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(54) Title: BIOMARKERS OF IMMUNE DYSFUNCTION IN RESPONSE TO CHRONIC STRESS, METHODS OF USE AND DIAGNOSTIC KITS

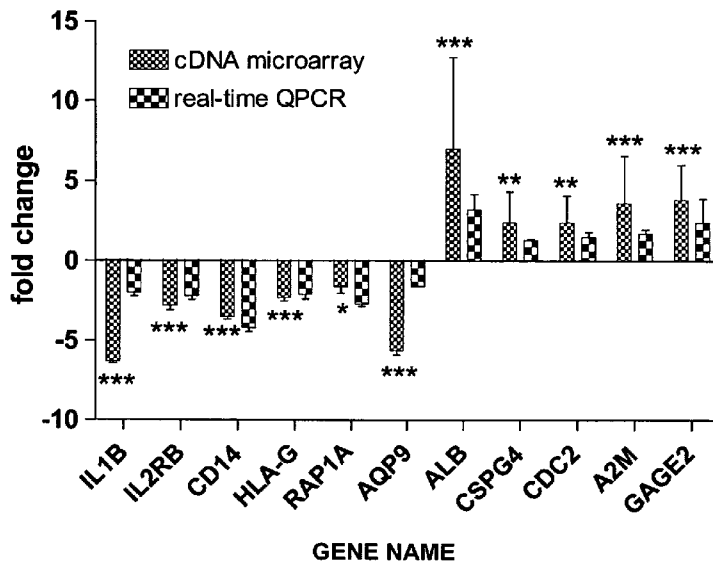
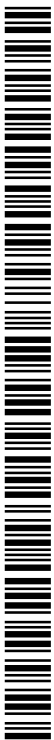


FIG. 4A

(57) Abstract: Diagnostic biomarkers for diagnosing immune suppression/dysfunction. The diagnostic biomarkers are genes and/or transcripts that are up or down regulated compared to normal expression when a subject has been stressed either mentally and/or physically. The invention also relates to a method of detecting comprised or suppressed immune response in a subject by comparing certain diagnostic biomarkers in the subject to a control set of diagnostic biomarkers.





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BIOMARKERS OF IMMUNE DYSFUNCTION IN RESPONSE TO CHRONIC STRESS, METHODS OF USE AND DIAGNOSTIC KITS

GOVERNMENT INTEREST

The invention described herein may be manufactured, used and licensed by or for the U.S. Government.

BACKGROUND OF THE INVENTION

1 FIELD OF THE INVENTION

The present invention relates to diagnostic biomarkers of immune suppression/dysfunction. The diagnostic biomarkers may be used to evaluate the capability of immune cells in subjects, and screen subjects for immune suppression/dysfunction in response to stress and/or pathogen exposure.

The present invention further relates to diagnostic biomarkers suitable for diagnosing Staphylococcus Enterotoxin B (SEB) exposure in a subject, and methods of using the same. These diagnostic biomarkers are suitable for diagnosing SEB exposure in the presence of comprised immune response or stress.

SUMMARY OF THE INVENTION

Diagnostic biomarkers for diagnosing immune suppression/dysfunction. The diagnostic biomarkers are transcripts that are up or down regulated compared to normal expression when a subject has been stressed either mentally and/or physically. The invention also relates to a method of detecting comprised or suppressed immune response in a subject by comparing certain diagnostic biomarkers in the subject to a control set of diagnostic biomarkers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a graph showing comparisons of before and after training of weights, temperatures and blood pressures of cadets;

Figure 1B) is a graph showing differential and complete leukocyte counts of trainees before and after training including complete and differential blood counts for pre- and post-Training subjects that include red blood cells, white blood cells, neutrophils and lymphocytes;, monocytes, eosophils and basophils;.

