Material and Methods (2035)

All studies were approved by the animal care committee at St. Michael's Hospital (Toronto, ON, Canada) in accordance with Canadian Council of Animal Care guidelines. All experiments were conducted in accordance to ARRIVE criteria[1] (check list included in supplemental material).

Polymicrobial Sepsis Models

Briefly, all studies used 8- to 14-week-old male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mice were randomized to caecum ligation and puncture (CLP) or sham surgery and at 6 hours post CLP were further randomized to receive a single intrajugular injection of 2 x 10^5 bone marrow derived MSCs or placebo (saline) as previously described (5). Animals were humanely sacrificed at 28 hrs and right lungs snap frozen for mRNA and miRNA profiling. Transcriptional microarray data have been deposited in GEO [Gene Expression Omnibus, *http://www.ncbi.nlm.nih.gov/geo* (accession number GSE40180)].

MicroRNA Analysis

Total RNA from lungs (five animals per group- sham/saline, CLP/saline, and CLP/MSCs) was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) and purified using RNeasy (Qiagen Inc., Chatsworth, CA) as per manufacturer specifications. RNA quality was ensured by spectrophotometric analysis (OD260/280) and gel visualization. miRNA profiling was performed using the Exiqon miRCURY LNA™ microRNA Arrays (Exiqon A/S, Vedbaek, Denmark). A total of 300 ng of total RNA from each sample was hybridized to the microRNA Array (6th gen) that measures the abundance of 1081 possible miRNAs. The variance-stabilizing normalization method was used to refine normalization using R/Bioconductor software [2]. Differential miRNA expression was performed using the LIMMA package[3]. A total of 391 miRNAs were deemed to be expressed in lungs, an FDR of ≤ 0.05 was used to identify differential expression. In parallel, we used the transcriptomic data from the original experiment to perform an *in silico* sequence based analysis to identify gene-sets that shared a 3'-UTR microRNA binding motif. Gene Set Enrichment Analysis (GSEA[4]) was used to analyze the 2-kb 3'UTR of all MSC-dependent mRNAs in the lung to predict enrichment for putative regulatory miRNAs. To identify candidates for future study we matched miRNAs identified *in silico* to those identified empirically.

Analysis of miRNA-target gene expression association

To identify significantly associated miRNA:mRNA pairs in MSC-treated versus non-treated septic lungs, we generated a gene by miRNA matrix table based on *in silico* predictions which we matched to our lung mRNA and miRNA experimental data (schematic of the animal experiment is shown in Figure 1A). Gene Set Enrichment Analysis (GSEA) was used to analyze the 2-kb 3'UTR of all MSC-dependent mRNAs in the lung to predict enrichment for putative regulatory miRNAs[4]. To identify significant association of miRNA:mRNA pairs differentially expressed in MSC-treated versus non-treated septic lungs, we obtained a list of predicted targets of the differentially expressed miRNAs from miRanda (http://mirdb.org/[5 6] score < -0.5, conservation score > 0.5) and TargetScan[7 8] (context score < -0.3) database with appropriate thresholds. We then generated a gene by miRNA matrix table which we matched to our lung mRNA and miRNA experimental data. A total of 22,111 probes (corresponding to 11,997 putative target genes) and 357 miRNAs whose foreground expression intensity values were over 1.2 times background were used to generate a statistical estimate of association or correlation between miRNAs and their putative mRNA targets using the equation:

$$Y(gene)=X\,(miRNA1)+X(miRNA2).....$$

(Where Y is the expression value of a given target gene and X is the expression of its miRNA)

We fitted a Bayes hierarchical generalized linear model [9] for the equation to identify the miRNAs significantly regulated the gene expression of the mRNA. We identified the significant association of miRNA:mRNA pairs from the analysis by controlling the genome-wide false discovery rate at 0.05 level. A total of 1,420 miRNA:mRNA pairs were found to be significantly associated.

Putative miR-27a-5p targets include VAV3 as well as other targets that were not identified by the pair analysis (miR:mRNA) but that are predicted to be miR 27a-5p targets by sequence complementarity (miRWalk13 http://mirwalk.umm.uni-heidelberg.de[10 11], Table 2). Of interest, various other putative targets of miR-27a-5p are known to play a role in ARDS such as nuclear factor 2 (Nrf2), angiotensin converting enzyme 1 (ACE1), epidermal growth factor (EGFR) and fibroblast growth factor 7 (FGF7, also known as Keratinocyte growth factor KGF). Ingenuity pathway was used to generate a putative interaction network using both predicted and

documented interactions between miR-27a-5p targets (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis Figure 1D).

