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Molecular Diagnostics in Sepsis: From Bedside to Bench

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- BACKGROUND:** Based on recent *in vitro* data, we tested the hypothesis that microarray expression profiles can be used to diagnose sepsis, distinguishing *in vivo* between sterile and infectious causes of systemic inflammation.
- STUDY DESIGN:** Exploratory studies were conducted using spleens from septic patients and from mice with abdominal sepsis. Seven patients with sepsis after injury were identified retrospectively and compared with six injured patients. C57BL/6 male mice were subjected to cecal ligation and puncture, or to IP lipopolysaccharide. Control mice had sham laparotomy or injection of IP saline, respectively. A sepsis classification model was created and tested on blood samples from septic mice.
- RESULTS:** Accuracy of sepsis prediction was obtained using cross-validation of gene expression data from 12 human spleen samples and from 16 mouse spleen samples. For blood studies, classifiers were constructed using data from a training data set of 26 microarrays. The error rate of the classifiers was estimated on seven de-identified microarrays, and then on a subsequent cross-validation for all 33 blood microarrays. Estimates of classification accuracy of sepsis in human spleen were 67.1%; in mouse spleen, 96%; and in mouse blood, 94.4% (all estimates were based on nested cross-validation). Lists of genes with substantial changes in expression between study and control groups were used to identify nine mouse common inflammatory response genes, six of which were mapped into a single pathway using contemporary pathway analysis tools.
- CONCLUSIONS:** Sepsis induces changes in mouse leukocyte gene expression that can be used to diagnose sepsis apart from systemic inflammation. (*J Am Coll Surg* 2006;203:585–598. © 2006 by the American College of Surgeons)
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The incidence of sepsis and the number of sepsis-related deaths is increasing at substantial cost (\$17 billion annually in the US),¹ as reported recently. The cornerstone of successful therapy is early, accurate diagnosis, coupled with eradication of the source of infection and appropriate antibiotic therapy.² Unfortunately, efforts to identify specific and sensitive diagnostic markers for sepsis have met with limited success.³ The diagnosis of sepsis in ICUs is especially

challenging because it is frequently difficult to distinguish between systemic inflammation and systemic infection. Clinicians are unable to identify the pathogen responsible for sepsis in up to 50% of patients or to determine whether patients are responding to antibiotic therapy¹; and the traditional means of identifying the organism responsible for bacterial infections are nonspecific (Gram stain), slow (culture), or insensitive (both Gram stain and culture).

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TD, DJM, and HD are employees of Partek Incorporated, a software company with expertise in microarray analyses, for example, nested cross-validation. GeneChip analyses were performed using Partek software as described herein; there were no monetary contributions.

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Abbreviations and Acronyms

CLP	= cecal ligation and puncture
FDR	= false discovery rate
EST	= expressed sequence tag
IP	= intraperitoneal
LPS	= lipopolysaccharide
PCA	= principal components analysis

There is compelling evidence that efforts to identify clinically important biomarkers in sepsis will prove successful ultimately,⁴⁻⁶ especially given the recent successes of high-throughput, genomic technologies in other fields that face diagnostic challenges (eg, leukemia,⁷ breast^{8,9} and colon cancer¹⁰). This molecular classification strategy involves searching for expression patterns in a subset of genes from tissues of known phenotype (the “training” data set), constructing a prediction rule, and then using these “biomarker” genes to predict the phenotype of new samples (the “test” data set). Studies that apply these genome-wide technologies to inflammation and sepsis in animal models and patients are now underway,¹¹⁻¹⁴ as reviewed recently.^{15,16} These reports suggest that genome-wide profiling of gene expression holds promise as a molecular diagnostic tool, capable of generating profiles from leukocytes that are sensitive, specific, and timely for pathogen detection.¹³ Despite provocative in vitro findings,^{4,5} there are few reports of using microarrays to study sepsis in vivo. These reports indicate that the transcriptional response during polymicrobial sepsis is organ-specific in mice¹² and rats.¹¹ There are no reports of using microarrays to make a diagnosis of sepsis, although very recent reports suggest that this will be feasible in patients.^{17,18} We hypothesized that leukocyte gene expression profiles obtained using DNA microarrays could be used to predict septic states; in particular, distinguishing between sterile and infectious sources of systemic inflammation, a common conundrum in caring for the critically ill or injured. Tissue samples from both septic patients and clinically relevant mouse models of sepsis were tested.

METHODS**Patient characteristics and samples**

Using an investigational protocol approved by the Washington University Human Studies Committee, informed consent was obtained to collect samples of splenic tissue intraoperatively or immediately postmor-

tem as described previously.¹⁹ Seven specimens for expression profiling were chosen retrospectively from patients with injury (trauma or operation) complicated by sepsis and organ dysfunction (Sepsis group). These were compared with six age- and gender-matched control specimens from patients with injury (trauma) requiring splenectomy (Injury group). A total of 13 patient spleen samples were collected.

Mice, experimental procedures, and samples

Care and use of laboratory animals were conducted in accordance with a protocol approved by the Washington University Animal Studies Committee. Seven- to nine-week-old, male C57BL/6 mice were purchased (Harlan, Inc) and allowed to acclimatize for at least 1 week. Male mice were used to avoid the confounding effect of the female estrous cycle and the well-characterized sexual dimorphism of the adaptive response to cecal ligation and puncture (CLP).²⁰ The seven experimental groups were designed to make classification difficult, reflecting clinically important distinctions relevant to care of ICU patients: lethal abdominal inflammation from a sterile source, lethal abdominal infection, and antibiotic-treated abdominal infection. Mice were assigned to the seven treatments listed in Table 1, grouped into those with no deaths (controls) and those that were “sick” with substantial associated mortality (sterile or infectious causes of systemic inflammation). Normal animals were untreated. Previously reported protocols were used to perform CLP and sham laparotomy.^{21,22} The ceca of some animals were punctured twice using a 25-gauge needle (CLP), and others were punctured using a 27-gauge needle to produce a “milder” sepsis that caused much lower mortality (Mild CLP). Animals that had laparotomy and cecal manipulation without ligation or puncture were included in the sham laparotomy group (Sham). Another group of animals had CLP and treatment with a standard antibiotic regimen for mouse abdominal sepsis: IP ceftriaxone 6.4 mg/kg and metronidazole 3.5 mg/kg, delivered 1, 12, and 24 hours after CLP (CLP + antibiotics group).²³ To induce severe systemic inflammation without infection, IP injection of *Escherichia coli* lipopolysaccharide (LPS) serotype 0111:B4 20 mg/kg (Sigma, Inc) was performed (LPS group).²⁴ The dose of LPS (20 mg/kg) was used because it reliably produces death over several days in the animals; smaller doses in pilot experiments tended to produce morbidity without mortality. Mice injected IP with

