Lymphatic Thrombosis and Impaired Lymph Drainage in Cigarette Smoke-Associated Emphysema

Barbara D. Summers,[1] Kihwan Kim,[1] Zohaib Khan,[2] Sangeetha Thangaswamy,[2] Cristina C. Clement,[2] Jacob McCright,[3] Katharina Maisel,[3] Sofia Zamora,[1] Stephanie Quintero,[1] Alexandra C. Racanelli,[1] Jisheng Yang,[5] Jeanine D'Armiento,[4] Laurel Monticelli,[1] Mark L. Kahn,[5] Augustine M. K. Choi,[1] Laura Santambrogio,[2] and Hasina Outtz Reed[1]

Online Methods Supplement

Lymph Harvest and Proteomic Analysis

Chemicals and other reagents

Acetic acid (ULC/MS grade), acetonitrile, formic acid, methanol, trifluoroacetic acid (TFA), and ULC/MS-grade water (for nano-LC analysis, 99% purity) were purchased from Fisher Scientific. TCEP-HCl, iodoacetamide, ammonium bicarbonate, glycine, urea, thiourea, KCl, KH_2PO_4, H_3PO_4, and Na_2CO_3 were of the highest grade available from Sigma-Aldrich Millipore. Porcine trypsin (20 mg, specific activity >5,000 units/mg sequencing grade modified), Lys-C (sequencing grade, 10 mg) and Glu-C, sequencing grade (10 mg) were purchased from Promega (Madison, WI). All solutions were prepared using MilliQ water purified by an Elix 3 UV Water Purification System (Millipore, Billerica, USA) and filtered through 0.2 mm pore membrane sterile filter units (Steritop™, Millipore). All methods were performed in accordance with the relevant guidelines and regulations. Total protein quantitation was performed using the Micro BCA™ Protein Assay Kit, (cat # 23235 from Thermofisher Scientific). Amicon Ultra-0.5 ml centrifugal filters (Ultracel-10K, cat#UFC501024) were purchased from Millipore-Sigma.

Extraction of endogenous peptides (peptidome) from lymph sample

Equal amounts of 50-100 ug of total protein from the lymph collected from mice exposed CS-exposed mice (n = 9) and room air controls (n = 11) were equilibrated in 0.4 ml of sterile PBS buffer supplemented with a cocktail of protease inhibitors (Roche). Peptides were extracted using 0.2% TFA. Samples were then filtrated through a 10,000-Da cutoff filter device (Amicon) at 10°C for 30 minutes, desalted using pepClean C-18 spin columns (Pierce), and eluted with 70% acetonitrile containing 0.1% TFA for further nanoLC/MS/MS analysis.

Processing of lymph samples for proteomics analysis

50-100 mg from each lymph were reduced with 25 mM TCEP-HCl (Thermo Scientific) in 50 mM ammonium bicarbonate (ABC) buffer, containing 8 M urea at pH 8.5 for 45 minutes at RT followed by alkylation with 100 mM iodoacetamide for 50 minutes in the dark at RT. The protein solutions were transferred on microcon-10kDa centrifugal filter units with Ultracel-10 membrane (catalog# MRCPRT010) from Millipore Sigma and washed with 50 mM ammonium bicarbonate buffer five times at 9000xg in a microcentrifuge, for 10 minutes each step. The reduced and alkylated samples were resuspended in 100 ml of 50 mM ABC buffer, pH 8.9 (urea <2M) and digestion was carried out at 37°C overnight (12 hours) using a combination of three enzymes: trypsin/LysC at 20:1 protein: enzyme ratio and GluC at 10:1 protein: enzyme ratio. The digestion was quenched with 0.5% acetonitrile and 1.5% formic acid. Processed peptides were then extracted through a 10-kDa MWCO (molecular weight cut-off) using 10kDa centrifugal filter units by spinning at 10,000xg for 15 minutes in a 20:1 microcentrifuge. The final peptide mixture, extracted from all enzymatic digestions, was desalted on C18 Prep clean columns (EMD Millipore) and reconstituted in 25 µl 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid for further nanoLC/MS/MS analysis.

Equal aliquots (ug) of endogenous peptides (MW<10 kDa) and/or tryptic peptides were analyzed in replicates (n=22 biological replicates for each sample set) by nano-LC-MS/MS using a combination of data dependent and independent analyses (DDA and DIA, respectively). We used a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer and applied a protocol developed and published previously (Clement CC et al., Immunity 2021).

Briefly, desalted peptides were injected onto an EASY-Spray PepMap RSLC C18 50 cm x 75 µm column (Thermo Scientific), which was coupled to the mass spectrometer. Peptides were eluted with a non-linear 180 min gradient of 5-30% buffer B (0.1% (v/v) formic acid, 100% acetonitrile) at a flow rate of 250 nL/min. The column temperature was maintained at a constant 50 ˚C during all experiments. For DIA analysis, survey scans of peptide precursors were performed from 350-1200 *m/z* at 120K FWHM resolution (at 200 *m/z*) with a 1 x 10^6 ion count target and a maximum injection time of 60 ms. The instrument was set to run in top speed mode with 3s cycles for the survey and the MS/MS scans. After a survey scan, 26 m/z DIA segments were acquired from 200-2000 *m/z* at 60K FWHM resolution (at 200 *m/z*) with a 1 x 10^6 ion count target and a maximum injection time of 118 ms. HCD fragmentation was applied with 27% collision energy and resulting fragments were detected using the rapid scan rate in the Orbitrap. The spectra were recorded in profile mode.