Functional Enrichment

Enrichr was used to identify functional enrichment amongst differentially expressed genes (http://amp.pharm.mssm.edu/Enrichr/)[12 13]. A positive Z-value indicates the score is above the mean while a negative score indicating a score below the mean (up or down regulation). For all Enrichment tables, gene sets were ranked using the combined score.

Cell Culture

Primary human pulmonary microvascular endothelial cells (HPMECs) were purchased from PromoCELL (Heidelberg, Germany) and cultured in Endothelial Cell Basal Medium MV 2 (EBM-2, CC-3202, Lonza) supplemented with EGM-2MV SingleQuot Kit Supplements and Growth Factors (CC-4176, Lonza) 37^{o}C with 5% CO_2. Primary human distal bronchial and small airway epithelial cells (Beas2b, ATCC CRL-9609) cultured in Dulbecco's Modified Eagle's Medium (DMEM, BE12-611F, Lonza) 37^{o}C with 5% CO_2. The RAW 264.7 cell line was gown in 90% Roswell Park Memorial Institute medium (RPMI + 10% h.i. FBS, Lonza). Murine MSCs (isolated from male C57Bl/6J mice, a gift from Dr. Darwin Prockop, Texas A&M Health Science Center, College Station, TX) were expanded in culture according to previously published literature. MSCs in all in vivo experiments were used between passages 8 and 11, and their differentiation capacity was described previously[14-16]. All *in vitro* experiments were performed with cells at passage 3 – 7 in 70-80% confluency. All *in vitro* experiments were performed in triplicate.

Treatments and Transfections

Cells (70 – 80% confluence) were transfected with 5 nM syn-hsa-miR-27a-5p miScript mimic (MSY0004501), 50 nM anti-hsa-miR-27a-5p miScript miRNA inhibitor (MIN0004501), 50 nM hsa-miRNA inhibitor negative control (1027271), 75nM VAV3 siRNA (AM16708), or 5 nM AllStar siRNA Negative Control (SI03650318), purchased from Qiagen, using HiPerfect Transfection Reagent (HPF, 301705 Qiagen) or Lipofectamine RNAiMAX (Thermo) as per manufacturer's specifications. Cells were treated with recombinant TNF (10 ng/ml; Life

Technologies) 24h after transfection. End points were analyzed 24h post-treatment. Luciferase reporter construct, pLightSwitch_3UTR (SwitchGearGenomics, Carlsbad, CA), containing either the wild type human VAV3-3'UTR, a 2 mutated version of the 3'UTR sequence of VAV3 (miR-27a-5p seed sequence deleted) were used. For reporter studies, HPMECs were grown to 70%–80% confluency, and transfected with 100 ng of the indicated reporter constructs. MiR-27a-5p mimics or inhibitors were co-transfected at 10 or 50 nM final concentrations respectively. After 24 or 48 hours of incubation, respectively, cells were treated with TNF for 24 hours. Empty vector and non-target sequence (ACTB and R01) were included as controls. Transfected cells were collected in 200 µl Reporter Lysis Buffer (Promega, Madison, WI). The activity of luciferase was measured with a spectrophotometer (Thermoscientic, Waltham, MA). Luciferase activity was normalized as per manufacturer's specifications (SwitchGearGenomics). For MSC conditioned medium experiments, HPMECs were grown and transfected as described above. TNF treatment was performed in serum-free EBM-2 media (Lonza, Burlington, On) supplemented with 20% (v/v) MSC conditioned medium and control medium, collected as described below.

Cell Viability Assay

Cells were seeded in a 96-well plate. After transfection and treatment with TNF-α, cells were incubated overnight. After washing twice with PBS, cells were given MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MTT assay (Sigma, St. Louis, MO)) solution (1 ml/well of 5 mg/ml solution in PBS) for 3 hours. Next, 100 µl/well DMSO (Sigma, St. Louis, MO) was added and the absorbance of formazan was measured at 570 nm using an ELISA reader (BioTek, Winooski, VT, USA). Absorbance values were normalized to the culture medium.