Table 1. Mouse Experimental Groups

Groups	Controls	"Sick"	Any-CLP
Normal	X		
Sham	X		
Saline	X		
CLP		X	X
Mild CLP		X	X
CLP + ABX		X	X
LPS		X	

ABX, antibiotic; CLP, cecal ligation and puncture; LPS, lipopolysaccharide.

normal saline vehicle (Saline group) acted as the concurrent control group for LPS treatment. The census of surviving mice was recorded at 24-hour intervals for 7 days. In a separate cohort, mice were sacrificed at 24 hours after injury by cervical dislocation under halothane general anesthesia. The 24-hours time point after injury was chosen because pilot experiments showed that the ability to distinguish between spleen expression profiles was greater at 24 hours than at 48 or 72 hours after injury.²⁵ Whole spleen tissue from eight CLP and eight Sham mice was harvested and flash-frozen in liquid nitrogen and stored at -80°C . In another cohort of animals, intracardiac blood from eight animals per group was pooled using the PAXgene Blood RNA System to derive total RNA from whole blood (PreAnalytiX GmbH). While 5 GeneChips from blood for each of the seven groups were prepared, there were a total of 35 mouse blood GeneChips from 280 animals: 28 GeneChips were in the training data set and 7 GeneChips were in the test (de-identified) data set.

White blood cell counts

Whole blood was collected for automated white blood cell counts from eight animals in each group over two replicates, each run performed with samples from concurrent control animals. A HemaVet Multispecies Hematology Analyzer (CDC Technologies) provided counts and an automated differential. Data were expressed as the mean \pm SEM.

Target cRNA and gene expression signal

Total RNA from human and mouse spleens was extracted per TRIzol protocol (Life Technologies, Inc). Total RNA from mouse blood was extracted using the PAXgene kit as recommended by the manufacturer. cRNA target for GeneChip hybridization was prepared from total RNA using the protocol recommended (Affymetrix, Inc). The human spleen cRNA was hybridized

with the human full-length GeneChip (approximately 7,000 probe sets). Mouse spleen and blood cRNA were hybridized with the U74Av2 GeneChip (approximately 12,400 probe sets). Fluorescent hybridization signal was detected using a GeneArray Scanner (Hewlett-Packard, Inc) at the Washington University Alvin J Siteman Cancer Center and, for the prospective mouse cohort, the GeneChip Scanner 3000 (Affymetrix). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE5663.

Tests for differential expression, class prediction, and pathway analysis

Expression values were calculated from GeneChip.cel files using DNA chip analyzer software (version 1.3) and default settings (only perfect match probes were used).²⁶ Principal components analysis (PCA) was used to visually explore global effects for genome-wide trends, unexpected effects, and outliers in the expression data (Partek software). Differentially expressed genes were identified using a mixed-model ANOVA and linear contrasts. We next determined whether it was possible to accurately distinguish tissue samples resulting from models of sterile systemic inflammation or models of systemic infection in human spleen, mouse spleen, and mouse blood. To produce unbiased estimates of prediction accuracy while also optimizing the set of predictor genes, classifier type, and classifier parameters, we used a two-level, nested cross-validation procedure that produces prediction estimates that are not biased by gene, classifier, and parameter selection. This procedure makes use of an "outer" cross-validation to produce the estimate of accuracy, and an "inner" cross-validation to perform classifier and gene selection.²⁷ For class prediction, we compared k-nearest neighbor, nearest centroid, and linear and quadratic discriminant analysis classifiers. Two complementary programs were used to query regulatory networks: PathwayAssist (Ariadne Genomics) and Pathway Analysis (Ingenuity Systems). Both PathwayAssist and Pathway Analysis use the published literature or publicly available databases to identify relationships between genes, small molecules, or other objects. This information in turn is used to map de novo, putative, interaction networks from a given list of input gene

Table 2. Patient Characteristics

Patient no.	Group	Age (y)/gender	Admitting diagnosis	Complications	MODS score	ISS	Positive cultures	Splenectomy	Disposition (LOS)
1	S	38/F	Metastatic colon adenocarcinoma and abdominal sepsis	Respiratory failure; septic shock; pneumonia; peritonitis; bronchopleural fistula	9	NA	Peritoneal fluid: <i>Pseudomonas aeruginosa</i> ; sputum: <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ; urine: vancomycin-resistant <i>Enterococcus</i>	Day 7 (abdominal reexploration)	Discharged (65 d)
2	S	51/M	Necrotizing fasciitis	Respiratory failure; sepsis	3	NA	Wound Gram stain with mixed organisms, including gram-negative rods and <i>Enterococcus</i>	Postmortem	Death (2 d)
3	S	19/M	Polysystem trauma	Respiratory failure; septic shock; pneumonia; peritonitis; renal failure; hepatic failure	9	50	Peritoneum: <i>Aspergillus fumigatus</i> ; sputum: <i>Pseudomonas aeruginosa</i>	Day 27 (abdominal reexploration)	Death (30 d)
4	S	83/F	Peritonitis secondary to colon anastomotic leak	Respiratory failure; sepsis; stroke; hepatic failure; DIC	4	NA	Peritoneal fluid Gram stain with gram-positive cocci, growth of <i>Bacteroides fragilis</i> ; urine: <i>Enterococcus</i> ; sputum: yeast	Postmortem	Death (8 d)
5	S	80/F	Perforated cecum and obstructing rectal cancer	Respiratory failure, septic shock	8	NA	Sputum: yeast	Postmortem	Death (43 d)
6	S	47/M	Necrotizing fasciitis of neck	Respiratory failure	7	NA	Tissue: Viridans group <i>Streptococci</i>	Postmortem	Death (3 d)
7	S	79/F	Perforated sigmoid colon	Respiratory failure, septic shock	4	NA	Blood: ORSA; urine: ORSA; sputum: ORSA	Postmortem	Death (32 d)
8	I	20/F	Polysystem trauma	Respiratory failure	1	29		Day of admission	Discharged (10 d)
9	I	80/F	Polysystem trauma	Respiratory failure	1	27		Postmortem	Death (7 d)
10	I	42/M	Splenic laceration sustained during assault		1	16	Sputum: <i>Staphylococcus aureus</i> , <i>Streptococcus pneumoniae</i>	Day of admission	Discharged (7 d)
11	I	20/F	Polysystem trauma		0	10		Day of admission	Discharged (5 d)
12	I	71/F	Polysystem trauma		1	29		Day of admission	Discharged (6 d)
13	I	18/M	Gunshot wound to abdomen		1	9		Day of admission	Discharged (20 d)

F, female; I, injury (control) patients; ISS, Injury Severity Score; LOS, length of stay in hospital; M, male; MODS, multiple organ dysfunction syndrome; ORSA, oxacillin-resistant *Staphylococcus aureus*; S, sepsis patients.

