDa cleavage product under

reducing conditions when TSLP was incubated with those cell lysates (not shown) or supernatants (Fig 3, **A**), although macrophage supernatants made a 10-KDa protein very weakly (Fig 3, **A**). We also incubated TSLP (M29-159) with NP extracts in the presence of various specific protease inhibitors and found that broad-spectrum serine protease inhibitors, Aprotinin and Nafamostat, almost completely blocked NP-dependent degradation of TSLP (Fig 3, **B**). This indicates that tissue serine proteases rather than proteases from inflammatory cells might be important for the truncation of TSLP in NPs.

Serine proteases can be classified into several clans.<sup>31,32</sup> The proteases of mixed nucleophile, superfamily A (PA) clan can be classified into 3 subfamilies, trypsin-like, chymotrypsin-like, and elastase-like proteases and approximately 75% of human serine proteases belong to this clan.<sup>31</sup> The serine protease B (SB) clan includes subtilisin-like proteases. Because the PA and SB clans are well studied and specific inhibitors are commercially available, we initially tested whether inhibitors specific to PA and SB clans block NP extract-dependent truncation of TSLP. We found that truncation of TSLP by NP extracts was inhibited by decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, the PCSK inhibitor), but not by TLCK (N $\alpha$ -Tosyl-L-lysine chloromethyl ketone hydrochloride; trypsin-like protease inhibitor), Tosyl phenylalanyl chloromethyl ketone (chymotrypsin-like protease inhibitor), or SSR69071 (elastase inhibitor) (Fig 3, **C**). This suggests that truncation of TSLP in NPs occurs in a PCSK family protease-dependent manner.

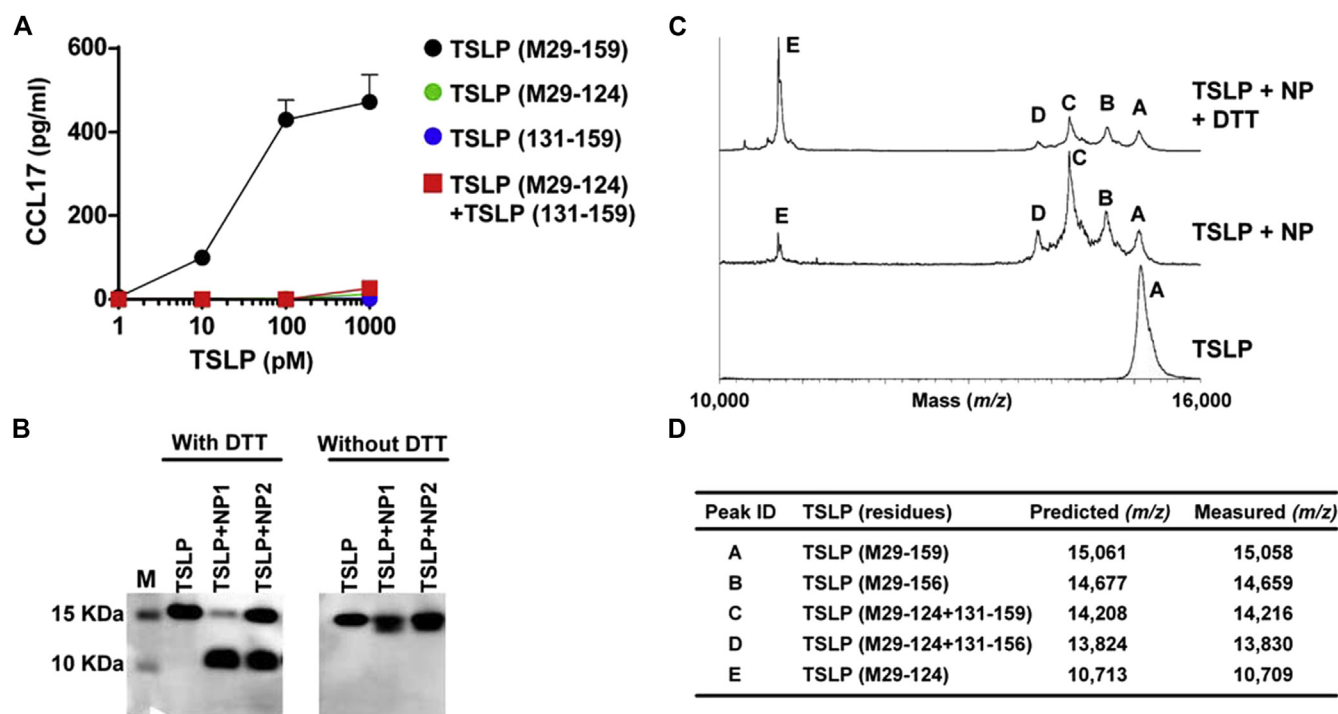


**FIG 2.** Cleaved TSLP is a heterodimeric protein. PBMCs were stimulated with TSLP (M29-159), TSLP (M29-124), and TSLP (131-159) for 48 hours. Concentrations of CCL17 protein in the culture supernatant were measured by ELISA (**A**). TSLP (M29-159) was incubated with 1 mg/mL NP extracts for 24 hours and TSLP proteins were separated by SDS-PAGE in the presence or absence of DTT (**B**). TSLP proteins were detected by Western blot using an anti-TSLP antibody. TSLP (M29-159) was incubated with 1 mg/mL NP extracts for 24 hours and truncated products were detected by MALDI-TOF MS in the presence or absence of 25 mM DTT (**C**). Summary table of identified truncated products assigned by comparison of measured with predicted *m/z* by MALDI-TOF MS (**D**). MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight.

The PCSK family consists of 9 members, with the first 7 members (PCSK1-7) recognizing shared motifs, (R/K) Xn(R/K)↓ (n = 0, 2, 4, or 6). The arrow indicates the cleaved site).<sup>33</sup> Because CMK inhibits PCSK1-7-mediated truncations, we used commercially available recombinant PCSKs in our TSLP truncation assay. We found that recombinant PCSK2, PCSK3, and PCSK7 dose dependently truncated TSLP and the cleaved product was similar in size to that generated by NP extracts (**Fig 3, D**). MS results under reducing conditions showed that recombinant PCSKs generated 2 truncated products, 11,565 *m/z* and 3,517 *m/z*, representing TSLP (M29-130) and TSLP (131-159) (**Fig 4, A**; see **Fig E3** in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) and data not shown). This indicates that the cleavage site for PCSKs is between residues 130 and 131, and PCSKs generate a heterodimeric metabolite TSLP (29-130 + 131-159).

### Carboxypeptidase N generates a stable heterodimeric metabolite of TSLP

Because NP extracts generated TSLP (M29-124 + 131-159), the C terminus of the longer subunit of heterodimeric TSLP generated by PCSKs seemed likely to be further digested by other NP proteases. To investigate this mechanism, we treated mature TSLP with PCSK3 for 24 hours and then incubated PCSK3-treated TSLP with NP extracts for up to 3 hours. We detected 7 peaks (*m/z* 11,561, 11,417, 11,282, 11,126, 10,966, 10,842, and 10,712), representing TSLP (M29-130) to TSLP

(M29-124), appearing after 30-minute incubation with NP extracts (**Fig 4, B and C**). Interestingly, TSLP (M29-124) was time-dependently generated by NP extracts and was the major product after 1-hour incubation (**Fig 4, B**). In the absence of DTT, we found a major peak having an *m/z* value of 14,224 representing TSLP (M29-124 + 131-159) by MS assay (not shown). Importantly, the C-terminal amino acid digestions occurred one by one and were not inhibited by Nafamostat or CMK, indicating that these cleavages occur in a serine protease-independent but a carboxypeptidase-dependent manner (**Fig 4, B**; see **Fig E4** in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Because NP extracts digested 6 amino acids representing KKRRKR from the C terminus of a longer peptide, we hypothesized that carboxypeptidase N (CPN), which removes lysine and arginine from the C terminus of many proteins, might be an enzyme responsible for this digestion. Interestingly, we found that NP extracts generated TSLP (M29-130 + 131-159), with an *m/z* value of 11,570 and 3,517 under reducing MS conditions, in the presence of a CPN inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (**Fig 4, D**, and data not shown). These results indicate that PCSK family proteases are rate-limiting enzymes in the truncation of mature TSLP (29-159) to generate the dimerized form TSLP (29-130 + 131-159) and then CPN further digests 6 amino acids from the C terminus of the longer subunit to generate a stable and active heterodimeric metabolite, TSLP (29-124 + 131-159), in NPs.

