

Endothelial eNAMPT Amplifies Preclinical Acute Lung Injury: Efficacy of an eNAMPT-Neutralizing mAb

SUPPLEMENTAL MATERIALS AND METHODS

Reagents. Recombinant human eNAMPT was purchased from Peprotech (Cranbury, NJ). The following antibodies- p-NFkB, pp-ERK, pp-p38, pp-JNK, IL-6, IL-8 (KC)- were purchased from Cell Signaling Technologies (Danvers, MA); β -actin from Invitrogen (Carlsbad, CA) (NFkB); and goat, rabbit, and mouse secondary antibodies from Life Technologies (Waltham, MA). Goat anti-human NAMPT polyclonal antibody (pAb) was custom-generated (17) by Lampire Biological Laboratories, Inc. (Pipersville, PA) by immunizing against full length rhNAMPT protein. All other reagents, including lipopolysaccharides (LPS, E. Coli 0127:B8 strain), were from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Generation of eNAMPT-neutralizing humanized monoclonal antibodies (mAbs). The eNAMPT-neutralizing humanized mAbs, ALT-100 and ALT-300 were provided by Aqualung Therapeutics Corporation (Tucson, AZ). ALT-100 and ALT-300 were derived from anti-human eNAMPT mAb-producing murine hybridomas (Abpro, Boston MA) which were extensively screened *in vitro* and *in vivo* and selected for high affinity eNAMPT binding and eNAMPT neutralization. Epitope-distinct murine mAbs (Abpro, Boston MA) from the two lead hybridomas underwent humanization (Fusion Antibodies, Belfast, UK) with generation of 50 humanized eNAMPT-neutralizing mAbs (25 mAbs per murine hybridoma). Each mAb was again screened *in vitro* using EC electrical resistance assays (34, 35), and NFkB activation biochemical assays (19) and by *in vivo* selection utilizing the preclinical murine lung injury models described below.

These screenings resulted in the selection of the three highest performing humanized mAbs (ALT-100, ALT-200, ALT-300). ALT-100 was selected for the *in vivo* therapeutic studies reported herein and ALT-300 chosen for incorporation into the tissue NAMPT-imaging probe, ProNamptorTM for studies to probe tissue NAMPT expression as described below (^{99m}Tc-ProNamptorTM). Extremely high NAMPT protein sequence homology between mice, rats, non-human primates and humans (95-99%), underscoring the utility of both the humanized ALT-100 and ALT-300 mAbs in the murine preclinical models reported in our studies.

Generation of the ProNamptorTM mAb imaging probe. The ALT-300 eNAMPT-binding humanized mAb was used for generation of fluorescent and ^{99m}Tc-labeled probes for imaging NAMPT expression in tissues. A non-specific human IgG was also fluorescently- or ^{99m}Tc-labeled and used as a control to verify the uptake specificity of ALT-300. For Cy5.5 labeling, the Cy5.5-NHS was dissolved in dimethyl sulfoxide (DMSO) (5 µg/µL). The antibodies (1 mg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.0) were reacted with Cy5.5-NHS at a molar ratio of 1:8. Following reaction in the dark (25°C, 2 h), the reaction mixture was processed using ZebaTM Spin Desalting Columns (7K MWCO) (Thermo Fisher Scientific, Waltham, MA) to separate unbound dyes from the dye-conjugated antibody (36, 37).

^{99m}Tc-ProNamptorTM was generated by radiolabeling ALT-300 with ^{99m}Tc by initial mAb conjugation with a heterobifunctional linker, succinimidyl-6-hydrazinonicotinate hydro-chloride (SHNH, in DMSO, 5 µg/µL) (Molecular Targeting Technologies, Inc., West Chester, PA) (36, 37) in 0.1 M sodium bicarbonate modification buffer (pH 8.0, 1:10 molar ratio, 25°C, 2 h). The SHNH-conjugated product (SHNH-antibody) was

purified and buffer-exchanged by conjugation buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 6.0) using the Zeba™ Spin Desalting Columns. Radiolabeling was completed by additions of $^{99m}\text{TcO}_4^-$ (15-20 mCi, 0.4-0.5 mL saline), Tricine solution (30 μL , 36 mg/mL), Tin(II) chloride dihydrate (10 μL , 2 mg/mL) in 0.1N HCl, and SHNH-antibody conjugate (16 μL , 100 μg) in 0.1M PBS pH 6.0 (25°C, 30 min). ^{99m}Tc -ProNamptor™ was purified using a G-25 Sephadex column and the radiochemical purity (RCP) was determined by size-exclusion high performance liquid chromatography (SEC-HPLC). The radiolabeling yield of ^{99m}Tc -ProNamptor™ was consistently greater than 90%. After gel filtration purification, all ^{99m}Tc -ProNamptor™ preparations used for animal injections exhibited greater than 98% RCP. In addition, a human IgG ^{99m}Tc probe was generated and served as control for the ^{99m}Tc -ProNamptor experiments.

^{99m}Tc -ProNamptor™ mAb imaging. A mouse model of skin inflammation induced by topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) was utilized to validate the ability of the Cy5.5-ALT-300 probe to detect NAMPT tissue expression *in vivo* (38, 39). Ear edema was induced in mice (10-15 weeks old) by topical application of 2 μg TPA dissolved in 20 μL of acetone, and administered through a micropipette to the inner and outer surface of the right ear (10 μL) with repeated application at 24 hours. The left ear was treated with 20 μL of acetone (vehicle) applied topically as a negative control. Three hours following the second TPA application, either Cy5.5-eNamptor™ or Cy5.5-IgG (15-20 μg) was injected intravenously (n=3 mice) and imaged (Lago Bioluminescence and Fluorescence Imaging System, Spectral Instruments Imaging, Tucson, AZ).

To assess the capacity of the ^{99m}Tc -ALT-300 probe to detect *in vivo* NAMPT tissue expression, ^{99m}Tc -ProNamptorTM (1.0-1.5 mCi, >98% radiochemical purity, 40, 41) was intravenously injected into two groups of C57BL/6J mice (n=3 each group) at 3h and 7h post intratracheal LPS instillation, i.e. the “one-hit” LPS ARDS model described in detail,below. An additional group received saline as a vehicle control (n=6). Mice were imaged at 30 min, 120 min and 240 min after radiotracer injection using a Quantum Imaging Detector (iQID) camera (42-44). This small-animal imaging system provides imaging of photon and particle emissions in small animals and tissues with high spatial resolution. After completion of the imaging, all mice were euthanized and the lungs were harvested for count activity-based measurements of ^{99m}Tc -ProNamptorTM biodistribution and for *ex vivo* autoradiography.

Mouse studies. *In vivo* experiments utilized either wild type male C57BL/6J mice (8–12 weeks, Jackson Laboratories, Bar Harbor, ME), EC-specific conditional NAMPT knockout mice (*EC-cNAMPT*^{-/-}) on a mixed 129/B6 background, or littermate NAMPT^{fl/fl} controls. All mice were housed under standard conditions (12h light-dark cycle, 25-27°C, ~40% humidity) in autoclaved micro-isolator cages with free access to food and water. All animal care procedures and experiments were approved by the Institutional Animal Care and Use Committee (University of Arizona). For the “one-hit” preclinical ARDS injury experiments, mice were challenged with LPS for 18h. For the “two-hit” preclinical ARDS injury experiments, mice were exposed to LPS for 18h with mechanical ventilation for the last 4 hours as we have described previously (46-48). In specific experiments, C57BL/6J mice received either intravenously-delivered PBS (vehicle control), a eNAMPT-neutralizing polyclonal antibody (pAb, 4mg/kg), or a

humanized eNAMPT-neutralizing monoclonal antibody (ALT-100 mAb, 0.4mg/kg) via the jugular vein.

Generation of conditional *NAMPT* knockout mice (*EC-cNAMPT*^{-/-}). Utilizing the University of Arizona Genetically-Engineered Mouse Model (GEMM) Core, a floxed *NAMPT* mouse was created (*NAMPT*^{fl/fl}). Briefly, a targeting vector was generated with a loxP site in intron 1, a FRT-flanked neo-containing positive selection cassette in intron 2, and second loxP site downstream of the neo-cassette. A thymidine kinase-negative selection cassette was inserted into intron 3. The targeting vector was electroporated into 129/S6 ES cells and selected for G418 resistance and gancyclovir sensitivity and the targeted ES cells injected into B6D2xB6 blastocysts. Positive expressing offspring were confirmed with the following genotyping primers: TCGAGCTATCATCATGCTTAACTTAC (common F), AAATCCCTCAGTGCACAGTAAATAG (wildtype R), CTGGCACTCTGTC GATACCC (neo R) creating band lengths of 244 bp (wildtype) and 372 bp (flox). *NAMPT*^{fl/fl} were in-crossed to produce mice homozygous for the floxed allele which were then crossed with a tamoxifen-inducible EC-specific Cre transgenic mouse line (Tek-cre/ERT2-1Soff) to create *EC-cNAMPT*^{-/-} mice (45). Mice were then backcrossed with the floxed homozygous *NAMPT* mouse to create an EC-specific conditional *NAMPT* KO mouse (*EC-cNAMPT*^{-/-}). To induce Cre activity, mice were injected for 5 consecutive days with tamoxifen (Sigma Aldrich) at a dose of 75mg/kg tamoxifen in corn oil. All *EC-cNAMPT*^{-/-} mice were utilized for experimentation after a minimum wait of two weeks after the final dose of tamoxifen. Mice were maintained on a mixed 129/B6 background and littermates that did not express the Cre recombinase gene were used as controls.

EC-specific KO mice carrying the NAMPT flox transgene did not display discernible differences in phenotypic traits compared to their wild-type littermates. Growth rates, fecundity, and fertility also did not differ from wild-type mice. Similarly, *NAMPT* flox mice crossed with the TIE2/ERT2 Cre mice to generate the conditional *NAMPT* KO line did not exhibit any phenotypic differences from either littermates or parental strains, both before and after tamoxifen injections.

Preclinical “one-hit” and “two-hit” preclinical ARDS models of LPS- and ventilator-induced murine lung injury. Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) with additional doses given as needed to ensure adequate anesthetic depth. Animals were intubated with a 20-G angiocatheter and received intratracheal LPS injection (*E. Coli* 0127:B8, 1 mg/ kg) with harvesting 18h (“one-hit” lung injury model). For the “one-hit” ARDS model, mice received an intratracheal LPS injection followed sacrificing at 18h as previously described (46-48). For the “two-hit” LPS/VILI-induced lung injury model, animals received intratracheal LPS (0.1 mg/kg) and after 18hrs, were reintubated and placed on mechanical ventilation for 4h (Advanced Ventilator System For Rodents, SAR-1000, CWE Incorporated, Ardmore, PA) as previously described (46-48). Mice were ventilated with room air for 4h using the following parameters: tidal volume (VT) 20 ml/kg, respiratory rate 90 breaths/min, and positive-end expiratory pressure 0 cm H₂O. Spontaneously breathing (SB) control animals were intubated and received intratracheal PBS but allowed to breathe spontaneously on room air during the duration of the experiment.

eNAMPT-neutralizing strategies in “one-hit” and “two-hit” preclinical ARDS models. Concomitantly with LPS challenge in either the “one-hit” or the “two-hit” model, specific groups of C57BL/6J mice received either an eNAMPT-neutralizing goat pAb (Lampire) or the humanized mAb (Aqualung Therapeutics, Tucson AZ) via i.v. injection (4mg/kg or 0.4mg/kg, respectively). An additional pAb or mAb dose was provided prior to the onset of mechanical ventilation in the LPS/ VILI “ two-hit” model.

Bronchoalveolar lavage (BAL) analysis. At the termination of each experiment, mice were euthanized by IACUC-approved exsanguination after anesthesia. BAL was performed with 1 ml of cold Hank’s buffered saline solution (HBSS) (Invitrogen) delivered intratracheally followed by slow recovery of the fluid as we have previously described (49). BAL fluid underwent centrifugation (500g, 20 min, 4°C), pellets re-suspended in 200µL cold HBSS and 1mL of RBC Lysis Solution (5 min). The pellet was recentrifuged (500g, 10 min, 4°C) and re-suspended in 200µL cold HBSS for total cell counting via an automated cell counter (TC20; Bio-Rad, Hercules, CA). PMN determinations were performed as previously described (49) by assessing the % of PMNs in BAL cytospins and multiplying by the total number of BAL cells retrieved/mL. The BAL supernatant was re-centrifuged (16,500g, 10 min, 4°C), and the supernatant collected for total protein measurements (Pierce BCA Protein Assay Kit, Thermo Scientific). BAL supernatant was stored at -80°C for further analysis.

Lung histology and immunohistochemistry (IHC) analyses. Hematoxylin and eosin staining: To assess alterations in the lung tissue morphology, lungs were fixed in 10% neutral buffered formalin for a minimum of 48 h, embedded in paraffin, sectioned and stained with hematoxylin-eosin (H & E). Routine H&E slides were

prepared using Richard-Allan hematoxylin, clarifier, bluing reagent and eosin and imaged (Olympus digital camera, 10x magnification) (50). **NAMPT staining:** The avidin-biotin-peroxidase method utilized a rabbit anti-human NAMPT polyclonal antibody (dilution of 1:1000, Bethyl Laboratories, Inc, Montgomery, TX) for IHC staining to visualize NAMPT expression in lung tissues (5 micron sections) as previously described (14, 17, 19). Results were compared to a rabbit IgG control (matched protein concentration, 1ug/ml, Vector Labs, Burlingame CA). Deparaffinized and rehydrated slides were ringed with an ImmunoPen rinsed in TBS, blocked for endogenous peroxidase (0.5% hydrogen peroxide, 20 min) and protein blocked (Vector Labs, 1 h, avidin D and biotin block, Vector Labs, 25⁰C). Slides were incubated overnight (4⁰C) in primary or IgG control followed by application of the biotinylated secondary Ab (1 h) with incubation with avidin-biotin complex (Vector Labs, 40 min, 25⁰C). The protein was visualized using DAB plus nickel (Vector Labs, 4 min) and counterstained with Mayers hematoxylin (Newcomer Supply, Middleton, WI) and bluing reagent added (Richard-Allan Scientific, San Diego CA). **NAMPT/CD31 co-staining in *EC-cNAMPT*^{-/-} mice:** Formalin-fixed, paraffin-embedded lung tissue sections from *EC-cNAMPT*^{-/-} mice were baked at 65⁰C overnight, washed in xylene (to remove paraffin), and passed through 100%, 75%, 50% isopropanol, and ddH₂O for rehydration. Antigen retrieval was performed using a sodium citrate buffer [10 mM sodium citrate, 0.05% Tween 20, pH 6.0] and heated at 97⁰C using decloaking chamber for 20 min. Slides were washed in washing buffer (0.1 M Tris-HCl, 0.3 M NaCl, 0.1% Tween 20, and 7.7 uM NaN₃, pH 7.6 at 25⁰C) followed by blocking buffer (5% v/v normal bovine serum, 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.6, 25⁰C, 30 min). Slides were next incubated overnight (4⁰C) with primary

rabbit anti-human NAMPT pAb (Bethyl Laboratories, Montgomery, TX) or the rat monoclonal anti-CD31 antibody (AbCam Cambridge, MA, #7388, MEC 7.46), diluted in blocking buffer in a humidified chamber. Slides were next washed 3 times in wash buffer and incubated with biotinylated secondary antibody (1h, 25°C, washed 3 times in washing buffer, mounted using ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, P36970) and stored in the dark (25°C) to cure the mountant. Specimens were imaged using Zeiss Axiovert Photomicroscope using a 10X objective (NA 0.4).

Evans Blue Dye extravasation assay: We evaluated lung vascular leakage by measuring extravascular Evans Blue Dye in the lung. Briefly, mice were injected Evans blue dye (0.05 mg, Sigma) i.v. 60 min before euthanasia. Lungs were then perfused to remove the intravascular dye, excised and homogenized in PBS. One volume of lung homogenate was incubated with 2 volumes of formamide and incubated at 60°C for 18 hrs before centrifugation. The optical density of the supernatant was measured at 620 nm and 740 nm using an Imark microplate reader. Concentrations of Evans blue were corrected for the presence of heme pigments using the following formula: $A_{620}(\text{corrected}) = A_{620}(\text{raw}) - (1.1927 \times A_{720}) + 0.0071$ (58). The extravasated Evans blue dye concentrations were then calculated against a standard linear curve as a reflection of vascular protein leak into lung tissues.