Figure 1C is a graph showing differential and complete leukocyte counts of trainees before and after training that include complete and differential blood counts for pre- and post-Training subjects included monocytes, eosophils and basophils;

Figure 2A is a table showing the analysis of differentially expressed genes in leukocytes of Ranger Trainees before and after Training;

Fig. 2B is a heat map that shows Hierarchical clustering of 288 genes that passed Welch's t-test with FDR correction ($q < 0.001$) and had expression alteration of ≥ 1.5 fold with each lane showing the 288 genes and their leukocyte expression level for each subject before (left panel) or after (right panel) training in comparison to human universal RNA;

Figures 3A-E are graphs showing correlation of real time PCR arrays with those from cDNA and oligonucleotide microarrays;

Figure 4A is a graph showing correlation of Real time QPCR and cDNA microarray analyses;

Fig. 4B) is a graph showing ELISA determination of plasma concentrations of proteins, and comparison with level of their transcripts from microarrays data;

Figure 5A is a heat map of expression patterns of immune response genes in leukocytes *in-vitro* exposed to SEB;

Figure 5B is a heat map of predicted and experimentally observed targets of RASP-regulated microRNAs;

Figure 5C is a sample PCA of differentially regulated microRNAs that passed Welch's Test ($p < 0.25$) and 1.3 fold change cut off;

Figure 5D is a map of regulatory interaction among stress-induced miRs, important transcription factors (NFkB1, NR3Ca, SATB1), inflammatory cytokines and antigen presenting molecules;

Figure 5E is a map showing seven stress-suppressed miRs targeting 48 mRNAs among differentially regulated mRNAs that passed $q < 0.001$ and 1.5 fold change;

Figure 6 is a graph showing predicted targets of miR-155 and let-7f families;

Figure 7A is a map of transcription factors predicted to be inhibited by battlefield stressors and their targets among stress-affected genes;

Figure 7B is a map showing transcription factors targeting RT-PCR assayed and differentially regulated genes;

Figure 8 is a map of functional network of differentially expressed genes connected by their sub-functions in the immune system;

Figure 9A is a map showing immune response transcripts involved in pattern recognition, viral, antibacterial and effector (humoral) responses;

Figure 9B is a diagram showing roles of stress down regulated genes in the cellular pathways of immune response;

Figure 9C is a diagram of action of secreted cytokines on other leukocytes;

Figure 10A is a diagram showing antigen presentation pathways;

Figure 10B is a diagram showing expression pattern of genes important for immunological synapse formation;

Figure 11 is a diagram showing Canonical pathways significantly associated with stress regulated genes that passed Welch's t-test and FDR correction ($p \leq 0.001$) and 1.5 fold change;

Figure 12 is a graph showing relative contribution (rank) of genes in classifying (predicting) control and stress groups of Ranger samples ranked using the Nearest shrunken centroid prediction approach;

Figure 13A is a graph showing stress specific genes differentiating stress from SEB, dengue virus and Yersinia pestis (plague) infections;

Figure 13B is also a graph showing stress specific genes differentiating stress from SEB, dengue virus and Yersinia pestis (plague) infections;

Figure 14 is a graph showing misclassification error rate vs threshold value; and

Figure 15 is a graph showing cross-validation of the prediction analysis of the invention.

DETAILED DESCRIPTION

Previous studies suggest that excessive or prolonged stress impairs protective immunity towards infection leading to increase susceptibility to illness. Comprehensive molecular explanations of the host's physiological stress response and the results of failed adaptation over time offer the potential to identify the debilitating pathophysiologic consequence of severe stress on health. More importantly, molecular approaches offer the opportunity to implement clinical strategies to differentiate immune impaired individuals from their normal counterparts.

Applicants examined the effects of long-term battlefield-like stressors of U.S. Army Ranger Training on genome wide expression profiles for biomarker identification of prolonged severe, stress-induced, compromised immune response. Applicants identified 59 differentially regulated transcripts using comparative Welch's T-test along with Bonferroni correction ($q < 0.01$) followed by 3-fold change. These 59 differentially

regulated transcripts are identified at Table 3 herein. Among the 59 differentially regulated transcripts identified, 48 were down regulated and 11 were up regulated. Most of the down-regulated transcripts were directly involved in protective immunity.

Differentially regulated transcripts identified and their cognate pathways were confirmed using quantitative real-time PCR arrays. Antigen preparation and presentation, chemotaxis, inflammation, and activation of leukocytes were among overrepresented immune response processes that were significantly associated with suppressed transcripts. Differentially regulated transcripts identified or genes from their corresponding pathway can serve as diagnostic biomarkers to differentiate/identify individuals with stress-induced immune suppression. cDNAs of some of these transcripts can be electrochemically tethered in the wells of micro- or nano-chips for quick diagnosis purpose.

