ORIGINAL ARTICLE

Stress-caused anergy of leukocytes towards *Staphylococcal enterotoxin* B and exposure transcriptome signatures

S Muhie1, R Hammamieh2, C Cummings3, D Yang4 and M Jett2

Leucocytes from soldiers exposed to battlefield-like stress (RASP; Rangers Assessment and Selection Program) were exposed *in vitro* to *Staphylococcal enterotoxin* B (SEB). We assayed SEB-induced regulation of gene expression, both in the presence and absence of severe stress, to generate two sets of gene profiles. One set of transcripts and microRNAs were specific to post-RASP SEB exposure, and another set were signatures of SEB exposure common to both the pre- and post-RASP leucocytes. Pathways and upstream regulatory analyses indicated that the post-RASP SEB-signature transcripts were manifestation of the anergic state of post-RASP leucocytes. These were further verified using expression-based predictions of cellular processes and literature searches. Specificity of the second set of transcripts to SEB exposure was verified using machine-learning algorithms on our and four other (Gene Expression Omnibus) data sets. Cell adhesion, coagulation, hypoxia and vascular endothelial growth factor-mediated vascular leakage were SEB-specific pathways even under the background of severe stress. Hsa-miR-155-3p was the top SEB exposure predictor in our data set, and C-X-C motif chemokine ligand 9 was SEB specific in all the analyzed data sets. The SEB-signature transcripts (which also showed distinct expression signatures from *Yersinia pestis* and dengue virus) may serve as potential biomarkers of SEB exposure even under the background of stress.

INTRODUCTION

Impact of select agents on severely stressed individuals, especially molecular responses of soldiers under extreme battlefield stress, are not well investigated. Molecular signatures of host responses to pathological conditions have the potential to be used as biomarkers and drug targets in addition to their use in differentiating patterns related to diseased/stressed vs baseline states.

Our studies focus on identifying host-response molecular signatures induced by select agents under the background of RASP (Rangers Assessment and Selection Program; the toughest training of the US Army Ranger Cadets designed to emulate extreme battlefield scenarios). The RASP cadets were pushed to their physical and psychological limits providing a rare opportunity for systems-level studies of the most severe form of battlefield-like stress along with *ex vivo* exposure to select agents. Previously, we showed that battlefield-like stress severely compromises the immune response.1 Transcript mediators of compromised immunity remained suppressed in post-RASP leucocytes that are *ex vivo* exposed to *Staphylococcal enterotoxin* B (SEB).1 But complete and differential counts of post-RASP white blood cells (WBCs) are within normal ranges.2 The compromised protective immunity and impaired response to the *in vitro* SEB challenge in spite of normal ranges of cell counts were considered to be indicators of the anergic state of post-RASP leucocytes to SEB.3

The SEB toxin is a category B select agent2 and a potent mitogen7 with affinity to polymorphic major histocompatibility complex class II molecules with potential to stimulate different clones of T-cells.4 SEB stimulation of T-cells lead either to an outburst of cytokines (*cytokine storm* or sepsis),5–9 cellular anergy or apoptosis depending on the nature of antigen-presenting cells (APCs), secreted cytokines and receptor T-cells.10 For example, *in vivo* anergized APCs such as monocytes with suppressed HLA-DR11 and radiation-exposed dendritic cells12 shown to form unstable immunological synapses *in vitro* and failed to initiate T-cell activation leading to T-cell anergy. These findings suggest that anergy in compromised accessory molecules of APCs induces defects in the earliest events of T-cell activation, immunological synapse formation and T-cell receptor (TCR)-mediated signaling.13 SEB is shown to promote further anergy in cells exposed to other stimuli. Memory CD4+ T-cells exposed to SEB became anergic,14 and ZAP-70 of such cells do not interact with the TCR/CD3 complex.15 But not all non-naive lymphocytes became anergic to SEB. SEB is shown to reactivate antigen-specific CD8+ memory T-cells.16 The non-naive nature of post-RASP leucocytes and their suppressed transcripts mediating antigen-presentation and TCR-signaling1 were favorable conditions for anergy. The RASP-suppressed immune response transcripts were further suppressed in SEB-exposed post-RASP leucocytes1—suggesting further anergy. In general, the types of previous stimuli seem to determine whether SEB challenge lead to anergy or antigen-specific activation.

Even though we assessed the expression patterns of RASP-suppressed immune response transcripts with SEB exposure,7 SEB-regulated host-response transcripts, associated pathways and molecular mediators of cellular anergy to SEB were not further investigated. And it is not also obvious about the presence of host-response molecular signatures that are persistently specific to SEB with the potential to discriminate SEB exposure under

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**Title:** Stress-caused anergy of leukocytes towards Staphylococcal enterotoxin B and exposure transcriptome signatures

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**Abstract:**
Leucocytes from soldiers exposed to battle??eld-like stress (RASP: Rangers Assessment and Selection Program) were exposed in vitro to Staphylococcal enterotoxin B (SEB). We assayed SEB-induced regulation of gene expression, both in the presence and absence of severe stress, to generate two sets of gene pro??les. One set of transcripts and microRNAs were speci??c to post-RASP SEB exposure, and another set were signatures of SEB exposure common to both the pre- and post-RASP leucocytes. Pathways and upstream regulatory analyses indicated that the post-RASP SEB-signature transcripts were manifestation of the anergic state of post-RASP leucocytes. These were further veri??ed using expression-based predictions of cellular processes and literature searches. Speci??city of the second set of transcripts to SEB exposure was veri??ed using machine-learning algorithms on our and four other (Gene Expression Omnibus) data sets. Cell adhesion, coagulation, hypoxia and vascular endothelial growth factor-mediated vascular leakage were SEB-speci??c pathways even under the background of severe stress. Hsa-miR-155-3p was the top SEB exposure predictor in our data set, and C-X-C motif chemokine ligand 9 was SEB speci??c in all the analyzed data sets. The SEB-signature transcripts (which also showed distinct expression signatures from Yersinia pestis and dengue virus) may serve as potential biomarkers of SEB exposure even under the background of stress.
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severe stress from other select agents. Identification of SEB-specific biomarkers is essential to introduce early mitigating measures against morbidity or toxic shock caused by SEB. Even in the case of RASP-suppressed inflammatory cytokines (upstream mediators of SEB-induced toxic shock), SEB-specific biomarkers under the background of severe stress are still needed: to ascertain whether the immune response is not further plummeted, and to differentiate SEB exposure from stress or infections in order administer mitigating or support schedules.

Hence this study is designed to determine whether host transcriptome responses to SEB can still predict such exposures despite stress-caused immune suppression. And to identify molecular mediators and features of stress- and SEB-caused anergy of leukocytes, in addition to assessing how the immune system of individuals under severe stress fares if the same individuals are also exposed to SEB toxin.

We employed experimental and computational approaches in identifying transcripts, pathways and processes involved in the host’s response to SEB. In the first approach, we used genome-wide transcriptome and microRNA (miR) profiling in SEB-exposed pre- and post-RASP leukocytes (collected from 15 RASP cadets). We analyzed SEB-regulated gene expression levels and miRs both in the presence and absence of severe battlefield-like stressors. Profiled transcripts were validated using quantitative real-time PCR (qRT-PCR).

In the second approach, we carried out computational identification of upstream regulatory modules (molecular networks) of SEB-regulated transcripts, expression-based prediction of cellular processes to determine activation status of immune processes in post-RASP SEB-exposed leukocytes and machine-learning algorithms (nearest shrunk centroid (NSC) and random forest (RF)) on our in-house data to identify and four other Gene Expression Omnibus (GEO) data sets to cross-validate the SEB-specific host-response biomarkers. The four GEO data sets were carefully selected to include (in addition to SEB exposure) other select agents (GSE4478), a different serotype of SEB or super-antigen (GDS3399), uses an in vivo system (GSE15571) and uses a bystander (indirect) activation of T-cells by SEB (GSE13718), so each complements the other.