Quantitative histologic analyses. Histological images from each group captured with light microscopy (Olympus digital camera) at 10x magnification, were randomly selected for quantification of H&E and NAMPT staining using ImageJ software (51) (different sections of each slide). For H&E image analysis, the percentage of area selected for measurement with all images processed and stored for statistical analysis.

For NAMPT staining image analysis, color segmentation plugin was utilized with POINTCROSS tool applied to each NAMPT staining image with a total of 3 color clusters. The area percentage of each color cluster was recorded and saved for statistical analysis.

Acute Lung Injury Severity Score (ALISS) quantification. The Acute Lung Injury Severity Score (ALISS) was utilized to integrate lung injury indices in the “one-hit” and “two-hit” preclinical ARDS models and to standardize the injury levels across the *in vivo* models. A ranking point system, incorporating published recommendations (59) objectively assigns a score to each study animal (1 to 4 points) for each of 4 injury severity readouts (H & E histology quantification, BAL total protein concentration, BAL total PMN cell count and plasma levels of the pro-inflammatory cytokine IL-6). The maximal score for each animal is 16 points/mouse. In general, an ALISS score of 1-4 reflects the complete absence of injury, scores of 5-8 points reflect mild injury, scores of 9-12 points reflect moderate injury, and scores >12 points reflect severe injury.

Measurements of electrical resistance across human lung endothelial cells- Trans-endothelial Electrical Resistance (TER). Human pulmonary artery endothelial cells (HPAEC or ECs) were obtained from Lonza (Walkersville, MD) and cultured as we described previously (52) in the manufacturer’s recommended endothelial growth medium-2 (EGM-2). Cells (passages 6 to 9) were seeded onto evaporated gold microelectrodes in polycarbonate wells and grown to confluence (37°C, 5% CO₂). Trans-endothelial electrical resistance (TER) measurements were performed using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as we have described previously in detail (53). Cells were monitored over time for TER

responses to agonist stimulation. TER values from each microelectrode were pooled as discrete time points and plotted versus time as the mean \pm SEM.

Biochemical tissue expression of NAMPT, NFkB, MAP kinases and IL-6.

Western blotting of lung homogenates obtained from “one-hit”- and “two-hit”-exposed mice was performed according to standard protocols as previously reported (23, 49). Lung tissues were homogenized in radioimmunoprecipitation assay lysis buffer (RIPA buffer) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 % NP-40, 1% sodium deoxy-cholate, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ protease inhibitors- 1mM AEBSF, 800nM aprotinin, 50 μM bestatin, 15 μM E64, 20 μM leupeptin, 10 μM pepstatin A). Following homogenization, lysates were centrifuged (12,000 rpm, 10 min, 4°C) and equal amounts of protein (40 μg) loaded onto 4-12% Bis Tris gels (Life Technologies). Western blotting was performed according to standard protocols (21, 47). Lung tissue expression levels of NAMPT, NFkB, MAP kinases, IL-6, and β -actin (total protein control) with densitometric quantification of lung tissue expression.

Murine plasma levels of eNAMPT by ELISA. eNAMPT plasma levels were measured in plasma by ELISA as we have previously reported (27, 31). Briefly, a 96-well plate (Nunc MaxiSorp) coated with proprietary goat anti-NAMPT pAb (100 $\mu\text{L}/\text{well}$, 8 $\mu\text{g}/\text{ml}$, diluted in coating buffer (1.5 g Na_2CO_3 , 2.93 g NaHCO_3 /1L distilled water, pH 9.6). After 24 h (4°C) the plate was warmed to 37°C (1 h), washed in 1x TBST (3 times, 0.1%) and incubated with 1% BSA-TBS (100 $\mu\text{L}/\text{well}$, 1 h, 37°C) to reduce non-specific binding. The plate was again washed with 1x TBS-T, followed by incubation with either the human rhNAMPT standard or the murine plasma sample diluted 1/10 in plasma

