Online Data Supplement

Title: Zinc-deficiency primes the lung for ventilator-induced injury

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Additional Methods:

Cell Culture

Human juvenile (5-year old donor) lung fibroblasts CCL-151 (ATCC, Manassas, VA), human fetal lung fibroblasts CCL-153 (ATCC), A549 lung adenocarcinoma (ATCC) and 16HBE14o- bronchial epithelial cells were grown in F12K medium (ATCC) supplemented with 10% FBS (Lonza, Basel, Switzerland), 100 IU/mL penicillin and 100 μg/mL streptomycin (Mediatech Inc, Manassas, VA). Human lung microvascular endothelial cells (HMVEC-L) from Lonza were grown in Clonetics™ EGM™-2-MV Bulletkit™ culture medium (Lonza).

Cell Stretch Studies

Tonic biaxial deformation of cells was achieved with a previously described stretch device (1). Briefly, it consists of a 6-well BioFlex plate (Flexcell International, Hillsborough, NC) tightly secured on a rigid platform which can slide downward at a pre-calibrated distance to stretch the silicon-bottom substrate of any single well over a 28-mm diameter Teflon indentor. Plate displacement is measured with a digital caliper and unstretched time-matched control wells are included in each plate. Cyclic strain regimen was delivered with a Flexercell Strain Unit FX-4000 (Flexcell International). All in-vitro experiments were carried out in collagen I-coated 6-well BioFlex plates (Flexcell International) after 24 hours culture in serum- or growth factor-free media.

RNA isolation, reverse transcription and quantitative PCR

Primers for gene of interest were designed with Primer Express 3.0 (Applied Biosystems, Foster city, CA): MT1M, forward 5'-CCTGATGTGGGAACAGCTCTTC-3' reverse 3'-CGTATT¬GAAAAAAAATC¬CAGGTTGT-5'; HMOX1, forward 5'-GCTTTCTGGTGGCGACAGTT-3' reverse 3'-GCCAGCATGCCTGCATTC-5'; HSPA1B, forward 5'-ACCAAGCAGACGCA¬GATCTTC-3' reverse 3'-CGCCCTCGTACACCTGGAT-5'; SLC30A1, 5'-TGGATC¬CGAGCCGAGGTA-3' reverse 3'-GCGAAACAGAGGCCAGTCA-5'; MT1G, forward 5'-CAAGTACAAATAGAGTGACCCGTAAAAT-3' reverse 3'-CACA-GAAAAAAGGGAATGTAGCAA-5'; GAPDH, forward 5'-ATGGAAATCCCATCACCATCTT -3' reverse 3'-CGCCCCACTTGATTTTGG-5'. Changes in gene expression were quantified with real-time PCR using SYBR green dye (Applied biosystems) in an ABI 7300 real-Time PCR systems (Applied Biosystems). Ct values were computed with manufacturer-supplied software ABI Prism 7000 Fast Sequence Detection (Applied Biosystems). Fold changes in transcripts were calculated using the "delta delta Ct" method and normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RNA microarray analysis

Total RNA (500 ng per microarray, per sample) was harvested with RNeasy Mini Kit (Qiagen, Valencia, CA), reverse transcribed to biotin labeled complementary DNA, then hybridized onto the Human Gene 1.0 ST microarray (Affymetrix, Santa Clara, CA) containing 33,297 probes representing 20,818 unique genes following standard Affymetrix protocol. The combined dataset of all samples was normalized using robust multiple array (RMA) background correction and the resulting probe signals were represented in logarithmic (base 2) scale. For each probe, we computed the average logarithmic signal fold change in the stretch relative to control samples (denoted AvgLF2) as the arithmetic average of the stretch sample signals minus the arithmetic average of control sample signals. We say that this fold change is significant if its magnitude exceeds both log2 (1.5-fold) and the maximum logarithmic fold change from signals within the stretch, or within the control sample groups (denoted NoisLF2) (2). NoisLF2 is computed by first sorting the sample signals within a treatment group in ascending order, and then calculating the arithmetic average of the upper 50% of sample signals minus the arithmetic average of the lower 50% of sample signals. Gene ontology enrichment analysis was performed using DAVID 6.7 (http://david.abcc.ncifcrf.gov) for select gene sets where the background gene set was all 20,818 unique genes measured by the microarray and the EASE score (modified Fisher exact test probability) for significance was set at <0.05 .