Dextran Permeability Assay

HPMECs were transfected with miR-27a-5p inhibitor or mimic, and seeded on 0.2% gelatin-coated 0.4 µm pore Transwell inserts (Corning, NY). After 24 hours incubation, serum-free culture medium containing saline or TNF-α was added, and cells were incubated overnight. The medium was removed, and 200 µl of PBS containing 2 mg/ml of FITC-labeled dextran (70 kDa)

was added to the upper chamber, while 600 µl of PBS alone was added to the lower chamber. Fluorescence was then measured from the lower chamber every 3 min for 3 hrs.

Scratch Migration Assay

HPMEC were transfected with miR-27a-5p inhibitor or mimic. After removing medium and washing the cells with PBS, a single scratch was made straight down the middle of the monolayer using a 100-µl pipette tip. Cells were washed with PBS to remove cellular debris and serum-free culture medium was added, with or without recombinant TNF-α. Each treatment group had 3 technical replicates. Two pictures of the scratch and surrounding cells were taken per well using a Nikon compound light microscope with a camera attached at time 0 and 24 hrs. Distances between the leading edges were measured at each time-point using ImageJ (National Institutes of Health).

Monocyte Adhesion Assay and Transendothelial Migration Assay

HPMEC were transfected with miR-27a-5p inhibitor or mimic for 24 hours as described. After washing serum-free culture medium containing saline or TNF-α was added, and the cells incubated overnight. Next, THP-1 monocytes were labeled with 2 µM of CellTracker™ Green 5-chloromethylfluorescein diacetate (ThermoFischer Scientific, Wilmington, DE) for 45 minutes. The HPMEC were washed with PBS, and 5×10^5 labeled monocytes (in RPMI-1640 medium) were added. For the monocyte adhesion assay, after 30 minutes, medium containing un-attached monocytes was aspirated, and the cells washed with PBS to remove unbound cells. The cells were then immediately imaged and measured for fluorescence (excitation/emission of 492/517 nm). For the transendothelial migration assay 600 µl of RPMI-1640 medium containing 10 ng/ml of monocyte chemoattractant protein-1 (MCP-1) was added to the lower chamber, and cells were allowed to migrate for 2 hours. Transwells were then removed and fluorescent images of the lower chamber are taken at 6 different sample locations.

Binding of miR-27a-5p to the human 3'UTR of VAV3

There are two seed miR-27a-5p binding sequences on the 3'UTR of *VAV3;* a proximal 7-mer and a more distal 6-mer sequence complementary to the heptameric and sextameric sequences in the 3'UTR region of the VAV3 gene. Two mutation constructs containing either a deletion of the

heptameric (mutant 1) or both the heptameric and sextameric (mutant 2) sequences were generated (Figure 3G and Supplemental Figure 4A). Transient co-transfection of a wild-type (WT) VAV3-3'UTR-luciferase reporter construct and mimic into HPMECs decreased expression of luciferase, which is not seen when the binding site of the seed sequences in the 3'UTR of VAV3 are mutated (Figure 3I). Co-transfection of the miR-27a-5p inhibitor with the WT or mutant VAV3-3'UTR luciferase construct (mutant 1 and 2) did not change luciferase activity from control levels, while TNF treatment decreased WT, but not mutant, luciferase activity. Importantly, miR-27a-5p inhibition in TNF-treated cells rescued luciferase activity indicating that inhibition of miR-27a-5p prevents binding of miR-27a-5p to the 3'UTR and VAV3 mRNA degradation. No significant changes in luciferase activity were seen when cells were transfected with a luciferase constructs containing the 3'UTR of the housekeeping gene actinomycin B (ACTB) or a non-targeting control R01 (Supplemental Figure 4B). Taken together, these data indicate VAV3 is a target of miR-27a-5p post-transcriptional regulation. MiR-27a-5p negatively regulates VAV3 expression by binding to its seed sequence on the 3'-UTR of VAV3 mRNA.

Real-Time PCR

Total RNA was isolated using Trizol (Ambion, Life Technologies) according to manufacturer's instructions. First Strand cDNA was synthesized with 2 ug RNA samples from tissues or 1 μg RNA samples from cells using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). Real-time PCR (RT-PCR) was performed with ViiA 7 Real Time PCR System (Applied Biosystems, Life Technologies) using Power SYBR Green Reagents (Applied Biosystems, Life Technologies). Primers were generated using PrimerQuest (IDT DNA Technologies) and purchased from IDT DNA Technologies. miRNA-specific primers are provided in Table A. The relative change in gene expression was calculated by the ΔΔCt method (Applied Biosystems) from triplicate determinations using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin or miR-191-5p as housekeeping genes or miRNA, respectively. Fold difference was calculated by normalizing all values to the average value of the respective control group. miR-191 has been previously reported to be the most suitable endogenous control gene owing to lower stability values and the least amount of intergroup variation in its expression level .