Diagnostic biomarkers within the scope of the present invention for use in identifying or screening individuals for immune suppression/dysfunction include five (5) or more, seven (7) or more, or ten (10) or more of the 59 differentially regulated transcripts identified herein or genes from their corresponding pathway. For example purposes, Applicants provide herein a subset of 14 of the 59 transcripts that can be used as a single batch of biomarkers (see Table 3A and 3B). The five (5) or more, seven (7) or more, ten (10) or more or twenty (20) or more of the differentially regulated transcripts or genes from their corresponding pathway may, for example, be selected from these. It is understood to one of ordinary skill in the art that there may be additional biomarkers, not yet identified, that can be used to screen individuals for immune suppression/dysfunction. This invention is not limited to the 59 biomarkers listed in Table 3.

These diagnostic biomarkers would be useful to diagnose immune suppression/dysfunction in a subject due to stress. The present invention further relates to diagnostic kits for use in screening immune function of a subject, where the kit employs the diagnostic biomarkers identified herein.

Applicants further conducted studies on the effect of stress on a patient's ability to respond to other pathogens. More specifically, Applicants studied the effect of Staphylococcus Enterotoxin B (SEB) on host response gene expression profiles, and identified genes that showed consistent differential expression towards SEB whether or

not the host had been exposed to stress. These transcripts or genes from their corresponding pathway were SEB-specific (independent of the physiologic and pathologic status of the host), and may serve as diagnostic markers of SEB exposure.

Therefore, this invention proposes a simple test to identify the capability of immune cells to respond to pathogenic agents in military personnel. This biomarker profile would allow for a semi-quantitative method to evaluate the immune system in terms of gene expression.

Transcriptomic characterization of immune suppression from battlefield-like stress

This invention identifies changes in transcriptome of human due to battlefield-like stress. Thorough understanding of stress reactions is likely to produce better strategies to manage stress, and improve health¹. Stress modulates gene expression, behavior, metabolism and immune function²⁻⁵. Chronic physiological and psychological stresses are major contributors of stress-induced suppression of protective immunity. For example, chronic stress impairs lymphocyte proliferation, vaccination efficacy⁶⁻⁹, NK cell activity, resistance to bacterial and viral infection¹⁰, and increases risk of cancer¹¹.

Yet, comprehensive descriptions of molecular responses to stress are needed to fully understand modulated networks and pathways, and hence to reduce and prevent pathophysiological effects of intense and prolonged stresses.

Here we report gene expression changes occurring in leukocytes collected from Army Ranger Cadets before and after eight-week Ranger Training. Ranger cadets are exposed to different and extreme physical and psychological stressors of Ranger Training Course, which is designed to emulate extreme battlefield scenarios: sleep deprivation, calorie restriction, strenuous physical activity, and survival emotional stresses – pushing cadets to their physical and psychological limits. The Ranger population provides a rare opportunity to study intense chronic battlefield-like stress, and to contribute to the understanding of intense chronic stress in general. Ranger Training has been shown to impair cognitive function, cause significant declines in 3,5,3'-triiodothyroxine and testosterone, and increase cortisol and cholesterol^{12, 13}.

Transcriptomic alterations, in this study, were assayed using cDNA microarrays. Results were corroborated with oligonucleotide, microRNAs, and real-time QPCR arrays,

and were confirmed using Quantitative RT-PCR and ELISA. Analyses of functional and regulatory pathways of differentially altered transcripts revealed suppression of immune processes due to battlefield-like stress. Some of stress induced microRNAs, and a number of stress inhibited transcription factors were found to regulate or be modulated by many compromised immune response transcripts. Suppressed immune response genes remained suppressed even after exposure of post-stress leukocytes to mitogenic toxin, SEB. This impaired activation is a clear indicator of anergy, and compromised protective immunity.

Results

Ranger Trainees experience an average daily calorie deficit of 1000-1200 kcal, restricted and random sleep of less than 4 hours per day, strenuous and exhaustive physical toiling and emotional survival stressors. Five of the initial fifteen Trainees enrolled in our study were replaced with five others due to attrition (to maintain 15 study subjects at both time points). All study subjects had complete and differential blood counts performed, and were observed for infections and injuries. By the end of training, Trainees showed significant average weight loss, decreased body mass index and diastolic blood pressure, and significant increase in average body temperature and systolic blood pressure (Fig 1A); and they showed metabolite patterns typical of severe stress. The vertical lines show the ranges of cell counts. (Normal Ranges are WBC $5 - 12 \times 10^3/\text{mm}^3$; NEU $2 - 8 \times 10^3/\text{mm}^3$; LYM $1 - 5 \times 10^3/\text{mm}^3$; MON $0.1 - 1 \times 10^3/\text{mm}^3$; EOS $0.0 - 0.4 \times 10^3/\text{mm}^3$; BAS $0.0 - 0.2 \times 10^3/\text{mm}^3$.)