RNA isolation, reverse transcription and quantitative PCR

Total RNA was harvested from cells and lung tissues after injury models with RNeasy Mini Kit (Qiagen). Messenger RNA was reverse-transcribed with TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster city, CA) and Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) following manufacturer's protocol. Please see supplement for list of primers.

RNA interference

Custom Stealth RNAi™ siRNA duplexes against MTF1 (sense: CACAAAGACUUUACGUUUCAGAUCU; and anti-sense: AGAUCUGAAACGUAAAGUCUUUGUG) and corresponding scrambled sequence controls were designed with the web-based BLOCK-iT™ RNAi Designer (Life Technologies, Carlsbad, CA).

Mouse models of VILI and LPS+VILI; Assessment of Physiologic Lung Injury

Under IACUC-approved protocols, *MT1/2*$^{(-/-)}$ mice and controls were anesthetized, tracheotomized, then subjected to ventilator-induced lung injury (VILI) on the mouse Flexivent ventilator (Scireq) using 24 ml/kg tidal volume with pressure-limited ventilation (30 cm H20), with a respiratory rate 150 breaths/minute, and PEEP 2.5 on ambient air for 2-8 hours. Zn-deficient and control mice were subjected to VILI using 15 or 24 ml/kg tidal volume. Animals were monitored continuously to maintain an appropriate plane of anesthesia. Hourly measurements of lung physiology (resistance, G; elastance, H) were undertaken after a

recruitment maneuver to limit atelectasis (3). For the dual model of lung injury, Zn-deficient and control animals were subjected to 10 mg nebulized endotoxin (LPS, Pseudomonas, Sigma) (3) followed 24h later by mechanical ventilation with tidal volume 15 ml/kg. At the completion of the protocol, BAL fluid was collected (see below), and lung tissue was harvested and stored for RNA analysis or fixed at 30 cm with paraformaldehyde for immunohistochemistry.

Immunohistochemistry analysis

Lung sections were analyzed by a lung pathologist blinded to experimental group.

Human Plasma Sample Analysis

Banked plasma samples (collected in EDTA-free tubes at the time of ICU admission) were analyzed from patients enrolled in our IRB-approved Brigham and Women's Hospital Medical Registry of Critical Illness (RoCI) (4, 5). Subjects were characterized as ARDS or ICU controls (without sepsis or ARDS), or as sepsis or SIRS (*75, 76*). Plasma samples were also collected from healthy non-ICU controls under a separate IRB-approved protocol. Plasma levels of interleukin (IL)-6 and 18 were carried out using commercially available kits according to the manufacturer's instructions (RayBiotech, Inc, Norcross, GA).

Lung tissue cytokine measurements

We performed a multiplex ELISA screen (Luminex Corporation, Austin TX) using lung homogenates (n=3/group) obtained by grinding 1 whole lung in 1 cc PBS without calcium or magnesium (6). The panel of cytokines included IL1A, IL-2, IL5, IL6, IL10, IL12p40, IL12p70, IL17, MCP1, IL1B, IL18, and IL33. Cytokine levelswere normalized to total protein levels determined by a BCA kit (Fisher Scientific).

Human Subject Statistical Analyses

Associations between zinc levels and other variables were tested using Spearman correlation. Further analyses included analysis of covariance with zinc level as the dependent variable and with final diagnosis ordered according to subject group (control, SIRS, Sepsis, ARDS) as the main grouping variable, allowing the inclusion of important covariates such as (APACHE II score, IL6 levels, malignancy, diabetes mellitus, and liver disease) in the model. Dunnett's adjustment for post hoc testing of ARDS versus each of the other groups was applied.

