Sphingosine-1-phosphate receptor 3 is a novel biomarker in acute lung injury

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ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS:

Reagents

Unless otherwise specified, reagents were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Rabbit and mouse anti-S1PR3 receptor antibodies were purchased from Exalpha Biologicals (Watertown, MA), mouse anti-nitrotyrosine (clone 1A6) antibody from Millipore (Millipore Corp., Bedford, MA). Rabbit anti-phosphoserine and rabbit anti-phosphothreonine antibodies were purchased from Zymed (South San Francisco, CA). Mouse anti-β-actin antibody and LPS were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was carried out as described earlier (E1). Briefly, HLMVECs lysates generated following LPS challenge (0 to 24 hrs, 1μg/ml), and S1PR3 was immunoprecipitated with anti-S1PR3 antibody, and eluted samples loaded on an IPG strip (Amersham Biosciences) which was rehydrated for 12 h followed by isoelectric focusing steps of 500 Vhr, 1000 Vhr, and 8000 Vhr using the IPGphor IEF system (Amersham Biosciences). The second dimension separation was run using XCell Surelock mini-cell system (Invitrogen) in 4–20% gels. Proteins were transferred onto Immobilon membranes, and developed with specific primary and secondary antibodies.

Construction and Delivery of siRNA against S1PR3

The siRNA sequence(s) targeting human S1PR3 receptor was generated using mRNA sequences from Gen-Bank™ (gi: 38788192). For each mRNA (or scramble), two targets were identified. Specifically, S1PR3 target sequence 1 (5'-AACAGGGACTCAGGGACCAGA-3'), S1PR3 target sequence 2 (5'-AAATGAATGTTCCTGGGGCGC-3'), scrambled sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3') and scramble sequence 2 (5'-AAGAACCCAATTAAGCGCAAG-3') were utilized. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). Human lung ECs were transfected with siRNA using siPORTamine™ (Ambion, TX) according to the manufacturer's protocol (~ 40% confluent). The transfected cells were then serum-starved for 1 hour and incubated with 3 μM (1.5 μM of each siRNA) of target siRNA (or control siRNA or no siRNA) for 6 hours in serum-free media. Complete media was then added (10% serum final concentration). Cells were then cultured for 42 h and used for biochemical experiments and/or functional assays.

In specific experiments for reducing S1PR3 *in vivo*, siSTABLE specifically modified siRNA, S1PR3 siRNAs or siRNA controls (Dharmacon) (10 mg/kg mouse, i.v.) were administered as previously described (20). Experimental groups included a spontaneously breathing (SB) with control siRNA, and SB challenged with S1PR3 siRNA, a high tidal ventilation (VILI), and a high tidal ventilation treated with S1PR3 siRNA (VILI-siS1PR3) (n= 4–6 for all groups). Bronchoalveolar lavage (BAL) fluid

was subsequently collected for protein/albumin measurements as described previously (E2).

RNA Isolation and Microarray Analysis

Mice were challenged with intratracheally administered LPS (2.5 mg/kg) or water (control). Eighteen hours after challenge, total RNA was isolated from whole lungs and subjected to expression profiling as described (E3) using Affymetrix Mouse 430 2.0 arrays and protocols (Affymetrix, Santa Clara, CA, USA). Chips were scanned using a GeneChip Scanner 3000 (Affymetrix) (E4, E5).

Microparticle Isolation

Cell culture medium derived S1PR3 from transfected EC cells was centrifuged at 200 x g for 10 min to obtain the cell pellet. The resulting supernatant was centrifuged twice at 500 x g for 10 min, twice at 1,500 x g for 15 min, and once at 10,000 x g for 30 min. Small microparticles were then pelleted by centrifugation for 1h at 70,000 x g. Pellets from above centrifugation were washed twice with PBS and resuspended in PBS and stored at −20°C until use. The amount of MPs was quantified by the total protein concentration measured using a Bio-Rad Protein Assay kit. The resuspended microparticles were applied to EC monolayers for TER measurements or for S1PR3 Western blot analysis.

Measurement of Transendothelial Electrical Resistance (TER)

EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements performed using an electrical cell-substrate impedance sensing system obtained from Applied Biophysics (Troy, NY) as described

previously (E6). TER values from each microelectrode were pooled for discrete time point and the mean ± S.E. plotted against time.

REFERENCES:

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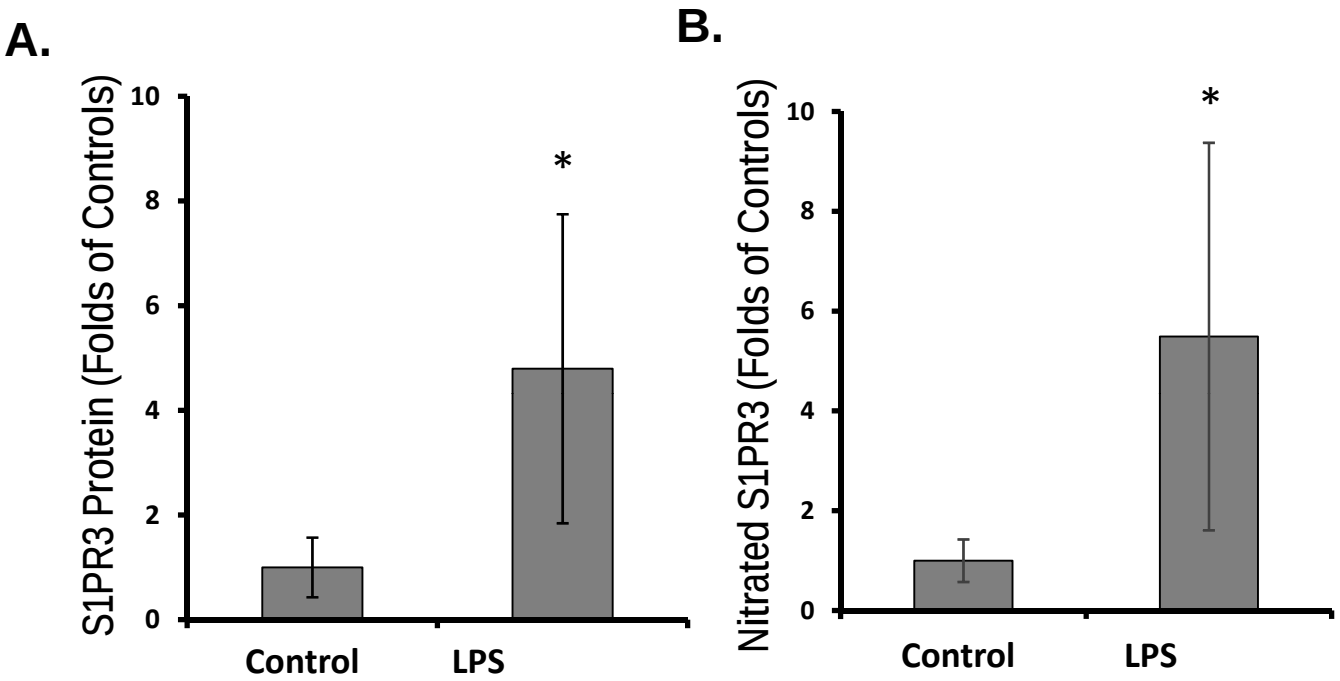
Figure 1S. Densitometry of immunoblotting for S1PR3 and nitrated S1PR3 protein levels in plasma from mice exposure to intratracheal LPS (2.5 mg/kg, n=10) or H2O (Control, n=8). S1PR3 protein in plasma was detected by immunoprecipitation and immunoblot with anti-S1PR3 (A) and anti-nitrotyrosine (B). The bands for protein were scanned for densitometry analysis. The levels of S1PR3 (A) and nitrated S1PR3 (B) in plasma were presented as folds of change, compared with controls (*$p<0.05$).

Figure 2S. After cells were transfected by S1PR3, exposed to LPS, thrombin (Thr), high molecular weight hyaluronic acid (HMW), low molecular weight hyaluronic acid (LMW), HMW with LPS, HMW with Thr, LMW with LPS, or ammonium chloride (NH4Cl), HPAECs were trypsinized and cell viability analysis was carried out by trypan blue exclusion under microscope .

Figure 3S. Comparison of APACHE II scores of patients in control, sepsis, and sepsis-induced ALI groups in ICU. The APACHE II scores of patients with sepsis and sepsis-induced ALI were significantly higher than control group (*$p<0.05$ vs. controls).

Figure 4S. Receiver operating characteristics (ROC) curve showing the relationship between sensitivity and specificity in determining the predictive value of S1PR3 and APACHE II for mortality in ICU patients. The data was analyzed with software MedCalc and the method was suggested by DeLong, et al (E7). The area under the empirical ROC curve (AUC) was 0.94 and 0.80, respectively. There is significant difference between AUC of S1PR3 and APACHE II (* $p<0.01$ vs. APACHE II).

Figure 1S


A.
S1PR3 Protein (Folds of Controls)
10
8
6
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B.
Nitrated S1PR3 (Folds of Controls)
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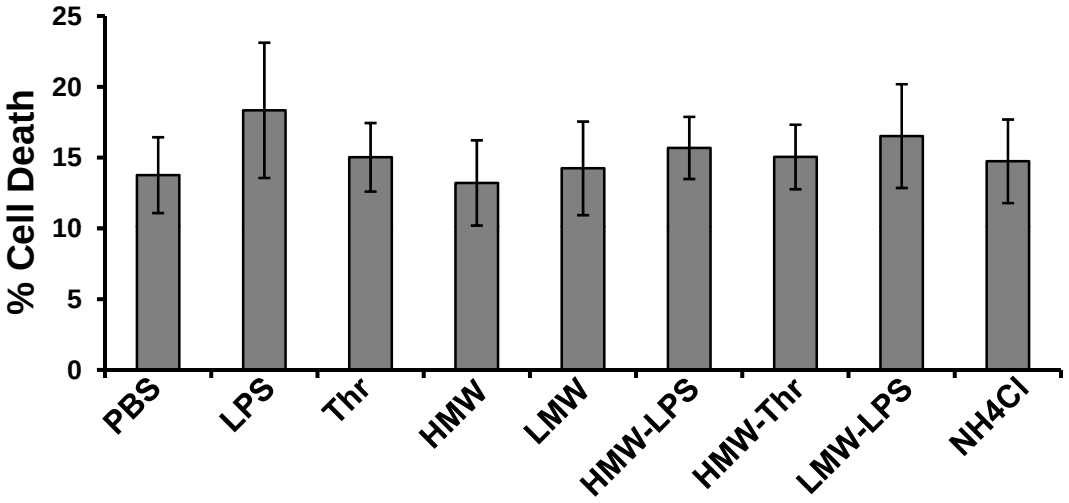
Figure 2S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25
20
15
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Thr
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Figure 3S

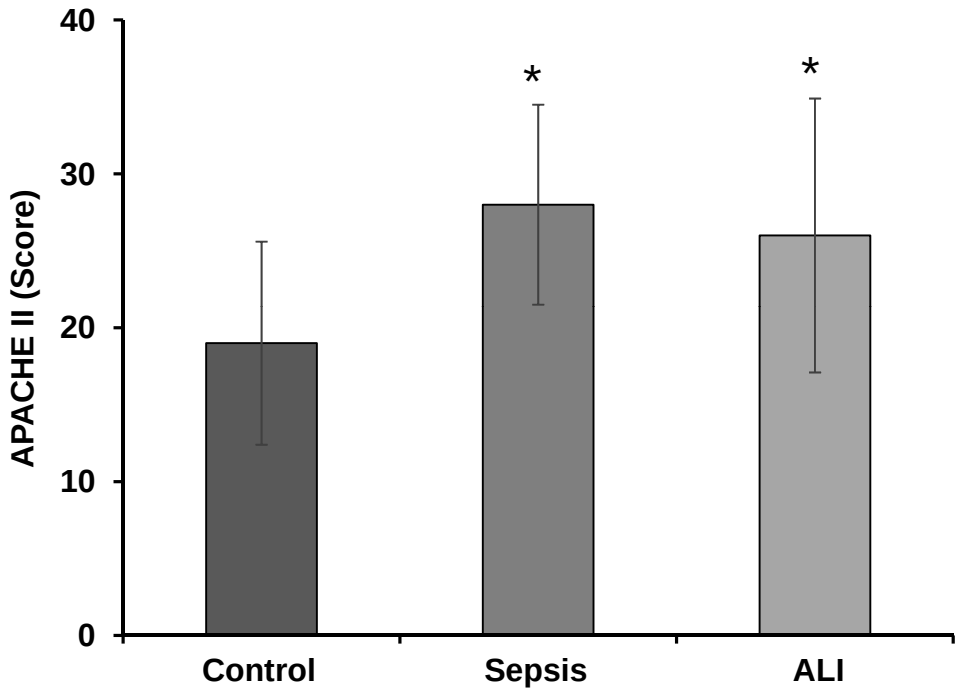
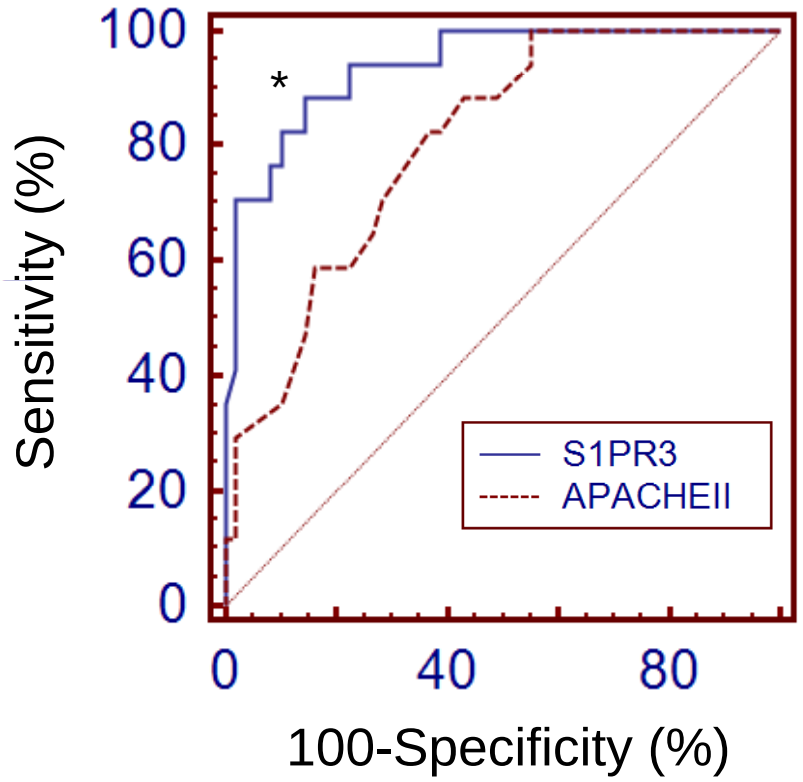


Figure 4S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80
60
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20
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Sensitivity (%)
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S1PR3
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