Type 2 deiodinase and host responses of sepsis and acute lung injury

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Online Supplement (Detailed Materials and Methods)

NCBI genome-wide expression profiles of preclinical murine ALI models. We evaluated four previously deposited microarray datasets: GSE2368 (1), GSE11662 (2), GSE9368 (3), and GSE14525 (4) in the National Center for Biotechnology Information Gene Expression Omnibus repository. These datasets were generated in spontaneously breathing (SB) mice; mice exposed to LPS (1 mg/kg, intratracheal delivery) for 1, 2, and 18 h or to MV with tidal volumes of 17 ml/kg for 6 h (GSE2368), 30 ml/kg for 4 h (GSE11662), or 40 ml/kg for 4 h (GSE9368, GSE14525). Expressions were profiled using Affymetrix platform and signal intensities normalized using Robust Multi-Array algorithm in R package affy (http://www.bioconductor.org) and expressed as $\log_2$ values. Spearman ranked correlation test was performed on LPS and VILI datasets where $p < 0.001$ is considered significant.

***In vivo* DIO2 silencing (siDIO2) and animal models**. For *in vivo* silencing, custom designed siRNA (5'-GAAGUUGGCUGGAGAAGAAUU-3') targeting murine *Dio2* was synthesized with siSTABLE modification by Dharmacon (Lafayette, CO). C57B6 mice were pre-treated intratracheally (i.t.) with 10 mg/kg siDIO2 for 72 h followed by 1 mg/kg LPS i.t. challenge for 18 h (LPS model) or by 30 ml/kg ventilation, 70 breaths/min and positive expiratory pressure of 0 cm H_2O for 4 h (VILI model). Vehicle control (CTRL) and scramble siRNA (siCTRL) were used as controls. BAL, lungs, blood and plasma were extracted for further analyses. All animal procedures conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the experimental use of animals and approved by University of Illinois at Chicago Animal Care and Use Committee.

BAL protein and leukocytes analysis in murine lungs. Mice underwent BAL extraction from both lungs with Hank's balanced salt solution (1 ml/mouse), and recovered BAL fluids were assayed as previously described (5). BAL protein concentrations were measured with a BCA Protein assay kit (Bio-Rad, Hercules, CA). Leukocytes in the cell pellet were counted by cytocentrifugation and Diff-Quik staining (Dade Behring Inc., Newark, DE).

Western blot analysis. Lung lysates were prepared as previously described (1-3). Immunoreactivity was assessed with an anti-D2 polyclonal antibody (1:500, H-165, Santa Cruz) or an anti-actin antibody (1:50000, A3854, Sigma). Proteins were detected using an enhanced chemoluminescence system (Pierce, Rockford, IL). Integrated densities of bands were quantified using ImageJ software (Research Services Branch, Bethesda, MD).

Prohormone thyroxine (T4) and thyroid stimulating hormone (TSH) levels in plasma. Mouse blood was harvested via the right carotid artery, and plasma was collected by centrifugation. T4 levels were measured using DRI® Thyroxine Assay kit

(Antech Diagnostics, Oak Brook, IL) following manufacturer's protocol. TSH levels were assessed via immunoradiometric assay with an iodine-labeled monoclonal antibody and a second monoclonal antibody recognizing a distinct TH epitope.

Endothelial Cell Cultures and Cyclic Stretch Studies. Human pulmonary microvascular ECs were obtained from Cambrex Corp. (Walkersville, MD) and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air as in prior studies ((6). For cyclic stretch experiments, confluent ECs were exposed to 18% cyclic stretch (CS) or static conditions for 24 h. D2 content in cell lysates was analysed by western blotting from control and 18% CS-challenged ECs as described previously (7).

Study populations and demographics. Genotyping was conducted in DNA obtained from 327 European-Americans (EAs)(188 controls, 139 cases with severe sepsis including 78 with ALI) and 261 African-Americans (AAs)(187 controls, 74 cases with severe sepsis including 41 with ALI). Severe sepsis and ALI were defined using the American–European Consensus Criteria (8) and the Society of Critical Care Medicine Consensus statements (9). Demographic and clinical information including source of infection, co-morbidities, and APACHE II scores were recorded to ensure comparability of the severity of illness between ALI and sepsis groups. Control subjects were drawn from the population and were free of lung inflammation, transplant, diabetes, and cancer based on medical record review. This study was approved by the University of Chicago IRB. Informed consent was obtained from all subjects.

SNP selection and genotyping. TagIT 3.03 software (10) was used to select a set of 10 cosmopolitan tagging SNPs (tSNPs) from HapMap phase II data (11-12). Genotyping was conducted using the iPLEX Gold™ Platform following manufacturer's protocol (Sequenom, San Diego, CA). A TaqMan™ allelic discrimination assay (Applied Biosystems, Foster City, CA) was used to validate the associated SNP.

