MATERIALS and METHODS

Ethical approval – All animal procedures were reviewed and approved by the animal care committee of the Hospital for Sick Children (Toronto, ON, Canada), in accordance with the Guidelines of the Canadian Council on Animal Care.

Reagents – Primary antibodies were as follows: phosphotyrosine (clone 4G10, Millipore, Temecula, CA), sAxl (AF854, R&D Systems, Minneapolis, MN), Axl (for immunoprecipitation, M20, Santa Cruz Biotechnology, Santa Cruz, CA; for Western blot, #4977 Cell Signaling Technology, Mississauga, ON, Canada), phospho-Akt (#4060) and Akt (#4691), both from Cell Signaling Technology. Pharmacologic agents used included: inhibitor of A Disintegrin and Metalloprotease (ADAM)-17: TAPI-2[1], blocks Axl shedding; rho-associated protein kinase inhibitor: Y27632[2], blocks stress fibre formation in response to cyclic stretch[3]; Ca^{2+} ionophore: ionomycin[4], elevates intracellular Ca^{2+} concentration (Calbiochem, Illerica, MA); stretch-activated channel inhibitor $GdCl_3$[5] (Sigma-Aldrich, Oakville, ON, Canada) and Axl antagonist R428[6] (AdooQ Bioscience, Irvine, CA).

Production of γ-Carboxylated Gas6 – HEK293T cells were transiently transfected with pCMV-SPORT6-mGAS6 (kindly provided by Dr. R. Birge, Rutgers University, NJ) using JetPRIME transfection reagent (Polyplus transfection, Illkirch, France), and cultured 72 h in serum-free media supplemented with 10 µg/mL vitamin K1 (Phytonadione injectable emulsion, Sandoz, Boucherville, QC, Canada). Conditioned media containing Gas6 was collected as described [7] and concentrated using a centrifugal filter device (Centriprep YM-10, Millipore, Bedford, MA). Gas6 concentration in concentrated conditioned media was determined by ELISA (mouse Gas6 Duoset, R&D Systems, Minneapolis, MN).

Murine model of VILI – C57BL/6J male mice (20-25 g, Charles River, St. Constant, QC, Canada) were anesthetized (ketamine 150 mg/kg, xylazine 15 mg/kg, I.P.), and ventilated *via* tracheotomy using a computer controlled small animal ventilator (SCIREQ, Flexivent, Montreal, Canada). Baseline (low stretch, protective) ventilation was with tidal volume (V_T) 10 mL/kg, PEEP 2.0 cmH_2O, frequency 135/min, and FiO_2 0.21. Lung compliance was measured at baseline and hourly thereafter. In Series 1, animals were randomized to continue baseline ventilation or

receive high tidal ventilation (V_T 20 mL/kg, PEEP 0 cmH_2O, frequency 45/min, FiO_2 0.21) for 4 h (n = 4/group)[8]. After completing the experiment, mice were sacrificed by exsanguination under anesthesia, bronchoalveolar lavage performed for protein analysis, and lungs removed and snap frozen.

Axl inhibition *in vivo* – In Series 2, C57/BL6J male mice were randomized to receive R428 (100 mg/kg in 1% DMSO, balance saline) or vehicle (n = 4/group), by I.P. administration[9]. Two hours later, mice were anesthetized, a tracheostomy performed, and high tidal mechanical ventilation performed as above for 4 h. Initial compliance measurements were taken before starting high tidal ventilation in all animals. Compliance measurements were taken hourly; sacrifice and sample collections were as above.

Endothelial cell culture, cyclic stretch and Axl activation by Gas6 – Rat pulmonary microvascular endothelial cells (RPMEC) were obtained from the Tissue and Cell Culture Core of the Center for Lung Biology, University of South Alabama and cultured in DMEM supplemented with 10% fetal bovine serum. Cells were seeded at 2×10^5 cells/cm^2 onto collagen-coated Bioflex 6-well plates (Flexcell International, Hillsborough, NC) for stretch experiments, or at 5×10^4 cells/cm^2 onto standard 6-well tissue culture plates for ionomycin experiments. Confluent monolayers were washed with PBS and starved for 4 h in serum-free media, followed by a further wash and replacement of serum-free media to minimize endogenous Gas6. Inhibitors (TAPI-2, 20 µM; Y27632, 5 µM, $GdCl_3$, 50 µM), or vehicle, were added 15-30 min before initiation of stretch. Cells remained under static conditions, or were subjected to cyclic stretch using a Flexcell FX-4000 Strain Unit (Flexcell International) for 30 min at a setting of 17% change in surface area, equibiaxial strain at 0.5 Hz. Conditioned media containing Gas6 (5 nM, final) was added and cells were incubated at 37 °C for 10 min under static conditions before harvest. Ionomycin (50 nM) was added 30 min before Gas6 in experiments using unstretched cells as indicated in the figures. Preliminary experiments using comparable amounts of conditioned media from HEK293T cells transfected with plasmid expressing an irrelevant protein (Green Fluorescent Protein) confirmed that activity in conditioned media was dependent on Gas6 transfection.