In the current study, we identified two sets of transcripts and miRs. One set of transcripts and miRs (regulated in SEB-exposed post-RASP leukocytes) revealed molecular patterns, regulatory networks and pathways that were mediators of the stress-caused anergy of leukocytes to SEB. Among important features of anergic responses of post-RASP leukocytes to SEB include highly suppressed transcripts mediating inflammation, chemotaxis, antigen presentation and TCR signaling. Particularly, APCs with suppressed transcripts critical for antigen presentation and chemotaxis (including soluble chemokines implicated in mediating indirect activation of T-cells) seem to be responsible for lack of both direct and indirect activation of post-RASP T-cells to SEB. Also, RASP-exposed (non-naive) T-cells with compromised antigen receptors, co-receptors and inhibited TCR signaling indicate T-cell anergy. Results from expression-based predictions further verified the anergic states of post-RASP leukocytes, in addition to accounting why post-RASP WBCs were within normal ranges (inhibited expansion and apoptosis) and yet were not responsive to SEB (inhibited viability, priming and activation).

The other sets of transcripts and miRs were specific to SEB exposure both in the presence and absence of severe stress. The SEB-specific set may serve as potential biomarkers of SEB exposure under the background of severe stress. Among the top SEB-classifier transcripts, C-X-C motif chemokine ligand 9 (CCL9) was predicted to be SEB specific in our data and the four GEO data sets, and the miR hsa-miR-155* was the top classifier in our miR data.

RESULTS
Leukocytes isolated from soldiers before and after US Army Rangers Training (RASP) were exposed to SEB toxin. 1 Normalized expression data from SEB-exposed leukocytes were analyzed to identify two groups of differentially regulated transcripts: indicators for the anergic states of post-RASP leukocytes to SEB, and signatures of SEB-exposure in both the pre- and post-RASP conditions (Supplementary Tables S1–S3). Potential upstream regulators of both groups of transcripts were identified computationally at the genome level (transcription factors (TFs)) and experimentally at the transcriptome level (miRs).

Transcripts, biological processes and pathways regulated by SEB in post-RASP leukocytes
Post-RASP SEB-specific transcripts and miRs were identified by comparing post-RASP SEB-exposed leukocytes (RASP+SEB) with pre-RASP SEB-exposed (SEB), unexposed post-RASP (RASP) or unexposed pre-RASP (control) leukocytes. A total of 460 transcript and 21 miRs were differentially regulated in post-RASP SEB-exposed leukocytes at P < 0.05 and ≥ 1.5-fold changes (Figures 1a and c, Table 1, Supplementary Tables S1 and S2). The 460 transcripts (representing 447 unique genes) were specific to post-RASP leukocytes exposed to SEB and did not show differential regulation in the pre- and post-RASP leukocytes exposed to Yersinia pestis or dengue virus (Supplementary Figure S1a).

Among the 460 transcripts, 238 were downregulated and 222 were upregulated in post-RASP SEB-exposed leukocytes compared with the SEB, RASP and control groups. Using Hypergeometric and Fisher’s enrichments along false discovery rate correction at q < 0.05, the 238 suppressed transcripts were significantly associated with pattern recognition, inflammation, chemotaxis, antigen presentation and lymphocyte activations (Figure 2, Supplementary Figure S2, Table 2 and Supplementary Table S3), indicating that SEB caused further compromised immune processes (Figure 1a and Supplementary Figure S1a). The 222 upregulated transcripts were largely associated with upstream modulators of immune responses (steroid hormones, insulin-like growth factors), sepsis-related cellular processes (vascular leakage, coagulation, integrin-cell surface interaction) and stress responses (Figure 2, Supplementary Figure S2, Table 2 and Supplementary Table S3).

Of the 238, 114 transcripts mediating protective immunity were common with RASP-regulated transcripts (Supplementary Figure S1c), and these were further suppressed by at least 1.5-fold in SEB-exposed post-RASP leukocytes (Supplementary Table S2). Over-represented immune response processes and pathways associated with post-RASP SEB exposure showed even much more extensive overlap with those identified for RASP-regulated transcripts, although they were regulated to different extents. 1 The concerted effect of stress and SEB showed further overall immune-suppression by at least 50% compared with stress alone (Supplementary Table S2). The immune-suppressive effect of RASP was aggravated in many of the immune response transcripts mediating inflammation, chemotaxis, antigen presentation and T-cell activations and immune-related regulatory networks in post-RASP SEB-exposed leukocytes (Figures 1a, b and 2, Supplementary Figure S1 and Supplementary Tables S2 and S5).

Stress-induced anergy of leukocytes towards SEB exposure
Using Agilent’s literature search tool (Cytoscape plugin; www. cytoscape.org), we identified 51 of the 460 post-RASP SEB-regulated transcripts reported to be features (mediators) of cellular anergy (Figure 1b and Supplementary Figure S1b). Enriched pathways associated with these 51 transcripts were reflective of compromised immunity of SEB-exposed post-RASP APCs and T-cells (Figure 2, Supplementary Figures S2–S5, and
It appears that post-RASP leukocytes became anergic to SEB, and possibly became more anergic upon SEB exposure as indicated by further suppression of these transcripts in SEB-exposed post-RASP leukocytes (Figures 1a and b, Supplementary Figure S1, Supplementary Table S2).

Identification of common regulatory motifs and potential TFs

Searching for common regulatory motifs for k-means clustered (post-RASP SEB-regulated) transcripts, we identified potential cis-binding sites for factors JUN, SRF, SP1, NFYA, KLF4 and MAFB. Regulatory interaction networks among these TFs and their downstream targets show both protein–DNA and protein–protein interactions (Figure 3). According to our regulatory interaction network, it seems that both regulatory interaction and functional co-localization of these factors and their downstream targets contribute to the immunosuppressive effect of battlefield-like stress (RASP) and SEB exposures (Figure 3).

Three of these trans-activators (NFYA, SRF and SP1) were upregulated, whereas the other two (JUN and MAFB) were downregulated though not significantly in post-RASP SEB-exposed leukocytes (Figure 3). The upregulated three factors showed protein–protein interaction (in our models), and they seem to interact in forming transcriptional complex in suppressing their downstream targets mediating in inflammation, chemotaxis, antigen presentation, T-cell proliferation and immune critical factors (NFKB1, RELA and JUN) (Figure 4, Supplementary Figure S6 and Supplementary Table S6).

miRs and TFs potentially targeting transcripts important in anergy and immune tolerance

A number of empirically and computationally identified TFs (SP1, NFYA, SRF, TSC22D3, GFI1, HDAC4, NLRCS, RORA, SIIT1 and BCL6)
regulating mRNAs stabilities) levels.

The genomic (transcription regulation) and transcriptomic (miRs regulators are potential upstream drivers of cellular anergy both at

were part of the 51 transcripts that are reported as molecular

fl suppress transcripts mediating in

upregulation of miR-155-3p (Figure 4).

a potential suppressor of miR-155-3p expression) seem to favor

on the other hand, downregulation of JUN (which is

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identified TFs (JUN, SRF, SP1, NFYA, KLF4 and MAFB) showed

Predicted activations of signaling pathways and cellular processes. As suppressed or induced expression of associated transcripts does not necessarily correspond to the activation or inhibition state of the corresponding pathway or process, we determined an activation state (by calculating activation z-scores based on the expression values of associated transcripts) for each signaling pathway or cellular process that are significantly associated with the 460 post-RASP SEB-regulated transcripts. This approach is important to identify which pathway or cellular process may have been activated (has activation z-score >1.5) or inhibited (has activation z-score < -1.5). The corresponding z-score values were used to color the nodes of the corresponding pathway or cellular process (blue: negative z-score values or inhibited; and orange or

without pathological conditions) but also it lacked the mechanisms of

SEB-exposed post-RASP APCs and T-cells (Supplementary Figure S4). Inhibited apoptosis indicates that the immune function (of

Predictions based on the expressions of both upstream modulators and downstream target transcripts indicated inhibition of TCR signaling, interleukin-2 expression, NFKB1 and JUN transcription activities (Supplementary Figure S3a). Our prediction analysis also suggest activation of LGR4, RORA, SIRT1, GFI1, CTLA4, TSC22D3, SIGIRR, NR1H2, NRLCS and HDAC4, which are potential upstream suppressors of inflammation, chemotaxis, formation of productive immunological synapse, TCR signaling and NFKB transcription activities (Supplementary Figure S3b).