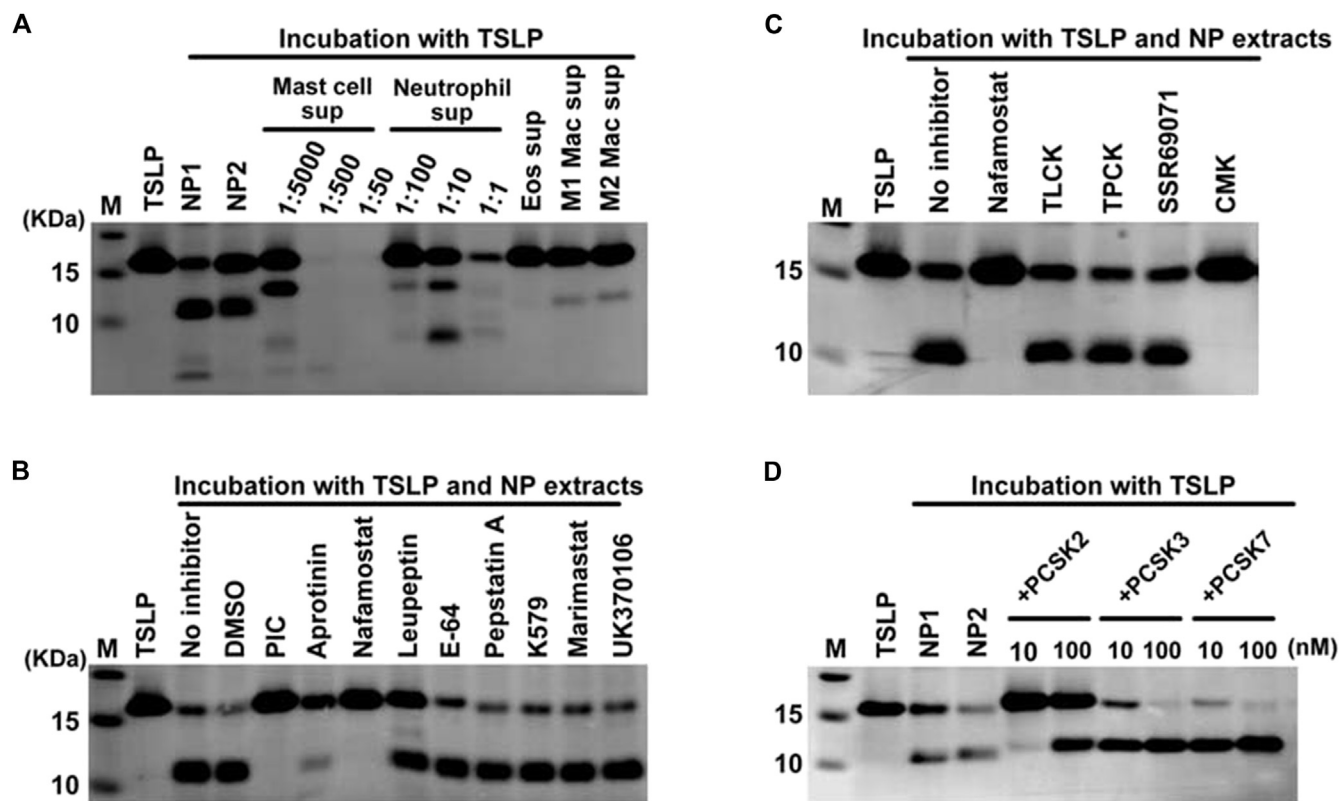


**FIG 3.** PCSKs are key truncation enzymes in NPs. TSLP (M29-159) was incubated with supernatants from activated mast cells, neutrophils, eosinophils (Eos), M1 macrophages (Mac), and M2 Mac for 24 hours (A). TSLP was incubated with 1 mg/mL NP extracts in the presence of 1% DMSO (vehicle control), 1% PIC, 1  $\mu$ M Aprotinin, 10  $\mu$ M Nafamostat mesylate, 10  $\mu$ M Leupeptin, 10  $\mu$ M E-64, 10  $\mu$ M Pepstatin A, 10  $\mu$ M K579, 20  $\mu$ M Marimastat, 10  $\mu$ M UK370106, 200  $\mu$ M TLCK, 200  $\mu$ M TPCK, and 1  $\mu$ M SSR69071 or 10  $\mu$ M CMK for 24 hours (B and C). TSLP was incubated with 1 mg/mL NP extracts, recombinant PCSK2, PCSK3, or activated PCSK7 for 24 hours (D). TSLP proteins were detected by Western blot using an anti-TSLP antibody. The results are representative of 3 separate experiments with separate donors (Fig 3, B and C). DMSO, Dimethyl sulfoxide; PIC, protease inhibitor cocktail; sup, supernatant; TLCK, N $\alpha$ -Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, Tosyl phenylalanyl chloromethyl ketone.

### Truncations of TSLP by PCSK and CPN may be homeostatic events in primates but not in rodents

Several PCSKs are known to be ubiquitously expressed, and CPN is known to be constitutively expressed in the liver and is secreted into the bloodstream.<sup>33,34</sup> We also found that levels of mRNA for most PCSKs in control sinus tissues were similar to levels in NPs, and were higher than in inflammatory cells (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This suggests that truncation of TSLP may occur in control sinus tissue. Therefore, we examined whether control sinus tissue also truncated TSLP. Interestingly, we found that tissue extracts from control uncinate sinus tissue and tonsil, as well as serum, were able to truncate TSLP and the truncation products were the same size as those generated by NP extracts (Fig 5). This suggests that truncation of TSLP may be a homeostatic but not a disease-specific event. Surprisingly, skin tissue extracts almost completely digested TSLP protein at 1 mg/mL, and at lower concentrations, skin extracts did not generate TSLP (29-124 + 131-159) (Fig 5 and data not shown). This indicates that TSLP protein may not be stable in the skin, and that the balance of proteases and protease inhibitors might control the stability of TSLP protein in tissues. Further study will be required to identify the enzymes responsible for the digestion of TSLP in skin.

We next tested whether the identified truncation events can occur in animal models. We found that a PCSK and CPN cleavage site that contains 7 basic amino acids (KKRRKRK) is present only in primate TSLP but not in rodent TSLP including mouse (Fig 6), rat, and rabbit.<sup>14,35</sup> This suggests that the identified truncation events of TSLP can occur only in primates.

### Effect of truncation on the functional activity of TSLP

We have previously reported that NP extract-treated TSLP has a higher activity than mature TSLP, which indicates that dimerized TSLP (29-124 + 131-159) is an active form.<sup>25</sup> However, the degree of activity due to dimerized TSLP metabolites was not clear. Because the major metabolite made is a dimerized form and adding a linker between the 2 peptides may affect the functional activity, synthesis of a recombinant heterodimeric metabolite was risky. In addition, we were unable to assemble the desired recombinant dimerized protein without a linker (not shown). We therefore generated a completely truncated TSLP metabolite, TSLP (M29-130 + 131-159), using recombinant PCSKs (Fig 7, A; see Fig E3), and investigated the functional activity on myeloid dendritic cells type 1 (mDC1s) using PBMCs.