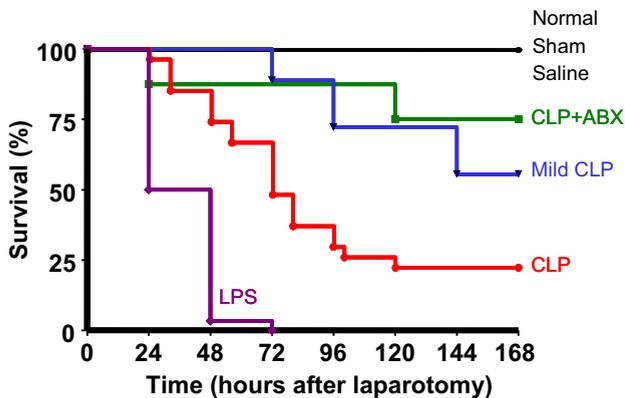


Figure 1. Survival in the mouse models. All control animals (Normal, Sham, Saline) survived. Cecal ligation and puncture (CLP) treatment decreased the proportion surviving compared with Sham controls ($p < 0.01$), with an approximate CLP-induced mortality of 80% at 7 days. Mice subjected to cecal ligation and puncture with the smaller, 27-gauge needle (Mild CLP) or with antibiotics (CLP plus antibiotics) had higher survival rates than animals challenged with 25-gauge needle punctures (CLP, $p < 0.01$ and $p < 0.0001$, respectively). Mice treated with lipopolysaccharide (LPS) all expired by 72 h after injury, a substantially higher mortality rate than the Saline control group and CLP ($p < 0.0001$). ABX, antibiotic.

identifiers. See Supplementary Information for additional details.

Statistical analysis

Survival data were calculated using the Kaplan-Meier method and survival curves were compared using log-rank test (Prism v3.03, GraphPad Software, Inc). CBC and cell differential data were analyzed by one-way ANOVA using the Tukey-Kramer post-test to correct for multiple comparisons (Prism v3.03). Modified multiple organ dysfunction syndrome (MODS) score²⁸ are reported as the mean \pm SEM and analyzed using the Mann-Whitney U test for nonparametric data (Prism v3.03). The significance of change in mouse blood relative RNA abundance was measured across all seven groups using two-way ANOVA for the effects of treatment and batch (time). From this analysis, a step-up false discovery rate (FDR) of 0.1 was used to identify a subset of discriminating genes for treatment effect, visualized using PCA.²⁹ For pair-wise group comparisons across mouse blood, a two-way ANOVA for the effects of treatment and batch was used. Because there was no batch effect for spleen, one-way ANOVA was used. An FDR of 0.1 was applied to the raw p value for each pair-wise comparison, giving a list of informational genes for each comparison.

RESULTS

Patient spleen differential gene expression and sepsis class prediction

The characteristics of the 13 patients sampled are found in Table 2. None of the Injury (control) patients had positive cultures or signs of infection or sepsis at the time of sample collection, and all but one recovered uneventfully after splenectomy. In contrast, all Sepsis patients had positive bacterial or fungal cultures or obvious signs of infection (pus or necrotizing fasciitis) complicated by sepsis-induced organ dysfunction at the time of operation (modified MODS score, $p = 0.001$ versus Injury). DNA chip analyzer hybridization signal analysis using default filters flagged 1 of 13 human GeneChips as a statistical outlier ($> 15\%$ probe pair expression values calculated as statistical outliers, suggesting unreliable hybridization signal). This sample (patient 7) was omitted from additional analysis. PCA was used to explore differences in gene expression for all genes across the remaining 12 microarrays, demonstrating considerable differences between the Sepsis and Injury classes (data not shown). Consistent with the large variance in expression observed across subjects, there were no genes identified as differentially expressed between these two classes using a FDR of 0.1. To estimate Sepsis class prediction accuracy among these samples, a 12×11 leave-one-sample-out nested cross-validation was performed. The average predictive accuracy was 67.1%.

Morbidity and mortality of sepsis and endotoxemia in mice

All control group animals (Saline, Sham, Normal) survived the duration of the experiment. In contrast, "sick" animals with systemic infection (CLP, CLP + antibiotics, Mild CLP, and LPS) exhibited typical signs of piloerection, anorexia, and lethargy followed by 7-day mortality rates of 78%, 25%, 44%, and 100%, respectively (Fig. 1). Absolute white blood cell counts varied among the seven experimental groups ($p < 0.0001$), in particular between control and sick animals (Fig. 2). There was no difference among animals with sterile or infectious causes of systemic inflammation (LPS, CLP, Mild CLP, or CLP + antibiotics). Likewise, there was no difference in cell differentials among controls or among animals with sterile or infectious causes of systemic inflammation, except for LPS versus CLP + antibiotics (neutrophils and lymphocytes, $p < 0.05$).

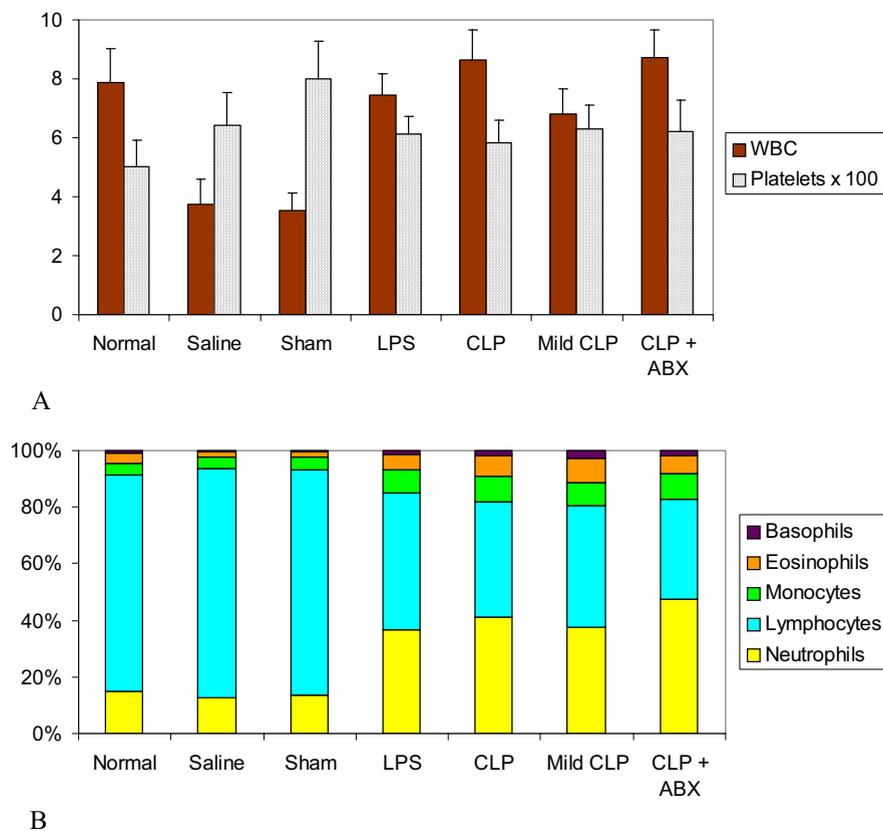


Figure 2. Complete blood counts and differentials of mice 24 h after challenge. Neither distinguished between the groups of “sick” animals: Lipopolysaccharide (LPS), cecal ligation and puncture (CLP), Mild CLP, and CLP + antibiotics (ABX).