Predictions at the level of cellular processes showed inhibited cellular viability, recruitment, priming, stimulation, activation, proliferation (clonal expansion) and apoptosis (attenuation) of SEB exposed post-RASP APCs and T-cells (Supplementary Figure S4). Inhibited apoptosis indicates that the immune function (of SEB-exposed post-RASP leukocytes) was not only compromised at the protective level (compromised protection against infection or pathological conditions) but also it lacked the mechanisms of

Table 1. A selection of the top SEB-specific transcripts in post-RASP leukocytes (among the 460 transcripts) (list of the 460 transcripts is given in Supplementary Table S2)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Symbol</th>
<th>Description</th>
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<th>S2 vs C2</th>
<th>S2 vs S1</th>
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<td>BG336318</td>
<td>CD74</td>
<td>CD74 molecule, MHC complex, class II invariant chain</td>
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<td>-114.9</td>
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<td>BF913224</td>
<td>GBP1</td>
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<td>-38.6</td>
<td>-87.7</td>
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<td>BG035651</td>
<td>SOD2</td>
<td>Superoxide dismutase 2</td>
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<td>-5.8</td>
<td>-63.2</td>
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<td>NM_000575</td>
<td>IL1A</td>
<td>Interleukin 1, alpha</td>
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<td>-10.3</td>
<td>-50.8</td>
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<tr>
<td>AV759427</td>
<td>HLA-DPA1</td>
<td>MHC complex, class II, DP alpha 1</td>
<td>-60.1</td>
<td>-8.9</td>
<td>-46.2</td>
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<tr>
<td>BG288796</td>
<td>IL1RN</td>
<td>Interleukin 1 receptor antagonist</td>
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<td>-10.5</td>
<td>-42.8</td>
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<td>BF795451</td>
<td>WARS</td>
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<td>AF085224</td>
<td>SLCO1A2</td>
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<td>AL033353</td>
<td>PTGS2</td>
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<td>CXCL9</td>
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<td>2.2</td>
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<tr>
<td>AW966037</td>
<td>MDK</td>
<td>Midkine (neurite growth-promoting factor 2)</td>
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<td>D90729</td>
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<td>G alpha 2</td>
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Abbreviations: C1, control; C2, RASP; RASP, Rangers Assessment and Selection Program; S1, pre-RASP SEB exposed; S2, post-RASP SEB exposed; SEB, Staphylococcal enterotoxin B.

(Figure 5 and Supplementary Figure S3b), trans-membrane signal receptors (LRG, CTLA4 and SIGIRR) (Supplementary Figure S3b) and miRs (miR-132-5p and miR-155-3p) (Figure 5) were identified as upstream suppressive regulators of transcripts mediating inflammation, chemotaxis, antigen presentation, TCR signaling and nuclear factor KB (NFKB) families of TFs.

Post-RASP SEB-regulated miRs (Figure 1c) and computationally identified TFs (JUN, SRF, SP1, NFYA, KLF4 and MAEBF) showed regulatory interaction (Figure 4). Assessing the interactome between these factors and differentially regulated miRs along with their downstream targets, the expression of NFYA, SRF and SP1 seemed to be favored by the downregulation of their upstream regulator miRs (which otherwise tend to suppress the stability of transcripts of these factors) (Figure 4). For example, suppression of miR-1224-3p seems to result in upregulation of SP1, and SP1 in turn induces growth hormone binding proteins and suppresses transcripts mediating antigen presentation, chemotaxis and inflammation (growth hormones and their receptors are also shown to suppress inflammation). Upregulated miRs (miR-132-5p and miR-155-3p) appear to mainly suppress transcripts mediating inflammation and chemotaxis (Figure 4). On the other hand, downregulation of JUN (which is a potential suppressor of miR-155-3p expression) seem to favor upregulation of miR-155-3p (Figure 4).

The post-RASP SEB-suppressed transcripts targeted by SEB-regulated miRs and computationally identified factors (Figure 4) were part of the 51 transcripts that are reported as molecular features of cellular anergy. Our analyses suggest that these regulators are potential upstream drivers of cellular anergy both at the genomic (transcription regulation) and transcriptomic (miRs regulating mRNAs stabilities) levels.
controlling its responses (it lacked the ability to attenuate to homeostasis when it is no longer needed). The latter normally manifests in apoptotic control of clonally expanded cells and clearance of unnecessary molecules to bring them back to the homeostasis level.

Expression-based prediction analyses also showed inhibited positive acute phase response and C-X-C receptor 3 (CXCR3) signaling (Supplementary Figures S3a and b). Based on our analyses of the GSE13738 GEO data, the CXCR3 signaling, which includes the post-RASP SEB-compromised chemokines of APCs (CXCL9 and CXCL10), seem to have a critical role in an indirect activation of T-cells. On the other hand, the transforming growth factor (TGF)-beta receptor signaling, which is an upstream effector of TOB-SMAD signaling in T-cells, hypoxia signaling and glucocorticoid receptor signaling were predicted to be activated, though these pathways were significantly associated with both upregulated and downregulated transcripts (Supplementary Figure S5).

Inhibited cellular processes seem to be important features of anergic cells, which can be potentially driven by stress-regulated TFs and miRs. Prediction analyses based on the expression patterns of upstream modulators and downstream targets of core transcripts of anergy-related regulatory networks and pathways were converged, pointing to the anergic response of post-RASP leukocytes to SEB. Here we have identified features of stress-induced anergy: transcripts, potential upstream drivers, and pathways and cellular processes leading to or features of cellular anergy.

Post-RASP SEB exposure predictor variables
To identify transcripts specific to SEB exposure under the background of severe stress, we used two prediction algorithms, RF$^{18}$ and the NSC$^{19,20}$ that are designed for biomarker discoveries.

Using NSC and RF predictors on the post-RASP SEB-exposed data in comparison to other conditions (control, RASP and in the pre- and post-RASP leukocytes exposed to Y. pestis or dengue virus), we have identified SEB-specific transcripts under the background of battlefield-like stress. Some of the top hits in post-RASP SEB-regulated transcripts include the soluble chemokines (CXCL9, CXCL10, CCL8), the immunomodulatory enzyme indoleamine-pyrorle 2,3 dioxigenase (INDO), the growth factors CSF1 and CSF2 and other T-cell response genes.

Using NSC (Supplementary Figure S7), 33 probes representing 32 genes were identified to be specific to post-RASP SEB-exposed leukocytes (Supplementary Figure S8). The 32 post-RASP SEB-specific genes were significantly associated with positive regulation of differentiation, activation and proliferations of leukocytes, response to wounding, anti-apoptosis, inflammation, hemopoiesis, stress response and protein kinase inhibitor activity.
Figure S11 and Supplementary Table S9). Other pathways significantly associated with upregulated and downregulated transcripts include immune response signaling, growth factors, catabolic processes, circadian clock and transcription regulations (Supplementary Figure S11, Table 4 and Supplementary Table S8).

Some of the SEB-specific pathways (hypoxia and vascular leakage) were predicted to be activated, whereas others (coagulation, CXC3 signaling, platelet activation and MYC suppressive signaling) were predicted to be inhibited. The TGF-beta and SMAD signaling (which are critical for the TOB signaling pathway) were also predicted to be activated in SEB-specific transcripts (in both pre- and post-RASP leukocytes) (Supplementary Figure S12).