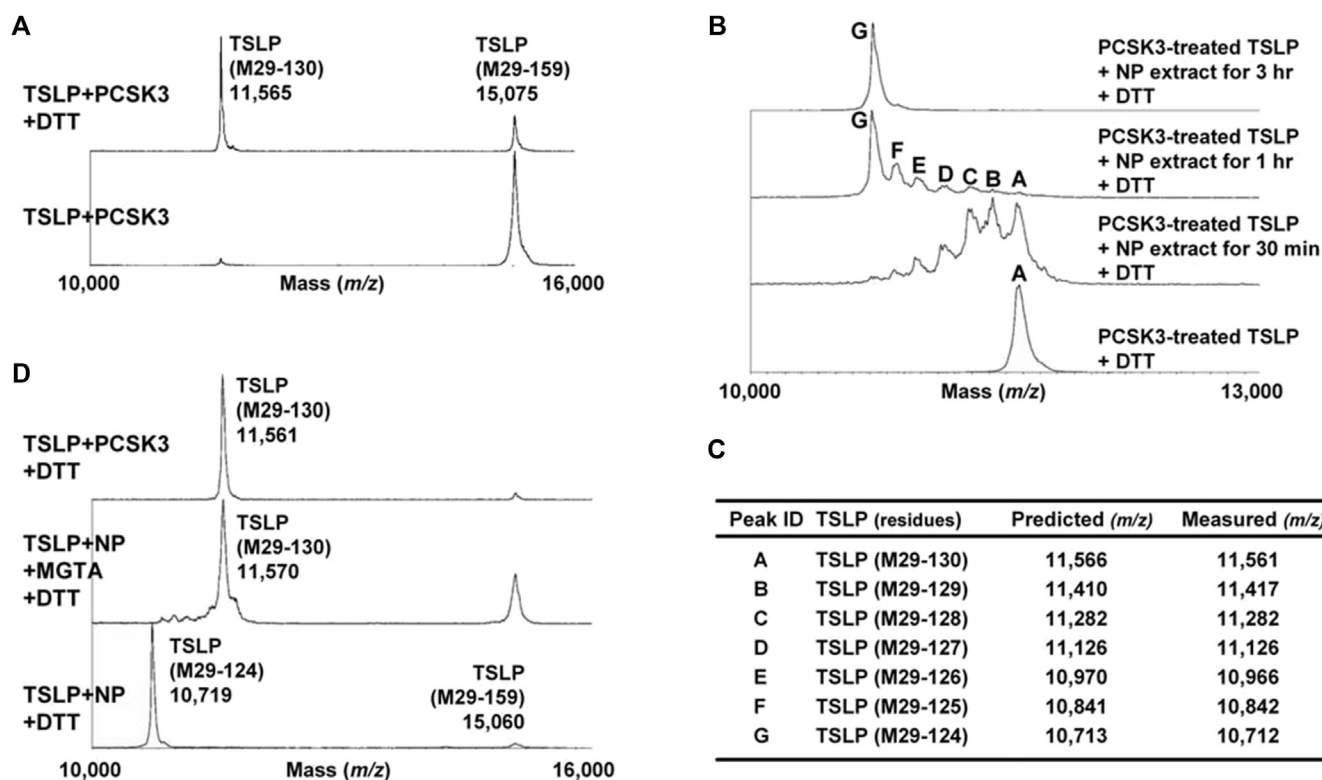


**FIG 4.** Carboxypeptidases generate a stable metabolite TSLP (M29-124 + 131-159). TSLP (M29-159) was incubated with 0.5  $\mu$ M recombinant PCSK3 for 24 hours (A). PCSK-treated TSLP was further incubated with 1 mg/mL NP extracts for 0.5 to 3 hours (B). TSLP was incubated with NP extract in the presence of 100  $\mu$ M MGTA (CPN inhibitor) for 6 hours (D). Truncated products were detected by MALDI-TOF MS with or without the presence of 25 mM DTT. Summary table of identified truncated products assigned by comparison of measured with predicted m/z by MALDI-TOF MS (C). MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid.

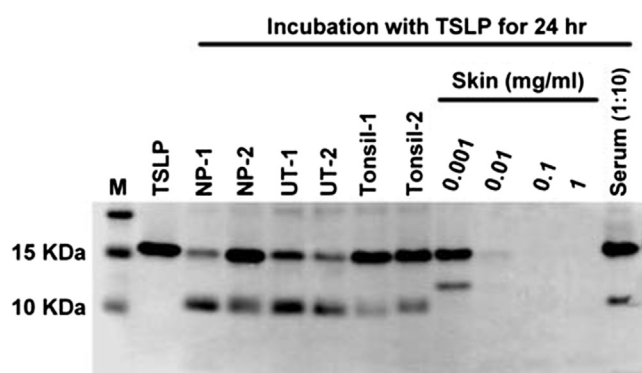


**FIG 5.** Truncation of TSLP by NP, control sinus, tonsil, skin, and serum. TSLP (M29-159) was incubated with 1 mg/mL NP extracts, 1 mg/mL control uncinat sinus tissue (UT) extracts, 1 mg/mL tonsil extracts, 0.001 to 1 mg/mL skin extracts, or 10% control serum for 24 hours. Truncated products were determined by Western blot under reducing conditions using anti-TSLP antibody.

We found that PCSK-treated TSLP (M29-130 + 131-159) dose dependently induced production of CCL17 and the potency was significantly higher ( $\sim$ 3-fold) than that of mature TSLP (Fig 7, B). Recently, Bartemes et al<sup>21</sup> reported that TSLP enhanced IL-33-mediated production of type 2 cytokines, IL-5 and IL-13, in PBMCs and this response was mediated via the activation of blood ILC2s. To test the effect of the truncation event on the

activation of ILC2s, we stimulated PBMCs with TSLP in the presence of IL-33. We found that PCSK-treated TSLP (M29-130 + 131-159) dose dependently enhanced IL-33-mediated production of IL-5 and the potency was also significantly higher than that of mature TSLP (Fig 7, C). These results indicate that the truncation event does not decrease, but rather enhances the functional activity of TSLP on TSLP-targeting cells including mDC1s and ILC2s.

## DISCUSSION

TSLP plays a critical role in type 2 inflammatory diseases as well as in host immunity. We initially discovered that TSLP was highly elevated in CRSwNP and that levels of TSLP positively correlated with markers of type 2 inflammation and eosinophilia in the US population.<sup>25</sup> This suggests that elevation of TSLP may control the accumulation of type 2 inflammation in NPs. However, we also have discovered that TSLP is truncated in NPs and truncated forms may retain activity.<sup>25</sup> This indicates more complicated TSLP regulation in humans. However, the structure and activity of the truncated forms of TSLP in NPs or other human tissue were not clear. We report here that TSLP protein is posttranslationally modified by 2 families of proteases in NPs and that a stable metabolite of TSLP demonstrates a heterodimerized structure and has increased biological activity compared with unmodified TSLP in TSLP receptor-mediated reactions.