Mouse differential gene expression

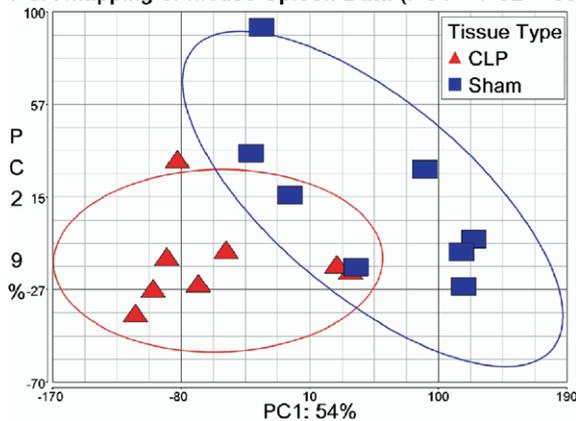
DNA chip analyzer hybridization signal analysis flagged 2 of the 35 mouse blood GeneChips as statistical outliers: one Saline and one Sham GeneChip from the training data set. These mouse outliers GeneChips were omitted from additional analysis. Thirty-three GeneChips remained in the mouse blood data set. PCA was used to visualize treatment differences in expression for all genes in blood and spleen, demonstrating batch and any-CLP treatment effects ($p < 0.05$, Fig. 3). We applied a stringent multiple test correction (Bonferroni, 0.05) to the p values from the two-way ANOVA to identify a small set of genes in blood differentially expressed between the seven treatment groups (25 probe sets corresponding to 24 genes, as probe sets for lipocalin 2 appeared twice [Table 3]).³⁰ PCA analysis of these 25 probe sets revealed that the seven experimental groups were clustered into three apparent phenotypes (Fig. 4): control animals, LPS-treated animals (sterile source of systemic inflammation), and those that had any CLP

treatment (Sepsis). Comparisons of gene expression across groups generated several informational gene lists (FDR = 0.1); each indicated apparent increases and decreases in gene expression induced by CLP or LPS (Fig. 5).

Sepsis class prediction in mice

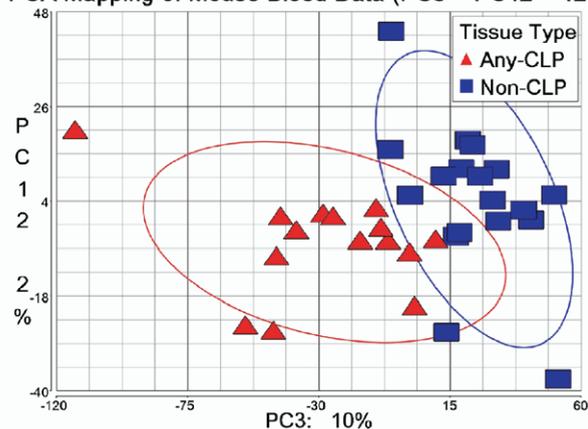
Using mouse spleen samples for gene expression analysis, we were able to classify the samples as CLP or Sham with 96.0% accuracy, estimated using cross-validation. For the blood data, the experimental design dictated that the first four replicates (batches) were used to train the classifiers. The best classifiers were trained on all 26 training samples (batches 1 to 4) and used to predict the seven de-identified test samples (batch 5). All seven test samples were predicted correctly as any-CLP (Septic) versus non-CLP. We also performed fivefold leave-one-batch-out cross-validation, which produced an overall accuracy estimate of 94.4% (Fig. 6). The prediction accuracy differentiating LPS from controls was 93.2%.

PCA Mapping of Mouse Spleen Data (PC1 + PC2 = 63%)



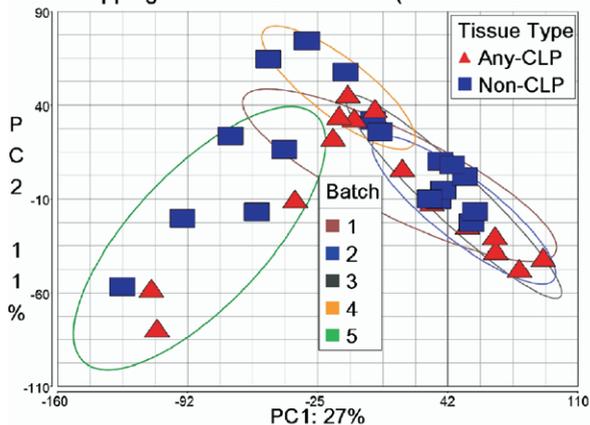
A

PCA Mapping of Mouse Blood Data (PC3 + PC12 = 12%)



B

PCA Mapping of Mouse Blood Data (PC1 + PC2 = 38%)



C

Figure 3. Principal components analysis (PCA) of mouse gene expression for all probe sets in 16 spleen and 33 mouse blood samples. In each case, profiled samples from septic subjects are shown in red triangles and samples from nonseptic subjects are shown in blue squares. (A) For mouse spleen samples ($n = 16$), differences between septic (cecal ligation and puncture [CLP]) and control

The ability to predict low versus high mortality after CLP was substantially lower (62.4%). To obtain the final mouse blood classifier, leave-one-batch-out cross-validation was performed for the purpose of classifier selection. Sixty-four classifiers tied for best prediction. The median number of genes for these 64 classifiers was 450. Of these 450 genes, only 61 genes showed changes greater than twofold, and the majority (86.4%) were altered by twofold or less.

Gene pathway analysis

There were nine genes that demonstrated increased RNA abundance across spleen and blood and across CLP and LPS (Fig. 5). We call this cluster of genes the “common inflammatory response cluster” (Table 4). Pathway analysis tools also were used to put this list into a biologic (functional) context. All but one of these genes is annotated and has been associated previously with a gene product, small molecule, or cellular process linked to inflammation (Fig. 7). A single expressed sequence tag (EST) completed the list of nine genes (GeneChip identifier, 99849_at). A BLAST search identified this sequence as retroviral. A search of the NCBI Gene Expression Omnibus microarray database showed that RNA for this Affymetrix probe set is increased in a number of models of inflammation, both animal and human.