Table 2. Some of the top biological processes associated with post-RASP SEB-regulated transcripts

<table>
<thead>
<tr>
<th>GO ID</th>
<th>FDR q-value</th>
<th>No. of genes</th>
<th>Biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>2376</td>
<td>1.57E-40</td>
<td>83</td>
<td>Immune system process</td>
</tr>
<tr>
<td>6950</td>
<td>2.23E-15</td>
<td>75</td>
<td>Response to stress</td>
</tr>
<tr>
<td>19882</td>
<td>3.47E-13</td>
<td>16</td>
<td>Antigen processing and presentation</td>
</tr>
<tr>
<td>6935</td>
<td>1.16E-10</td>
<td>21</td>
<td>Chemotaxis</td>
</tr>
<tr>
<td>9611</td>
<td>1.91E-10</td>
<td>35</td>
<td>Response to wounding</td>
</tr>
<tr>
<td>45321</td>
<td>9.93E-10</td>
<td>23</td>
<td>Leukocyte activation</td>
</tr>
<tr>
<td>1819</td>
<td>4.06E-09</td>
<td>16</td>
<td>Positive regulation of cytokine production</td>
</tr>
<tr>
<td>51251</td>
<td>5.51E-09</td>
<td>16</td>
<td>Positive regulation of lymphocyte activation</td>
</tr>
<tr>
<td>6954</td>
<td>1.14E-08</td>
<td>25</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>2684</td>
<td>1.47E-08</td>
<td>23</td>
<td>Positive regulation of immune system process</td>
</tr>
</tbody>
</table>

Among the 32 probes, 11 (CCL8, CSF1, CSF2, CXCL9, HLA-DMB, INDO, LOC152415, PLEK, UBD, WARS and YWHAH) were specific to both pre- and post-RASP SEB-exposed leukocytes (Supplementary Figure S8). Of these, CXCL9 was the top predictor as confirmed by the qRT-PCR (Supplementary Figure S8). Of the 374 SEB-regulated transcripts, four transcripts (two downregulated: CXCL9, INDO; and two upregulated: CSPG2, TSC22D1) were assayed using individual qRT-PCR. The direction of expression in both the microarray and qRT-PCR matched for the four assayed transcripts, though the deviation of TSC22D1 was large across biological replica (Figure 7).

Among the 374 SEB-regulated transcripts, four transcripts (two downregulated: CXCL9, INDO; and two upregulated: CSPG2, TSC22D1) were assayed using individual qRT-PCR. The direction of expression in both the microarray and qRT-PCR matched for the four assayed transcripts, though the deviation of TSC22D1 was large across biological replica (Figure 7).

The four GEO data sets (GSE15571, GDS3399, GSE13738 and GSE15571) were analyzed to identify SEB-specific transcriptome signatures. We used both parametric and non-parametric four comparative tests on our data set (Supplementary Table S1) and two independent machine-learning classifiers (NSC and RF/varSelRF (variable selection random forest)) across five different data sets to identify SEB-specific biomarker transcripts even under the background of extreme battlefield-like stress.

We compared the SEB-exposed (pre- and post-RASP) groups with the control and RASP groups (at P ≤ 0.05 and 1.5-fold filter) to identify 374 transcripts that were specific to SEB exposure in both the pre- and post-RASP leukocytes (Figure 5a, Table 3, Supplementary Tables S1 and S7). The SEB-regulated 374 transcripts were not observed as differentially regulated in the pre- and post-RASP leukocytes that were exposed to Y. pestis or dengue virus (Supplementary Figure S10). Many (283) of the 374 transcripts were upregulated, whereas 91 were downregulated in both SEB-exposed pre- and post-RASP leukocytes.

Functional interaction networks of pathways significantly associated with SEB-regulated transcripts were related to hypoxia, vascular leakage (vascular endothelial growth factor (VEGF) and VEGFR signaling), cell adhesion (integrin activities and extracellular matrix organization), coagulation (platelet activation and coagulation signaling), activation of T-cells (CXCR3 signaling), stress responses (intron splicing, glucocorticoid receptor signaling, MYC repressive signaling) and apoptosis (Figure 6, Supplementary Table S9).

Abbreviations: FDR, false discovery rate; GO, Gene Ontology; RASP, Rangers Assessment and Selection Program; SEB, Staphylococcal enterotoxin B.

The four GEO data sets (GSE15571, GDS3399, GSE13738 and GSE4478) were used for our classification were carefully selected, each providing a unique feature (in addition to the SEB-induced genome-wide host-response commonality). The GSE15571 is an in vivo SEB exposure in SEB-sensitive (HLA-DR3 transgenic) mouse model, GDS3399 from peripheral blood mononuclear cells (PBMCs) treated with recombinant SEB or Staphylococcal enterotoxin I (to check if the SEB specific transcripts can also
discriminate SEB from its closely related toxin, serotype I), GSE13738 examines indirect activation of memory CD4+ T-cells using an in vitro system of bystander human T-cell activation (in the absence of TCR cross-reactivity), and GSE4478 gene expression response of PBMCs to various pathogens and toxins, including SEB, at different time points (providing the possibility of selecting features discriminating SEB from other select agents). Each of these GEO data sets was used to assess whether the identified features also were consistently specific to SEB under different conditions and under the background of other confounding factors.

Applying NSC and RF/varSelRF machine-learning algorithms on the four GEO data sets, we identified 29 genes that were common with the 374 SEB-specific transcripts (Figure 9). The 29 transcripts (common across our in-house generated data and 3 GEO data sets: GDS3399, GSE13738 and GSE15571) were specific to SEB in the presence of RASP stressors and were distinct from the expression patterns of pre- and post-RASP leukocytes exposed to dengue virus or Y. pestis (Figures 9 and 10 and Supplementary Figures S14 and S15). Also, these transcripts were specific to SEB in comparison to other pathogens or toxins (GEO data set: GSE4478) (Figure 10 and Supplementary Figure S15).

In addition to the seven SEB-specific classifier transcripts, we have identified SEB-specific classifier transcripts (WARS, CXCL9, MYO9B, CD200, TSPAN17, UBE2G1, SLC03A1 and PDCD4) from GEO data sets and our data (Figure 9).

Both sets of transcripts were cross-validated using machine-learning classifiers on the GEO data sets and our data, respectively, and found to be specific to SEB exposure under different pathophysiological conditions. Particularly, the transcript CXCL9 was significantly regulated in the four GEO data sets, and it was one of the top classifiers of SEB in all the data sets we run classification algorithms (Supplementary Figures S13–S19).

The hsa-miR-155* (hsa-miR-155-3p) miR was specifically upregulated in the SEB-exposed pre- and post-RASP groups and was the top SEB classifier miR in our miR data (Supplementary Figures S20 and S21). The SEB-induced hsa-miR-155-3p was identified as a potential upstream regulator of SEB-regulated transcripts, including CXCL10 (member of the CXCR3 signaling which is implicated for the indirect activation of T-cells by SEB) (Figure 8).

Among the top classifiers, CXCL9 was common in the analyzed data sets: our data and the four GEO data sets (Figure 10, Supplementary Figures S7–S9 and S13–S19). The CXCL9 has >88% AUC (area under the receiver-operating characteristic (ROC) curves), and >83% specificity and >93% sensitivity (in our data and GSE4478 GEO data; Supplementary Figure S13b). The miR miR-155-3p was the top classifier in our data, with 100% specificity and sensitivity (Supplementary Figure S21). Since we are underpowered in terms of miR data sample size (8 cases and
also, at the time of writing this paper, there were no SEB summaries of the experimental conditions of the GEO data sets are given under the 'Materials and Methods' section.

Activation of lymphocytes through secreted cytokines (interleukins and chemokines) without direct TCR ligation of SEB toxin

Comparative analysis of the GSE13738 (GEO data set) in which three types of cells were exposed to SEB (resting T-cells, bystander T-cells and directly activated T-cells) shows that a number of transcripts, including CXCL9, were upregulated in the bystander T-cells compared with resting and directly activated T-cells (Supplementary Figure S18). This observation suggests the critical role of CXCL9 (and other chemokine ligands of CXCR3) in the SEB-triggered activation of T-lymphocytes, even in the absence of direct antigen–TCR ligation.