Detection of sAxl shedding – To detect Axl ectodomain shedding *in vitro*, RPMEC monolayers on Bioflex plates were serum-starved and subjected to stretch as above in the presence of 10 μM TAPI-2 (or vehicle). Conditioned media was harvested and concentrated using a centrifugal filter device. Cell monolayers were rinsed and lysates prepared as above. Conditioned media and matched cell lysates were analyzed by SDS-PAGE and Western blot as described below to detect sAxl and Axl, and shedding expressed as sAxl in conditioned media divided by total Axl (sAxl in media + Axl in lysate).

Quantitation of mRNAs – RNA was isolated from mouse lung tissue using Purelink RNA Minikit (ThermoFisher Scientific, Carlsbad, CA) and changes in gene expression measured using relative quantitative real-time PCR with an ABI7900HT Detection System (Applied Biosystems, Foster City, CA) and Power SYBR Green (Thermofisher Scientific) reaction mix. Gene expression was calculated relative to 18S rRNA using the comparative cycle threshold (ΔΔCt) method using cDNA from a non-ventilated control lung as calibrator. Primers used were: SOCS1: forward – 5'-CACCTTCTTGGTGCGCG-3'; reverse – 5'- AAGCCATCTTCACGCTGAGC; SOCS3: forward – 5'-GCTCCAAAAGCGAGTACCAGC-3'; reverse – 5'- AGTAGAATCCGCTCTCCTGCAG; IL-6: forward – 5'-ACAAAGCCAGAGTCCTTCAGAGAGA-3'; reverse – 5'- GGAGAGCATTGGAAATTGGGGTAGG; IL-1β: forward – 5'- AGCTCTCCACCTCAATGGACAGAAT-3'; reverse – 5'- TCCTTTGAGGCCCAAGGCCACA; TNFα: forward – 5'-CCCTCACACTCAGATCATCTTC-3'; reverse – 5'- GCTACGACGTGGGCTACAG-3'; MIP-1α: forward – 5'-CCCAGCCAGGTGTCATTTTCC-3'; reverse – 5'-GCATTCAGTTCCAGGTCAGTG-3'.

Immunoprecipitation and Western blots – Cell monolayers were washed with ice-cold phosphate-buffered saline and lysed on ice in a buffer containing 50 mM Tris-HCl pH7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.27 M sucrose, 0.1% β-mercaptoethanol, and protease and phosphatase inhibitors. For direct immunoblot analysis, an aliquot of cell lysate or conditioned media was denatured in SDS-PAGE sample loading buffer. For immunoprecipitation, the remaining lysate was incubated overnight at 4 °C with Axl antibody (1 μg), after which protein G-agarose (Thermofisher Scientific) was added for 2 h. Agarose beads were collected by low speed centrifugation and washed 3 times with lysis buffer containing 0.1M NaCl and once with 50 mM Tris-HCl pH7.4. Immunoprecipitates were eluted in SDS-PAGE

sample loading buffer at 95 °C. Protein samples were electrophoresed through polyacrylamide gels, transferred to PVDF membranes and blocked with TBS (50 mM TrisHCl pH7.6, 0.14 M NaCl) containing 0.25% Tween-20 and 5% bovine serum albumin (BSA) (for phosphotyrosine detection) or 0.1% Tween-20 and 5% non-fat dry milk (other targets). Membranes were incubated overnight with primary antibodies diluted in TBS with Tween-20 as above and 5% BSA. Secondary antibody (horse-radish peroxidase-conjugated) was diluted 1:10,000 in blocking buffer and incubated for 1 hour at room temperature. Following washes in TBS plus Tween-20, enhanced chemiluminescence detection was carried out according to the manufacturer's recommendations (Perkin Elmer, Waltham, MA) using Xray film. Blots were stripped in 25 mM glycine-HCl pH2, 1% SDS before being blocked and re-probed for normalization. Band intensities were quantitated using ImageJ (National Institues of Health, USA http://imagej.nij.gov.ij), normalized to total Axl (for phospho-tyrosine blots) or actin (all others) and expressed relative to the average of controls on each blot when combining data from multiple blots for statistical analysis.

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