DISCUSSION

The ability to diagnose sepsis more accurately would allow appropriate treatment to be instituted earlier, thereby improving the likelihood of survival.^{31,32} We hypothesized that expression profiles could distinguish between septic and nonseptic states in vivo, and that expression profiles could define lists of common responder genes using a systematic, unbiased approach.^{15,16} Recent

(Sham) samples are evident in principal components (PCs) 1 and 2. (B) For mouse blood samples ($n = 33$), technical batches are indicated by ellipses drawn at 2 SDs from the centroid of each batch. The first two PCs reveal that batch-to-batch variability is larger than the biologic variability when all genes are considered. (C) The treatment effect of any-CLP (CLP, Mild CLP, and CLP + antibiotics) versus non-CLP (lipopolysaccharide [LPS], Saline, Sham, and Normal) is apparent, but less notable than the batch effect (appearing on PC3 and PC12, explaining 10% + 2% of variance, respectively). No PCs besides PC3 and PC12 showed a substantial difference between any-CLP and non-CLP groups (determined by *t*-test on each PC). Note that the GeneChip scanner used for batches 1 to 4 was different than that used for batch 5 (latest generation), which is one explanation why batch 5 is most different from the other four batches.

Table 3. Genes that Differentiate Between Seven Experimental Groups

SAA3	Serum amyloid A 3
S100A8	S100 calcium-protein protein A8 (calgranulin A)
LCN2	Lipocalin 2 (oncogene 24p3)
ARG1	Arginase, liver
FCER1G	Fc fragment of IgE, high-affinity I, receptor for; γ polypeptide
TGTP	T-cell-specific GTPase
GBP2	Guanylate-binding protein 2, interferon-inducible
GHRL	Ghrelin precursor
ARPC1B	Actin-related protein 2/3 complex, subunit 1B, 41 kDa
1700093E07RIK	RIKEN cDNA 1700093E07 gene
API5	Apoptosis inhibitor 5
KIAA 1892	WD repeat domain 40A
LST1	Leukocyte-specific transcript 1
CCL6	Chemokine (C-C motif) ligand 6
GABARAPL2	GABA(A) receptor-associated protein-like 2
IL4R	Interleukin 4 receptor
MARCKS	Myristoylated alanine-rich protein kinase C substrate
HCK	Hemopoietic cell kinase
CHPF	Chondroitin polymerizing factor
CTSZ	Cathepsin Z
SPI1	Spleen focus forming virus proviral integration oncogene spi 1
RAB4B	RAB4B, member RAS oncogene family
CLECSF6	C-type (calcium-dependent, carbohydrate-recognition domain) lectin, superfamily member 6
IFITM6	Interferon induced transmembrane protein 6

reports using microarray technology *in vitro* indicated that inflammatory and infectious insults produce distinct transcriptional signatures.^{4,5} The current study is the first examining the ability of microarray gene expression profiles to distinguish sterile from infectious causes of systemic inflammation *in vivo*.

Molecular diagnostics in patients and mice

We examined gene expression profiles of splenic tissue from patients with injury versus those with injury complicated by sepsis and organ dysfunction. Differences in apparent gene expression between the Sepsis and Injury (control) phenotypes were used to construct a classifier, the accuracy estimate of which was 67.1%. This small, exploratory clinical study provided “bedside” proof of feasibility using human transcriptional profiles to model

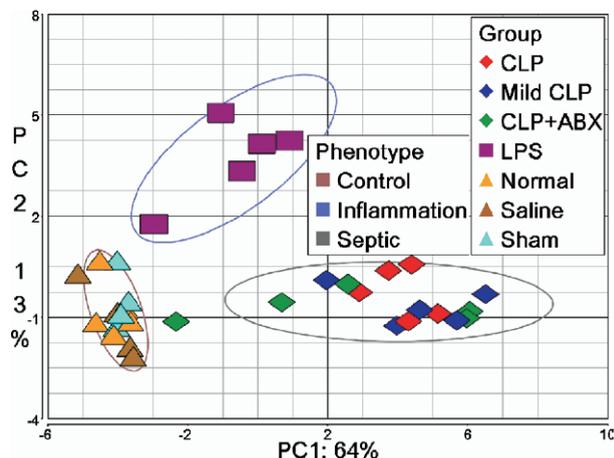
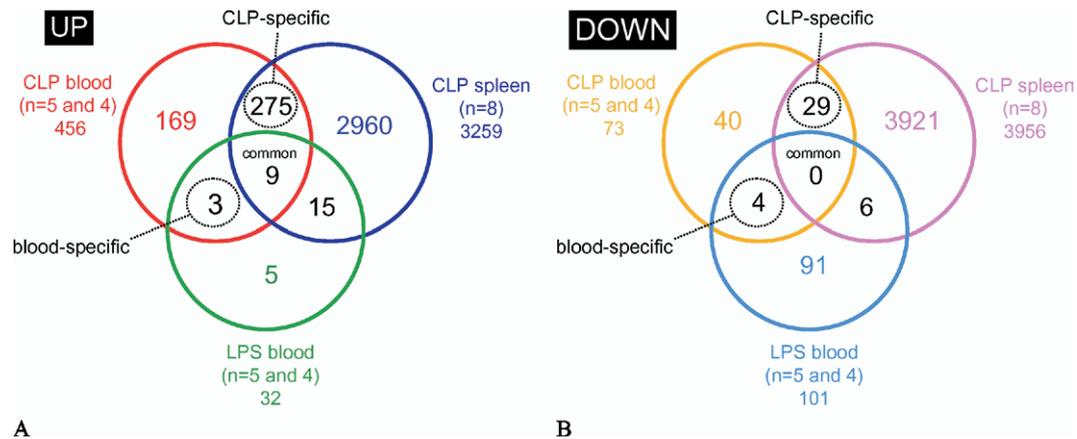


Figure 4. Class discovery in mouse blood. A two-way ANOVA identified genes that exhibit differential expression across the seven experimental groups. A stringent Bonferroni cutoff of 0.05 identified 25 probe sets that were notably different between groups, corresponding to 24 genes (lipocalin 2 was present twice), including one EST. Principal components analysis of these 25 probe sets grouped the 33 arrays into only 3 phenotypic classes: septic (any cecal ligation and puncture [CLP]), lipopolysaccharide (LPS), and controls (Saline, Sham, and Normal). The list of these 24 differentiating genes is in Table 3. ABX, antibiotics.