Results from GSE13738 GEO data suggest that expression of the soluble chemokines (CXCL9 and CXCL10 belonging to the CXCR3 signaling) may induce activation of some subpopulation of T-cells without direct antigen–TCR ligation. Transcripts of CXCL9 and CXCL10 were also highly suppressed in SEB-exposed post-RASP leukocytes, potentially causing impaired indirect activation of (post-RASP) T-cells upon SEB exposure. Suppressed cell adhesion molecules may also contribute to the inhibited state of CXCR3 signaling, as these classes of molecules implicated as SEB-recognition molecules mediating stimulation of APCs to secret soluble chemokine ligands of CXCR3, which can indirectly activate T-cells. Cell adhesion molecules were among the significantly SEB-regulated transcripts (both in our data and in the four GEO data sets). T-cell-expressed cell adhesion molecules are also important part of the co-receptors complex for SEB-induced T-cell activation as evidenced by antibody blocking of intercellular cell adhesion molecule 1 (ICAM1), which led to inhibited production of pro-inflammatory mediators and inhibited T-cell proliferation. ICAM1 is also among the top classifier of SEB in our NSC classification of the GSE4478 GEO data (Figure 10, Supplementary Figure S13b).

The cellular anergy of the post-RASP leukocytes toward SEB may be contributed by suppressed expression of CXCL9 (including the other CXCR3 ligands) and ICAM1 in addition to suboptimal antigen presentation and inhibited TCR signaling (inhibited formation of productive immunological synapse).

DISCUSSION

Previously, we identified molecular mediators of compromised protective immunity caused by battlefield-like stress (RASP). Post-RASP leukocytes were anergic to the potent mitogen, SEB. Yet differential counts of post-RASP WBCs were within normal ranges. In this study, data from ex vivo SEB-exposed leukocytes (from both the pre- and post-RASP groups) were analyzed to identify
two sets of transcriptome signatures: (i) mediators of cellular anergy (induced by battlefield-like stress) to SEB, and (ii) SEB-specific markers both in pre- and post-RASP leukocytes. We have identified molecular mediators of anergy in post-RASP leukocytes, including potential upstream modulators, pathways and cellular processes, coalescing in forming anergy (molecular features and drivers of stress-caused cellular anergy) and molecular signatures of SEB exposure, including SEB-specific pathways and processes that were shared between the pre- and post-RASP conditions. The specificity of SEB-regulated transcripts to SEB exposure were further verified using four different GEO data sets (to have potential utility to discriminate SEB toxicity from stress and infections with other pathogens).

Stress-caused anergy of leukocytes towards SEB
Using multiple and independent analyses approaches (on data from the post-RASP SEB-exposed leukocytes), we identified new mechanistic and molecular evidences why leukocytes exposed to severe stress were anergic to SEB. We used these molecular evidences (in SEB-exposed post-RASP leukocytes) in connecting signaling pathways and cellular processes to features of cellular anergy.

Figure 5. (a) SEB signature transcripts in both the pre- and post-RASP leukocytes. Shown here are the 374 differentially regulated transcripts specific to SEB exposure compared with the unexposed pre- and post-RASP groups (at $P < 0.05$ and fold change $> 1.5$). (b) SEB-specific miRs in both pre- and post-RASP leukocytes. Five miRs were differentially regulated (three upregulated and two downregulated) in both the SEB-treated groups compared with the unexposed samples. Note: the use of miR name hsa-miR-720 was discontinued since 2 August 2012, according to the NCBI website (http://www.ncbi.nlm.nih.gov/gene/100302198), and there were no downstream targets for hsa-miR-720.
experimental replica of the assays. Availability of enough total RNA to carry out all the biological and pre- and post-RASP samples, and samples were selected based on among genes that showed consistent regulation by SEB in both the exposed and stress plus SEB) were used. Transcripts were picked replica samples from each group: control, SEB-treated, stress-

Comparison of transcript levels using qRT-PCR and cDNA microarrays for SEB-regulated genes, both in the pre- and post-RASP groups. CXCL9 and INDO were downregulated, whereas the other microarrays for SEB-regulated genes, both in the pre- and post-RASP groups. CXCL9 and INDO were downregulated, whereas the other

Prediction-based inhibition of TCR complex formation and transcription activities of NFKB1 and JUN (Supplementary Figure S3a) predicted activation of TGF-beta receptor signaling (Supplementary Figure S5a) (an upstream modulator of TOB-SMAD inhibitory activities of T-cell proliferation: G1 arrest), along with predicted inhibition of interleukin-2 expression, also confirms impaired formation of productive immunological synapse and suboptimal TCR signaling (Figure 11). These inhibited signaling pathways were further verified at the level of cellular processes. Predicted inhibitions of cellular processes explain why complete and differential counts of post-RASP WBCs were within normal ranges (inhibited expansion or absence of significant apoptosis), and yet they were not responsive to the potent mitogen, SEB (inhibited viability, stimulation and activation). Apoptotic response inflammatory responses of APC (monocytes) and induce immune tolerance. A number of upstream regulatory molecules (computationally identified TFs and differentially regulated miRs) were identified as potential upstream drivers of immune suppression leading to anergy. Enriched pathways also showed involvement of suppressed transcripts in cellular activation, mirroring their role in the manifestation of cellular anergy. Pathways and processes associated with suppressed transcripts signifies inhibited recruitment of APCs, inhibited formation of productive immune synapses and suboptimal TCR activation ultimately leading to curtailed priming and stimulation of T-cells and hence T-cell anergy. The post-RASP APCs with compromised chemokines and antigen presentation molecules possibly transduce T-cell anergy as shown in the case of anti-HLA-DR antibody-treated tolerogenic monocytes, and radiation-exposed dendritic cells transmit anergic behavior leading to hyposensitive T-cells.

Most suppressed transcripts were important mediators of protective immunity, and many of these seemed to be manifestation of the anergic state of SEB-exposed post-RASP leukocytes, for example, transcripts listed in Figure 1b. Some of the upregulated transcripts such as insulin-like growth factors (Figure 2) seemed to induce immune tolerance or anergy, insulin-like growth factors and their receptors are shown to suppress