Differential and complete blood counts showed small but significant differences between pre- and post-Training cells, yet all were within normal ranges (Fig. 1B and 1C). To normalize for cell count differences, equal number of pre- and post-Training leukocytes were used for isolation of RNA, and equal amounts of isolated RNAs were used for microarrays, and RT-QPCR assays.

As shown in Figures 1B-1C, differential and complete leukocyte counts of soldiers before and after RASP are presented. Differential and complete blood counts for pre- and post[RASP subjects included red blood cells (RBC), white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS) and baseophils, (BAS). Using comparative t-test, only RBC ($P < 0.006$) and BAS ($p < 0.02$)

were significantly changed (reduced) after RASP. The ranges of cell counts including RBC and BAS (shown by the vertical lines) were within normal ranges. Normal ranges are WBC $5-12 \times 10^3 \text{ mm}^{-3}$; NEU $2-8 \times 10^3 \text{ mm}^{-3}$; LYM $1-5 \times 10^3 \text{ mm}^{-3}$; MON $0.1-1 \times 10^3 \text{ mm}^{-3}$; EOS $0.0-0.4 \times 10^3 \text{ mm}^{-3}$; BAS $0.0-0.2 \times 10^3 \text{ mm}^{-3}$.

Transcriptome profiling of Pre- and Post-Training Leucocytes

We used three transcriptome profiling techniques to cross-validate our findings: cDNA and oligonucleotide microarrays, and quantitative real time PCR arrays. Expression profiles were done on total RNAs isolated using two different methods: Trizol (Invitrogen, Inc) and PAXgene, (Qiagen, Inc).

cDNA microarrays analyses

To analyze gene expression profiles of leukocytes of Ranger Cadets collected before and after eight-week Training, we used custom cDNA microarrays that contained ~10 000 well-characterized cDNA probes of 500 to 700 base pairs representing ~9 000 unique human gene targets. Welch's (unpaired unequal variance) t-test along with false discovery rate (FDR) correction was used on normalized expression data to identify 1 983 transcripts that were significantly changed ($q \leq 0.05$), with 1 396 showing ≥ 1.5 fold change in expression level between pre- and post-Training samples (Table 4). Among 1 396 differentially regulated genes, 288 genes Fig. 2B were significantly changed at $q \leq 0.001$, and 87 of these were differentially regulated by > 3 -fold change. Of these 87 genes, 72 were down-regulated, and 68 of 72 genes have direct role in immune response, including 23 of the 25 most down-regulated genes. These results strongly suggest that Ranger Training stressors suppress the immune response, and this finding was corroborated by functional and pathway enrichments.

Functional enrichments of significantly regulated genes using both hypergeometric test (FDR correction, $q \leq 0.05$), and Fishers exact test identified the immune system as the most affected biological process. Apoptosis, stress response, response to wounding, metabolism, hormone receptor signaling (peptide and steroid), cell cycle and unfolded protein response signaling were also significantly associated with altered transcripts. Yet, immune system process was most significantly over-represented ($q < 1.7E-16$), and was associated with 177 differentially regulated genes. Of the 177

genes, 151 were down-regulated, and 26 were up-regulated. Further functional enrichment of the 151 genes indicated that these genes were significantly associated with microbial recognition, inflammation, chemotaxis, antigen presentation, and activation of lymphocytes, mast cells and macrophages (Tables 1). The 26 Up-regulated immune response genes were associated with response to steroid hormone stimulus, regulation of leukocyte activation, complement activation, negative regulation gene expression, and negative regulation of phosphorylation (Table 1).

Table 1: Functions significantly associated with differentially regulated immune response genes that passed Welch's t-test and FDR correction ($q < 0.05$ and showed > 1.3 fold change in post RASP leukocytes compared with pre-RASP leukocytes.

