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

Julie A. Poposki, MS[a]; Aiko I. Klingler, PhD[a]; Whitney W. Stevens, MD, PhD[a]; Anju T. Peters, MD[a]; Kathryn E. Hulse, PhD[a]; Leslie C. Grammer, MD[a]; Robert P. Schleimer, PhD[a, b]; Kevin C. Welch, MD[b]; Stephanie S. Smith, MD[b]; Douglas M Sidle, MD[b]; David B. Conley, MD[b]; Bruce K. Tan, MD[b]; Robert C. Kern, MD[a,b]; Atsushi Kato, PhD[a, b*].

[a] Division of Allergy-Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA.

[b] Department of Otolaryngology - Head and Neck Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL, USA.

***** Address correspondence to Atsushi Kato, Ph.D.,
Division of Allergy-Immunology, Northwestern University Feinberg School of Medicine,
240 E. Huron, Room M305, Chicago, IL 60611 USA.
Phone: 312-503-0086, FAX: 312-503-0078
E-Mail: a-kato@northwestern.edu

Methods

Patients and biopsies

Patients with chronic rhinosinusitis (CRS) were recruited from the Otolaryngology clinic and the Northwestern Sinus Center of Northwestern Medicine. Nasal polyp 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.[E1, 2] 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 procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine (IRB Project Number: STU00080917).

Reagents and recombinant proteins

BSA, Tween 20, DMSO, protease inhibitor cocktail (PIC, PN; P8340), Aprotinin, Leupeptin, E-64, Pepstatin A, Tosyllysine Chloromethyl Ketone Hydrochloride (TLCK), Tosyl

phenylalanyl chloromethyl ketone (TPCK), PMA and A23487 were purchased from Sigma-Aldrich (St. Louis, MO), Nafamostat mesylate, K579, Marimastat, UK370106, SSR69071, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK), recombinant PCSK2, PCSK3, PCSK7, IL-4, IFN-γ, M-CSF and GM-CSF were purchased from R&D systems (Minneapolis, MN), DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) was purchased from EMD Millipore (Billerica, MA) and recombinant mature TSLP (M29-159) and IL-33 were purchased from BioLegend (San Diego, CA). 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 min at 4°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 min at 4°C and the supernatants were stored at -80°C. Before analysis, tissue homogenates were centrifuged at 16,000*g* for 15 min 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% DMSO (vehicle control), 1% PIC, 1 µM Aprotinin (Serine protease inhibitor), 10 µM Nafamostat mesylate (Serine protease inhibitor), 10 µM Leupeptin (Serine/cystein protease inhibitor), 10 µM E-64 (Cystein protease inhibitor), 10 µM Pepstatin A (Aspartyl protease

inhibitor), 10 μM K579 (DPP-IV inhibitor), 20 μM Marimastat (Broad spectrum MMP inhibitor), 10 μM UK370106 (MMP3/12 inhibitor), 1 μM SSR69071 (elastase inhibitor), 200 μM TLCK (trypsin-like protease inhibitor), 200 μM TPCK (chymotrypsin-like protease inhibitor) or 10 μM 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.[E3] In some experiments, TSLP was incubated with 40 U/ml tryptase (Enzo Life Sciences, Farmingdale, NY), 23 U/ml chymase (Enzo Life Sciences), 100 μg/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.[E4] 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-γ 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 μM PMA and 1 μM 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 μg/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 LDL sample buffer (Invitrogen). To detect TSLP, reduced samples were resolved on a 4-12% NuPAGE Bis-Tris Gel (Invitrogen) in an

MES buffer system and then proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). In some experiments, samples were dissolved in DTT free LDL 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 anti-human TSLP polyclonal antibody (R&D systems) or 0.5 µg/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 minutes incubation at room temperature with IRDye 700DX conjugated streptavidin (1:50,000, Rockland Immunochemicals, Gilbertsville, PA) or IRDye 800 conjugated donkey anti-rabbit IgG antibody (1:10,000, Rockland Immunochemicals). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Protein sequencing and mass spectrometry

5 µM mature recombinant TSLP (M29-159) 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 µM Nafamostat mesylate, 10 µM CMK or 100 µMMGTA. 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.

115

116 ***Cell culture***

117 Buffy coats from human peripheral blood were obtained from American Red Cross (St.
118 Paul, MN). Human PBMCs were isolated by centrifugation on a Ficoll-Paque PREMIUM
119 density gradient (GE Healthcare, Piscataway, NJ). Monocytes and mDC1s were separated from
120 human PBMC using the MACS system (Miltenyi Biotec, Auburn, CA) with anti-CD14
121 MicroBeads (Miltenyi Biotec) or a CD1c+ Dendritic Cell Isolation Kit (Miltenyi Biotec)
122 respectively. The purity of the monocytes (CD14+) and mDC1s (CD1c+, CD19-, CD11c+) was
123 determined by flow cytometry and always exceeded 90%. The cells were suspended in RPMI
124 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, and
125 100 μg/ml streptomycin (Invitrogen). The cells were stimulated with TSLP (M29-159), TSLP
126 (M29-124), TSLP (131-159), PCSK-treated TSLP and 10 ng/ml IL-33 for 48 (CCL17) or 72 (IL-
127 5) hours. To generate TSLP (M29-130 + 131-159), 10 μM recombinant TSLP mature (M29-159)
128 was incubated with 1 μM PCSK3 or 150 nM active PCSK7 for 24 hours. The effect of truncation
129 was determined by SDS-PAGE, western blot and MALDI-TOF MS under reducing conditions.
130 We used PCSK-treated TSLP in our *in vitro* cell culture study if the efficiency of truncation
131 exceeded 98%. The CCL17 and IL-5 concentrations in the supernatant were measured with
132 DuoSet ELISA kits (R&D Systems).

133

134 ***Real-time RT-PCR***

135 A portion of nasal tissues for isolation of RNA was transferred to RNAlater (Ambion,
136 Austin, TX) and stored at -20°C. Total RNA from sinus tissue was extracted using QIAzol
137 (Qiagen, Valencia, CA) and was cleaned and treated with DNase I using NucleoSpin RNA

(Clontech Laboratories, Mountain View, CA) according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using a RNA 6000 Nano LabChip (Agilent Technologies). RNA in which RIN 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, CA). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Real-time RT-PCR was performed with a TaqMan method using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) in 20 μl reactions (10 μl 2x TaqMan Fast Advanced Master Mix (Applied Biosystems), 1 μl 20x primer and probe mixture for target gene, 1 μl 20x primer and probe mixture for β -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, IA). Primer and probe set for β -glucuronidase (GUSB; Human β -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 ± 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, CA). A *p*-value of less than 0.05 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,[E4] the functional activity of the isolated truncation products of TSLP was not known. Ito *et al.*, previously reported that TSLP induced production of CCL17 in myeloid DCs (mDCs).[E5] Therefore we stimulated PBMCs, mDC1s, CD14+ 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 (Supplementary Fig. S1A). We also found that NP homogenate treated TSLP induced more CCL17 than mature TSLP in PBMCs (Supplementary Fig. S1B). Since CD1c positive cell (mDC1)-depleted PBMCs did not produce CCL17 upon 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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FIGURE LEGENDS

Supplementary Fig. S1. Myeloid DC mediated production of CCL17 by TSLP in PBMCs. PBMCs, mDC1s, monocytes and CD1c+, CD14+ and CD19+ cell-depleted PBMCs were stimulated with medium control or 100 pg/ml recombinant mature TSLP for 48 hours (A). PBMCs were stimulated with 10-1000 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 ± SEM of 3 (A) or 4 (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 (B). * $p < .05$.

Supplementary Fig. S2. 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 (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 ± SEM of 3 independent experiments.

Supplementary Fig. S3. PCSKs generate TSLP (29-130 + 131-159) in NPs.

Recombinant mature TSLP (M29-159) was incubated with 1 μM PCSK3 for 24 hours and truncated products were detected by MALDI-TOF MS in the presence or absence of 25 mM

DTT.

Supplementary Fig. S4. 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 μM CMK or 10 μM 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 mass to charge ratio (*m/z*).