### Table 3. Expression fold-changes of some of the top SEB-regulated transcripts both in the pre- and post-RASP leukocytes (of the 374 transcripts listed in Supplementary Table S7)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Symbol</th>
<th>Description</th>
<th>S1 vs C1</th>
<th>S2 vs C1</th>
<th>S1 vs C2</th>
<th>S2 vs C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4458648</td>
<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>−5.4</td>
<td>−77.7</td>
<td>−3.7</td>
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<td>AI688905</td>
<td>YWHAH</td>
<td>Tyrosine 3-monooxygenase</td>
<td>−4.5</td>
<td>−1.7</td>
<td>−6.4</td>
<td>−2.3</td>
</tr>
<tr>
<td>AW197081</td>
<td>CXCL9</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
<td>−2.8</td>
<td>−45.9</td>
<td>−2.7</td>
<td>−43.8</td>
</tr>
<tr>
<td>B256993</td>
<td>LOC152415</td>
<td>Similar to chemokine (C-C) receptor 1</td>
<td>−2.5</td>
<td>−4.4</td>
<td>−1.7</td>
<td>−3.0</td>
</tr>
<tr>
<td>AW408137</td>
<td>TFEC</td>
<td>Transcription factor EC</td>
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<td>−1.7</td>
<td>−2.8</td>
<td>−2.2</td>
</tr>
<tr>
<td>AKO26053</td>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>−2.2</td>
<td>−2.0</td>
<td>−2.3</td>
<td>−2.2</td>
</tr>
<tr>
<td>AB015331</td>
<td>INDO</td>
<td>Indoleamine-pyrolyse 2,3 dioxygenase</td>
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<td>−29.3</td>
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<td>−31.5</td>
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<td>XM.006526</td>
<td>CCL8</td>
<td>Chemokine (C-C motif) ligand 8</td>
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<td>−25.3</td>
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<td>−32.6</td>
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<tr>
<td>NM.013309</td>
<td>WARS</td>
<td>Trytophanyl-tRNA synthetase</td>
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<td>−55.8</td>
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<tr>
<td>AW007710</td>
<td>ANKRD12</td>
<td>Ankyrin repeat domain 12</td>
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<td>−1.9</td>
<td>−1.5</td>
</tr>
<tr>
<td>XM.012865</td>
<td>CASQ2</td>
<td>Calsequestrin 2 (cardiac muscle)</td>
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<td>−1.7</td>
<td>−1.7</td>
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<tr>
<td>NM.021138</td>
<td>HLA-DMA</td>
<td>MHC complex, class II, DM beta</td>
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<td>−3.6</td>
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<td>−2.9</td>
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<tr>
<td>AI740449</td>
<td>BMP2K</td>
<td>BMP2 inducible kinase</td>
<td>−1.9</td>
<td>−1.9</td>
<td>−1.6</td>
<td>−1.7</td>
</tr>
<tr>
<td>AF007128</td>
<td>C5F1</td>
<td>Colony-stimulating factor 1</td>
<td>−1.7</td>
<td>−6.9</td>
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</tr>
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<td>NM.003670</td>
<td>BHLHB2</td>
<td>Basic helix-loop-helix domain containing, class B, 2</td>
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<td>−1.5</td>
<td>−1.7</td>
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</tr>
<tr>
<td>NM.002993</td>
<td>CSF2</td>
<td>Colony-stimulating factor 2</td>
<td>−1.6</td>
<td>−1.3</td>
<td>−2.4</td>
<td>−2.0</td>
</tr>
<tr>
<td>AL043594</td>
<td>LRRC32</td>
<td>Leucine-rich repeat containing 32</td>
<td>−1.5</td>
<td>−1.5</td>
<td>−1.9</td>
<td>−1.9</td>
</tr>
<tr>
<td>BE644965</td>
<td>SEC11A</td>
<td>SEC11 homolog A (S. cerevisiae)</td>
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<td>2.1</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>AI144469</td>
<td>NDUFB3</td>
<td>NADH dehydrogenase 1 beta subcomplex, 3</td>
<td>2.7</td>
<td>2.2</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td>NM.006867</td>
<td>YEATS2</td>
<td>YEATS domain containing 2</td>
<td>3.1</td>
<td>1.8</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>AW953364</td>
<td>GPR126</td>
<td>G protein-coupled receptor 126</td>
<td>2.7</td>
<td>1.9</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>BE208451</td>
<td>LOC91316</td>
<td>Ig lambda-like polypeptide 1</td>
<td>2.1</td>
<td>1.5</td>
<td>2.2</td>
<td>1.6</td>
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<tr>
<td>BG05177</td>
<td>EVL</td>
<td>Enah/Vasp-like</td>
<td>2.2</td>
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<td>2.2</td>
<td>1.6</td>
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<td>BG036342</td>
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<td>NM.000032</td>
<td>QPCT</td>
<td>Glutaminyl-peptide cyclotransferase</td>
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<tr>
<td>BE748274</td>
<td>SORD</td>
<td>Sorbitol dehydrogenase</td>
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<tr>
<td>AL033529</td>
<td>FAM62A</td>
<td>Family with sequence similarity 62 member A</td>
<td>2.6</td>
<td>2.3</td>
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</tr>
<tr>
<td>NM.014025</td>
<td>TSC2D1</td>
<td>TSC2D2 domain family, member 1</td>
<td>2.3</td>
<td>3.4</td>
<td>1.7</td>
<td>2.5</td>
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<tr>
<td>AI762790</td>
<td>HTRA1</td>
<td>Htra serine peptidase 1</td>
<td>2.5</td>
<td>3.2</td>
<td>2</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Abbreviations: C1, control; C2, RASP; RASP, Rangers Assessment and Selection Program; S1, pre-RASP SEB exposed; S2, post-RASP SEB exposed; SEB, Staphylococcal enterotoxin B.
of non-naive (for example, memory) T-cells towards SEB is probably due to the action of SEB-induced secretion of soluble cytokines by APCs and may not be through direct antigen–TCR ligation. However, if the APCs are anergic and unable to secrete soluble cytokines, non-naive T-cells may not respond to SEB (resulting in an insignificant level of apoptosis), as seen in post-RASP leukocytes.

Taken together, the fore mentioned features were indicators of the anergic state of APCs, with curtailed cytokine secretion and antigen-presenting molecules, and anergic T-cells with compromised antigen receptor, co-receptors and TCR signaling molecules. It appears that RASP-induced suppression of transcripts mediating antigen presentation and chemotaxis may lead to the anergic state of APCs, which may be responsible for lack of both direct and indirect activation of post-RASP T-cells towards SEB.

SEB exposure signature transcripts were consistent despite physiological and pathological state of the host

Despite the complications due to suppressed immune responses seen at the end of the intensive battlefield-like stress, other groups of host response transcripts and miRs showed consistent differential expression towards SEB both in pre- and post-RASP leukocytes. Using parametric and non-parametric comparative tests, and two machine-learning classifiers, we identified SEB-specific transcripts from our data and used four other GEO data sets for cross-validation. Identified transcripts were found to be specific to SEB exposure under different pathophysiological conditions (Supplementary Figure S10) and may serve as candidate biomarkers for SEB exposure under severe stress and infection with other bio-threat agents.

Even though most of the initial phase of SEB toxicity (the ‘cytokine storm’) seemed to be inhibited in severely stressed RASP cadets, functional interaction networks and pathways suggestive of remnants of SEB-induced sepsis or toxic shock such as activated hypoxia, and vascular leakage, integrin activation, angiotensin maturation and TGF-beta receptor signaling and inhibited platelet activation, coagulation, glucocorticoid receptor signaling and cellular adhesion) persisted in both the pre- and post-RASP leukocytes (Supplementary Figure S12). But these may not translate to phenotypic manifestations of sepsis or toxic shock owing to the inhibited inflammatory response that are responsible for SEB-induced sepsis or toxic shock.

In spite of the suppressed upstream mediators of SEB toxicity (inflammatory cytokines), we still need SEB-specific biomarkers under the background of severe stress: (i) to assess the extent of further immune depletion (if any) caused by the additional insult due to infection or exposure to select agent; and (ii) to differentiate SEB exposure from stress or other infections in order to administer mitigating or support schedules. The SEB-specific transcripts were distinct from the expression patterns of infection with other select agents (such as Y. pestis and dengue virus) and can be good candidates as potential diagnostic markers of SEB exposure in the presence of severe stress. Particularly, CXCL9 and hsa-miR-155-3p were identified as top classifier features for SEB exposure under different pathophysiological conditions.

Possible scenarios and outcomes of SEB-induced stimulation of APCs and T-cells

Based on our observations and reports from others, we have proposed the following scenarios and outcomes from SEB-exposed lymphocytes. (i) Analyses of the GEO data sets indicate that both direct and indirect activation pathways may act synergistically (for example, the direct ligand–TCR activation signaling acting along with the CXCR3 receptor signaling) to result in the activation of naive T-cells culminating in toxic shock or sepsis. On the other hand, suppression of CXCR3 signaling (suggested by our analysis to be involved in an indirect activation) may lead to partial stimulation (anergy), as well as impaired formation of productive immunological synapse, and inhibited TCR signaling may lead to suboptimal direct activation (anergic T-cells). It seems that if either of these activation routes compromised, SEB exposure may lead to anergy. The outcome of SEB-induced response of non-naive APCs and T-cells (weather it leads to anergy or not) seems to depend on the type and nature of the previous stimulus: (ii) If the previous stimulus is non-specific (general) stimulus such as stress or radiation, the SEB exposure of such non-naive T-cells may lead to anergy. And also immune response molecules compromised by previous non-specific insult or general stimulus (such as stress, radiation or any immune compromising challenge, including antibody blocking) may lead to cellular anergy. It is less likely that such non-naive lymphocytes keep memory of the non-specific insult. It is also possible that the anergic state of post-RASP leukocytes to SEB is mainly due to suppression of the immune important transcript and may not necessarily be due to their non-naive state. It seems that anergy is a more likely outcome of suppressed molecular mediators of protective immunity. Hence, the immune-suppressive effect of these non-specific insults may be responsible for the anergy outcome rather than the non-naive natures of APCs and T-cells. (iii) However, if the previous stimulus is antigen specific resulting antigen-specific memory cells, then SEB can reactivate these.
memory T-cells leading to reactions similar to responses caused by the previous stimulus. Previous antigen-specific reaction leading to antigen-specific memory T-cells result in antigen-specific response as shown by others that SEB reactivates influenza virus-specific CD8+ memory cytotoxic T cells largely via direct SEB–TCR engagement. But it needs further investigation if host-response signaling related to viral infections are shared with those of SEB induced. From our analysis, we have observations suggesting the presence of overlapping or shared pathway between SEB toxin and intracellular pathogens such as viral infections as shown by the significantly enriched interferon signaling pathways (Figure 2), the chemokine ligands of CXCR3 (Groom and Luster) and shared classifiers of SEB with brucella (from NSC classification of GSE4478, Figure 10). If the ability of SEB to reactivate antigen-specific memory cells is independently proofed, then SEB can be modified to specifically target antigen-specific memory lymphocytes for use as a general immunization booster (instead of tinkering specific antigens and adjuvants).