Assessment of population stratification among case-control samples. EAs were genotyped for 93 ancestry informative markers (AIMs) covering the largest features of the European North-Northwest–South-Southeast axis of differentiation (13). AAs were genotyped for 96 AIMs selected with average allele frequency differences >0.6 among European, West African and Native American/Asian populations (14-15). EIGENSOFT was used to perform principal component analysis (PCA) (16). Previously published reference data from 163 Swedish, 57 Polish, 76 English, 119 Italians, 68 Greeks, and 55 Spanish (13), as well as from Utah residents with ancestry from northern and western Europe (CEU) (11) were used for EA PCA analysis, whereas data from CEU and Yoruba in Ibadan, Nigeria (YRI) were for AA PCA analysis (11).

Statistical analysis. *In vivo* animal results were analyzed using one-way ANOVA; paired groups were compared by Newman-Keuls test. Results are expressed as mean $\pm$ SE. Three to five mice were used in each experimental group. Demographics were compared by two-tailed Mann-Whitney test, and all groups were compared across diagnosis groups by one-way ANOVA. Survival rates were compared by chi-square tests. Departures from Hardy-Weinberg equilibrium (HWE) were calculated using the Exact test (17) and significant departures were set at $p \leq 0.006$ and $p \leq 0.005$ for EAs and AAs respectively, based on the number of tests performed. Direct association of tSNPs with disease was assessed using the Cochran-Armitage trend test assuming an additive model and the empirical *p*-value obtained by 1000 permutations.

Both HWE and trend test statistics were computed by means of a custom script for STATISTICA (StatSoft Inc., Tulsa, OK). Multiple logistic regression analysis was used to adjust for age and sex and for population stratification including the first principal component (PC1) scores derived from PCAs using SPSS 14.0 (SPSS Inc., Chicago, IL). SPSS was also used to estimate the odds ratio (OR) and 95% confidence intervals (CI). Testing UNtyped Alleles software (TUNA) ver. 1.1 (18) was used to estimate allele frequencies of untyped SNPs in all samples and to perform indirect association testing of untyped SNPs with minor allele frequency (MAF) ≥5% and multilocus LD values (M_D) ≥ 0.7. PHASE 2.1 (19) was used to estimate the *DIO2* haplotypes from CEU and YRI data that were subsequently employed by TUNA as a reference for the underlying linkage disequilibrium (LD) across gene variants in the two populations. To judge the significant SNP associations in the context of the multiple comparisons performed, a False Discovery Rate was assessed by QVALUE (20), considering the two conditions tested (*i.e.* severe sepsis and sepsis associated-ALI) and the two populations. EPIDAT 3.0 (http://dxsp.sergas.es) was used to perform a joint analysis using Mantel-Haenszel test. Haplotype associations were tested using Hapstats software (21). Statistical significance was set at $p \leq 0.05$.

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SUPPLEMENT FIGURE LEGEND

Figure E1. Increased DIO2 protein expression in human lung microvascular endothelial cells (HLMV) upon stretch challenge. Panel A. DIO2 protein and β-actin expression levels were detected by Western blotting from control (static) and 18% cyclic stretch (CS), 24 h challenged HLMV. **Panel B.** Graphical presentation of normalized DIO2 (D2) protein levels to β-actin depicting significant increases of D2 in CS compared with static controls. N=3, * denotes $p < 0.05$.

Supplement Table E1. Completion rates and Hardy-Weinberg equilibrium *p*-values for the genotyped *DIO2* tSNPs.

				European Americans			African Americans		
tSNP	Position*	Location	CR†(%)	Controls	SS‡	ALI§	Controls	SS‡	ALI§
rs225019	79733709	3' flanking	99.8	NA	NA	NA	1.000	0.278	1.000
rs17110436	79735086	3' flanking	99.1	NA	NA	NA	0.572	0.583	1.000
rs225017	79736979	3' flanking	99.8	0.769	**0.005**	**0.007**	0.197	1.000	1.000
rs225014	79739333	Ala92Thr	99.8	0.649	0.427	0.770	0.657	0.060	0.531
rs225011	79741961	Intron 2	99.7	0.769	**0.005**	**0.009**	0.140	1.000	1.000
rs2267872	79743426	Intron 1	99.7	0.638	0.098	0.420	0.700	**0.020**	0.086
rs2267873	79743567	Intron 1	99.8	0.408	0.068	0.255	0.356	0.331	0.508
rs8009555	79744267	Intron 1	99.5	0.650	0.119	0.420	0.347	1.000	1.000
rs6574551	79744287	Intron 1	97.4	0.665	0.147	0.433	0.134	1.000	0.450
rs12885300	79748019	5' flanking	99.5	0.525	**0.004**	**0.016**	0.102	0.545	1.000

*According to NCBI build 36.3.
†Completion rate.
‡Severe sepsis.
§Acute lung injury.
Nominal significant departures from HWE in bold.
NA, Not assessed since the SNP was found monomorphic in the EA population.