the septic phenotype, but also demonstrated a large degree of variance in gene expression between subjects, because of both technical and biologic differences. To control more of this variance we moved from the bedside to the bench, performing a systematic examination of the diagnostic potential of spleen and blood gene expression profiling in inbred mice. Consistent with the human data, spleen samples from mice after CLP exhibited microarray patterns that could be modeled to predict the septic phenotype. The nested cross-validation accuracy estimate of 96.0% for sepsis prediction using mouse spleen was considerably better than that found using human spleen, likely because of the mouse experimental design that exploited fresh tissues and identical age, gender, and genotype across subjects. Because the clinical use of gene expression analysis using splenic tissue is severely limited, we explored next the use of circulating blood for class prediction in our mouse models. The combined accuracy of the predictions for any-CLP versus non-CLP and LPS versus controls was high at 94.4% and 93.2%, respectively. The accuracy estimate for distinguishing between the high and low mortality CLP groups (CLP versus Mild CLP and CLP plus antibiotics) at this 24-hour time point was much less at 62.4%. These conclusions are consistent with the PCA analysis in Figure 4, in which we sought differences in apparent



A

B

Figure 5. Differential gene expression in mouse data sets. Shown on the outside of each circle is the number of probe sets altered in either spleen or blood by the treatment compared with its respective control: cecal ligation and puncture (CLP) versus Sham or lipopolysaccharide (LPS) versus Saline. (A) Probe sets increased in response to treatment. (B) Probe sets decreased in response to treatment. There were 275 genes increased in spleen and blood by CLP compared with the Sham control. The intersection of the genes increased by CLP and LPS in blood and spleen defines a nine-gene common inflammatory response cluster (protein-protein interactions mapped in Fig. 7).

gene expression across the seven experimental groups. Interestingly, the only samples that were cluster outliers were in the CLP plus antibiotics group, consistent with an effect of antibiotics to change the septic phenotype toward the control phenotypes. We conclude that circulating blood gene expression profiles can be used to predict CLP and non-CLP phenotypes in prospective cohorts, in particular, distinguishing controls from lethal endotoxemia (LPS) from lethal infection (CLP). It is important to note that at this 24-hour time point, there were no substantial differences between LPS or any of the CLP groups in clinical presentation or complete blood counts. Microarray analysis could make the diagnosis of sepsis (distinguishing between sterile and infectious sources of systemic inflammation) when clinical criteria and white blood cell counts could not, a frequent occurrence in ICUs.³³ In addition, the differences between groups as measured by absolute fold-changes in individual gene expression were small (eg, in the final model for blood, 86.4% of genes were altered less than twofold), yet the changes in the patterns of expression across hundreds of genes were robust predictors of phenotype.

Biology of inflammation and sepsis

To discover changes in expression generic to the inflammatory and septic responses, we used the intersection of gene lists identified by the pair-wise group comparisons (Fig. 5). Nine genes were commonly increased, validated across two

tissue types (spleen and blood) and two insults (CLP and LPS). In contrast, there were no genes that were commonly decreased. Given that this list of nine genes was based on changes in relative RNA abundance across a number of cell types, the network analysis performed served as an exploratory tool, validating *in silico* the role of six of nine genes in canonical pathways for inflammation, apoptosis, and signal transduction: inhibitor of DNA binding 2, calgranulin A and B, interferon regulatory factor 7, lipocalin 2, and formyl peptide receptor-like 1 (Table 4). Several characteristics of these nine common inflammatory response genes are notable. Inhibitor of DNA binding 2 is required for normal mouse immune development, especially of lymph nodes and Peyer's patches.³⁴ Calgranulin A and B, which belong to a recently described group of proinflammatory molecules, form extracellular complexes that bind to and activate endothelial cells, promoting chemotaxis and phagocytic adhesion in a positive feedback manner.^{35,36} Interferon regulatory factor 7 is a key regulatory of monocyte development, essential to differentiation of monocytes to macrophages.³⁷ Lipocalin 2 is a secreted protein that undergoes transcriptional induction after cytokine withdrawal and induces leukocyte-specific apoptosis.³⁸ Lipocalin 2 transcription, translation, and secretion are induced by ligation of Toll-like receptors on leukocytes, with secreted lipocalin 2 acting to sequester siderophores, thereby limiting bacterial growth.³⁹ Formyl peptide receptor-like 1 is a member of the chemoattractant subfamily of G protein-

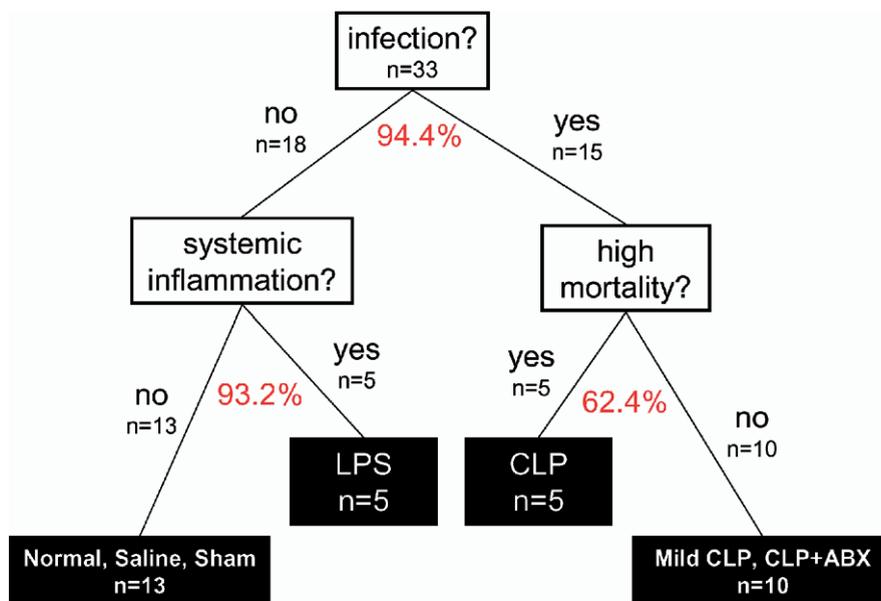


Figure 6. Class prediction in mouse blood. Using microarray gene expression profiles, classification exhibited high accuracy (94.4%) between samples from animals with infection (cecal ligation and puncture [CLP], Mild CLP, and CLP with antibiotics). Among animals with infection (any-CLP), accurate distinction could not be achieved between high and low mortality rates (62.4%). Among noninfected animals, high prediction accuracy (93.2%) was achieved between sterile systemic inflammation (lipopolysaccharide [LPS]) and controls. ABX, antibiotics.

coupled receptors that are involved in controlling leukocyte migration.⁴⁰ The other two annotated genes not listed in the network, neutrophilic granule protein and serum amyloid A3, have also been associated with inflammation and cellular defense, although less is known about their functions and protein interactions. Neutrophilic granule protein is a cysteine protease inhibitor that has been associated with myeloid differentiation.⁴¹ Serum amyloid A3 is a high-density apolipoprotein, the only amyloid made by both hepatocytes and peripheral monocytes and macrophages.⁴² It is believed to function by retargeting transported lipids, including cholesterol, in the disposal of toxins.⁴² The function of the ninth gene, a retroviral species that is increased in a number of different models of infection and inflammation, is not known.