GO-ID	Function	Gene symbol
Functions of down-regulated immune response genes		
45321	leukocyte activation	MICA,CD8A,CD8B,ELF4,TLR4,ADA,CD74,CD93,CD2,FCER1G,CD4,SYK,IL4,KLF6,PTPRC,CD3D,IL8,CD3E,RELB,SLAMF7,CD40,LAT,LCK,CD79A,LCP2
6954	inflammatory response	CXCL1,ITGAL,TNF,TLR2,NFKB1,ITGB2,TLR4,CCL5,CD97,CC L20,KRT1,IL1B,IL1A,CEBPB,IL8,IL1RN,GRO3,CD40,CCL18,CD180,C8G,SCYA7,CCL13,CCR7,CYBB,CCR5,CRH,CD14
19882	antigen processing and presentation	HLA-DQB1,MICA,CD8A,HLA-DRB1,RELB,HLA-C,FCGRT,HLA-B,HLA-G,CD74,B2M,FCER1G,HLA-DPA1,HLA-DPB1,HLA-DOB,AP3B1,HLA-DRA
46649	lymphocyte activation	IL4,PTPRC,KLF6,MICA,CD3D,CD8A,ELF4,CD3E,CD8B,RELB,CD40,SLAMF7,CD74,ADA,LCK,CD2,CD4,CD79A,SYK
30097	hemopoiesis	IL4,PTPRC,KLF6,CD3D,LYN,HCLS1,RELB,IFI16,MYH9,CD164,CD74,LCK,CD4,SPIB,CD79A,MYST1,SYK,MYST3
52033	pathogen-associated molecular pattern recognition	PF4,CHIT1,TLR2,TLR4,SCYA7,CD14,PF4V1,CLP1,TICAM1,FPR L1,FPR1
6935	chemotaxis	IL4,CXCL1,C5AR1,IL8,GRO3,ITGB2,PF4,CCL5,CCL18,SCYB5,SCYA7,CCL13,CCR7,CCR5,PPBP,CCL20,IL1B,FCER1G,SYK
42110	T- cell activation	PTPRC,MICA,CD3D,CD8A,CD3E,CD8B,ELF4,RELB,CD74,ADA,LCK,CD2,CD4,SYK
2274	myeloid leukocyte activation	LAT,IL8,CD93,RELB,FCER1G,TLR4,LCP2
50778	positive regulation of immune response	PTPRC,MICA,SLK,FYN,KRT1,TLR2,FCER1G,CD79A,C8G,SYK
6959	humoral immune response	PSMB10,CD83,ST6GAL1,TNF,HLXB9,POU2F2,KRT1,AIRE,C8G
1934	positive regulation of phosphorylation	TNF,CCND3,LYN,HCLS1,IL1B,CD4,SYK
45087	innate immune response	CYBB,IL1R1,SARM1,CLP1,KRT1,TLR2,TLR4,SLAMF7,CD180,C8G
2252	immune effector process	PTPRC,LAT,MICA,FCN2,KRT1,FCER1G,SLAMF7,CD74,C8G
30593	neutrophil chemotaxis	IL8,FCER1G,IL1B,ITGB2,SYK
7229	integrin- signaling	LAT,ITGAL,ITGAX,ITGB2,MYH9,ITGAM,SYK

45058	T- cell selection	CD3D,CD4,CD74,SYK
1816	cytokine production	IL4,CD4,ISGF3G,CD226,LCP2
6909	phagocytosis	CD93,FCN2,CLP1,FCER1G,CD14
2460	somatic recombination for adaptive response	IL4,RELB,FCER1G,TLR4,CD74,C8G

Functions associated with up-regulated immune response genes

48545	response to steroid hormones	CEBPA,CAV1,HMGB2,PRKACA,CD24
42326	negative regulation of phosphorylation	CAV1,PRKACA,INHA
6956	complement activation	C4B,C3,C2
10817	regulation of hormone levels	DHRS2,ACE,FKBP1B
43434	response to peptide hormones	HHEX,PRKDC,PRKACA
2762	negative regulation of myeloid leukocyte differentiation	FSTL3,INHA
32088	negative regulation of NFkB activity	POP1,SIVA
51384	response to glucocorticoids	CEBPA,CAV1,PRKACA
16481	negative regulation of transcription	CEBPA,HHEX,CAV1,HMGB2,FST,HEL LS

Oligonucleotide microarrays

Gene expression alterations in leukocytes of Rangers before and after Training were also analyzed using PAXgene RNA isolation and oligonucleotide microarrays representing 24 650 human gene probes. This different RNA isolation procedure and microarray assay again showed that the immune system was most significantly affected process. Normalized expression levels were analyzed using Welch's t-test ($p < 0.05$, without multiple correction), and fold change filter (≥ 1.5 fold). Among 1570 genes (that passed these filters), 104 genes were associated with the immune response processes including microbial recognition, chemotaxis, inflammation, antigen presentation, and T-cell, B-cell and NK-cell activations (Figs. 3A-E & Table 5).