DDA nano-LC/MS/MS for generation of spectral libraries

The sample specific spectral library (SSL) was generated by pooling 1/10 aliquots from each biological sample and DDA method for peptide MS/MS analysis. Survey scans of peptide precursors were performed from 400 -1500 *m/z* at 120K FWHM resolution (at 200 *m/z*) with a 4 x 10^5 ion count target and a maximum injection time of 50 ms. The instrument was set to run in top speed mode with 3 s cycles for the survey and the MS/MS scans. After a survey scan, tandem MS was performed on the most abundant precursors exhibiting a charge state from 2 to 6 of greater than 5 x 10^3 intensity by isolating them in the quadrupole at 1.6 Th. CID fragmentation was applied with 35% collision energy and resulting fragments were detected using the rapid scan rate in the ion trap. The AGC target for MS/MS was set to 1 x 10^4 and the maximum injection time limited to 35 ms. The dynamic exclusion was set to 60 s with a 10-ppm mass tolerance around the precursor and its isotopes. Monoisotopic precursor selection was enabled. The remaining half of each sample was run using DIA method as described above.

Generation of Spectral Libraries

To generate the spectral libraries, the acquired DDA raw files corresponding to the pooled samples from the biological replicates (aliquots of 1/10 from each CS-exposed and room air control sample) were searched with PEAKS X+ and then filtered with Scaffold software (version 4.6.2). The enzyme restriction was set up as "*no enzyme*" option in PEAKS X+ to fit the endogenously processed peptides. Then, the spectral library was exported as a *".blib"* file using the built-in available option from the Scaffold software.

An independent analysis of the MS/MS DDA raw files was performed using the MSFragger (The Nesvizhskii Lab, 1301 Catherine, 4237 Medical Science I, Ann Arbor, MI 48109: version 3.2). MSFragger was set up to search a reverse concatenated uniprot-filtered-organism Mus musculus (mouse) database (April 2021, 36,902 entries) assuming the digestion enzyme trypsin for proteomics samples. MSFragger was searched with a fragment ion mass tolerance of 20 PPM and a parent ion tolerance of 20 PPM. Deamidated of asparagine and glutamine, oxidation of histidine, methionine, and tryptophan and carbamidomethyl of cysteine were specified in MSFragger as fixed modifications. Scaffold (version Scaffold_5.0.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Then, the spectral library was exported as a ".blib" file using the built-in available option from the Scaffold software.

DIA Analysis of peptidomes and proteomics data

DIA data were analyzed using Scaffold DIA (1.2.1) (Proteome Software Inc., Portland) which had the raw data files converted to mzML format using ProteoWizard (3.0.11748). The analytic samples were aligned based on retention times and individually searched against "DDA.blib spectral library" with a peptide mass tolerance of 10 to 15 ppm and a fragment mass tolerance of 15 to 50.0 ppm. Variable modifications were imported from the DDA based spectral library as follow: methionine, lysine, proline, arginine, cysteine, and asparagine oxidations (+15.99 on CKMNPR), deamidation of asparagine and glutamine (NQ-0.98) and pyro-Glu from glutamine (Q-18.01 N-term). The *"no enzyme*" option was used in Scaffold DIA with variable allowed 8-12 missed cleavage site(s) for peptidomics analysis. Only peptides with charges in the range [2-8] and length in the range [5-25] were exported for further quantitation. For the proteomics analysis, "trypsin" restriction was used for the enzyme digestion and one "allowed missed cleavage" Peptides identified in each sample were filtered by Percolator (3.01. nightly-13-655e4c7-dirty) to achieve a maximum FDR between 0.01-0.05. Individual search results were combined, and peptide identifications were assigned posterior error probabilities and re-filtered to FDR thresholds of 0.01-0.05 by Percolator (3.01. nightly-13-655e4c7-dirty). Peptide quantification was performed by Encyclopedia (0.7.2). For each peptide, the 5 highest quality fragment ions were selected for quantitation. The intensities for the proteins were calculated and normalized by summation of the peptide intensities using the Scaffold DIA's built-in normalization algorithm.

Independent analysis of DIA data files from peptidomes and proteomics with PEAKS X+/pro

Raw files from each biological replicate were filtered using the DIA built-in option in PEAKS X+ /Pro(Bioinformatics Solutions, Waterloo, Canada), de novo sequenced, and assigned with protein ID using by searching against the mouse Swiss-Prot database (April 2021; 36,902 entries) and the following search parameters trypsin/LysC/Glu-C, as restriction enzymes in the case of proteomics data set and "*no enzyme*" for searching the peptidomics dataset; two allowed missed cleaves at one peptide end was applied for tryptic peptides. The parent mass tolerance was set to 13 ppm using monoisotopic mass, and fragment ion mass tolerance was set to 0.03 Da. Carbamidomethyl cysteine (+57.0215 on C) was specified as a fixed modification. Methionine, lysine, proline, arginine, cysteine, and asparagine oxidations (+15.99 on CKMNPR), deamidation of asparagine and glutamine (NQ-0.98), and pyro-Glu from glutamine (Q-18.01 N-term) were set as variable modifications. We also performed an additional analysis of peptidomes and proteomics DIA files using the "*Spectral Libraries*" search option enabled by the PEAKS X/Pro, using the spectral libraries generated from DDA raw files as described above.

Data were validated using the false discovery rate (FDR) method built in PEAKS X+, and protein identifications were accepted with a confidence score (−10lgP) >15 for peptides and (−10lgP) >15 for proteins; a minimum of one peptide per protein was allowed after data were filtered for <5% FDR for endogenous peptides and FDR<1% for tryptic peptides; and <3% FDR for proteins identifications ($P < 0.05$).