Supplement Table E2. Association of *DIO2* SNPs (tagging and untyped) with severe sepsis and severe sepsis-associated ALI.

				European Americans					African Americans					Joint analysis	
				Minor allele frequency			*p*-value (*q*-value)		Minor allele frequency			*p*-value (*q*-value)		*p*-value	
#	SNP	Position*	Location†	Controls (N=188)	SS‡ (N=139)	ALI§ (N=78)	SS	ALI	Controls (N=187)	SS (N=74)	ALI (N=41)	SS	ALI	SS	ALI
1	rs225019	79733709	3'flanking	<0.01	0.00	0.00	NA	NA	0.08	0.07	0.07	0.710 ((0.317)	0.824 (0.317)	NA	NA
2	rs17110436	79735086	3'flanking	<0.01	0.00	0.00	NA	NA	0.15	0.12	0.10	0.375 (0.317)	0.273 (0.317)	NA	NA
3	rs225017	79736979	3'flanking	0.46	0.42	0.38	0.328 (0.317)	0.083 (0.140)	0.17	0.14	0.19	0.359 (0.317)	0.714 (0.317)	0.728	0.112
4	rs225015	79737332	3'flanking	0.34	0.26	0.22	**0.009** (0.030)	**0.003** (0.020)	0.39	0.34	0.34	0.288 (0.317)	0.365 (0.317)	**0.015**	**0.010**
5	rs7140952	79738025	3'flanking	0.09	0.09	0.08	0.841 (0.317)	0.882 (0.327)	0.24	0.19	0.16	0.129 (0.184)	0.084 (0.140)	0.450	0.197
6	rs225014	79739333	Exon 3 (Thr92Ala)	0.40	0.31	0.26	**0.009** (0.030)	**0.004** (0.020)	0.44	0.44	0.46	0.926 (0.337)	0.726 (0.317)	0.057	**0.038**
7	rs225013	79739682	Intron 2	0.50	0.40	0.35	**0.028** (0.080)	**0.001** (0.020)	0.24	0.24	0.27	0.949 (0.339)	0.657 (0.317)	0.057	**0.024**
8	rs225011	79741961	Intron 2	0.46	0.41	0.39	0.267 (0.317)	0.109 (0.168)	0.33	0.35	0.37	0.615 (0.317)	0.541 (0.317)	0.241	0.119
9	rs225010	79742032	Intron 2	0.43	0.35	0.31	**0.033** (0.083)	**0.002** (0.020)	0.33	0.35	0.37	0.617 (0.317)	0.598 (0.317)	0.204	0.088
10	rs2267872	79743426	Intron 1	0.09	0.09	0.08	0.783 (0.317)	0.787 (0.317)	0.11	0.10	0.12	0.739 (0.317)	0.566 (0.317)	0.943	0.906
11	rs2267873	79743567	Intron 1	0.10	0.11	0.12	0.612 (0.317)	0.560 (0.317)	0.39	0.40	0.38	0.838 (0.317)	0.817 (0.317)	0.679	0.884
12	rs8009555	79744267	Intron 1	0.09	0.10	0.08	0.695 ((0.317)	0.745 (0.317)	0.26	0.29	0.24	0.552 (0.317)	0.641 (0.317)	0.517	0.594
13	rs6574551	79744287	Intron 1	0.09	0.10	0.09	0.700 (0.317)	0.827 (0.317)	0.33	0.34	0.30	0.666 (0.317)	0.594 (0.317)	0.599	0.575
14	rs1388378	79744725	Intron 1	0.09	0.10	0.09	0.707 (0.317)	0.836 (0.317)	0.26	0.29	0.24	0.560 (0.317)	0.639 (0.317)	0.499	0.714
15	rs12885300	79748019	5' flanking	0.36	0.37	0.41	0.676 (0.317)	0.305 (0.317)	0.06	0.10	0.12	0.073 (0.140)	**0.039** (0.087)	0.300	0.083

*Nomenclature used in Figure 5.
†According to NCBI build 36.3.
‡Severe sepsis.
§Severe sepsis-associated acute lung injury.
tSNPs in bold and underlined; nominal significant associations in bold.
NA; not tested for association since MAF was below 5% in the sample.

Supplement Table E3. Genotyped *DIO2* SNPs, MAFs and unadjusted *p*-values for association with survival among severe sepsis patients.