We compared this common inflammatory response cluster with the list of proteins recently reported to diagnose intra-amniotic infection in patients,¹⁸ as another means of validating the importance of these nine genes. Of the 11 proteins and polypeptides detected in that study, 3 were also identified in our study, specifically calgranulin A and B and lipocalin 2 (neutrophil gelatinase-associated lipocalin). Calgranulin A (S100A8) was also the most differentially expressed gene in blood in a small microarray study comparing eight septic patients with four surgical controls with-

out systemic inflammation.¹⁷ Serum amyloid A protein was identified as a plasma proteome biomarker in patients with coronavirus (severe ARDS).⁴³ Together, these studies support our hypothesis that there are a group of common inflammatory response genes that can be used as novel biomarkers to diagnose inflammation across species, tissue, and different types of infecting organisms, at either the RNA or protein level.

Limitations

Because of the large degree of variation (noise) in human spleen gene expression, no individual genes surveyed passed the FDR filter. Nevertheless, the data from these patients have proved invaluable for the study of immune dysfunction in human sepsis² and provided proof-of-principle here that molecular profiles of human lymphoid tissues could be used to distinguish between septic and nonseptic phenotypes. Because cellular populations of mammalian tissues are heterogeneous, use of microarray profiles to study the cellular response to a given stimulus must be understood in the context of changes in cell populations. This substantially limits conclusions about whole spleen data, given our reports and those of others that sepsis accelerates splenocyte apoptosis.^{2,19,44} In contrast, changes in blood cellular heterogeneity are measured

Table 4. Mouse Common Inflammatory Response Cluster of Nine Genes

Gene	Name	GO and functional annotations	Mouse cell type*	Observed mean fold increase
Saa3	Serum amyloid A 3	Acute phase response activity, lipid transporter activity	Macrophage	7.1 in CLP blood 5.1 in LPS blood 7.3 in CLP spleen
NGP	Neutrophilic granule protein	Antimicrobial peptide activity, enzyme inhibitor activity	Neutrophil	4.1 in CLP blood 3.8 in LPS blood 1.7 in CLP spleen
S100A8	S100 Calcium-binding protein A8 (calgranulin A)	Metal ion binding	Neutrophils, B cells, dendritic cells	3.7 in CLP blood 4.8 in LPS blood 3.5 in CLP spleen
S100A9	S100 Calcium-binding protein A9 (calgranulin B)	Metal ion binding, signal transducer activity	Neutrophils, B cells, dendritic cells, monocytes	4.4 in CLP blood 5.2 in LPS blood 3.0 in CLP spleen
IRF7	Interferon regulatory factor 7	RNA polymerase transcription factor activity	T cells	1.8 in CLP blood 1.9 in LPS blood 1.3 in CLP spleen
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Nucleus, development; required for NK cell commitment; negatively regulates B-cell differentiation	B cells, NK cells	2.5 in CLP blood 2.8 in LPS blood 2.1 in CLP spleen
LCN2	Lipocalin 2 (oncogene 24p3)	Binding, transporter activity	Neutrophils	7.2 in CLP blood 8.5 in LPS blood 7.7 in CLP spleen
FPRL1	Formyl peptide receptor-like 1	Receptor activity	Neutrophils, splenocytes	5.7 in CLP blood 5.7 in LPS blood 1.3 CLP spleen
EST	Unknown	Unknown	Unknown	3.0 in CLP blood 2.3 in LPS blood 1.8 in CLP spleen

*Ingenuity Pathways Knowledge Base.

CLP, cecal ligation and puncture; LPS, lipopolysaccharide; NK, natural killer.

routinely. Although CLP and LPS stimuli changed both absolute WBCs and cell differentials compared with the controls, among the four groups of “sick” animals cell counts were indistinguishable (Fig. 2). This mirrors the clinical situation where differentiating between sterile (LPS) and infectious (any-CLP) sources of systemic inflammation is not possible based on clinical grounds or cell counts. Microarray profiles, as we have discussed, were very successful at making this distinction.

Many questions remain unanswered. What are the optimal computational methods to identify robust predictors from microarray or proteomic data? Can gene or protein expression profiles be used to diagnose sepsis in other animal models? If so, are there predictive gene sets that are common to different types of infection (eg, gram-positive versus gram-negative infections)? Once the diagnosis has been made, are there markers that indicate response to therapy or prognosis? There are sufficient preclinical and preliminary patient data to justify testing these hypotheses. Because of the heterogeneity of expression profiles, large-scale collaborative studies will

be required to enroll sufficient patients to identify robust sepsis markers, and in the process, untangle the biology of infection from inflammation.¹³

Back to the bedside—the promise of molecular profiling for sepsis diagnosis

In conclusion, our *in vivo* data corroborate *in vitro* findings indicating that microarray analysis holds promise as a means of identifying distinct expression profiles (“molecular fingerprints”) that can diagnose the septic phenotype. Our human spleen data join recent blood data from septic patients¹⁷ and serum and amniotic fluid data from patients with intra-amniotic fluid infection,¹⁸ indicating that transcriptome and proteome studies will deliver on the promise of novel inflammation diagnostics.^{15,16} A single inflammation gene, calgranulin A (S100A8), was detected in all three studies at either the RNA or protein level. Our data are unique in that they show that transcriptome molecular profiles can distinguish between sterile and infectious causes of systemic inflammation and can make a diagnosis of sepsis in pro-