Supplemental Table 1

Tissue cytokines in zinc deficient and control mice

	BASELINE				MV 15ml/kg				LPS + MV 15 ml/kg			
pg/ug/ul	**Control**		**Zn Def**		**Control**		**Zn Def**		**Control**		**Zn Def**	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1α	1.47	0.1	2.29	0.51	12.56	7.8	1.67	0.31	80.55	19.37	**173.9***	24.3
IL-2	0.19	0.18	0.68	0.064	nd		nd		0.13	0.07	0.13	0.07
IL-4	nd		nd		nd		nd		nd		nd	
IL-5	1.32	0.1	1.09	0.15	1.16	0.1	1.19	0.15	1.29	0.07	1.2	0.16
IL-6	0.98	0.1	0.88	0.006	14.03	3.89	18.4	6.5	134.6	49.1	**387.7****	63.3
IL-10	1.89	1.44	2.21	0.03	1.66	0.04	1.37	0.07	2.53	0.085	1.37	0.07
IL-12p40	18.6	2.28	22.7	1.49	3.8	0.87	9.99	2.14	103.1	24.9	**197.68***	37.75
IL-12p70	1.88	0.04	1.75	0.09	1.75	0.09	1.81	0.43	1.94	0.09	1.81	0.15
IL-13	nd		nd		nd		nd		nd		nd	
IL-17	0.503	0.025	0.46	0	0.54	0.049	0.52	0.09	0.56	0.031	0.54	0.042
γ-INF	nd		nd		nd		nd		nd		nd	
TNF-α	nd		nd		nd		nd		nd		nd	
MCP-1	4.16	0.699	4.02	0.052	6.89	0.2	12.72	6.33	26.45	14.94	51.05	27.86
GMCSF	nd		nd		nd		nd		nd		nd	
IL-1β	6.59	4.1	21.87	2.69	2.23	0.35	1.89	0.83	6.3	1.87	8.99	0.6
IL-18	36.3	9.3	42	1.3	68.9	5.04	47.6	9.7	38.5	6.7	24.4	0.8
IL-33	150.1	12.8	128.7	7.1	253.77	57.89	368.6	101.4	675.1	21.9	653.8	47.03

p<0.01 two-way ANOVA vs. LPS + MV Control

****p<0.05 two-way ANOVA vs. LPS + MV Control***

nd denotes levels of cytokine were not detectable in sample.

Supplemental Table 2. Baseline patient characteristics (see Figure 7B)

Variable	Cohort (n=112)
Age (years)	61 [28, 94]
Gender (% male)	54%
Race	
White	81%
Black	11%
Hispanic	4%
Asian	2%
Other	0%
APACHE II scores	23 [6, 39]
In-Hospital Mortality	22% (25/112)
Co-morbidities	
CAD	15% (17/112)
CHF	9% (10/112)
COPD	13% (15/112)
Liver disease	7% (8/112)
CKD	22% (25/112)
Cancer	
Hematological	27% (30/112)
Solid tumor	26% (29/112)
DM	21% (23/112)

*Age and APACHE II scores are expressed in medians [min, max 95% CI].

*Excludes 4 healthy controls, included in the zinc assay.

Supplemental Table 3. Patient characteristics according to ICU Diagnosis (See Figure 7B)

	ICU Control (n=3)*	SIRS (n=26)	SEPSIS (n=62)**	ARDS (n=21)^
APACHE	22 [14, 30]	19 [4, 34]	21 [4, 37]	27 [13, 41]
In hospital mortality	0%	8%	21%	48%
Vasopressors	0%	12%	48%	57%
Invasive ventilation	33%	23%	27%	76%
Transfusions	0%	15%	18%	33%
Glasgow Coma Scale	15 [14,15]	15 [7, 15]	15 [7, 15]	8 [3,15]
Lactate (mEq/l)	2 [2]	2 [0, 5]	2 [0, 6]	3 [0,7]
Plasma Zinc (µM)	8.3 [5.9-10.7]	6.2 [3.1-12.6]	5.9 [1.2-14.9]	4.4 [0.3-8.5]
Heart disease	33%	19%	47%	33%
Heart Failure	0%	7%	9%	9%
CAD	33%	3%	19%	14%
Acute Kidney Injury	33%	11%	34%	48%
Chronic Kidney disease	66%	27%	16%	29%
Chronic renal replacement	33%	11%	6%	0%
DM	33%	19%	19%	24%
Liver disease	33%	7%	3%	14%

Obesity	0%	11%	3%	4%
TPN	0%	0%	1%	0%
COPD	33%	19%	15%	0%
Malignancy	66%	50%	53%	52%
Solid Tumor	33%	35%	24%	19%
Hematologic Tumor	33%	15%	29%	33%
Plasma IL6	158.3 [0-335.3]	151.5[0-825]	600.4[0-3937]	1045[0-4410]
Plasma IL18	615.5 [592-653]	449.5 [36-1101]	688 [235-2121]	695.7[320-1307]

*Excludes 4 healthy controls, included in the zinc assay.

**None of the sepsis patients progressed to ARDS development.

^Etiology of ARDS was sepsis-induced ARDS in 18 subjects, idiopathic pneumonia syndrome in 2 subjects, and acute interstitial pneumonia in one subject.

APACHE II scores, GCS, Lactate, Zinc, IL6, and IL18 are expressed in medians [min, max 95% CI].

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