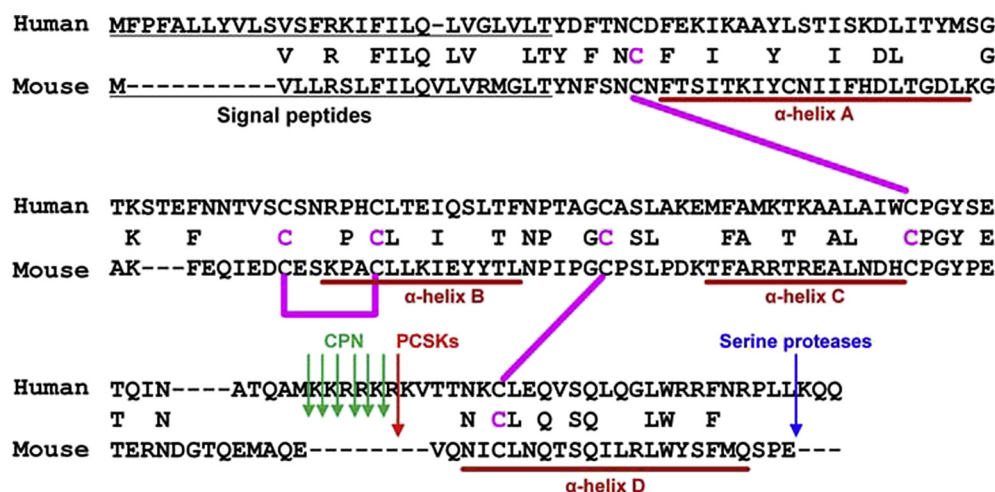


**FIG 6.** Sequence alignments for TSLP protein from human and mouse. Pink lines indicate the putative disulfide bonds based on the structure of mouse TSLP.<sup>35</sup> Arrows indicate identified cleavage sites.

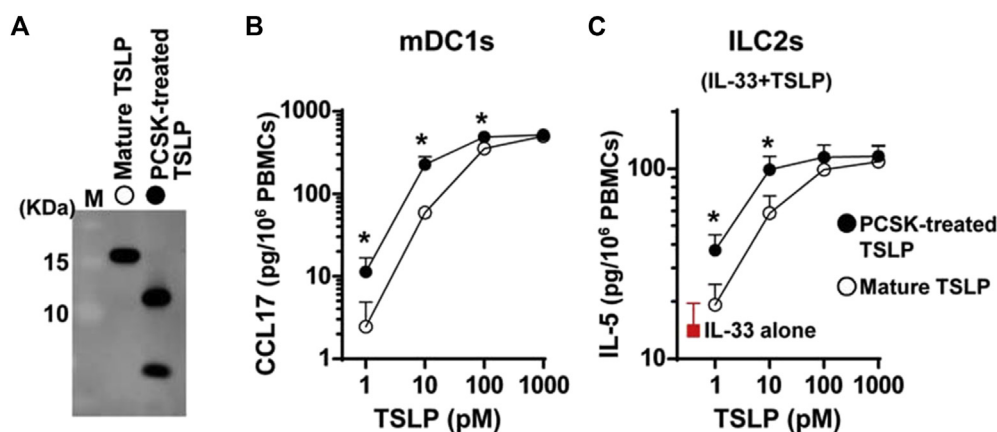


**FIG 7.** TSLP (29-130 + 131-159) activates mDCs and ILC2s. TSLP (M29-159) was incubated with 1  $\mu$ M recombinant PCSK3 for 24 hours and truncation efficiency was determined by Western blot in reducing conditions using anti-TSLP antibody (**A**). PBMCs were stimulated with 1 to 1000 pM mature TSLP or 1 to 1000 pM PCSK-treated TSLP (29-130 + 131-159) for 48 hours (**B**). PBMCs were stimulated with 1 to 1000 pM mature TSLP (M29-159) or 1 to 1000 pM PCSK-treated TSLP (29-130 + 131-159) in the presence of 10 ng/mL IL-33 for 72 hours (**C**). Concentrations of CCL17 and IL-5 proteins in the culture supernatant were measured by ELISA (Fig 7, **B** and **C**). Results shown are means  $\pm$  SEMs of 5 (Fig 7, **B**) or 4 (Fig 7, **C**) independent experiments. Differences between TSLP and PCSK-treated TSLP were analyzed using the paired *t* test and the Wilcoxon matched-pairs signed rank test. mDC, Myeloid dendritic cells; mDC1, mDC type 1. \**P* < .05.

Although PTM is a well-known mechanism controlling the activity of cytokines, it had not been investigated whether TSLP protein is also regulated by PTM. This is the first evidence that TSLP is posttranslationally modified by 2 families of enzymes in NPs. We first determined that PCSK family proteases are rate-limiting enzymes in the truncation of mature TSLP and in the generation of a heterodimeric structure. The mammalian PCSK family comprises 9 serine proteases. The first 7 PCSKs have shared recognition motifs and are responsible for the activation of a large number of proteins including peptide hormones and growth factors.<sup>33,36</sup> Because CMK completely blocked NP extract-mediated truncation of TSLP and recombinant PCSK2, 3, and 7 truncated TSLP (Fig 3), it was deemed likely that PCSK1-7 must be responsible for the truncation of TSLP in NPs. PCSK1 and PCSK2 are known to be mainly localized within

secretory granules of neural and endocrine cells.<sup>33</sup> PCSK4 is exclusively expressed in testicular germ cells, placenta, and ovary. In contrast, PCSK3, 5, 6, and 7 are widely expressed.<sup>33</sup> We also found that expression of PCSK3, 5, 6, and 7 was higher than that of PCSK1, 2, and 4 in NPs (see Fig E5). This indicates that PCSK3, 5, 6, and 7 are the main truncation enzymes likely to metabolize TSLP in NPs. We found the presence of mRNAs for PCSK3 and PCSK7 in mast cells by PCR, although we could not detect 10-KDa protein after treatment with mast cell supernatants (Fig 3 and Fig E5). However, we found that other mast cell proteases, including chymase and cathepsin G, digested TSLP within 24 hours (see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). These results indicate that mast cells may be able to truncate TSLP between residues 130 and 131 by PCSKs but other proteases immediately digest the

derived products to smaller peptides. We also detected some PCSKs in macrophages and PCSK5 in neutrophils. However, the levels of PCSKs in inflammatory cells were lower than in NPs (Fig E5). This indicates that PCSKs from inflammatory cells probably play a minor role in the truncation of TSLP in NPs. Future studies will be required to investigate whether all these PCSKs contribute to the truncation of TSLP in NPs and to identify the local distribution of PCSKs in NP tissue.

We determined that the second step of TSLP metabolism in NPs was mediated by a 6 amino acid digestion from the C terminus of the longer peptide, TSLP (29-130), by CPN within 1 hour (Fig 4) and this generated a major heterodimeric metabolite TSLP (29-124 + 131-159). This indicates that TSLP (29-130 + 131-159) is an unstable intermediate metabolite having less than a 1-hour half-life and that TSLP (29-124 + 131-159) is a stable metabolite in NPs. CPN is a tetrameric enzyme consisting of 2 catalytic subunits (CPN subunit 1) and 2 regulatory subunits (CPN subunit 2).<sup>34</sup> We found that mRNAs for CPN subunit 1 and CPN subunit 2 were almost undetectable in NPs and sinus tissues compared with the liver (Fig E5). This indicates that CPN is not synthesized locally in NPs. NPs are known to be characterized by intense edema with accumulation of plasma proteins.<sup>37</sup> This indicates that CPN may be coming from the bloodstream via vascular leakage. Future studies will be required to identify the source of CPN in NPs.

We determined the functional activity of TSLP (M29-130 + 131-159) compared with mature TSLP (M29-159) due to technical problems making recombinant TSLP (M29-124 + 131-159). We have found that truncation between residues 130 and 131 does not diminish, but rather enhances the functional activity, although TSLP (M29-124) did not activate the TSLPR complex (Figs 2 and 7). This indicates that the C-terminal end of TSLP is necessary for the activation of the TSLPR complex. Because CPN is known to be present in serum,<sup>34</sup> TSLP (M29-130 + 131-159) might be very quickly further digested to TSLP (M29-124 + 131-159) during PBMC culture. Therefore, the identified activity of TSLP (M29-130 + 131-159) might be from TSLP (M29-124 + 131-159). Further study will be required to identify the time-kinetics of the 6 amino acid digestion from TSLP (M29-130 + 131-159) in serum containing culture medium.