Real Time Quantitative PCR Array

We used real time quantitative PCR (QPCR) arrays to confirm differential expression of genes identified by cDNA and oligonucleotide microarrays, and to survey additional immune related genes. Assay results of PCR arrays that contained more than 160 genes in antigen presentation and NFkB signaling pathways (RT² ProfilerTM PCR Arrays, SABioscience, MD) verified down-regulation of 116 immune response genes, consistent with microarray data (Tables 3A, 3B and 4). The vast majority of the genes important for microbial pattern recognition, inflammation, antigen presentation, T-cell

activation and transcription factors related to immune response were suppressed across cDNA, oligonucleotide and PCR arrays (Fig. 3A and 3B)

Referring to Figs. 3A-E, genes are shown that are associated with pattern recognition receptors (Fig. 3A); inflammatory response (to scale the graph, fold changes of -15.2 and -23.8, labeled * and **, respectively, were assigned a values of ~5 and 6, respectively (Fig. 3B); antigen preparation and presentation (*fold change: -12.3; assigned value ~ -5 for scaling the graph) (Fig. C); transcription factors (* fold change: -12.6; ** fold change: -12.3; *** fold change: -14; these were adjusted to around -5 for scaling the graph) (Fig. 3D); T-cell activation, differentiation and proliferations. Expression profiles of genes shown in pannels A-E were assayed using SABiosciences RT² Profiler™ (PAHS 406 and PHAS 25) PCR Arrays, cDNA microarrays, and oligonucleotide microarrays (Fig. 3E). Total RNA samples were isolated using Trizol reagents for cDNA microarray analysis, and total RNA samples used for PCR and oligonucleotide arrays were isolated from blood samples collected in PAXgene tubes. (Note: PCR arrays were carried out on subjects participated throughout our study, and fold changes for these figures were calculated on data from both round subjects).

Real Time Quantitative PCR

Additional quantitative real-time PCR assays were carried out using specific primer pairs to confirm 10 representative genes among 1396 significantly altered genes shows number of genes that passed Welch's t-test at different q-values (FDR corrected p-values) and Fold Change cut-offs) (Fig. 2A)(Table 2). Real-time QPCR Assayed and confirmed genes included IL1B, IL2RB, CD14, HLA-G, RAP1A, AQP9, ALB, CSPG4, CDC2, A2M, and GAGE2. Individual real-time QPCR results confirmed and validated these differentially expressed genes identified by cDNA arrays (Fig. 4A).

Fig. 4A shows Real time PCR reactions for each gene were carried out with three or more replicates. The microarray data were from Trizol RNA isolation and cDNA microarrays (* p-values < 10⁻⁵, ** p-values < 0.0002, *** p-value < 0.02). The p-values given here were taken from the microarray analyses obtained after FDR correction.

Genes associated with microbial recognition

Genes associated with microbial pattern recognition were significantly suppressed in post-Training leukocytes (Table 5, & Tables 1 & Fig. 5D). These genes include Toll-like receptors (TLR 2, 3, and 4), CD14, CD93, chitinase 1 (CHIT1), formyl peptide receptor 1 (FPR1), formyl peptide receptor like 1 (FPRL1), dicer1 (DICER1), cleavage and polyadenylation factor I subunit (CLP1), platelet factor 4 (PF4), platelet factor 4 variant 1 (PF4V1), toll-like receptor adaptor molecule 1 (TICAM1), and myeloid differentiation primary response gene 88 (MYD88). TLR6 was down-regulated but it did not pass the FDR correction filter.

CD14, along with TLR4/TLR4 and TLR2/TLR6, recognize lipopolysaccharides and peptidoglycans, respectively. TLR3, CLP1 and DICER1 bind to double stranded viral RNAs. TLR9 and CD93 recognize unmethylated CpG dinucleotides of bacterial DNA, and patterns of apoptotic cells, respectively. FPR1 and FPRL1 bind bacterial N-terminal formyl-methionine peptides. CHIT1 recognizes fungal and pathogens with chitin patterns. PF4 and PF4V1 recognize patterns of plasmodium and tumor cells. TICAM1 and MYD88 are important cytosolic adaptor molecules of microbial pattern recognitions. Transcripts of these genes were down-regulated suggesting a compromised innate immune response with regard to microbial recognition.