Table A. microRNA specific primers for qRT-PCR

Primer	Sequence
miR-27a-3p Reverse Transcription Primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGGCGGAACT
miR-27a-5p Reverse Transcription Primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTGCTCACA
miR-27a-3p Forward qRT-PCR Primer	ACACTCCAGCTGGGTTCACAGTGGCTA
miR-27a-3p Reverse qRT-PCR Primer	TGGTGTCGTGGAGTCG
miR-27a-5p Forward qRT-PCR Primer	ACACTCCAGCTGGGAGGGCTTAGCTGCT
miR-27a-5p Reverse qRT-PCR Primer	TGGTGTCGTGGAGTCG

*miR-191 was used as the house keeping miR for normalization of miR amplification. This miRNA was found to be statistically superior to other most commonly used reference RNAs used in miRNA qRT-PCR experiments, such as 5S rRNA, U6 snRNA, or total RNA[17].

Immunoblotting

Protein concentration was measured using Bradford protein assay. Protein expressions were determined by Western blot, as previously described. Western blot bands were processed using Bio-Rad Gel Doc 2000. Densitometry was performed using GelQuantNet software. Antibodies used are as follows: VAV3 (#2398, Cell Signaling), GAPDH (#2118, Cell Signaling), and B-actin (sc-47778, Santa Cruz).

In vivo inhibition of miR-27a-5p in murine lungs

Mice were randomized (random number generator) to receive miR-27a-5p inhibitor (INH), negative control (NC), or equal volume saline combined with transfection reagent (HPF). The oligonucleotide mixtures were co-administered intra-tracheally with LPS (10 mg/kg body weight) or equal volume saline up to a total volume of 60 uL. Animals were anesthetized with inhaled isofluorane. A small incision was made near the anterior aspect of the neck. The platysma and anterior tracheal muscles were bluntly dissected. Using a 31-gauge insulin syringe (BD Sciences), LPS and oligonucleotide mixture were co-administered directly into the trachea. The surgical area was sutured and animals were allowed to recover in a bedding free cage. All

animals received subcutaneous (s/c) fluid resuscitation with 50ml/kg saline and 0.2mg/kg Buprenorphine (Buprenex) for pain. Endpoints were measured at 24h post-instillation.

***Assessment of Cell Infiltration from Bronchoalveolar Lava*e**

Bronchial alveolar lavage by cannulating the trachea and lavaged with three 0.5 mL aliquots of PBS. Total cell counts were obtained by ViCell. BALf cells were spun down, fixed onto slides, and stained with haematoxylin and eosin (H&E). Slides were imaged using Olympus Upright BX50 microscope – 10 fields per slide were imaged. Macrophages and neutrophils were counted on all 10 fields to obtain differential cell counts. Total protein was measured in BALf supernatant by Bradford (Bio-Rad). IgM levels in BALf supernatant was measured by ELISA (E90-101, Bethyl BioSciences).

Immunohistochemistry

Detection of VAV3 was performed. Briefly, slides were deparaffinised by xylene incubation and hydrated with subsequent ethanol washes. Antigen retrieval was performed using hot citrate buffer (10 mM, pH 6). Slides were blocked with 10% bovine serum albumin (BSA) for 1h, washed, and incubated with primary antibody (VAV3 1:50) overnight at 4^{o}C. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide before incubating with secondary antibody (anti-rabbit) for 1h at room temperature. Slides were developed with DAB substrate for 15 minutes and counterstained with hematoxylin. 3,3′-Diaminobenzidine (DAB) peroxidase staining shows decreased VAV3 antibody binding after LPS treatment in both distal bronchial epithelial and microvascular endothelial cells (Figure 6F), indicating that there is a decrease in VAV3 expression following LPS instillation. Quantification analysis showed that miR-27a-5p inhibitor co-instillation attenuates the decrease in VAV3 protein expression levels (Figure 6H). Expression of VAV3 also decreased in lung tissues following LPS instillation in mice that received the negative control but was preserved in mice that received the miR-27a-5p inhibitor (Figure 6I).