	European Americans			African Americans		
	Minor allele frequency			Minor allele frequency		
dbSNP ID	Alive (N=88)	Dead (N=51)	*p*-value*	Alive (N=46)	Dead (N=28)	*p*-value*
rs225019	0.00	0.00	NA	0.08	0.05	0.513
rs17110436	0.00	0.00	NA	0.12	0.13	0.872
rs225017	0.41	0.44	0.557	0.15	0.11	0.468
rs225014	0.30	0.31	0.792	0.46	0.41	0.557
rs225011	0.40	0.44	0.475	0.33	0.39	0.482
rs2267872	0.10	0.09	0.692	0.12	0.07	0.347
rs2267873	0.11	0.12	0.804	0.40	0.39	0.874
rs8009555	0.10	0.10	0.907	0.31	0.25	0.382
rs6574551	0.10	0.10	0.960	0.39	0.25	0.103
rs12885300	0.34	0.43	0.161	0.11	0.09	0.589

**p*-value calculated using allelic trend test.

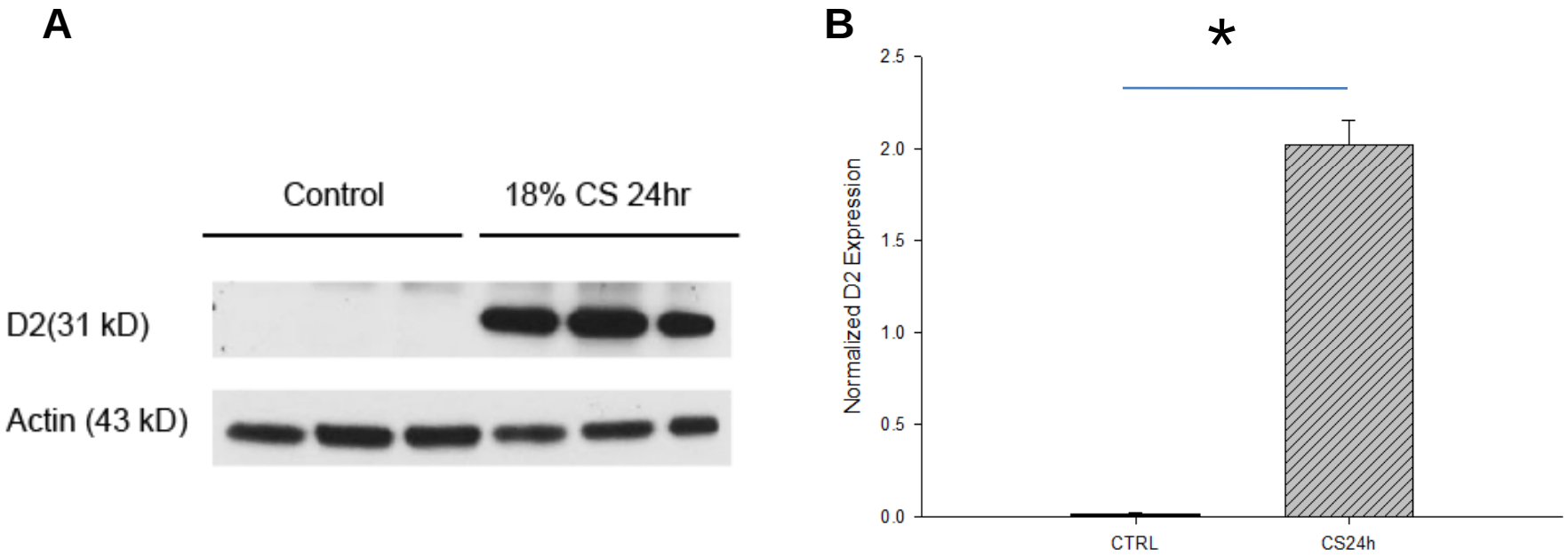
Supplement Table E4. Association of haplotypes (>5%) of *DIO2* SNPs rs225014 and rs12885300 with Severe Sepsis and Severe Sepsis-Associated ALI.

	European American					African American				
	Frequency			p-value†		Frequency			p-value†	
Haplotype*	Control (N=190)	SS (N=143)	ALI (N=77)	CvsSS	CvsALI	Control (N=188)	SS (N=75)	ALI (N=41)	CvsSS	CvsALI
A-T	0.328	0.360	0.394	0.398	0.134	0.056	0.088	0.098	0.111	0.073
A-C	0.271	0.334	0.343	0.069	0.089	0.502	0.478	0.439	0.639	0.293
G-C	0.373	0.292	0.246	**0.026**	**0.005**	0.441	0.421	0.439	0.733	0.885

*SNP order is rs225014-rs12885300

†*p*-value is unadjusted

Figure E1.


A
Control
18% CS 24hr
D2(31 kD)
Actin (43 kD)
B
*
Normalized D2 Expression
2.5
2.0
1.5
1.0
0.5
0.0
CTRL
CS24h