In contrast to TSLP (M29-130 + 131-159), we could not find any activity in the mixture of TSLP (M29-124) and TSLP (131-159) (Fig 2). Recently, Verstraete et al<sup>35</sup> identified the structure of the mouse TSLP-TSLPR-IL-7R $\alpha$  complex by X-ray crystallography. TSLP adopts a short-chain 4-helix bundle ( $\alpha$ -helix A to D). Helix A, helix D, and the overhand AB loop contact the TSLPR. In contrast, helix A and helix C engage with IL-7R $\alpha$ .<sup>35</sup> Based on the protein sequence homology of TSLP between human and mouse, helix D of human TSLP exists on residues 134 to 153, which is located within the shorter subunit, TSLP (131-159) (Fig 6).<sup>35</sup> Because TSLP (M29-124) completely lacks helix D and mixing TSLP (M29-124) and TSLP (131-159) cannot form the 3D structure with correct disulfide bonds, TSLP (M29-124), TSLP (131-159), and their mixture may not be able to bind to the TSLPR complex. PCSKs and CPN remove 6 amino acid residues between 125 and 130 from human TSLP. This sequence is located in the long CD overhand loop, which does not contribute to any binding interface between TSLPR and IL-7R $\alpha$ .<sup>35</sup> This may be a reason why heterodimeric TSLP metabolites do not lose their bioactivity after

the 6 amino acid removal from mature TSLP. This also indicates that cleavage of the CD overhand loop probably does not destroy the structure but may instead induce a small conformational change. This change may result in a higher binding affinity to the TSLPR complex. Further studies will be required to identify the crystal structure of the truncated human TSLP-TSLPR-IL-7R $\alpha$  complex.

Although TSLP is involved in type 2 inflammation and disease in both mice and humans, the mechanism of induction of type 2 inflammation by TSLP might differ in the 2 species. For example, TSLP can directly promote T<sub>H</sub>2 differentiation in mice from naive T cells in the absence of antigen-presenting cells.<sup>38,39</sup> In humans, however, TSLP-dependent T<sub>H</sub>2 differentiation requires antigen-presenting cells.<sup>17,22</sup> These data suggest that distribution of the TSLP receptor and activation mechanisms by TSLP might be different in humans and mice, and therefore human studies are important to understand the complex biology of TSLP. Importantly, we report here that human TSLP protein is controlled by PTM. Although we initially hypothesized that PTM of TSLP is a disease-specific event, our data clearly show that it is a homeostatic event in primates (Figs 5 and 6). This result suggests that the pathogenic role of TSLP might be controlled by overexpression and overproduction rather than by PTM. However, understanding PTM of human TSLP may be very important in clinical situations. PTM affects conformational change and functional activity of TSLP protein and TSLP-targeting drugs will need to block both mature and truncated active TSLP metabolites. However, this mechanism cannot occur in rodent species that are used in preclinical studies for drug development. These data suggest that human studies will be necessary for clinical development of TSLP-targeting drugs. A recent clinical trial indicated that AMG 157, an antihuman TSLP antibody, reduced allergic responses.<sup>26</sup> This suggests that AMG 157 may bind and inhibit both mature and truncated active TSLP metabolites. Future study will be required to determine whether AMG 157 inhibits TSLP metabolite-mediated responses.

In summary, we report here that TSLP is controlled by PTM in NP tissues, that PCSKs are rate-limiting enzymes in the truncation of TSLP, and that CPN immediately generates an active heterodimeric metabolite. Our findings indicate that the effects of TSLP overproduction in CRSwNP may be further amplified by PTMs. Future efforts to target TSLP in disease will need to account for this conformational variability and the mechanisms of PTM.

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## METHODS

### Patients and biopsies

Patients with CRS were recruited from the Otolaryngology Clinic and the Northwestern Sinus Center of Northwestern Medicine. NP tissue was obtained during routine endoscopic sinus surgery performed on patients with CRS. All subjects met the criteria for CRS as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 and the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force.<sup>E1,E2</sup> Patients with an established immunodeficiency, pregnancy, coagulation disorder, or diagnosis of classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study. Ethmoid and uncinate tissues from normal control subjects without a history of CRS were obtained during procedures for conditions other than CRS (septoplasty for nasal obstruction, transnasal endoscopy skull base procedures, repairs of facial fractures, treatment of nasal disorders in obstructive sleep apnea, etc). Tonsil tissue was obtained during tonsillectomy and normal skin tissues were obtained from facelift procedures at the Otolaryngology Clinic of Northwestern Medicine. All subjects signed informed consent forms and the protocol governing the procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine (Institutional Review Board Project Number STU00080917).

### Reagents and recombinant proteins

BSA, Tween 20, dimethyl sulfoxide, protease inhibitor cocktail (PIC, PN; P8340), Aprotinin, Leupeptin, E-64, Pepstatin A, Tosyllysine Chloromethyl Ketone Hydrochloride (TLCK), N $\alpha$ -Tosyl phenylalanyl chloromethyl ketone, PMA, and A23487 were purchased from Sigma-Aldrich (St Louis, Mo), Nafamostat mesylate, K579, Marimastat, UK370106, SSR69071, decanoyl-Arg-Val-Lys-Arg-chloromethylketone, recombinant PCSK2, PCSK3, PCSK7, IL-4, IFN- $\gamma$ , M-CSF, and GM-CSF were purchased from R&D Systems (Minneapolis, Minn), DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid was purchased from EMD Millipore (Billerica, Mass), and recombinant mature TSLP (M29-159) and IL-33 were purchased from BioLegend (San Diego, Calif). Recombinant TSLP (M29-124) was synthesized at BioLegend. TSLP (131-159) peptide was synthesized at RS Synthesis (Louisville, Ky).

### Tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 mL of PBS was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C and the supernatants were stored at -80°C. Before analysis, tissue homogenates were centrifuged at 16,000g for 15 minutes at 4°C and we used those supernatants for each assay.

### TSLP cleavage assay and Western blot analysis

Recombinant mature TSLP (M29-159) was preincubated with 1 mg/mL tissue extracts for 6 or 24 hours. In some experiments, TSLP was incubated with NP extract in the presence of 1% dimethyl sulfoxide (vehicle control), 1% PIC, 1  $\mu$ M Aprotinin (serine protease inhibitor), 10  $\mu$ M Nafamostat mesylate (serine protease inhibitor), 10  $\mu$ M Leupeptin (serine/cystein protease inhibitor), 10  $\mu$ M E-64 (cystein protease inhibitor), 10  $\mu$ M Pepstatin A (aspartyl protease inhibitor), 10  $\mu$ M K579 (Dipeptidyl peptidase-4 inhibitor), 20  $\mu$ M Marimastat (broad-spectrum MMP inhibitor), 10  $\mu$ M UK370106 (MMP3/12 inhibitor), 1  $\mu$ M SSR69071 (elastase inhibitor), 200  $\mu$ M TLCK (trypsin-like protease inhibitor), 200  $\mu$ M Tosyl phenylalanyl chloromethyl ketone (chymotrypsin-like protease inhibitor), or 10  $\mu$ M CMK (subtilisin-like proprotein convertase inhibitor) for 6 hours. In some experiments, TSLP was incubated with cell-free supernatants from activated mast cells, eosinophils, neutrophils, M1 macrophages, and M2 macrophages for 24 hours. Eosinophils and neutrophils were purified from human peripheral blood.<sup>E3</sup> In some experiments, TSLP was incubated with 40 U/mL tryptase (Enzo Life Sciences, Farmingdale, NY), 23 U/mL chymase (Enzo Life Sciences), 100  $\mu$ g/mL cathepsin D (Enzo Life Sciences), and 0.2 U/mL cathepsin G (EMD Millipore)

