I. Experimental Design:

1. The goal of the experiment:

To elucidate the genome level expression signatures that differentiate pediatric SIRS, sepsis, and septic shock.

2. Brief description of the experiment:

Objectives: To advance our biological understanding of pediatric septic shock, we measured the genome-level expression profiles of critically ill children representing the systemic inflammatory response syndrome (SIRS), sepsis, and septic shock spectrum.
Design: Prospective observational study involving microarray-based bioinformatics.
Setting: Multiple pediatric intensive care units (PICU) in the United States.
Patients: Children ≤ 10 years of age: 18 normal controls, 22 meeting criteria for SIRS, 32 meeting criteria for sepsis, and 67 meeting criteria for septic shock on PICU day 1. The available PICU day 3 samples included 20 patients still meeting sepsis criteria, 39 patients still meeting septic shock criteria, and 24 patients meeting the exclusive day category, SIRS resolved.
Interventions: None other than standard care.
Measurements and Main Results: Longitudinal analyses were focused on gene expression relative to control samples and restricted only to patients having paired day 1 and 3 samples. The longitudinal analysis focused on upregulated genes revealed common patterns of upregulated gene expression, primarily corresponding to inflammation and innate immunity, across all patient groups on day 1. These patterns of upregulated gene expression persisted on PICU day 3 in patients with septic shock, but not to the same degree in the other patient classes. The longitudinal analysis focused on downregulated genes demonstrated gene repression corresponding to adaptive immunity-specific signaling pathways and was most prominent in patients with septic shock on days 1 and 3. Gene network analyses based on direct comparisons across the SIRS, sepsis, and septic shock spectrum, and all available patients in the database, demonstrated unique repression of gene networks in patients with septic shock corresponding to MHC antigen presentation. Finally, analyses focused on repression of genes corresponding to zinc-related biology demonstrated that this pattern of gene repression is unique to patients with septic shock.
Conclusions: Although some common patterns of gene expression exist across the pediatric SIRS, sepsis, and septic shock spectrum, septic shock is particularly characterized by repression of genes corresponding to adaptive immunity and zinc-related biology.

3. Keywords:

Inflammation; sepsis; innate immunity; T cells; MHC antigen; zinc

4. Experimental factors:

Parameter: Normal children and children with SIRS, sepsis, or septic shock.
Sample type: RNA
Source name: Whole blood
Organism: Homo sapiens
Strain: not applicable
Extracted molecule: Total RNA was extracted from whole blood using the PaxGene system. Controls had one sample taken. Children with SIRS, sepsis, or septic shock had one sample taken within 24 hours of admission to the pediatric intensive care unit (i.e. day 1) and when possible 48 hours later (i.e. day 3). RNA was hybridized to the Human Genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA)

II. Samples used, extract preparation, and labeling:

Origin of biological samples: Whole blood from the above named patient categories.

Manipulation of biological samples: None

Experimental factor value: Samples = control, SIRS, sepsis, or septic shock

Technical protocols: Total RNA was extracted from whole blood samples using the PaxGene™ Blood RNA System (PreAnalytiX, Qiagen/Becton Dickson) according the manufacturer's specifications. Labeling involved an in vitro transcription reaction using the ENZO BioArray HighYield RNA Transcript Labeling Kit (Affymetrix) according to manufacturer's instructions.

External controls: Control Oligonucleotide B2 (Affymetrix), 20X Eukaryotic Hybridization Controls (1.5 pM bioB, 5 pM bioC, 25 pM bioD, 100 pM cre, Affymetrix).

Table 1: Patient demographics for longitudinal analysis.

	Controls	SIRS	Sepsis	Septic Shock
Number of patients	18	14	14	56
Median age in years (IQR)	1.4 (0.2 – 3.8)	3.1 (1.8 – 7.1)	2.2 (1.2 – 5.5)	2.3 (0.8 – 6.1)
Median PRISM score (IQR)	n/a	12 (10 – 17)	10 (5 – 12)	18 (12 – 23)[a]
% mortality (28 day)	n/a	0	7	27[b]
Median # of organ failure (IQR) [c]	n/a	1 (0 – 1)	1 (0 – 1.2)	2 (2 – 3)[a]
Number of Males/Females	10/8	8/6	10/4	27/29
No. with documented infection	n/a	0	32[d]	43[e]
% with co-morbidity [f]	n/a	38	37	40
% immunosuppressed[g]	n/a	33	22	37
Day 3 classification				
SIRS	n/a	4	0	1
SIRS resolved	n/a	9	5	8
Sepsis	n/a	1	8	5
Septic shock	n/a	0	1	33
Death prior to day 3	n/a	0	0	9

[a]P < 0.05 versus SIRS and sepsis (ANOVA Kruskall Wallis).

[b]P < 0.05 versus SIRS and sepsis (Chi square).

[c]Refers to the maximum number of organ failures over the initial 7 days in the PICU.

[d]Gram negative bacteria (38%); gram positive bacteria (36%); other (26%).

[e]Gram negative bacteria (22%); gram positive bacteria (54%); other (24%).

[f]Refers to any co-morbidity or chronic condition other than the primary diagnosis leading to PICU admission.

[g]Refers to the presence of an intrinsic immunodeficiency, or a patient receiving immunosuppressive medications (including steroids).

III. Hybridization procedures and parameters:

The protocol and conditions used during hybridization, blocking and washing: Create a hybridization cocktail for a single probe array that contains 0.05 μg/μL fragmented cRNA, 50 pM Control Oligonucleotide B2 (Affymetrix), 20X Eukaryotic Hybridization Controls (1.5 pM bioB, 5 pM bioC, 25 pM bioD, 100 pM cre) (Affymetrix), 0.1 mg/mL Herring Sperm DNA (Promega), 0.5 mg/mL Acetylated BSA (Invitrogen), and 1X Hybridization Buffer. Heat hybridization cocktail to 99°C for 5 minutes, to 45°C for 5 minutes, and spin at maximum speed in a microcentrifuge for 5 minutes. Fill probe array with 200 μL of 1X Hybridization Buffer. Incubate at 45°C for 10 minutes in the GeneChip Hybridization Oven 640 (Affymetrix) rotating at 60 rpm. Remove 1X Hybridization Buffer and fill probe array with 200 μL of the hybridization cocktail. Incubate at 45°C for 16 hrs in the Hybridization Oven rotating at 60 rpm.

IV. Measurement specifications:

The image file was captured on an Affymetrix GeneChip Scanner 3000 and initially processed with Microarray suite 5.0 (Affymetrix).

Standard Affymetrix internal control genes were used to check the quality of the assay by the signals of the 3' probe set to the 5' probe set of the internal control genes, GAPDH and beta-actin, with acceptable 3' to 5' ratios between 1 and 3. Eukaryotic Spike controls were used to determine that the hybridization of target RNA to the array occurred properly.

Each array was normalized the same. Global scaling is used by adjusting the average intensity or signal value of each probe array to the same Target Intensity Value (TGV) of 1500. Genechip Operating Software 1v4 (Affymetrix) was then used to generate CEL files from each experiment, which were then imported into GeneSpring for all further data analysis.

GeneSpring 7.2 (Agilent technologies Inc. Palo Alto, California) was used for normalization, clustering, filtering, and statistical analyses. The Raw CEL files were processed using the RMA (Robust Multichip Average) built in GeneSpring software. All the samples were then normalized to the median of the controls.

V. Array Design

Commercial Affymetrix Human Genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA).