Late Multiple Organ Surge in Interferon-Regulated Target Genes Characterizes Staphylococcal Enterotoxin B Lethality

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Abstract

Background: Bacterial superantigens are virulence factors that cause toxic shock syndrome. Here, the genome-wide, temporal response of mice to lethal intranasal staphylococcal enterotoxin B (SEB) challenge was investigated in six tissues.

Results: The earliest responses and largest number of affected genes occurred in peripheral blood mononuclear cells (PBMC), spleen, and lung tissues with the highest content of both T-cells and monocyte/macrophages, the direct cellular targets of SEB. In contrast, the response of liver, kidney, and heart was delayed and involved fewer genes, but revealed a dominant genetic program that was seen in all 6 tissues. Many of the 85 uniquely annotated transcripts participating in this shared genomic response have not been previously linked to SEB. Nine of the 85 genes were subsequently confirmed by RT-PCR in every tissue/organ at 24 h. These 85 transcripts, up-regulated in all tissues, annotated to the interferon (IFN)/antiviral-response and included genes belonging to the DNA/RNA sensing system, DNA damage repair, the immunoproteasome, and the ER/metabolic stress-response and apoptosis pathways. Overall, this shared program was identified as a type I and II interferon (IFN)-response and the promoters of these genes were highly enriched for IFN regulatory matrices. Several genes whose secreted products induce the IFN pathway were up-regulated at early time points in PBMCs, spleen, and/or lung. Furthermore, IFN regulatory factors including Irf1, Irf7 and Irf8, and Zbp1, a DNA sensor/transcription factor that can directly elicit an IFN innate immune response, participated in this host-wide SEB signature.

Conclusion: Global gene-expression changes across multiple organs implicated a host-wide IFN-response in SEB-induced death. Therapies aimed at IFN-associated innate immunity may improve outcome in toxic shock syndromes.
all potential agents and are likely immunogenic. Furthermore, strategies that seek to prevent the first step in T-cell and APC co-activation may be ineffective in post-exposure treatment [20]. Patients with de novo toxic shock are generally diagnosed after the onset of cytokine storm, as would be the case for casualties from an act of bioterrorism. Targeting host-responses downstream from superantigen exposure is therefore appealing clinically. However, some cytokines linked to toxic shock pathogenesis, such as TNFα and IL-1β, are released hyper-actively [6,21,22]. By analogy, targeting these and similar mediators in septic shock syndrome failed to improve survival [23]. Candidate host responses that are both slow to develop and central to outcome have been elusive.

An obvious step in developing new therapeutic approaches for SEB-induced toxic shock is finding relevant models that mimic important aspects of human disease. Compared to humans, mice are much less susceptible to SEB due to its decreased affinity for mouse MHC class II molecules [24,25,26]. Therefore, mouse models of SEB-induced shock have used potentiating agents such as lipopolysaccharide [27], viruses [28], D-galactosamine [29], or actinomycin D [30] to amplify the toxic effects of SEB. However, the sensitizing agents themselves often activate similar cell populations in vivo through different signaling pathways, and therefore, potentially interact with the effects of SEB in unpredictable ways [31,32]. To overcome these limitations, a “double-hit” low dose SEB model was developed in C3H/HeJ mice, a lipopolysaccharide (LPS) resistant mouse strain with defective Toll-like receptor 4 (TLR-4) signaling, to investigate the pathological consequences of SEB-induced shock without the use of synergistic agents [31]. This “SEB-only” toxic shock model relies on the intranasal (i.n.) administration of SEB followed by a second intraperitoneal (i.p.) dose 2 hours later. These two relatively small SEB challenges trigger intense inflammation in the lung and systemic cytokine release that culminate in death more than 3 days after the initial exposure. Importantly, cytokine release, pathological lesions, and time to lethality in C3H/HeJ mice resemble findings in primate studies [30,33,34,35] and clinical staphylococcal toxic shock syndrome in patients [1]. Furthermore, respiratory system challenge with the induction of a pulmonary inflammatory response simulates the manner in which SEB would be used as a bioweapon [15].

In this study, oligonucleotide microarrays were used to examine the temporal pattern of SEB-induced gene expression in peripheral blood mononuclear cells (PBMC), spleen, lung, liver, kidney, and heart to identify common molecules and pathways that might serve as post-exposure therapeutic targets. The extent to which SEB responses, independent of synergistic agents, are distinct or similar across different organs at the transcriptional level has not been previously investigated. The murine toxic shock model employed here does not require high doses of SEB or second agents to produce lethality [31]. Furthermore, mice become moribund relatively late, better reflecting the time course of human toxic shock syndromes.

**Materials and Methods**

**Mouse model of SEB-mediated shock**

The Institutional Animal Care and Use Committee (IACUC) of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) approved the protocols, AP06-063 and AP10-002, under which this study was conducted. All reported research was performed in compliance with the Animal Welfare Act and other federal statutes and regulations relating to experiments involving animals and adhered to the principles in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. This research was conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Male C3H/HeJ mice (National Cancer Institute, Frederick, MD), weighing ~20 g each (7–10 weeks old), were housed in conventional microisolator cages. Food and water were freely available at all times. Purified SEB was procured from Toxin Technology (Sarasota, FL) and diluted in sterile, endotoxin-free phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO). Frozen (~70°C) aliquots of toxin were used for all subsequent studies. SEB was administered i.n. (5 μg/dose/mouse in 50 μl) with a micropipet and i.p. (2 μg/dose/mouse in 200 μl) with a tuberculin syringe (26G–3/8 inch needle), given 2 h apart. This was the optimal timing and dose previously determined to cause shock and death without the use of synergistic agents [31]. Control C3H/HeJ mice were given two doses of saline (i.n. and i.p.) 2 h apart similar to the SEB-exposed mice. An intramuscular-injected mixture of ketamine (2.4 mg/kg), acepromazine (0.024 mg/kg), and xylazine (0.27 mg/kg) was used to anesthetize mice prior to all i.n. challenges. Mice exposed to both doses of SEB succumbed to death between 72 and 120 h.

Blood was collected into EDTA-treated tubes from anesthetized and subsequently euthanized mice by cardiac puncture at 2.75 h, 5 h, and 24 h after i.n. SEB. Blood was then immediately diluted with an equal volume of PBS and layered over Nycoprep 1.077 (Sigma-Aldrich). PBMCs were collected at the interface after centrifugation at 700 g for 30 min. Organs were excised from matched, euthanized animals at the same times post-challenge as blood samples. Tissue samples for microarray were thinly sliced and preserved in RNA later Stabilization Reagent (Qiagen, Valencia, CA) and stored at −80°C.

**Total RNA isolation and microarrays**

PBMCs were lysed with RLT buffer (Qiagen) and homogenized (Qiashredder column). Total RNA was extracted using RNeasy mini kits (Qiagen), following the manufacturer's instructions. Organ samples were disrupted utilizing a TissueLyser (Qiagen), and total RNA was isolated using RNeasy mini kits (Qiagen). The quality of total RNA was evaluated using RNA 6000 Nano LabChips (Agilent 2100 Bioanalyzer, Santa Clara, CA). All samples had intact 18S and 28S ribosomal RNA bands with RNA integrity numbers (RIN) between 7.1 to 9.4, and RNA A260/ A280 ratios between 1.9 and 2.0.

Double-stranded cDNA was synthesized from total RNA (2 μg) using GeneChip Expression 3’-Amplification Reagents One-Cycle cDNA Synthesis kits (Affymetrix, Santa Clara, CA, USA). Purified cDNA was then used for in vitro synthesis of biotin labeled cRNA (IVT GeneChip Expression 3’-amplification kits; Affymetrix). Labeled cRNA was purified and fragmented (20 μg) for 35 min at 95°C in fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate). Fragmented cRNA was evaluated using Flash Gel System (Lonza, Walkersville, MD) after which fragmented cRNA (15 μg) was hybridized to Mouse Genome 430 2.0 arrays (Affymetrix) for 16 h at 45°C. Arrays were washed and stained using GeneChip Hybridization, Wash, and Stain kit (Affymetrix) on the Affymetrix Fluidics Station 400. Microarrays were then scanned in an Affymetrix 7G scanner.

**Microarray analysis**

After scanning, CEL files were transferred to the NIH AGCC database for archival storage and later analyzed using Affymetrix Expression Console and the MAS5 algorithm for the Mouse 430_2 microarray. Resulting Signal Intensity values and Present-Absent
calls for each of 45,101 probesets were processed in JMP statistical package (SAS, Cary, NC) using the Mathematical and Statistical Computing Laboratory (MSCl) Analyst’s Toolbox [36], written by and freely available (http://abs.cit.nih.gov/MSCLtoolbox/) from one of the authors (PJM). The quantile-normalizing, variance-stabilizing “S10” transform was applied to the data, separately for each tissue (PBMC, spleen, lung, liver, kidney, and heart). Separate normalization by tissue was performed because gene expression was expected to be widely divergent across different tissues. Data for each tissue was subjected to principal components analysis (PCA). Possible outlier microarrays were first identified by PCA plot inspection; quality control parameters of potential outliers (cRNA yield, percent present, raw Q [noise], scaling factor actin 3’/5’ ratio) were examined to further adjudicate possible censure. The microarray data used in our analysis including CEL files has been deposited in the Gene Expression Omnibus Database (GEO) (http://www.ncbi.nlm.nih.gov/gds) and is available under accession number GSE52474.

After removal of outliers, 154 of 168 microarrays were analyzed using a one-way, seven level ANOVA, with levels C0, C2, C5, C24, S2, S5 and S24, corresponding to baseline animals (C0), and controls at 2.75, 5 and 24 h, and SEB challenged animals at 2.75, 5 and 24 h. Each level had at least 3 biological replicates for each tissue and time point. Selection of probesets as differentially expressed in any individual tissue required a false discovery rate (FDR) of less than or equal to 5%, fold-change ≥1.5 between SEB-challenged and control samples at one or more time points, and a present call in ≥50% of samples at any level. Requiring that these criteria were met across all six tissues retained 103 probesets that annotated to 85 unique gene identifications. Because these transcripts were subsequently recognized as interferon (IFN) response genes (see below), a list of 26 secreted IFN pathway initiators was constructed from a search of PubMed and other online sources. Of these, 19 mapped to probesets on our mouse microarrays and 8 were differentially regulated (FDR ≤5%, fold-change ≥1.5 and present call ≥50% at any level) in at least one tissue and time point.

In a separate analysis to test for the effects of time, stress and anesthetic agent use in the absence of SEB challenge, baseline animals at time 0 were compared to controls at 2.75, 5 and 24 h, applying the same selection criteria used in our primary analysis. Within the controls, no differentially expressed genes were found for spleen, liver, kidney and heart, while PBMC and lung returned only 3 transcripts each. Furthermore, the 6 transcripts that changed significantly over time under control conditions in PBMC and lung showed no overlap with the SEB-induced gene signature reported here.

Quantitative real-time PCR (qRT-PCR)

High Capacity cDNA Reverse Transcription kits and gene specific primers and probes for Cxcl11 (cat # mm00444662_m1), Herc6 (cat # mm0134963_m1), Irf1 (cat # mm01288350_m1), Irf3 (cat # mm00492567_m1), Irgm1 (cat # mm00492596_m1), Parp12 (cat # mm00556509_m1), Stat1 (cat # mm00439518_m1), Xaf1 (cat # mm00776505_m1), and Zbp1 (cat # mm00457979_m1) were purchased from Applied Biosystems (Foster City, CA). Transcript levels were then measured using TaqManH Universal PCR master mix, and the ABI Prism 7900 sequence detection system (Applied Biosystems). GAPDH was significantly affected by SEB challenge, precluding its use as a housekeeping gene to normalize expression [37]. Fold-change from control measured by qRT-PCR was compared directly with microarray results for the same target gene. Because microarrays tend to underestimate the magnitude of differential expression, only the probeset with the largest fold-change is shown for target genes with multiple retained probesets. Thematic analysis.

Probesets differentially expressed across all tissues were uploaded into Ingenuity Pathway Analysis (IPA®) and examined using the Bio Function, Canonical Pathway and Upstream Regulator applications. Significant upstream regulators were chosen to construct a network of gene interactions based on the known molecular mechanism of cellular activation by SEB and the predominant signature for IFN-regulated genes. These included the T-cell receptor (TCR), IFN pathway initiators expressed early in PBMCs, spleen and lung (TNF, IL-1β, IL-2, IFNγ and IL-12B), and any upstream regulator differentially expressed across all tissues. The resulting network connected 70 of 79 genes recognized by IPA®. The remaining 9 genes and their connections were manually curated using published articles [38,39,40] from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and STRING version 9.05 (http://string-db.org/newstring.cgi?show_input_page=p?UserId=xYMVCkf5LzA&SessionId=1, a database of known and predicted protein-protein interactions [41].

The apparent enrichment of IFN-regulated genes among our transcripts differentially regulated across all tissues was further explored using Interferome v2.01 (http://interferome.its.monash.edu.au/interferome/). This database contains a comprehensive list of IFN-regulated genes manually curated from publicly available microarray datasets, as well as tools for analyzing IFN-regulated gene signatures in experimental results [42]. Genes regulated across all tissues were examined for evidence of an IFN-regulated gene signature. IFN subtype, concentration and timing were unrestricted (default settings), as were biological system and cell-type. Both in vivo and in vitro experimental results were permitted, but the analysis was restricted to mouse data, corresponding to our animal model. The database recognized 85 uniquely annotated genes from the all-tissue list as IFN-regulated genes, which were then classified by IFN subtype.

Promoter analysis

The promoters of genes regulated across all tissues were initially analyzed within Interferome v2.01 for IFN pathway-driven regulatory binding sites [42]. This tool relies on TRANSFAC® Professional (2012) matrices and the MATCH™ algorithm with settings to minimize false positives. Next, ExPlain 3.1 (BIOBASE Knowledge Library: http://www.biobase-international.com/; Beverly, MA) was used to determine if IFN-driven transcription factor binding sites were truly over-represented relative to other matrices. In the F-Match module, 492 mouse housekeeping gene promoters were used as the “No-set”. The “vertebrate_all” minimize false positives profile of position weight matrices was chosen to identify the most promising potential binding sites. Using only the best-supported promoters (n = 84) from our all-tissue list of differentially expressed genes, the maximum promoter window was set at -500 to +100 bp, with cut-off and window optimization, and a p-value threshold of 0.01.

Histopathology and immunohistochemistry

Organs were excised from euthanized control and SEB-exposed C3H/Hij mice at the indicated times and emersion-fixed in 10% neutral-buffered formalin. Formalin fixed specimens were embedded in paraﬃn before sectioning at 5 micron and mounting on glass slides. Slides were deparafﬁnized with xylene, hydrated through graded alcohols to water and stained with hematoxylin and cosin. Stained samples were dehydrated through graded alcohols to xylene and sealed using Permount™.
Only lung tissue showed characteristic SEB-associated histopathological changes at 24 h post-SEB exposure, the last time point corresponding to our microarray analysis and was subjected to further analysis. For TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay, sections were deparaffinized with xylene, hydrated and pretreated with protease K followed by EDTA and BSA blocking. Samples were then stained with anti-DIG 1:1000 (Roche Applied Science, USA; cat # 1093274) and fuchsin and counterstained with hematoxylin followed by dehydration through graded alcohols to xylene prior to sealing with Permount™. For immunohistochemistry, sections were deparaffinized and heated in a steamer at 90°C for 20 min to retrieve antigen. Sections were blocked with hydrogen peroxide and BSA, and then washed with Tris-Buffered Saline and Tween 20 (TBST). This was followed by overnight incubation at room temperature with primary antibody, either rabbit polyclonal anti-nitrotyrosine (Abcam, Cambridge, MA; cat # ab42789) or anti-polyADP-ribose (BD Bioscience, San Jose, CA; cat # 551813), each diluted 1:200. Slides were subsequently washed with TBST and incubated with secondary goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA; cat # BA-1000) diluted 1:500 for 30 min followed by streptavidin-HRP 1:400 for 30 min, both at room temperature. Finally, slides were developed with DAB (diaminobenzidine tetrahydrochloride) and counterstained with Carazzi’s hematoxylin.

Histopathology and TUNEL assays were initially read by one author (MFS) blinded to challenge and time point. Lung tissue was available from three control and three SEB-challenged animals at 24 h, and one control and two SEB-challenged animals at 48 h. For TUNEL analysis, lung sections were macroscopically divided into five regions within which five high-powered fields (HPFs) were examined in each for a total of 25 HPFs per slide. The number of apoptotic cells in each HPF was quantitated and overall results were subjected to a one-way analysis of variance (ANOVA) combining the four controls, followed by post-hoc contrasts using unpaired t-tests and Bonferroni corrections.

**Results**

SEB-induced a shared genomic response in PBMCs and all organs

Over time in control animals, no transcripts significantly changed in spleen, liver, kidney and heart, and only three each were altered in PBMC and lung (data not shown). Importantly, none of these time-affected transcripts in controls overlapped with the SEB gene signature described below.

SEB challenge compared to control had its earliest and strongest effects in PBMCs (Tables 1 and 2). Responses in spleen and lung, and finally liver, kidney, and heart were progressively delayed (Table 1), involved fewer transcripts, and became increasingly biased toward gene induction compared to suppression (Table 2). However, the number of affected transcripts in each tissue continued to increase with time and some met criteria for differential regulation (≥5% FDR; ≥1.5-fold-change compared to control; and ≥50% present call) in all tissues. These latter transcripts were uniformly induced and accounted for a substantial proportion of all differentially regulated probesets (>50%) in liver, kidney and heart (Table 2). See Table S1 for a complete organ-by-organ list of differentially expressed transcripts.

Focusing on the shared SEB-response from Tables 1 and 2, 103 probesets representing 85 uniquely annotated transcripts (Table 3) were identified as differentially expressed across all six tissues. Notably, six transcripts induced in all tissues had maximal changes in excess of 100-fold and somewhat unexpectedly, these large effects were seen in heart (Igtp), kidney (Igpl, Gbp6, and Gbp6/10), liver (Igpl/2), and lung (Cxc9) rather than tissues primarily composed of lymphocytes (PBMCs and spleen). Many of the genes significantly induced in all tissues have not been previously associated with SEB responses. SEB challenge affected DNA/RNA sensors such as Ifih1 (Mda5) and Zbp1; IFN-induced genes (Ifi1, Ifii2, Ifi3, Ifi47, Ifi202b, Igtp, Igp1 Igp2, Stat1, Ifi17, Ifi17, and Ifi18) including those with direct antiviral activity (Dlx59, Herc6, Ig, Oasl2, Oasl3, Samh1); apoptosis/DNA damage-related molecules (Ddx31, Parp9, Parp12, Parp14, Tnfsf10, and Xa11); signal transduction effectors (Gbp1, Gbp2, Tgtp, and Igrn1); innate inflammatory response mediators and regulators (Cxc9, Cxc10, Cxc11, I15b, and Traf6); cell receptors (Cd274, H2T10, Fcgr4, Ly6a, Ly6c, Ly6e); immunoproteasome components (Psme2, Psmb8, Psmb9, and Psmb10); and ER/ metabolic stress pathway genes (Eif2ak2, Erp1, and Ubd). Note these functional classifications are offered as examples and are neither complete nor mutually exclusive. Overall, inspection of the 85 transcripts that were uniformly up-regulated across all tissues (Table 3), suggested that lethal SEB challenge in this model

### Table 1. Tissue and time specific counts of differentially expressed probesets shown at each time point.

| Tissue | Time = 2.75 | Time = 5 | Time = 24 |
|--------|------------|----------|-----------|
| PBMC   | Down       | Up       | Down & Up | Total |
| 193    | 119        | 1017     | 843       | 1843  | 1437  |
| Spleen |            |          |           |       |       |
| 3      | 36         | 10       | 204       | 378   | 460   |
| Lung   | 11         | 10       | 182       | 275   | 632   |
| Liver  | 3          | 4        | 27        | 23    | 229   |
| Kidney | 0          | 0        | 3         | 19    | 203   |
| Heart  | 3          | 0        | 4         | 11    | 12    |
| All*   | 0          | 0        | 0         | 0     | 101   |

* a Ifih1 (probeset ID 1416714_at) met selection criteria at 5h in PBMC and spleen, and at 24 h in lung, liver, heart, and kidney. Cxc9 (probeset ID 1456907_at) met selection criteria at 5 h in PBMC, spleen, lung, and kidney, and at 24 h in spleen, lung, liver, kidney, and heart. Therefore, these probesets are not counted in all-tissue totals at specific time points. b Tgtp1/2 (probeset ID 1449009_at) met all-tissue criteria at both 5 h and 24 h, and, therefore, is counted in this table at both time points.

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### Table 2. Tissue specific counts of differentially expressed probesets aggregated across time points.

| Tissue | Across All Times* |
|--------|-------------------|
| PBMC   | Down | Up | Down & Up | Total |
| 2219   | 1905 | 116| 4008 | 3% |
| Spleen | 384  | 574| 950  | 11% |
| Lung   | 290  | 669| 10   | 949 | 11% |
| Liver  | 32   | 243| 9    | 266 | 39% |
| Kidney | 23   | 315| 3    | 335 | 31% |
| Heart  | 18   | 267| 5    | 280 | 37% |
| All    | 103  | 103| 103  | 100%|

* a In each individual tissue, some probesets met selection criteria for down-regulation at one time point and up-regulation at another. Row totals count each probeset once using the following formula: (Down) + (Up) – (Down & Up).

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## Table 3. Eighty-five annotated genes were up-regulated in all six tissues.

| Gene Symbol | Gene Title | Entrez Gene ID | Tissue and Time (hours) of Maximum Fold Change | Maximum Fold Change |
|-------------|------------|----------------|-----------------------------------------------|---------------------|
| Igtp        | interferon gamma induced GTPase | 16145 | Heart 24 | 168.88 |
| Iigp1       | interferon inducible GTPase 1 | 60440 | Kidney 24 | 132.73 |
| Tgtp1; Tgtp2| T cell specific GTPase 1; T cell specific GTPase 2 | 21822; 100039796 | Liver 24 | 127.34 |
| Gbp6*       | guanylate binding protein 6 | 100702 | Kidney 24 | 118.55 |
| Gbp6; Gbp10*| guanylate binding protein 6; guanylate-binding protein 10 | 100702; 626578 | Kidney 24 | 114.21 |
| Cxcl9       | chemokine (C-X-C motif) ligand 9 | 17329 | Lung 24 | 103.17 |
| Irgm2       | immunity-related GTPase family M member 2 | 54396 | Heart 24 | 49.79 |
| Cxcl10      | chemokine (C-X-C motif) ligand 10 | 15945 | PBMC 5 | 49.25 |
| Gbp1; LOC100047734| guanylate binding protein 1 (interferon-induced) | 14468 | Kidney 24 | 45.03 |
| Gbp2        | guanylate binding protein 2 | 14469 | Kidney 24 | 39.18 |
| Gbp8        | guanylate-binding protein 8 | 76074 | PBMC 24 | 35.59 |
| Ly6a        | lymphocyte antigen 6 complex, locus A | 110454 | PBMC 24 | 34.74 |
| Psmb8       | proteasome subunit, beta type 8 (large multifunctional peptidase 7) | 16913 | Kidney 24 | 27.10 |
| Irgm1       | immunity-related GTPase family M member 1 | 15944 | Heart 24 | 27.05 |
| Cd274       | CD274 antigen | 60533 | Heart 24 | 25.27 |
| Stat1       | signal transducer and activator of transcription 1 | 20846 | Kidney 24 | 24.37 |
| Ubd         | ubiquitin D | 24108 | Kidney 24 | 20.07 |
| Serpina3g   | serine (or cysteine) peptidase inhibitor, clade A, member 3G | 20715 | Lung 24 | 18.66 |
| Psmb9       | proteasome subunit, beta type 9 (large multifunctional peptidase 2) | 16912 | Heart 24 | 17.99 |
| Fam26f      | family with sequence similarity 26, member F | 215900 | Lung 24 | 17.39 |
| Gbp3        | guanylate binding protein 3 | 55932 | Kidney 24 | 16.91 |
| Ifi47       | interferon gamma inducible protein 47 | 15953 | Kidney 24 | 16.01 |
| Psmb10      | proteasome (prosome, macropain) subunit, beta type 10 | 19171 | Kidney 24 | 15.49 |
| Gbp7        | guanylate binding protein 7 | 229900 | Heart 24 | 14.91 |
| Cxcl11      | chemokine (C-X-C motif) ligand 11 | 56066 | Lung 24 | 14.07 |
| Ifi1        | interferon-induced protein with tetratricopeptide repeats 1 | 15957 | PBMC 24 | 13.98 |
| Oas12       | 2'-5' oligoadenylate synthetase-like 2 | 23962 | Spleen 24 | 13.11 |
| H2-23; C920025E04Rik| histocompatibility 2, T region locus 23; RIKEN cDNA C920025E04 | 15040; 667803 | Heart 24 | 12.89 |
| Ifi18bp     | interleukin 18 binding protein | 16068 | PBMC 24 | 12.63 |
| Zbp1        | Z-DNA binding protein 1 | 58203 | Lung 24 | 12.00 |
| Isg15; Gm9706b| ISG15 ubiquitin-like modifier; predicted gene 9706 | 100038882; 677168 | PBMC 24 | 11.68 |
| Ifi202b; LOC100044068c| interferon activated gene 202B; interferon-activable protein 202-like | 26388; 100044068 | Lung 24 | 11.68 |
| Ifi3        | interferon-induced protein with tetratricopeptide repeats 3 | 15959 | Heart 24 | 11.02 |
| Ifi202b     | interferon activated gene 202B | 26388 | Lung 24 | 10.63 |
| Usp18       | ubiquitin specific peptidase 18 | 24110 | PBMC 24 | 10.34 |
| Ifi44       | interferon-induced protein 44 | 99899 | Lung 24 | 9.56 |
| Ifi2        | interferon-induced protein with tetratricopeptide repeats 2 | 15958 | Lung 24 | 9.50 |
| AW112010    | expressed sequence AW112010 | 107350 | Lung 24 | 9.11 |
| If7         | interferon regulatory factor 7 | 54123 | Heart 24 | 8.40 |
| Gvin1; Gm4070| GTase, very large interferon inducible 1; predicted gene 4070 | 74558; 100042856 | Kidney 24 | 8.27 |
| Fcgr4       | Fc receptor, IgG, low affinity IV | 246256 | PBMC 24 | 8.03 |
| Wars        | tryptophanyl-tRNA synthetase | 22375 | Heart 24 | 7.78 |
| Plac8       | placenta-specific B | 231507 | Liver 24 | 7.49 |
| Bst2        | bone marrow stromal cell antigen 2 | 69550 | Lung 24 | 7.19 |
| Herc6       | hect domain and RLD 6 | 67138 | PBMC 24 | 7.11 |
| Rtp4        | receptor transporter protein 4 | 67775 | Kidney 24 | 7.06 |
triggered a widespread genetic program characterized by IFN, stress and damage pathway responses.

Pattern and magnitude of the all-tissue shared response

A heatmap of the 103 probesets participating in this shared-response across tissues is shown in Figure 1. Gene symbols are shown when available; duplicate labels denote that more than one
probeset was annotated to the same transcript. As shown, all-tissue shared-response genes tended to be more highly expressed (red) in immune tissue (PBMCs and spleen) at baseline. Furthermore, responses as early as 5 h post-challenge are only apparent on this heat map in PBMCs and spleen, and involve relatively few leading-edge transcripts. Otherwise, the shared genomic response to SEB was highly consistent across all-tissues and most apparent at 24 h. Notably, these changes in gene expression precede the first deaths in this model by more than 48 h [31]; 24 h was chosen for study here because it is the latest known time point at which initiation of immune modulator therapy still affords protection [43].

To better illustrate the commonality of this shared-response in PBMCs and across multiple organs, parallel plots were generated within tissue, normalizing each of the 103 probesets to its own 0 h baseline expression level (Fig. 2). As seen, expression changes were generally maximal at 24 h in all tissues. However, 12 probesets (shown in red) representing 11 leading-edge transcripts (Cxcl9, Cxcl10, Cxcl11, Cd274, Fam26f, Irf1, Irf8, Irgm2, Parp14, Srpma3g, Stat1) reached their peak response in PBMCs and/or spleen at 5 h post-SEB challenge. Notably, 3 of these 11 genes are transcription factors (Irf1, Irf8, and Stat1) downstream from traditional IFN signaling, and 3 are recognized as early/ immediate IFN-induced chemokines (Cxcl9, Cxcl10, and Cxcl11). These results suggest that an IFN-type response might have been initiated in PBMCs and spleen with later generalization to other tissues.

Finally, this coordinated genomic response was examined in a correlation matrix to determine which tissues displayed the most similarity in expression patterns at 24 h (Fig. 3). Comparisons with the highest correlation coefficients are shown in red and the lowest are in blue. Not unexpectedly, the two tissues comprised largely of lymphocytes, PBMCs and spleen, correlated closely with each other (r = 0.74). Interestingly, lung displayed a response most similar to liver (r = 0.84), possibly reflecting the large number of tissue macrophages in both organs or that SEB challenges were delivered into the bronchial tree and peritoneal cavity, respectively. Perhaps least anticipated were the shared-responses at 24 h in kidney and heart (r = 0.82; Fig. 3). These anatomically, histologically, and functionally dissimilar organs were both relatively distant from the sites of SEB challenge. Nonetheless, maximum fold-changes for a substantial number of transcripts occurred in these two organs (Fig 3 and Table 3).

Quantitative real-time PCR (qRT-PCR) confirmation of microarray results

Nine genes differentially regulated across all tissues (Cxcl11, Herc6, Irf1, Irf8, Irgm1, Parp12, Stat1, Xaf1, Zbp1) were selected for validation by qRT-PCR. Each gene was tested in 4 separate samples from each of 6 tissues obtained at 24 h after SEB challenge. Microarray tended to underestimate fold-change from control compared to qRT-PCR results, as shown by the systematic deviation from the line of identity in Fig. 4A. Overall, qRT-PCR confirmed that every differentially regulated gene was induced >1.5 fold compared to control in all six tissues (Fig 4B to 4J), with the exception of Irf8 (Fig. 4E). Irf8 had met inclusion on the all-tissue list of differentially regulated transcripts. However, 8/19 genes were found to be significantly up-regulated in PBMCs, spleen and/or lung prior to 24 h (Table 5), suggesting that the products of these genes might be contributing to the global IFN-type response seen here.

Finally, the Upstream Regulators application in IPA® was used to construct a network of gene interactions that encompassed activation of TCRs by SEB and the IFN pathway activating molecules identified above (Fig. 6). Notably, 79 genes recognized by the database, 70 were found to be significantly up-regulated in PBMCs, spleen and/or lung prior to 24 h (Table 5), suggesting that the products of these genes might be contributing to the global IFN-type response seen here.

Pathology and immunohistochemistry

At 24 h comparing SEB-challenged animals to controls, inflammatory cellular infiltrates were only seen in lung tissue. Therefore, gene expression changes in liver, kidney and heart 24 h after SEB exposure were not due simply to the influx of immune cells. In lung tissue at 24 h, SEB-challenge caused a multifocal indicated, top functional categories for these genes included inflammatory response and antigen presentation. The all-tissue gene list was also significantly associated with liver, kidney and heart toxicity (data not shown). A canonical pathway analysis identified an IFN-biased innate immune response that seemed more appropriate for viral infection rather than a bacterial toxin (Fig. 5A). Top, highly significant canonical pathways included IFN signaling, antigen presentation, interferon regulatory factor (IRF) activation by cytosolic pattern recognition receptors, retinoic acid-mediated apoptosis signaling, protein ubiquination pathway, pathogenesis of multiple sclerosis, and role of retinoic acid-inducible gene (RIG)-like receptors in anti-viral immunity. Because of the overwhelming IFN-response signature, the all-tissue gene list was next analyzed in a custom database (Interferome v2.0) of curated IFN-regulated genes from publically available microarray experiments [42]. Using mouse-specific IFN-regulated gene lists and default 2.0 fold-change cutoff values, 85 uniquely annotated transcripts were classified as belonging to type I, type II, or both type I and II, IFN-response subtypes (Fig. 5B).

To further test whether IFN-regulated binding sites were truly over represented among genes up-regulated by SEB across all tissues in our animal model, these promoters were next compared to a large set of mouse housekeeping genes (BIOBASE Knowledge Library® using an all vertebrate promoter matrix profile from TRANSFAC® Professional (see Methods). Of 33 significantly enriched promoter matrices (matched promoter FDR<0.05), the top 11 ranked from lowest to highest FDR were all regulated by IFN activated and/or induced transcription factors (Table 4). Notably, none of these genes appeared on our all-tissue list of differentially regulated transcripts. However, 8/19 were found to be significantly up-regulated in PBMCs, spleen and/or lung prior to 24 h (Table 5), suggesting that the products of these genes might be contributing to the global IFN-type response seen here.

To better illustrate the commonality of this shared-response in PBMCs and across multiple organs, parallel plots were generated within tissue, normalizing each of the 103 probesets to its own 0 h baseline expression level (Fig. 2). As seen, expression changes were generally maximal at 24 h in all tissues. However, 12 probesets (shown in red) representing 11 leading-edge transcripts (Cxcl9, Cxcl10, Cxcl11, Cd274, Fam26f, Irf1, Irf8, Irgm2, Parp14, Srpma3g, Stat1) reached their peak response in PBMCs and/or spleen at 5 h post-SEB challenge. Notably, 3 of these 11 genes are transcription factors (Irf1, Irf8, and Stat1) downstream from traditional IFN signaling, and 3 are recognized as early/ immediate IFN-induced chemokines (Cxcl9, Cxcl10, and Cxcl11). These results suggest that an IFN-type response might have been initiated in PBMCs and spleen with later generalization to other tissues.

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Figure 1. Heatmap of 103 probesets differentially regulated in all tissues. Probesets (≤5% FDR; ≥1.5-fold-change compared to control; and ≥50% present call within at least one condition/time point, across all tissues) are displayed on the vertical axis and designated, when available, by the
symbol of the gene to which each is annotated, including duplicates. Tissue and time points are denoted on the horizontal axis. Each probeset has been normalized to its mean value across all times and tissues within one row. Red signifies expression above and green below the mean value within an individual row. As shown, baseline expression of these differentially expressed transcripts tends to decrease from PMBC > Spleen > Lung > Liver > Kidney, Heart. In contrast, all of these genes are induced by staphylococcal enterotoxin B (SEB) challenge with most reaching their highest levels of expression at 24 h across all tissues. Three unannotated probesets, identified only by Affymetrix® probeset IDs; Predicted gene Gm9706 of unknown function; second probeset annotated to Gm9706 is also annotated to the gene symbol Ig51; while these probesets do not cluster together, peak expression for both were seen in PMBCs at 24 h, suggesting that they may interrogate the same gene, but with different efficiencies; cProbably detecting Gbp6 with which it clusters, but this probeset retains its annotation to both Gbp10 and Gbp6 as shown; dProbably detecting Ifi202b with which it clusters, but this probeset retains its annotation to both LOC100044068 and Ifi202b as shown; eProbably detecting Gbp1, but this probeset retains its annotation to both LOC100047734 and Gbp1 as shown.

Furthermore, the walls of some small vessels at 48 h contained neutrophilic fragments consistent with vasculitis (Fig. 7 inset). Because these SEB-associated changes could serve as a quality control measure, lung tissue was chosen to further study apoptosis, free radical injury, and polyADP ribosylation.

SEB challenge compared to control produced a significant increase (2.93±0.12 versus 0.06±0.06 cells/HPF; p<0.001) in apoptotic cells associated with bronchioles as measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Fig. 7; arrows). Nitrotyrosine staining, a measure of peroxynitrite-mediated oxidant injury, did not differ between the two groups with all specimens showing similar amounts of faint staining (Fig. 7; arrows) in alveolar epithelium, small vessel endothelium and alveolar macrophages. In contrast, immunohistochemistry staining for polyADP-ribose (PAR), a product of poly[ADP-ribose] polymerase (PARP) enzymatic activity, showed increased staining associated with SEB exposure that was largely cytosolic and proportional to the increase in inflammatory cellularity. At 48 h, hypertrophied alveolar epithelial cells (Fig. 7; arrows) stained prominently for PAR. Notably, three macro-PARP genes, Parp9, Parp12 and Parp14, were significantly up-regulated across all tissues in mice challenged with a lethal dose of SEB.

Discussion

Oligonucleotide microarrays were used to analyze the global, host-wide response to SEB in a murine model of superantigen-mediated shock that uses relatively low-dose challenges without the need for priming agents and does not cause rapid death [31]. Although relevance to human disease is not certain, our finding of a late type I/II IFN response across multiple organs and tissues lends support to the possible importance of this pathway in toxic shock syndromes. Eighty-five genes that annotated to an IFN antiviral response were uniformly up-regulated in PMBC, spleen, lung, liver, kidney, and heart. Potential initiators of IFN signaling such as IL-2, IL-12B and IFNγ were only modestly up-regulated at early time points in some tissues, while IFN regulatory factors and Zbp1, a DNA sensor/transcription factor that directly elicits IFN innate responses, were notable components of the host-wide SEB signature. These results suggest that therapies aimed at IFN-associated innate immune responses may improve outcome in human toxic shock.

Despite important clinical distinctions between staphylococcal menstrual [1] and non-menstrual [46,47,48], and streptococcal [2] forms of toxic shock, the various exotoxins associated with each of these syndromes including SEB share a common mechanism that leads to intense activation of the host immune system. In each case, the putative exotoxin binds directly to TCRs on T-cells and to major histocompatibility complex class II molecules on antigen-presenting cells, forming a bridge [5]. Co-stimulatory receptors on both cells, such as CD28 and CD80, also bind to each other triggering a massive polyclonal inflammatory response [11,12].
The ensuing cytokine storm with the rapid release of TNFα, IL-1β, IL-2 and IFNγ has been generally held responsible for all of the subsequent, clinical consequences of toxin exposure including hypotension, multiple organ injury and death [6,21,22]. Here, a shared genomic response across all tissues and organs was found that annotated to 85 up-regulated genes. While this shared response represented only a small fraction of all differentially expressed transcripts in PBMCs (3%), its dominance as a proportion of involved genes grew from spleen and lung (11% each) to liver, kidney and heart (all >30%) and over time. The simultaneous, concordant expression of transcripts across multiple organs suggested a common transcriptional regulatory mechanism that might be central to pathogenesis and possibly provide insights into treatment. Ultimately, all of these shared genes could be mapped to a dominant type I and type II IFN-signature. Notably, other investigators using animal models [26,49,50,51] or studying the cytokine response in patients with toxic shock syndrome [22] have provided evidence for the possible importance of IFN signaling in the pathogenesis of superantigen-mediated disease. Recently, Tilahun et al found that Ifnγ knockout conferred significant protection from lethal SEB challenge in a HLA-DR3 transgenic mouse model of toxic shock syndrome [51]. Collectively with previous work, our results suggest that IFN targeted therapeutic approaches warrant investigation.

The molecular mechanisms that drive this host-wide IFN-response 24 h after SEB exposure are not clear, but several possibilities are suggested by the list of affected genes. While, IFNα/β was not up-regulated at any time point in any tissue, other IFN pathway inducers, including IL-2, IL-12β, and IFNγ, were induced early, but only modestly so, in PBMCs, spleen and lung, and may have contributed to the shared IFN-response. Also supporting this notion, a number of the transcripts up-regulated across all tissues are known to amplify IFN-regulated gene transcription. PARP9 and PARP14 remodel chromatin and NMI interacts with STAT (signal transducer and activator of transcription) proteins to increase transcriptional responses to IL-2 and IFNγ [52,53,54]. An alternative explanation for the general IFN-response found in our model is suggested by the all tissue induction of Zbp1, a transcription factor activated by cytosolic, double-stranded DNA...
Figure 4. Quantitative real-time PCR (qRT-PCR) confirmation of tissue-wide changes in gene expression. Nine genes were quantitated by qRT-PCR across all 6 tissues at 24 h. (A) Scatter plot of all genes and tissues tested comparing microarray and qRT-PCR fold-change from control. As shown by the line of identity (x = y), qRT-PCR typically returned higher fold-change results than microarray. Gene specific results, colored by tissue (see Legend), are shown as follows: (B) Cxcl11; (C) Herc6; (D) Irf1; (E) Irf8; (F) Irgm1; (G) Parp12; (H) Stat1; (I) Xaf1; and (J) Zbp1. All qRT-PCR results met the >1.5 fold-change cut-off for gene selection, except for measurements of Irf8 in PBMCs and spleen. However, Irf8 similarly failed selection by microarray in these tissues at 24 h. Four samples were tested per tissue. Each PBMC sample represented a pool of multiple mice while each organ sample came from an individual mouse.

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Figure 5. Thematic analysis, interferon (IFN) response subtype classification, and promoter analysis for binding matrices responsive to IFN. (A) Canonical pathways significantly associated with the all-tissue response to staphylococcal enterotoxin B (SEB) challenge. Seventy-nine unique genes were recognized by the Ingenuity Pathway Analysis® (IPA®) database and mapped to IFN signaling, antigen presentation,
and activation of IFN regulatory factor (IRF) by cytosolic pattern recognition receptors, among the other canonical pathways shown. (B) Classification of genes significantly up-regulated across all tissues by IFN response subtype. Note that for Mus musculus, the Interferome v2.01 database contained 1655 Type I genes, 1413 Type II genes, and no Type III genes. (C) IFN pathway-driven regulatory binding sites identified in the promoters of genes regulated across all tissues. Of 81 promoter regions analyzed (from +500 to −1500 bp), 68 were found to contain IFN-driven regulatory matrices as shown. Results generated by Interferome v2.01 using TRANSFAC® Professional (2012) matrices and the MATCH™ algorithm.

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fragments, whether microbe- or host-derived, that can induce type I IFN genes independent of IFNα/β signaling [55,56]. Several poly [ADP-ribose] polymerases (Parp9, Parp12 and Parp14) and Dtx3l, an E3 ubiquitin ligase co-regulated with Parp9 through a shared promotor, were also up-regulated in every tissue tested and functionally annotate to DNA repair [57,58]. Together the expression of Zbp1, a DNA sensor, and multiple DNA damage/repair genes suggests that the host-wide IFN-signature reported here might be driven by multi-organ cellular injury. Nuclear or mitochondrial DNA leakage into the cytoplasm of injured cells could trigger damage[danger]-associated molecular pattern (DAMP) recognition, resulting in an IFN-response [59,60,61]. However, some caution is warranted in drawing this conclusion. PARP12 has recently been recognized as a cytoplasmic, post-transcriptional regulator with antiviral activity [62,63] and may not function primarily in DNA damage/repair. Importantly, immunohistochemical staining for poly-ADP-ribose (PAR) was largely cytosolic and widespread tissue damage was not seen by histopathology at 24 h post-exposure.

Epigenetic remodeling may also serve to activate IFN-type antiviral gene responses independent of or at least additive with IFNα/β signaling in non-immune cells. Fang and colleagues found that blocking histone 3 lysine 9 di-methylation (H3K9me2) led to a robust IFN signature and viral resistance in fibroblasts found that blocking histone 3 lysine 9 di-methylation (H3K9me2) led to a robust IFN signature and viral resistance in fibroblasts [57]. Notably, the IFN-regulated genes that were restricted by H3K9me2 overlap extensively with our all-tissue list. Furthermore, H3K9me2 epigenetic marks in neurons, cardiac myocytes, and other parenchymal cells are thought to suppress IFN responses and protect diverse cell types from IFN-induced tissue injury [64,65].

The antiviral effector molecules induced by SEB in every organ tested included Ddx58, Eif2ak2, Herc6, Ifih1 (Mda5), Isg15, Oas1a, Oas2, and Samhd1. DHX58 binds viral RNA and regulates RIG1 (retinoic acid-inducible gene 1), an intracellular pattern recognition receptor and viral sensor [66]. Likewise, IIFH1 (MDA5) is a RIG1-like receptor (RLR) family member that participates in viral defense [67]. HERC6 was recently identified as the main E3 ligase that catalyzes ISG15 conjugation (ISGylation) of proteins in mice to restrict the replication of a wide variety of viruses [68]. EIF2AK2 is activated by double-stranded RNA to block protein synthesis [69]. OAS1A and OAS12 are 2′-5′A synthetase family members that also bind double-stranded RNA and activate latent ribonuclease L, which degrades viral RNA [70]. Finally, SAMHD1 depletes the pool of nucleotides available to viral reverse transcriptases and thus prevents replication of HIV and other viruses [71]. Although latent virus activation cannot be entirely ruled out in our murine model of SEB challenge, viral cytopathic effects have not been seen on histopathology [31]. Nonetheless, further studies are needed to better understand the implications of this previously unrecognized, broad SEB-associated antiviral signature. At least superficially, this antiviral response would seem to be maladaptive in the setting of a bacterial infection with superantigen production. Another somewhat surprising genetic signature found in our host-wide, shared response was the induction of immunoproteasome components (Psme2, Psmbl8, Psmbl9, and Psmbl10) along with two ubiquitin ligases (Uba7 and Ubd), a deubiquitinating protease (Usp18), and Erap1, an aminopeptidase involved in antigen processing [72]. The protein products of these genes are integral parts in the machinery needed for the proper function of dendritic cells and other professional APCs [73]. Notably, UBD is

| Promoter Matrix Name | Associated Transcription Factors | Yes (sites/1000 bp) | Yes/No* | From | To | Matched promoters FDR |
|----------------------|---------------------------------|-------------------|--------|------|--------|------------------------|
| V$\text{SISRE}_01$   | If7, If8, Isgf3g, Stat1, Stat2  | 0.2778            | infinity | −500 | 100   | 4.6E-09                |
| V$\text{SIF7}_01$   | If7, If8, Isgf3g, Stat1, Stat2  | 0.3373            | infinity | −500 | 100   | 4.6E-09                |
| V$\text{SIRF1}_06_01$ | If1                | 0.2579            | infinity | −400 | 100   | 9.2E-08                |
| V$\text{SIRF}_06$   | If1, If2, If3, If4, If5, If6, If7, If8, Isgf3g, Stat1, Stat2 | 0.2183            | infinity | −400 | 100   | 5.3E-07                |
| V$\text{SLIMP1}_06$ | Prdm1b              | 0.1786            | infinity | −400 | 100   | 2.4E-05                |
| V$\text{SIRF1}_06$   | If1, If2            | 0.4365            | 6.78    | −200 | 100   | 3.3E-05                |
| V$\text{SICSBP}_06$ | If7, If8, Isgf3g, Stat1, Stat2 | 0.1786            | infinity | −200 | 100   | 1.1E-04                |
| V$\text{SIRF7}_03$  | If7                | 0.7143            | 4.05    | −200 | 100   | 6.3E-04                |
| V$\text{SIRF8}_06$  | If8                | 0.2778            | 9.11    | −200 | 100   | 7.6E-04                |
| V$\text{SIRF3}$     | If3                | 0.1786            | 17.57   | −500 | 100   | 1.2E-03                |

*aRatio of the abundance of each promoter matrix in genes differentially regulated across all six tissues compared to 492 mouse housekeeping genes (see Methods).

bPrdm1 (Blimp1), a transcriptional repressor essential for B- and T-cell differentiation and homeostasis, is regulated by Irf4. Prdm1 and interferon regulatory factors bind to similar DNA sequences. Some promoters contain overlapping motifs where Prdm1 and Irf family members may competitively interact.

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Table 5. Interferon pathway inducing genes (fold change, SEB control).

| Gene ID | Gene Title | PMBC | Spleen | Lung | Liver | Kidney | Heart |
|---------|------------|------|--------|------|-------|--------|-------|
| 16175   | interleukin 1 beta | 1.02 | 1.07  | 1.12 | 1.11  | 1.09   | 1.10  |
| 15978   | interferon gamma | 1.71 | 1.51  | 2.50 | 1.27  | 1.17   | 0.92  |
| 21926   | tumor necrosis factor | 1.76 | 7.04  | 1.75 | 1.17  | 1.28   | 1.14  |
| 21927   | colony stimulating factor 1 | 1.87 | 1.97  | 1.80 | 1.75  | 1.75   | 1.37  |
| 12977   | colony stimulating factor 3 | 1.01 | 1.94  | 1.26 | 1.09  | 1.37   | 1.09  |

Tissues meeting all criteria (5% FDR; 1.5 FC) 2.75 h 5 h 24 h 2.75 h 5 h 24 h 2.75 h 5 h 24 h 2.75 h 5 h 24 h 2.75 h 5 h 24 h 2.75 h 5 h 24 h

| Fold Change | PMBC | Spleen | Lung | Liver | Kidney | Heart |
|-------------|------|--------|------|-------|--------|-------|
| 2.75 h 5 h 24 h | 2.75 h 5 h 24 h | 2.75 h 5 h 24 h | 2.75 h 5 h 24 h | 2.75 h 5 h 24 h | 2.75 h 5 h 24 h | 2.75 h 5 h 24 h |

known to promote the expression of the immunoproteasome component Psmb9, and plays an important role in dendritic cell maturation [74]. The induction of these genes might be anticipated in PBMCs and spleen, Likewise, lung and liver tissue have substantial numbers of macrophages and resident dendritic cells. In contrast, the strong expression of these genes in the kidneys and heart has not been reported previously. However, numerous studies have demonstrated that non-professional, antigen presenting-like cells can arise in non-myeloid cells of various tissues and organs, an occurrence that has been associated with autoimmunity [75]. Cells from the fibrous cap of human atherosclerotic lesions express the immunoproteasome component Psmb8 in response to IFNγ sensitization [76]. Finally, human endothelial cells exposed to either TSST-1 or SEB have been shown to express class II MHC molecules and to function as competent superantigen-presenting cells, possibly contributing to the vascular injury seen in patients with toxic shock syndrome [77].

The potential relevance of the shared, host-wide genomic program reported here to toxic shock syndrome is an important question. Recently, chronic infusion of low dose SEB in HLA-DQ8 transgenic mice was shown to produce a lupus-like syndrome involving multiple organs (lung, liver and kidney) [49]. STAT4 or IFNγ deficiency prevented this autoimmune-like tissue injury, supporting a pathogenic role for the Th1-type cytokines, IL-12 and IFNγ, in this model. More directly related to toxic shock and perhaps septic shock syndrome, mice with knockout of interferon-alpha receptor-1 (Ifnar1), and thus incapable of responding to type I IFNs (IFNα/β), were remarkably resistant to TNFα-induced inflammatory shock and death [78]. Importantly, IFNAR1 deficient mice were also protected from lethal S. aureus pneumonia compared to their wild-type counterparts [79]. This is notable as most immune system gene-knockouts are less fit to challenge with viable infectious agents. Collectively, these findings suggest that type I IFN responses, crucial in viral and intracellular pathogen defense, may be quite harmful in certain bacterial infections, at least in mice. Whether the late, host-wide, IFN-signature seen here is similarly detrimental and therefore a viable therapeutic target requires further investigation.

Dexamethasone and rapamycin have both been beneficial in our murine model of SEB lethality [43,80]. Given very early (within 2 h) after SEB-challenge and then continued for a full 96 h, dexamethasone rescued animals from death and inhibited many inflammatory mediators, including several IFN-pathway initiators [80]. However, dexamethasone does not improve survival when given more than 5 h after SEB challenge. Whether late (after 5 h) administration of dexamethasone also fails to quell the host-wide IFN-signature reported here is not known. Unlike dexamethasone, very high-dose rapamycin prevented deaths, even when given as late as 24 h after SEB [43], the last time point we analyzed by microarray. The mammalian target of rapamycin (mTOR) has very complex effects on immunity. Inhibition of mTOR by rapamycin decreases T-cell proliferation and suppresses type I IFN responses [81], but can increase monocye/macrophage-mediated inflammation [82,83]. Lethality and IL-1β levels were increased by rapamycin in LPS-challenged mice, probably because mTOR suppression of NFκB and caspase-1 was blocked [84]. Interestingly, several genes on our all-tissue list were blocked by rapamycin in CpG oligodeoxynucleotides/TLR9-activated dendritic cells including Cxcl9, Gbp7, Ifit1, Igsf, Oasl1, and Rtp1 [84]. Overall, the beneficial effects of dexamethasone and rapamycin in our lethal mouse model of SEB-challenge [43,80] support the potential pathogenic importance of the host-wide IFN-signature found here, but this hypothesis requires further testing.
In summary, a host-wide, innate IFN-response was seen across all tissues and organs in a lethal mouse model of SEB challenge. Whether this unexpected shared genomic program is primarily driven by the induction of IFN pathway inducers and amplifiers, DNA damage, and/or the recruitment of non-professional APCs into the generalized inflammatory response requires further study. Nonetheless, this multi-organ response to SEB exposure may contribute to the pathophysiology of SEB-induced shock and provides a rational for the specific interruption of these pathways to reduce inflammation and tissue injury. Because of the common mechanism of immune activation by all superantigens, therapeutics based on these findings could also be useful for the management of de novo toxic shock syndrome. Importantly, the generalized response seen here, characterized by IFN-inducible transcripts and intracellular sensors, unfolds in a manner such that an adequate window of time may exist for interventions based on this new view of superantigen lethality.

![Functional network of selected upstream-regulators and differentially expressed genes across all tissues.](image)

**Figure 6.** Functional network of selected upstream-regulators and differentially expressed genes across all tissues. From among the significant nodes identified using the Ingenuity Pathway Analysis® (IPA®) Upstream Regulator tool, the following were selected for inclusion in the displayed network: 1) the T-cell receptor (TCR), as this is the primary target of staphylococcal enterotoxin B (SEB)-mediated cell activation (colored orange at the network center); 2) TNF, IL-1β, IL-2, IFNγ and IL-12B, as these are known interferon (IFN) pathway initiators that were expressed early in the peripheral blood mononuclear cells and/or spleens of the SEB challenged mice (colored blue and positioned as the inner most ring of the network); and 3) any upstream regulator that was also present on our all-tissue list of differentially expressed genes (colored in shades of red proportional to fold-change) and positioned as the next ring moving outward. The resulting network connected 70 of 79 genes recognized by IPA®. The remaining 9 genes (outside of the outermost ring) were connected manually (see text) using PubMed and STRING (http://string-db.org/newstring_cgi/) version 9.05, a database of known and predicted protein-protein interactions. A key defining colors, shapes, and relationships is shown. In addition, changes in gene symbols from those in Figure 1 and Table 3 are provided for clarity. Also note that IPA® frequently defaults to all-capital gene symbols that denote human genes, while elsewhere the mouse format is followed of only capitalizing the first letter.

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![Pulmonary pathology: hematoxylin and eosin (H&E) stain, TUNEL assay and immunohistochemistry staining for nitrotyrosine and polyADP-ribose.](image)

**Figure 7.** Pulmonary pathology: hematoxylin and eosin (H&E) stain, TUNEL assay and immunohistochemistry staining for nitrotyrosine and polyADP-ribose. Compared to control animals at 24 h, staphylococcal enterotoxin B (SEB) challenge caused a multifocal, minimal to mild perivascular, peribronchiolar, interstitial and subpleural lymphohistiocytic inflammatory infiltrate. At 48 h a coalescing, neutrophil-predominant infiltrate was seen in SEB exposed animals that now extended into alveoli. Multiple vessel walls 48 h after SEB exposure contained neutrophilic fragments (arrows) consistent with vasculitis (H&E inset, SEB 48 h). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay demonstrated an increase in bronchiolar apoptotic cells (arrows) after SEB challenge compared to control that was significant at 24 h post-exposure (2.93 ± 0.12 versus 0.06 ± 0.06 cells/HPF; p < 0.001). Immunohistochemistry for nitrotyrosine was not different comparing SEB to control with all specimens showing faint staining (arrows) of alveolar epithelium, small vessel endothelium and alveolar macrophages. In contrast, immunohistochemistry for polyADP-ribose (PAR) showed increased staining associated with SEB exposure that was mostly proportional to the increase in inflammatory cellularity. At 48 h, hypertrophied alveolar epithelial cells (arrows) stained prominently for PAR.

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Supporting Information

Table S1 All probesets in any tissue meeting selection criteria (≤5% FDR; ≥1.5-fold-change compared to control; and ≥50% present call in at least one condition/time point).

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Author Contributions

Conceived and designed the experiments: TK RLD PJM GAF. Performed the experiments: GAF MB. Analyzed the data: CYD PJM JME MFS RLD. Contributed reagents/materials/analysis tools: TK RLD PJM. Wrote the paper: RLD TK.

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