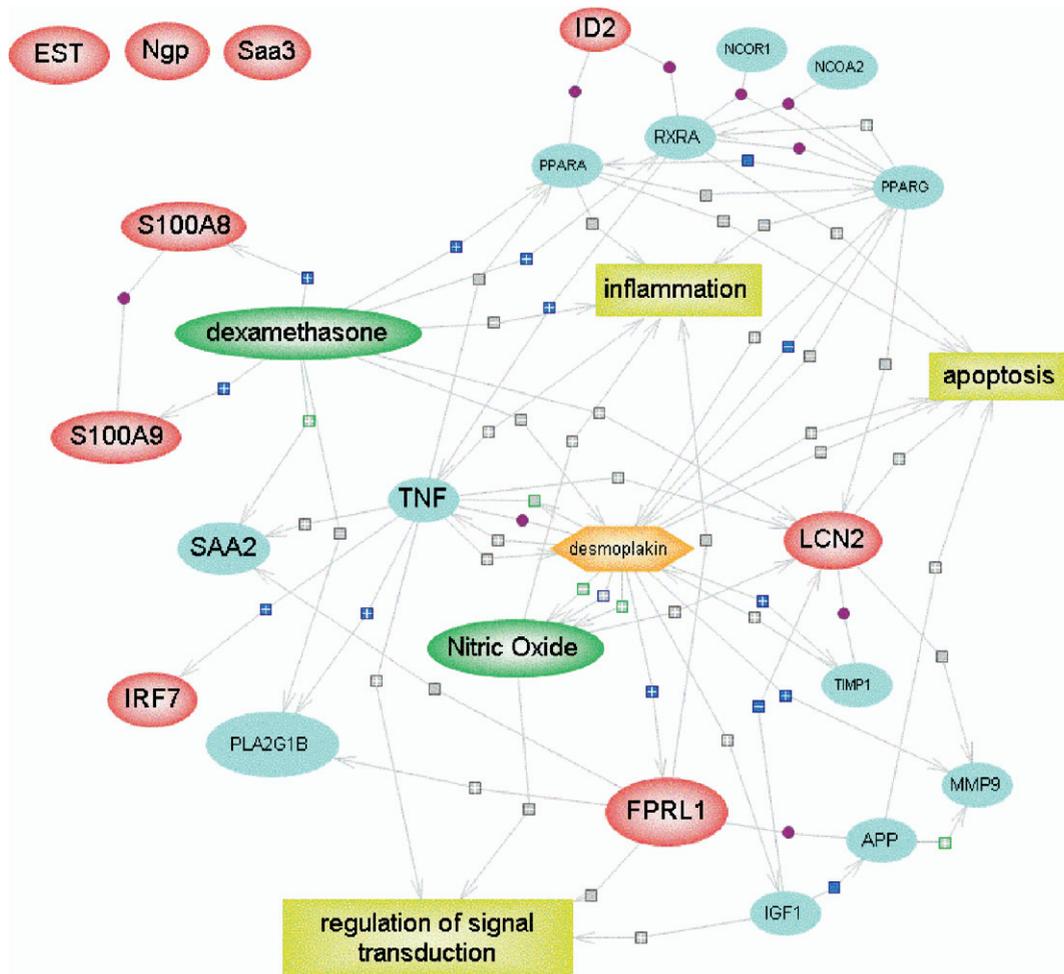


Figure 7. Mouse common inflammatory response cluster. Nine probe sets (red genes) were commonly altered regardless of tissue or insult. A contemporary pathway analysis tool was used to automatically create a network of interactions among these nine genes, based on previously reported interactions in the literature. Eight of the nine probe sets are known inflammation genes; the ninth probe set is an expressed sequence tag. This network validates *in silico* that six of these genes are involved in canonical pathways of inflammation, apoptosis, and regulation of signal transduction.

spective cohorts. Importantly, we observed that the magnitude of change in gene expression that was needed to predict the septic phenotype was very small. It was the pattern of these small changes in expression that were predictive, not the magnitude of any single change. We and others reported recently validated clinical protocols for blood gene expression profiling used to characterize the human systemic inflammatory response.^{13,14} The data presented here suggest that these protocols should be extended to clinical trials, testing the efficacy of microarray gene expression profiling to diagnose human sepsis. We expect that these studies will provide new insight into how specific pathogens uniquely perturb the physiology of cir-

culating leukocytes and how the host successfully mounts pathogen-specific defenses.

Author Contributions

Study conception and design: Chung, Laramie, Meyer, Downey, Buchman, Karl, Stormo, Hotchkiss, Cobb
 Acquisition of data: Chung, Laramie, Buchman, Stormo, Hotchkiss, Cobb
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 Drafting of manuscript: Chung, Downey, Buchman, Stormo, Hotchkiss, Cobb

Critical revision: Chung, Downey, Buchman, Stormo, Hotchkiss, Cobb

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SUPPLEMENTARY INFORMATION

Tests for differential expression, class prediction, and pathway analysis: For the mouse blood study, a mixed-model ANOVA was used to detect differential expression between treatment groups, with a linear contrast between the any-CLP and non-CLP groups. The ANOVA model was chosen to partition treatment group and technical batch variability from variability due to biological and experimental noise. The following linear mixed model was used to detect differential expression on a gene-by-gene basis in the mouse blood data:

$$y_{gij} = \mu_g + T_i + B_j + \varepsilon_{gij}$$

where y_{gij} is the expression of the g th gene for i th treatment and j th batch. The mean expression for the g th gene is given by μ_g . The symbols T and B represent effects due to treatment and batch respectively. The error for the g th gene for sample ij is designated as ε_{gij} . For the mixed-model analysis of variance, treatment is a fixed effect and batch is a random effect. A batch constitutes 7 samples (one from each treatment group) which were processed and hybridized at the same time. In the case of the last batch (batch 5), the GeneChips were scanned on a different scanner. For the mouse and human spleen studies samples were processed in a single batch, so a simple one-way analysis of variance with a contrast between any-CLP and non-CLP was used to identify differentially expressed genes. The linear contrast between any-CLP and non-CLP is given by:

$$\frac{1}{3}(\mu_{CLP} + \mu_{CLP+ABX} + \mu_{MildCLP}) - \frac{1}{4}(\mu_{LPS} + \mu_{Saline} + \mu_{Sham} + \mu_{Normal})$$

where μ_{CLP} is the mean of the CLP group, $\mu_{CLP+ABX}$ is the mean of the CLP+ABX group, *etc.*

Where possible, the following competing classifiers were considered for all tasks, and the optimal classifier was selected: number of genes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 5000, and 10000), prior probabilities for nearest centroid (equal and proportional), functions for discriminant analysis (linear and quadratic), prior probabilities for discriminant analysis (equal and proportional), number of neighbors (k) for k -NN (1, 3, and 5), and distance functions for k -NN (Euclidean distance, Pearson's linear correlation, and absolute value distance). Thus as many as 426 classification models were considered for each classification task.

For the mouse blood data, we used a leave-one-batch-out (5-fold, one for each of the 5 batches) outer cross-validation, while the inner cross-validation is leave-one-sample-out. We refer to this as nested cross-validation with an outer "leave-one-batch-out" layer and an inner "leave-one-sample-out" layer.(27) Using this method, the determination of how many and which genes to use for classification were determined using only the training samples. In addition, all additional classifier parameters (*e.g.*, number of neighbors and distance measure) were determined using only the training samples. For each held-out batch in the outer 5-fold cross-validation, the classifier and genes that performed best on inner cross-validation were selected and applied to the 6 or 7 held-out test samples (two batches only had 6 samples due to removal of an outlier sample). For the mouse and human spleen data where all samples were processed in a single batch, both the outer and inner cross-validation used full leave-one-sample-out.