for 24 hours. Mast cells were generated from human CD34+ hematopoietic progenitor cells, as described previously.<sup>E4</sup> M2 macrophages were differentiated from monocytes by culturing in the presence of 10 ng/mL GM-CSF for 6 days and then in the presence of 100 ng/mL LPS and 20 ng/mL IFN- $\gamma$  for 24 hours. M1 macrophages were differentiated from monocytes by culturing in the presence of 25 ng/mL M-CSF for 6 days and then in the presence of 20 ng/mL IL-4 for 24 hours. Eosinophils, neutrophils, mast cells, and macrophages were stimulated with 1  $\mu$ M PMA and 1  $\mu$ M A23487 for 6 hours and we used cell-free supernatants in the assay. In some experiments, TSLP was incubated with recombinant PCSK2, PCSK3, or active PCSK7 for 6 or 24 hours. Recombinant PCSK7 (R&D Systems) was in the proprotein form. To generate an active form, recombinant PCSK7 was incubated with 2  $\mu$ g/mL thermolysin (Sigma) for 4 hours at 37°C and the enzymatic reaction was stopped by addition of 10 mM 1,10 phenanthroline (Sigma).

Samples for Western blotting were dissolved in LDS sample buffer (Invitrogen, Carlsbad, Calif). To detect TSLP, reduced samples were resolved on a 4% to 12% NuPAGE Bis-Tris Gel (Invitrogen) in an 2-(N-Morpholino) ethanesulfonic acid buffer system and then proteins were transferred onto a Polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif). In some experiments, samples were dissolved in DTT-free LDS sample buffer to examine the effect of the reduction of TSLP. The membranes were blocked in blocking buffer for fluorescent Western blotting (Rockland, Gilbertsville, Pa) for more than 2 hours and subsequently incubated overnight at 4°C with 50 ng/mL biotinylated sheep antihuman TSLP polyclonal antibody (R&D Systems) or 0.5  $\mu$ g/mL rabbit anti-TSLP polyclonal antibody (Peprotech, Rocky Hill, NJ). Following primary antibody incubation, the membranes were washed repeatedly in 0.1% Tween 20/PBS and then labeled by 45-minute incubation at room temperature with IRDye 700DX-conjugated streptavidin (1:50,000, Rockland Immunochemicals, Gilbertsville, Pa) or IRDye 800-conjugated donkey antirabbit IgG antibody (1:10,000, Rockland Immunochemicals). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Neb).

### Protein sequencing and mass spectrometry

Mature recombinant TSLP (M29-159) (5  $\mu$ M) was incubated with 1 mg/mL NP extracts for 6 or 24 hours. In some experiments, TSLP was incubated with NP extract in the presence of 1% PIC, 10  $\mu$ M Nafamostat mesylate, 10  $\mu$ M CMK, or 100  $\mu$ M DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid. The cleaved products were separated by SDS-PAGE and then proteins were transferred onto a PVDF membrane. The N-terminal protein sequences of each product were determined by an Edman-based Applied Biosystems 492 Procise Protein Sequencer at Texas A&M University, within the Protein Chemistry Laboratory. Mass spectrometry was performed on a Shimadzu Axima CFR MALDI-TOF MS at the Texas A&M University. In some experiments, samples were treated with 25 mM DTT for 15 minutes before analyzing by MALDI-TOF MS.

### Cell culture

Buffy coats from human peripheral blood were obtained from American Red Cross (St Paul, Minn). Human PBMCs were isolated by centrifugation on a Ficoll-Paque PREMIUM density gradient (GE Healthcare, Piscataway, NJ). Monocytes and mDC1s were separated from human PBMCs using the MACS system (Miltenyi Biotec, Auburn, Calif) with anti-CD14 MicroBeads (Miltenyi Biotec) or a CD1c+ Dendritic Cell Isolation Kit (Miltenyi Biotec), respectively. The purity of the monocytes (CD14+) and mDC1s (CD1c+, CD19-, CD11c+) was determined by flow cytometry and always exceeded 90%. The cells were suspended in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Invitrogen). The cells were stimulated with TSLP (M29-159), TSLP (M29-124), TSLP (131-159), PCSK-treated TSLP, and 10 ng/mL IL-33 for 48 (CCL17) or 72 (IL-5) hours. To generate TSLP (M29-130 + 131-159), 10  $\mu$ M recombinant TSLP mature (M29-159) was incubated with 1  $\mu$ M PCSK3 or 150 nM active PCSK7 for 24 hours. The effect of truncation was determined by SDS-PAGE, Western blot, and MALDI-TOF MS under reducing conditions. We used PCSK-treated TSLP in our *in vitro*

cell culture study if the efficiency of truncation exceeded 98%. The CCL17 and IL-5 concentrations in the supernatant were measured with DuoSet ELISA kits (R&D Systems).

### Real-time RT-PCR

A portion of nasal tissues for isolation of RNA was transferred to RNeasy lysis buffer (Qiagen, Germantown, Md) and stored at  $-20^{\circ}\text{C}$ . Total RNA from sinus tissue was extracted using QIAzol (Qiagen, Valencia, Calif) and was cleaned and treated with DNase I using NucleoSpin RNA (Clontech Laboratories, Mountain View, Calif) according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif) using a RNA 6000 Nano LabChip (Agilent Technologies). RNA in which RNA Integrity Number was greater than 7.0 was used for cDNA synthesis. Total RNA from 5 donor pooled human adult skin, lung, and liver tissues was purchased from BioChain Institute (Newark, Calif). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time RT-PCR was performed with a TaqMan method using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, Calif) in 20  $\mu\text{L}$  reactions (10  $\mu\text{L}$  2 $\times$  TaqMan Fast Advanced Master Mix [Applied Biosystems], 1  $\mu\text{L}$  20 $\times$  primer and probe mixture for target gene, 1  $\mu\text{L}$  20 $\times$  primer and probe mixture for  $\beta$ -glucuronidase [GUSB] plus cDNA equivalent to 10 ng of total RNA). Primer and probe sets for PCSK1 (Hs.PT.56a.20110761), PCSK2 (Hs.PT.56a.3835659), PCSK3 (FURIN: Hs.PT.56a.21271756), PCSK4 (Hs.PT.56a.39453462), PCSK5 (Hs.PT.56a.28190137), PCSK6 (Hs.PT.56a.3163847), PCSK7 (Hs.PT.56a.22452484), CPN1 (Hs.PT.58.1675481), and CPN2 (Hs.PT.58.21352543.g) were purchased from Integrated DNA Technologies (Coralville, Iowa). The primer and probe set for  $\beta$ -glucuronidase (GUSB; Human  $\beta$ -glucuronidase endogenous control, PN: 4326320E) was purchased from Applied Biosystems. The mRNA expression levels were normalized by the expression of a housekeeping gene, GUSB. Expression of GUSB was not significantly different between control ethmoid tissues and NPs (data not shown).

### Statistics

All data are reported as the mean  $\pm$  SEM. Differences between groups were analyzed using the paired *t* test and the Wilcoxon matched-pairs signed rank test. All statistical analyses were performed using GraphPad prism 6.04 software (La Jolla, Calif). A *P* value of less than .05 was considered significant.