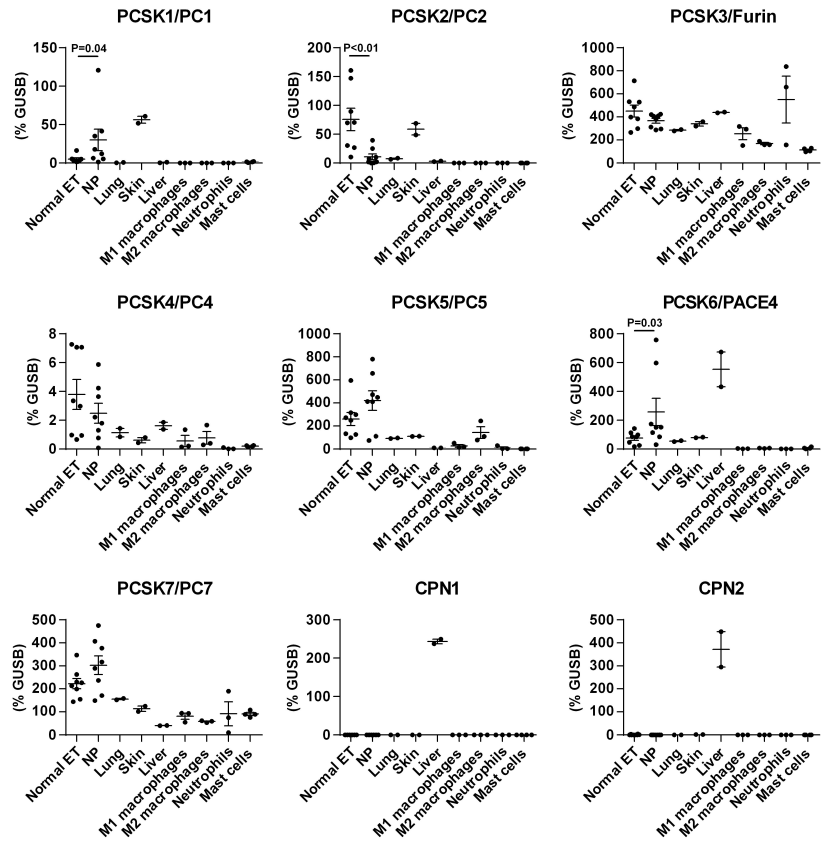
Supplementary Fig. S5. Expression of PCSKs and CPN in NPs.

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), carboxypeptidase N subunit 1 (CPN1) and 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.

Supplementary Fig. S6. 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 µg/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.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CPN2
(% GUSB)
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P<0.01
P=0.03
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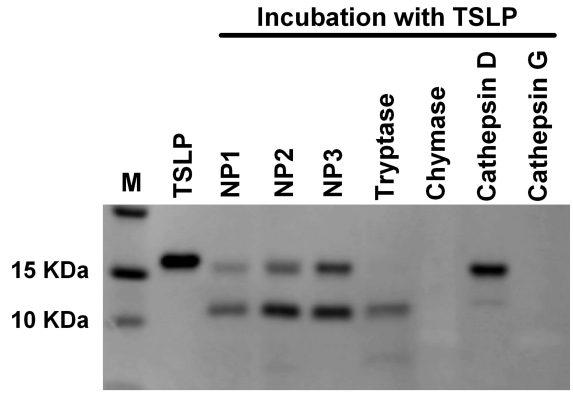
NP
Lung
Skin
Liver
M1 macrophages
M2 macrophages
Neutrophils
Mast cells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NP2
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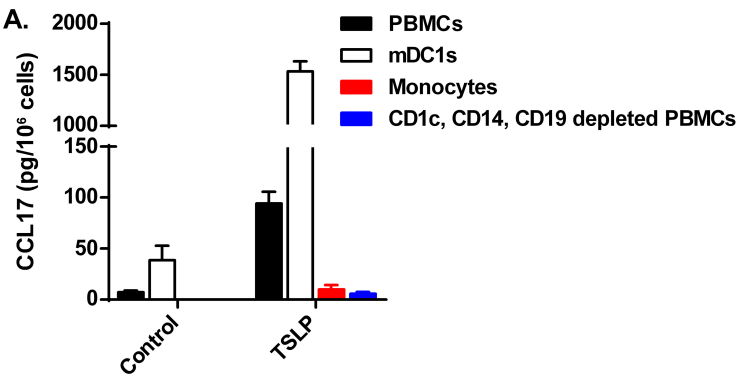
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1000
150
100
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PBMCs
mDC1s
Monocytes
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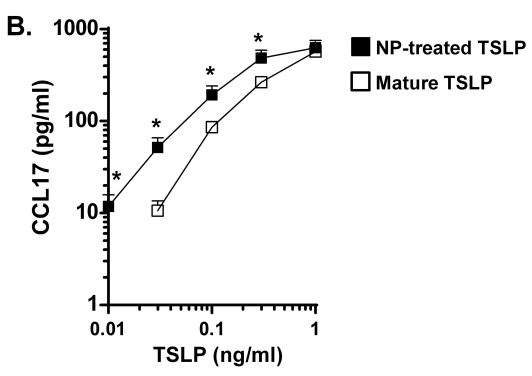
Control
TSLP


B.
1000
100
10
1
CCL17 (pg/ml)
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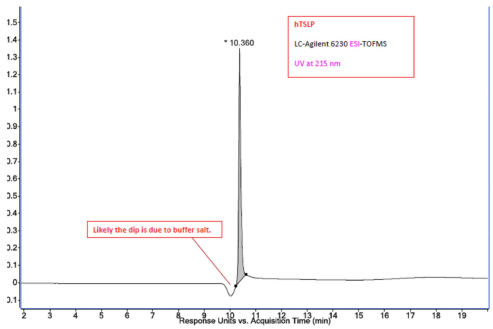
0.1
1
TSLP (ng/ml)
NP-treated TSLP
Mature TSLP
*
*
*
*

A. HPLC analysis



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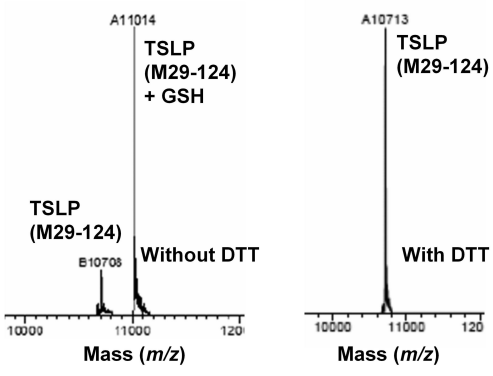
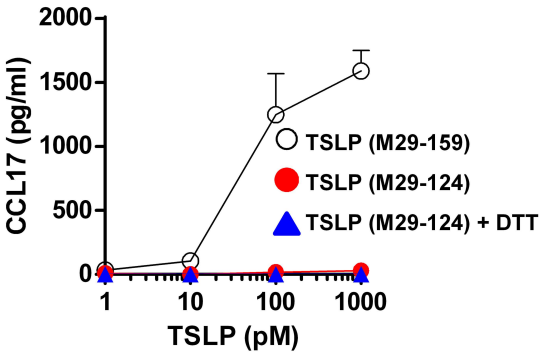
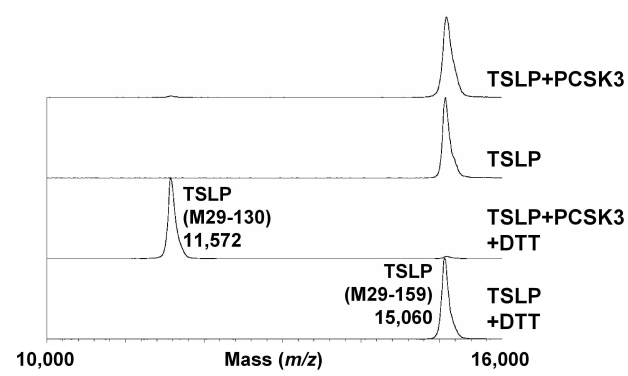


C. PBMC-based bioassay

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+DTT
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(M29-159)
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+DTT
10,000
Mass (m/z)
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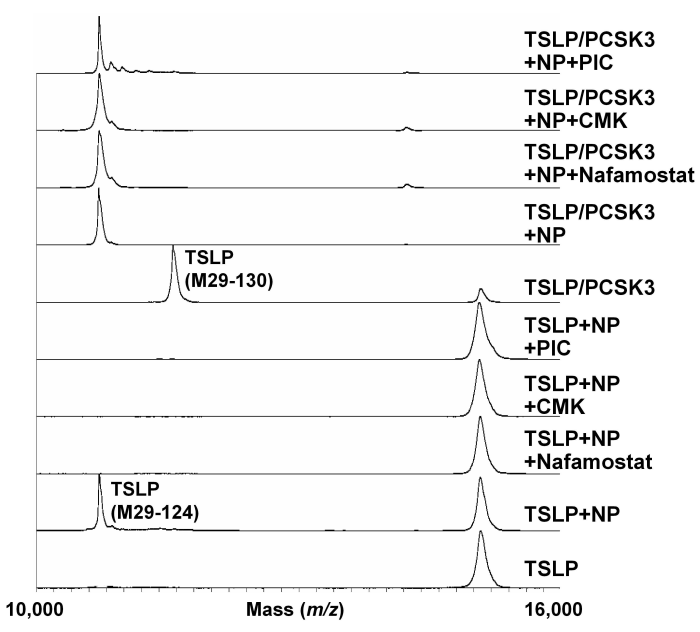
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Mass (m/z)
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