Formalin Fixed Paraffin Embedded Human ARDS Samples

Formalin-fixed, paraffin-embedded 8 um sections of lung autopsy samples from patients who died with ARDS, with or without histology-proven diffuse alveolar damage (DAD) were

obtained from the Critical Care Department at the University Hospital of Getafe, Spain. RNA was isolated from the section slides using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion, Life Technologies) per manufacturer's protocol. RNA was pooled from 3 - 5 slides to obtain sufficient amount. cDNA synthesis and qRT-PCR for miR-193b-5p were performed as described above.

Statistics

Mice were randomized to treatment groups using a random number generator. Observers assessing end-points were blinded to group assignment. Where Kolmogorov-Smirnov (K-S) test demonstrated normal distribution [based on p-value ≥0.05 for statistic (D)] data was used to determine normal distribution of the data. Data is presented as individual values (symbols) for individual mice, means ± standard deviation (SD) and corrected using Tukey's test for multiple comparisons. Non-parametric data are presented as median ± interquartile range (IQR) and Dunn's post-hoc test used to correct for multiple comparisons.

Supplemental Figure Legends

Supplemental Figure 1: Differential expression of other miR-27a-5p targets in: A) Relative change in gene expression in A) Raw 264.7 cell (murine macrophage cell line) stimulated with TNF (10 ng/ml, 24 hrs); B) Primary Human Bronchial Epithelial Cells (BEAS2b) stimulated with TNF (10 ng/ml, 24 hrs); **C)** Primary Human Pulmonary Microvascular Endothelial Cells (HPMECs) exposed to TNF (10 ng/ml, 24 hrs); D) lung tissues from mice randomized to LPS (10 mg/kg) or equal volume saline 24 hrs later lungs were collected for qRT-PCR. E) Western blot quantification of ACE1 and EGFR normalized to β-actin in HPMECs exposed to TNF (10 ng/ml, 24 hrs). F) ELISA for FGF7 (KGF) using lung tissue protein lysates from mice following 24 hrs LPS stimulation. Genes and proteins profiled were VAV3, fibroblast growth factor 7 (FGF7; alias Keratinocyte growth factor KGF), epithelial growth factor receptor (EGFR), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin. Data is normally distributed. Presented as mean ± SD (n = 3-5 per group; * $p \leq 0.05$; unpaired Student T-test).

Supplemental Figure 2: Response of VAV3, ACE 1 and NRF2 to miR-27a-5p inhibition: HPMECs were transfected with miR-27a-5p inhibitor (INH; 25 nM), mimic (MIM; 25 nM) or negative control (NC; 25 nM) for 24h and then stimulated for 24h with TNF (10 ng/ml). **A**) Fold change in the expression of VAV3 and ACE1 normalized to β-actin following transfection of miR-27a-5p inhibitor or negative control. B) Fold change in the expression of ACE1 in HPMECS stimulated with TNF transfected following transfection with miR-27a-5p mimic or C) miR-27a-5p inhibitor compared to negative control (control) normalized to GAPDH (n=3). D) Fold change in the expression of NRF2 in HPMECS stimulated with TNF transfected following transfection with miR-27a-5p mimic or C) miR-27a-5p inhibitor compared to negative control (control) normalized to GAPDH (N=3). K-S test demonstrated normal distribution. Data is presented for individual experiments as means ± SD (* $p \leq 0.05$; two-way ANOVA, Tukey correction for multiple comparisons).

Supplement Figure 3: HPMEC Cell MTT Viability Assay. MTT cell viability assay show no change in viability of HPMECs after 24 hrs stimulation with TNF (10ng/ml) or transfection with miR-27a-5p inhibitor (INH; 25 nM), mimic (MIM; 25 nM) or negative control (NC; 25 nM) for 24h and then stimulated for 24h with TNF (10 ng/ml). Normally distributed OD intensity

normalized to control is presented as means ± SD, normalized to SAL+HPF control (*$p \leq 0.05$; two-way ANOVA).

Supplemental Figure 4: Luciferase construct (SwitchGear Genomics) showing addition of the wildtype and mutant 3'UTR of VAV3. Luminescence of positive controls, showing that addition of wildtype or mutant VAV3 3'UTR does not affect luciferase activity, compared to an empty 3'UTR or 3'UTR containing positive controls, R01, β-actin, and empty 3'UTR.

Supplemental References

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