Figure 7. Functional interaction network based upon the expression and significance of genes specific to SEB (among the 374 SEB-regulated transcripts). Larger nodes are more significant, and reds are upregulated in both the pre- and post-RASP SEB-exposed leukocytes compared with the unexposed pre- and post-RASP leukocytes; green were downregulated. Detailed list of pathways and processes are given in Supplementary Table S9.

Figure 8. Regulatory interaction networks among SEB-regulated miRs (both in pre- and post-RASP leukocytes) and target mRNAs. Highlighted nodes are miR nodes, and faded nodes are transcripts, which are not directly attached with miRs. The mature miR-155* (hsa-miR-155-3p) was upregulated in SEB-exposed pre- and post-RASP leukocytes.

Gene expression fold change

-2.5  0  2.5

log2 (fold change)

p-value

node size

0.1  0.74

Functional interaction network based upon the expression and significance of genes specific to SEB (among the 374 SEB-regulated transcripts). Larger nodes are more significant, and reds are upregulated in both the pre- and post-RASP SEB-exposed leukocytes compared with the unexposed pre- and post-RASP leukocytes; green were downregulated. Detailed list of pathways and processes are given in Supplementary Table S9.

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Our data also suggest that if individuals under severe battlefield-like stress are also exposed to SEB, they may not be affected by the mitogenic factor of SEB that results from the ‘cytokine storm’ of the initial immune reaction. Instead, they may be highly susceptible to any (opportunistic) infection due to further suppression of protective immunity. Under severe stress background, symptomatic recognition of SEB exposure or other select agents seems difficult and may cause more damage (without being suspected) on the health of soldiers under battlefield scenarios. That is, SEB-caused further suppression of transcripts mediating the initial inflammatory response to SEB toxicity may have more general implication. Individuals exposed to severe battlefield-like stress may not show a symptomatic high fever if they are also infected or poisoned with other select agents, pathogens or toxins.

Future directions and study limitations
The sample size for our data was moderate and, for GEO data sets, are small for reliable biomarker identification and cross-validation. Hence, the SEB-specific transcripts and miRs have to be taken as initial and yet positive indicators of the existence of persistent candidate biomarkers for SEB, even in the presence of severe stress, which were distinct from transcript signatures of bacterial (Y. pestis) and viral (dengue virus) infections. Such potential SEB biomarkers need further verification at the functional (protein) level and then in humanized mouse model of SEB.

This approach of identifying specific host response biomarkers (for toxins or pathogens) can be made very powerful by increasing the pools of samples (biological replica) and target probes used in such investigations. The identified host origin pathogen-specific or stress fingerprints have the potential to be used as specific markers if they are used in groups (similar to DNA fingerprinting patterns) on a chip, for example.29

This study was restricted to male subjects owing to the all male composition of the RASP (Army Rangers) population. Some of our findings may not be applicable to female subjects (as female subjects may react differently towards RASP or SEB or both).

MATERIALS AND METHODS
Subjects and blood collection
Our study subjects were 15 male informed and consented US Army RASP cadets. The 15 biological replicas, and at least three experimental replicas for each biological replica, was chosen to gain moderate effect size. Details
are given in another paper. Briefly, our subjects underwent extremely challenging three-phased training involving intense physical exertion, survival psychological stress and caloric and sleep restrictions.

Pre- and post-RASP leukocytes were collected and processed as mentioned in our previous work. Isolated leukocytes were treated with SEB toxin, Y. pestis or dengue virus.

Treatment of leukocytes with SEB, dengue virus serotype 2 (DEN-2) and Y. pestis

Treatment with SEB toxin. Exposure of leukocytes to SEB toxin was carried out as mentioned before. Briefly, ~1 x 10⁶ cells ml⁻¹ in RPMI 1640 with 10% human AB serum medium were treated with SEB to a final SEB concentration of 100 ng ml⁻¹ and incubated for 6 h at 37 °C and 5% CO₂.

Treatment with Y. pestis. Y. pestis strain and growth conditions: Y. pestis KIM5 Pgm-negative mutant bacteria laboratory stock was obtained from Dr Luther Lindler while at Walter Reed Army Institute of Research (Silver Spring, MD, USA), and bacteria were grown on Brain Heart Infusion (BHI, Invitrogen, Rockville, MD, USA) agar plates at 30 °C for 48 h. Preculture of Y. pestis bacteria was grown in BHI broth at 26 °C overnight in shaker incubator at 180 r.p.m., and bacteria were diluted 1/25 in fresh BHI medium. The organism was then grown at 26 °C for 5 h (OD₆₀₀ ~ 0.5) and used for infection. To determine the multiplicity of infection (MOI), we counted serial dilutions of the bacterial culture by plating on (BHI) agar plates followed by incubation at 30 °C for 48 h.

Leukocytes were suspended to ~1 x 10⁶ cells ml⁻¹ with 1 x balanced salt solution (BSS) without divalent cations (Gibco) and centrifuged at 350 x g for 15 min. Two ml of 1 x BSS was added, cells were incubated with Y. pestis to a final MOI of 1 (1 bacterium per cell) for 30 min and then diluted and centrifuged as before. Cell pellets were re-suspended in 7 ml of 1 x BSS and treated with Y. pestis to a final MOI of 1 and incubated for 4 h at 37 °C and 5% CO₂.

Treatment with DEN-2. DEN-2 (strain 16681, provided by Dr Alex Birk of Baruch S Bloomberg Institute, Doylestown, PA, USA) was propagated in Vero cells and collected at 4 days postinfection. Titers were determined using a plaque assay in Vero cells. All infections were carried out at a MOI of 5 (five viral particles per cell) for 1 h, after which virus was removed and cells were incubated for an additional 48–72 h.

Leukocytes were processed in the same manner as mentioned for Y. pestis treatment and incubated with DEN-2 at a MOI of 1 for 2 h preplating and then diluted and centrifuged as before. Cells were then incubated in plates for 4 h to a final DEN-2 MOI of 1 in 1 x BSS media at 37 °C and 5% CO₂.

Toxin- or pathogen-treated leukocytes (at the end of each incubation period) were centrifuged at 350 g for 15 min, treated with 2 ml TRIzol reagent (Invitrogen, Inc., New York, NY, USA) and were stored at −80 °C for RNA isolation.

RNA isolation

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s instructions. Quality assessment of isolated RNAs and related processes were done as described in another manuscript.

Microarray Chip preparation, printing and hybridization

Human cDNA array chips were processed, and microarray slides were hybridized following the published methods.

Quantitative real-time PCR

Reverse transcriptase reagent (iScript; Bio-Rad, Inc., Hercules, CA, USA) and RT-PCR master mix (QuantiTect SYBR Green PCR Kit; QIAGEN Inc., Valencia, CA, USA) were used for qRT-PCR on three to five biological replicates (based on sample availability) for each primer pair using iCycler iQ Real-Time PCR Detection System (Bio-Rad, Inc.). The custom oligonucleotide primers (Supplementary Table S10) were designed using the Primer3 software (www.basic.nwu.edu/biotools/Primer3.html) or were based on those from UniSTS (http://www.ncbi.nlm.nih.gov/genome/UniSTS) and Universal Probe Library Set for Human (Roche Applied Science, www.roche-applied-science.com). Parallel amplification reactions using 18S rRNA primers were done as a housekeeping gene control. Fold changes for each primer pair were calculated using the equation: $\Delta\Delta Ct = \frac{\Delta Ct_{\text{GOI}}}{\Delta Ct_{\text{HKG}}}$.
where ΔCt is the difference between the Ct values of control and treated samples of a given gene (gene of interest (GOI) or housekeeping genes (HKG)), and E is the primer efficiency obtained from the slope of the best fitting standard curve for each pair of primers.