Genes associated with chemotaxis and inflammation

Stress suppressed transcripts associated with chemotaxis and inflammation included interleukins (IL1A, IL1B, IL4, IL8,), interleukin receptors (IL1R1, IL1RN, IL2RB, IL10RA,), chemokine (C-X-C motif) ligands (CXCL1,), chemokine (C-C motif) ligands (CCL13, CCL18, CCL20), tumor necrosis factor alpha (TNF α), TNF receptor super-family members 1B, 10B and 10C (TNFRSF1B, TNFRSF10B and TNFRSF10C), TNF superfamily members 3, 8, (LTB, TNFSF8), complement component 8 gamma (C8G), cytochrome b-245 beta (CYBB), CD97 and interferon gamma receptor (IFNGR2) (Tables 1 & 5).

Genes associated with activation of myeloid leukocytes

Tables 1 & 5 show suppressed transcripts associated with activation of mast cells and macrophages. These included toll-like receptors (TLR4), TNF, LAT, lymphocyte

cytosolic protein 2 (LCP2), SYK, CD93, and IL4 RELB. Suppressed genes associated with inflammatory responses (IL1, CD14, INFR1) were also significantly associated with activation of myeloid cells. Differentiations of myeloid leukocytes were significantly associated with interferon gamma inducible proteins 16 and 30 (IFI16), myosin heavy chain 9 (MYH9), IL4, Spi-B transcription factor (SPIB), NFkB3, MYST histone acetyltransferases (MYST1 and 3), TNF, PF4, hematopoietic cell-specific lyn substrate 1 (HCLS1), V-src-1 Yamaguchi sarcoma viral related oncogene homolog (LYN) and V-maf (musculoaponeurotic fibrosarcoma) oncogene homolog b (MAFB). Down-regulation of hemopoietic transcription factors (MAFB and HCLS1) and CSF1R may indicate less viability of myeloid cells to expand or to replenish. Suppression of mRNAs of these genes suggests poor activation, differentiation and proliferation of myeloid leukocytes in response to infection, and hence poor innate and adaptive immune responses.

Genes associated with antigen presentation

Genes associated with antigen preparation encompass MHC classes (I & II), CD1s, B-cell co-receptors and integrins (Tables 1 and 5). Transcripts of MHC class I (HLA-B, HLA-C, HLA-G, beta-2-microglobulin (B2M)), MHC class II (HLA-DRB1, HLA-DRA, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, CD74, HLA-DOB), B-cell co-receptors (CD79A, CD79B), Ig heavy constant gamma 1 (IGHG1), Ig heavy constant alpha 1 (IGHA1), MHC class I polypeptide related sequence A (MICA), adaptor-related protein complex 3 beta1 (AP3B1), intercellular adhesion molecules 1, 2 and 3 (ICAM1, ICAM2, ICAM3) were down-regulated implying poor antigen preparation and presentation, and hence impaired adaptive immune response.

Genes associated with activation of lymphocytes

Suppressed transcripts associated with T-cell activation, differentiation and proliferation included TCR co-receptors (CD4, CD8 α , CD8 β , CD3 ϵ , CD3 δ , CD247), linker for activation of T cells (LAT), TCR signaling molecules [protein kinase c theta (PRKCQ), protein tyrosine phosphatase receptor type C (PTPRC), C-SRC tyrosine kinase (CSK), spleen tyrosine kinase (SYK) lymphocyte specific protein tyrosine kinase

(LCK)], integrins CD2, CD44, integrin alpha L, M and X (ITGAL, ITGAM, ITGAX), and cyclin D3 (CCND3) (Tables 1 & 5).

Interleukin 4, SYK, PRKCD, CD40, PTPRC, cyclin-dependent kinase inhibitor 1A (CDKN1A), Kruppel-like factor 6 (KLF6), SLAM family member 7 (SLAMF7), and killer cell Ig-like receptor three domains long cytoplasmic tail1 (KIR3DL1) were significantly associated with activation, differentiation and proliferation of B-cells, and NK-cells (Tables 1 & 5).