## RESULTS

### Development of PBMC-based TSLP bioassay

Although we previously found that NP extract-pretreated TSLP (a mixture of mature and truncated TSLP) had higher activity than mature TSLP using a mast cell-based bioassay,<sup>E4</sup> the functional activity of the isolated truncation products of TSLP was not known. Ito et al<sup>E5</sup> previously reported that TSLP induced production of CCL17 in myeloid dendritic cells. Therefore, we stimulated PBMCs, mDC1s, CD14<sup>+</sup> monocytes, and CD1c, CD14, CD19-positive cell-depleted PBMCs with mature TSLP and monitored the production of CCL17 in those cell types. We found that PBMCs and mDC1s but not monocytes or PBMCs depleted of CD1c, CD14, and CD19 produced CCL17 after stimulation with TSLP (Fig E1, A). We also found that NP homogenate-treated TSLP induced more CCL17 than did mature TSLP in PBMCs (Fig E1, B). Because CD1c-positive cell (mDC1)-depleted PBMCs did not produce CCL17 on treatment with TSLP, we concluded that the production of CCL17 by TSLP in PBMCs was mediated by the activation of blood mDC1s. Therefore, we used PBMCs for the TSLP bioassay instead of mast cells and monitored the production of CCL17 as a marker of TSLP-mediated activation of mDC1s in PBMCs.

### REFERENCES

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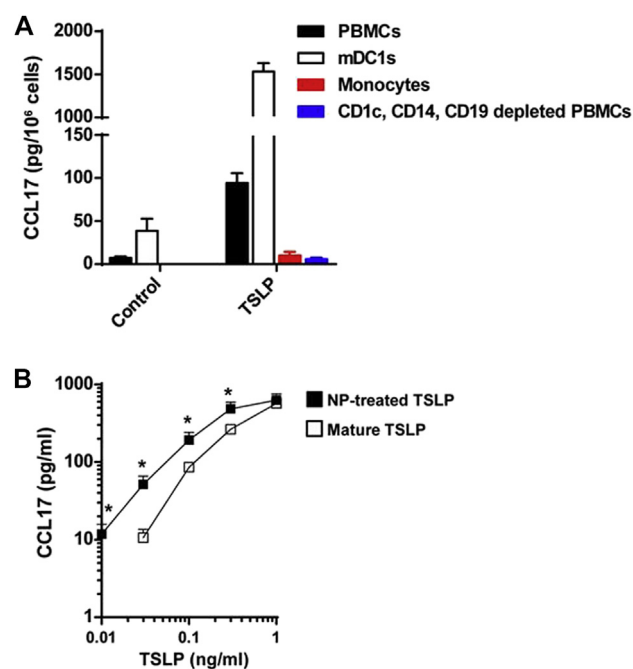


**FIG E1.** Myeloid DC-mediated production of CCL17 by TSLP in PBMCs. PBMCs, mDC1s, monocytes, and CD1c<sup>+</sup>, CD14<sup>+</sup>, and CD19<sup>+</sup> cell-depleted PBMCs were stimulated with medium control or 100 pg/mL recombinant mature TSLP for 48 hours (**A**). PBMCs were stimulated with 10 to 1000 pg/mL mature TSLP or NP-treated TSLP for 48 hours (**B**). Concentrations of CCL17 protein in the culture supernatant were measured by ELISA. Results shown are mean  $\pm$  SEM of 3 (Fig E1, **A**) or 4 (Fig E1, **B**) independent experiments. Differences between TSLP and NP-treated TSLP were analyzed using the paired *t* test and the Wilcoxon matched-pairs signed rank test (Fig E1, **B**). DC, Dendritic cell; mDC1, myeloid DC type 1. \**P* < .05.

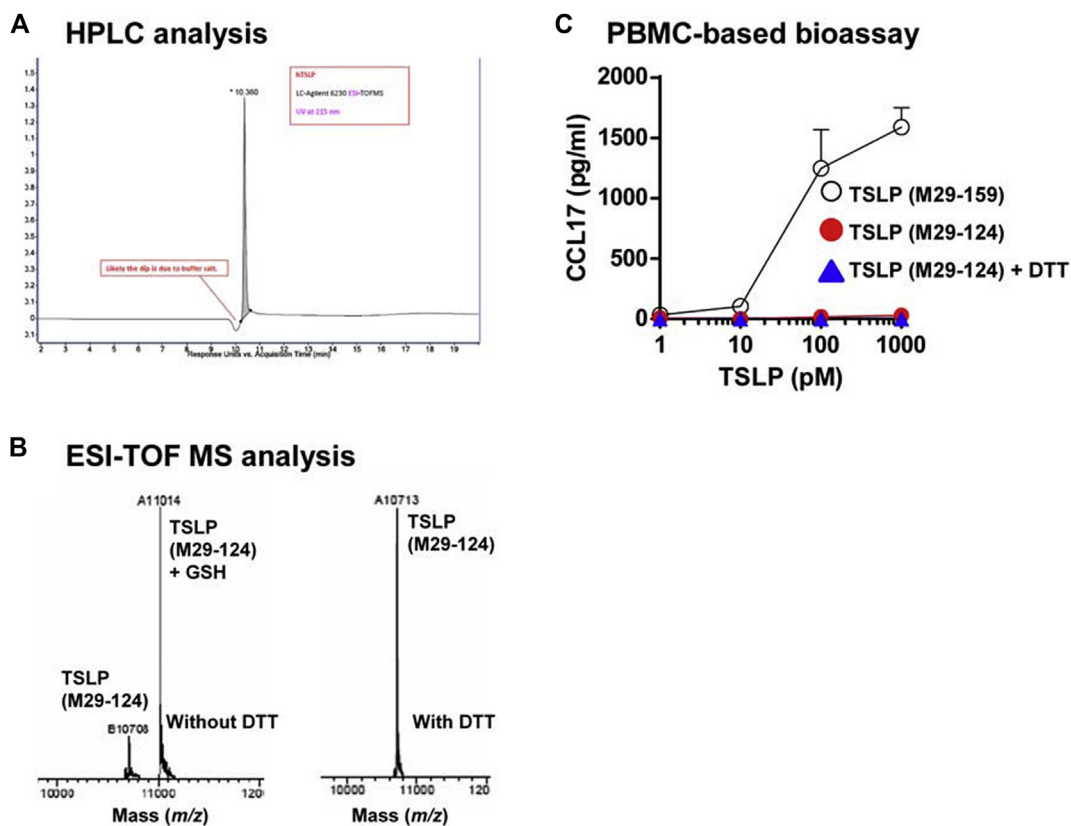


**FIG E2.** TSLP (M29-124) does not activate mDC1s in PBMCs. Recombinant TSLP (M29-124) was synthesized at BioLegend. The purity of recombinant TSLP (M29-124) was greater than 98% as analyzed by HPLC (**A**) and SDS-PAGE (not shown). Molecular mass was detected as 11,014 by ESI-TOF MS (**B**). 11,014  $m/z$  protein converted to 10,713  $m/z$  in the presence of DTT (Fig E2, **B**). PBMCs were incubated with TSLP (M29-159), TSLP (M29-124), and DTT-pretreated TSLP (M29-124) for 48 hours (**C**). Concentrations of CCL17 protein in the culture supernatant were measured by ELISA. Results shown are mean  $\pm$  SEM of 3 independent experiments. *ESI-TOF*, Electrospray ionization-time of flight; *GSH*, glutathione.

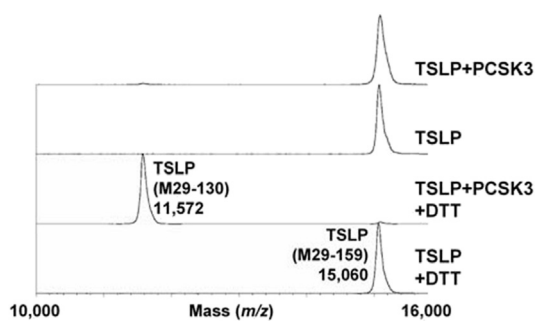


**FIG E3.** PCSKs generate TSLP (29-130 + 131-159) in NPs. Recombinant mature TSLP (M29-159) was incubated with 1  $\mu$ M PCSK3 for 24 hours and truncated products were detected by MALDI-TOF MS in the presence or absence of 25 mM DTT. *MALDI-TOF*, Matrix-assisted laser desorption/ionization-time of flight.