Microarray data analyses
Background and foreground pixels of the fluorescence intensity of each spot on the microarrays were segmented using ImaGene 8.0 (BioDiscovery Inc., El Segundo, CA, USA) as published, including local background corrections, and sub-grid based Lowess normalizations were performed for each chip independently.

MicroRNA analysis
Expression profiles of miRs were assayed using Agilent’s human microRNA v3 microarray (Agilent Technologies, Inc., Santa Clara, CA, USA) consisting of 15 K targets representing 939 human and (human) viral miRs. Differentially expressed miRs were analyzed using Quicore Omics Explorer v2.3 (Quicore, Lund, Sweden) and GeneSpring GX 12.0 (Agilent Technologies, Inc.). Target transcripts of profiled miRs were identified using the TargetScan of GeneSpring GX 12.0 (Agilent Technologies, Inc.), and Ingenuity Pathway Analysis v9 (IPA) (Ingenuity Systems, Inc., Redwood City, CA, USA). Networks among differentially expressed miRs and their target miRNAs were constructed using IPA.

Identification of mRNA targets of differentially regulated miRs.
Messenger RNA targets of significant miRs were identified using TarBase, miRecords, and TargetScan databases via IPA (Ingenuity Systems, Inc.).

Molecular interactions and gene ontology networks
Cytoscape 3.2 (www.Cytoscape.org) and IPA v9 were used for analyses and visualization of enriched gene ontologies and pathways. Hypergeometric gene ontology enrichments were carried out using BiNGO 2.44 (Maere et al.) of Cytoscape. Pathways associated with stress, SEB-regulated transcripts and altered miRs were analyzed using Fisher’s exact test of IPA.

Identification of SEB-specific transcripts and potential anergy-inducer genes
Differentially expressed genes specific to SEB both in the pre- and post-RASP leukocytes were identified using GeneSpring GX v12.1 (Agilent Technologies, Inc.), Quicore Omics Explorer v2.3 (Quicore), Limma package of R (www.cran-r.org) and common probes from four different comparative tests. Genes potentially associated with anergy were identified using Agilent Literature Search v 1.77, BiNGO 2.44 (both Cytoscape plugins) and IPA.

Microarray significance analyses and identification of class predictors
Normalized expression data (from SEB-exposed leukocytes) were analyzed using four different comparative tests (moderated t-test, Welch’s test, Mann–Whitney unpaired test) (Supplementary Table S1) at P<0.05 without correction for multiple comparisons. These comparison analyses were selected to represent different variance conditions and assumptions of data distributions. The first three assumes normal distribution of the data, and the last does not assume normal distribution of the data. The unpaired test assumes equal variance, whereas Welch’s does not. Moderated test implements Bayes’ linear fitted normalization. Taking the common outputs of these tests potentially minimize type I error in lieu of adjusting for multiple comparison tests. Each of these comparative tests was used to identify two groups of differentially regulated transcripts: SEB-regulated transcripts in post-RASP leukocytes, and SEB-regulated transcripts in both the pre- and post-RASP groups. Transcripts that passed all four tests and showed a fold change of at least 1.5 were used for downstream functional and prediction analyses. Supervised class predictions were done using the NSC and RF, followed by selection of the top predictors using variable selection on RF (varSelRF).

Figure 11. Overly simplified signaling processes leading to inhibited T-cell stimulation, activation and proliferation. TGF-beta receptor signaling targeting TOB-SMAD complexes inhibits expression of interleukin-2, and also TOB may contribute to G1 arrest leading to inhibited T-cell proliferation. Together with non-productive immunological synapse (inhibited TCR signaling) and suppressed immune response TFs (NFKB1 and JUN) may gave rise to inhibition of T-cell activation and curtailed clonal expansion. SEB is shown to bind MHC-II and beta of TCR as well as to CD24 and B7 in case of productive immunological synapse formation. But in SEB-exposed post-RASP leukocytes, these were suppressed with predicted inhibition of their activities. Note: blue-colored nodes were inhibited and red/orange-colored were activated.
For selection of classifier variables (transcripts, microRNAs and TFs), we used Rmagpie, pamr, RF and varSelRF packages of Bioconductor (www.bioconductor.org) and R (www.r.org).

Microarray (expression) data-based prediction of TFs, regulatory binding sites and identification of downstream targets among genes with SEB-altered expression

Using a range of machine-learning (prediction) approaches such as the ensemble of decision trees or RF and NSC, we have identified SEB-specific features (mRNAs, miRs and TFs). For both identification and cross-validation of SEB-exposure predictors, randomization and permutation analyses of classifiers were conducted, and appropriate scores were generated.

Potential regulatory sites for genes with SEB-altered expression were identified using HumanGenome9999 (Agilent Technologies, Inc.) containing partial human genome sequences (9999 bp upstream regions for 21,787 genes). Statistically significant (P ≤ 0.05) common binding motifs (5–12 base pairs) of similarly expressed (k-clustered) genes were identified searching upstream regions (1–1000 nt upstream of transcription start sites). A number of other prediction and repository databases were used for motif search and identification of cognate TFs. These include MATCH (http://www.gene-regulation.com/pub/programs.html), TFSEARCH (http://www.cbcr.jp/research/db/TFSERARCH.html), DBD (www.transcriptionfactor.org), JASPAR (http://jaspar.cgb.ki.se), TRANSFAC 7.0 - Public (http://www.gene-regulation.com/pub/databases.html), ChipMAPPER (http://genome.ufl.edu/mapper/mapper-main), ConTra v2 (http://bioit.dmbr.ugent.be/contrav2), Pscan (http://159.149.160.51/pscan) and IPA. Prediction Z-scores and regulatory targets were generated using RF and displayed using IPA. TF–target interaction networks and pathways were generated and visualized using Cytoscape and IPA.

Specificity and sensitivity of SEB classifier transcripts

The ROC curves for the top SEB classifiers (CXCL9, INDO, CCL8, ICAM1, ERBB3, CXCL10, CDKN1A and HRGAI) were constructed using pROC and verification packages of R programming language (www.r-cran.org). AUCs, sensitivity and specificity were also determined using the same packages.

Identification of SEB-specific transcripts from four GEO data sets

Publicly available GEO data sets (GDS 3399, GSE 4478, GSE 13738, and GSE 15571) related to SEB exposure and our in-house generated expression data were analyzed to identify SEB-specific transcripts. Conditions and experimental design of the four GEO data sets are:

(i) GDS3399 is generated from the analysis of PBMCs treated with the recombinant staphylococcal superantigen toxins Staphylococcus enterotoxin I or SEB.
(ii) GSE15571 is DNA microarray data from a study of gene expression changes induced by SEB in a HLA-DR3 transgenic mouse model.
(iii) GSE13738 is designed to examine the possibility of the activation of memory CD4+ T-cells via mechanisms, which act independently of direct TCR ligation, using an in vitro system of bystander human T-cell activation in the absence of TCR cross-reactivity.
(iv) GSE4478 addresses gene expression responses of human PBMCs to various select agents and toxins, including SEB, at different time points.

Analyses summary

Four different comparative tests and two prediction approaches (NSC and RF) were used to identify SEB signatures from aggregated and yet different data sets (our in-house generated expression data and the four other GEO data sets).

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HUMAN SUBJECTS PROTECTION

Research was conducted in compliance with IRB-approved human subjects protocol —no.1014 for initial collection of samples and A-16815 for continuation of data evaluation. The human use approval was obtained from the local Protection of Human subjects Office and further approved by the Human Research Protection Office, Office of Research Protections, US Army Medical Research and Materiel Command, Fort Detrick, MD, USA.

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REFERENCES


Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)