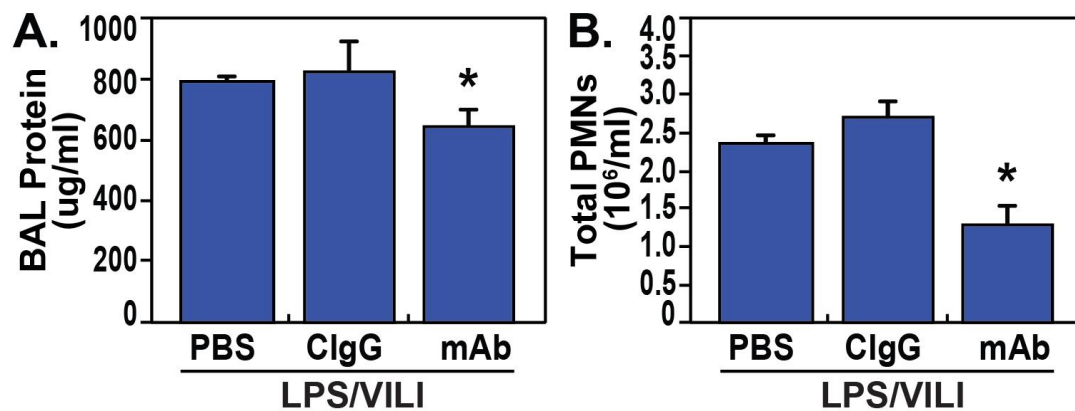
diluent buffer (100 μ L/well) (Cygnus). The plate was again incubated overnight (4°C), warmed 37°C, and washed 3 times in 1x TBS-T. To detect plasma eNAMPT, the plate was incubated with the rabbit anti-NAMPT pAb (Bethyl, 100 μ L/well, dilution 1:10,000 in 1% BSA-TBS) for 1 h at 37°C followed by washing 3 times in 1x TBS-T. The plate was next incubated with the secondary donkey anti-rabbit HRP-labelled pAb (100 μ L/well, 1:10,000 in 1% BSA-TBS) at 37°C for 1 h, followed by 1x TBS-T washing. The plate was finally developed with the HRP substrate (SIGMAFAST OPD) for 5 min at 25°C with the reaction stopped with 10% H₂SO₄, followed by absorbance reading 490 nm.

Murine plasma inflammatory cytokine levels via meso scale discovery (MSD) platform. A meso-scale ELISA platform was utilized (Meso Scale Diagnostics, Rockville, MD) for measurements of plasma levels of IL-6 and IL-8 (KC). Each biotinylated antibody (10 μ g/ml, specific for each analyte, was mixed with a different linker for each analyte and incubated for 30 min at 25°C. The reaction was terminated with 200 μ l of free biotin solution and 600 μ l of the 10x U-PLEX linked biotinylated antibody solution with 50 μ l of coating solution was added to each well in 96 well plate and incubated for 1 h (800 rpm shaking, 25°C). After washing, each well was supplemented with 25 μ l of diluent and 25 μ l of calibrator or samples/standards, incubated for 1 h (800 rpm shaking, 25°C). After washing (TBS-T), each well was supplemented with 50 μ l/well of 1x detection antibody solution, again incubated for 1h, washed and supplemented with 2x Read Buffer T followed by plate imaging and calculation of the absolute concentration values based on standards.

Statistical analysis. Continuous data were compared using nonparametric methods and categorical data by chi square test. Where applicable, standard one-way

ANOVA was used and groups compared using the Newman-Keuls test. Two-way ANOVA was used to compare the means of data from two or more different experimental groups. If a significant difference was present by ANOVA ($p < 0.05$), a least significant differences (LSD) test was performed post hoc. Statistical tests were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical significance was considered at $p < 0.05$.

Supplemental Figure 1



Supplemental Figure 1. The specific eNAMPT-neutralizing mAb, but not a non-specific human IgG, attenuates “two-hit” preclinical ARDS/VILI injury. The significant increases in BAL protein (**Panel A**) and BAL PMNs (**Panel B**) in mice exposed to LPS (0.1mg/kg,18h) followed by mechanical ventilation (4h, tidal volume 20 mL/kg) were significantly reduced in mice receiving the eNAMPT-neutralizing humanized ALT-100 mAb (0.4mg/kg, at time 0 with LPS) but not injection of **non-specific human IgG antibody** (* $p < 0.05$ vs. LPS/VILI-PBS).