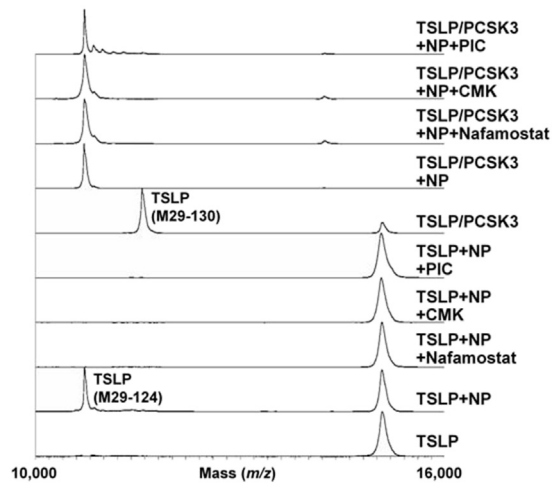


**FIG E4.** Serine protease inhibitor does not block 6 amino acid deletions from PCSK-treated TSLP by NP extracts. Recombinant mature TSLP (M29-159) and PCSK3-treated TSLP (TSLP/PCSK3) were incubated with NP extract in the presence of 1% PIC, 10  $\mu$ M CMK, or 10  $\mu$ M Nafamostat for 3 hours. Truncated products were detected by MALDI-TOF MS in the presence of 25 mM DTT. The x-axis of the mass spectra represents  $m/z$ . *MALDI-TOF*, Matrix-assisted laser desorption/ionization-time of flight.

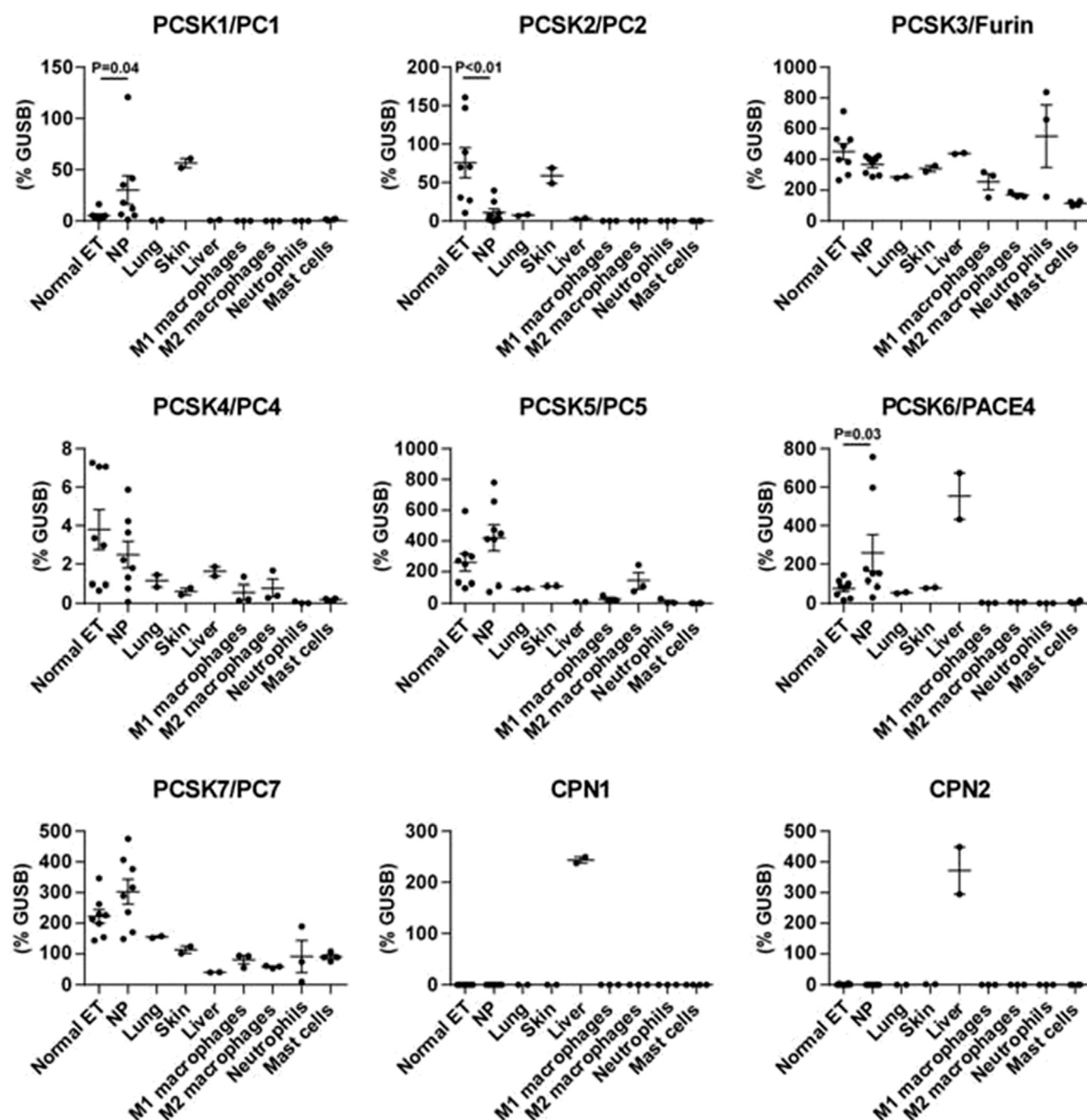


**FIG E5.** Expression of PCSKs and CPN in NPs. The gene expression of PCSK1-7 (also known as proprotein convertase 1 [PC1], PC2, furin, PC4, PC5, paired basic amino acid cleaving enzyme 4 [PACE4], and PC7, respectively), CPN subunit 1 (CPN1), and CPN subunit 2 (CPN2) in normal ethmoid sinus tissues (ET) ( $n = 8$ ), NP tissues ( $n = 8$ ), lung (pooled), skin (pooled), liver (pooled), M1 macrophages ( $n = 3$ ), M2 macrophages ( $n = 3$ ), neutrophils ( $n = 3$ ), and mast cells ( $n = 4$ ) was measured by using real-time RT-PCR. Gene expression was normalized by a housekeeping gene, GUSB, and expression levels were shown as % expression of GUSB. Pooled RNA from human liver was used as a positive control of the expression of CPN1 and CPN2. Differences between ET and NP were analyzed using the Mann-Whitney test.

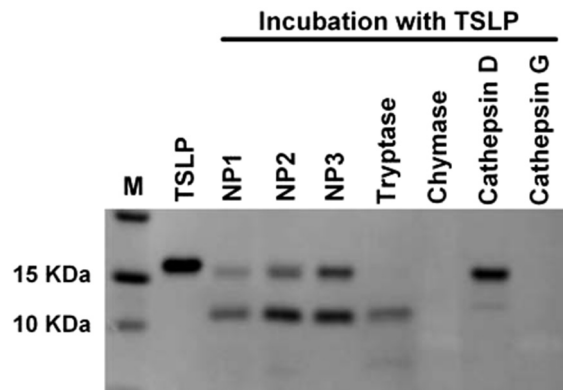


**FIG E6.** Digestion of TSLP by mast cell proteases. Recombinant mature TSLP (M29-159) was incubated with 1 mg/mL NP extracts, 40 U/mL tryptase, 23 U/mL chymase, 100  $\mu$ g/m cathepsin D, and 0.2 U/mL cathepsin G for 24 hours. Truncated products were determined by Western blot under reducing conditions using anti-TSLP antibody.