Transcription factors associated with immune responses

Transcription factors that are important regulators of immune response genes were down-regulated. Suppressed factors included nuclear factor kappa B family (NFkB1, NFkB2, RELA, RELB), interferon regulatory factors 1, 5, 7, 8 (IRF1, IRF5, IRF7 and IRF8), signal transducer and activator of transcription (STAT2, STAT6), and SP transcription factors (SP1, SP140) (Tables 1 & 5). In addition, transcription factors GA binding protein alpha (GABPA), POU class 2 homeobox 2 (POU2F2), p53 (TP53), p53 binding protein 1 (TP53BP1), early growth response 2 (EGR2), splicing factor 1(SF1), and hypoxia inducible factor 3 and alpha subunit (HIF3A) were down-regulated. Up-regulated transcription factors included hepatocyte nuclear factor 4 alpha (HNF4A) hepatic leukemia factor (HLF), sterol regulatory element binding transcription factor 2 (SREBF2) transcription factor AP-2 alpha (TFAP2A), transcription factor 7-like 2 (TCF7L2)and NF-kappa-B inhibitor-like 2 (NFKBIL2) (Tables 1 & 5).

ELISA Assays of Plasma Proteins

Plasma concentrations of insulin-like growth hormones 1 and 2 (IGF1 and IGF2), prolactin (PRL), tumor necrosis factor alpha (TNF), and enzymatic-activity of superoxide dismutase 1 (SOD1) were determined by ELISA to examine gene expression alterations at the protein level. Relative quantities of proteins, and levels of transcripts profiled by cDNA and oligonucleotide microarrays were compared (Fig. 4B). Reduced IGF1 has been shown to be a biomarker of negative energy balance under conditions of multiple Ranger Training stressors¹², and IGF1 transcript in leukocytes and protein in plasma are reduced after Training. Plasma concentration of PRL was up-regulated while

transcriptome profiling showed down-regulation by microarray analyses, suggesting differential regulation of prolactin at transcription and translation levels.

Fig. 4B shows plasma concentrations of prolactin (PRL), insulin-like growth factors I and II, tumor necrosis factor alpha (TNF α) and enzymatic activity of superoxide dismutase 1 (SOD1) were assayed using nine biological replicates and three experimental replicate samples corresponding to each biological replicate for each of these proteins. The IGF-I depletion is consistent with other studies that measured its plasma concentration on similar subjects¹³ (* p-values < 0.003, ** p-values < 0.04, *** p-value < 0.0002).

Response of Leukocytes to ex vivo Treatment of Staphylococcal enterotoxin B

Staphylococcus enterotoxin B (SEB) is a superantigen, and a potent T cell activator known to induce proinflammatory cytokine release in vitro¹⁴. Leukocytes of Ranger Trainees collected before and after Training were challenged ex vivo with SEB and immune response transcripts were analysed. In pre-Training leukocytes, SEB toxin induced majority of immune response genes (Fig. 5A). However, in post-Training leukocytes, stressed suppressed immune response genes showed no sign of re-activation even after ex vivo exposure to SEB (Fig. 5A). Rather SEB seemed to further suppress expression of many of these transcripts. Impaired response of post-Training leukocytes to SEB is consistent with suppression of immune response pathways and networks revealed by transcriptome analyses.

In Fig. 5A, expression of immune response genes in leukocytes exposed ex vivo to SEB is shown. Leukocytes isolated from whole blood were treated with SEB ($\sim 10^6$ cells ml⁻¹ in RPMI 1640 and 10% human AB serum at a final concentration of 100 ng ml⁻¹ SEB). Total RNA was isolated using Trizol and expression levels were profiled using cDNA microarrays. Shown here are the 151 RASP-suppressed immune response genes that passed Welch's test and FDR correction ($q < 0.05$). (a) Lanes left to right: pre-RASP samples not exposed to SEB (control), pre-RASP samples exposed to SEB, post-RASP samples not treated with SEB, post-RASP samples exposed to SEB. For comparative visualization purpose, expression values of the other groups were transformed against the Pre-RASP control samples (black lane). Heat map of the same

data without transformation is given in the supplement. (b) Expression values in SEB exposed leukocytes (in both the pre- and post-RASP conditions) were compared with the corresponding SEB untreated groups (pre-RASP control and post-RASP stressed groups). (c) Heat map of 151 immune response genes in SEB treated groups (in both pre- and post-RASP leukocytes) clustered after subtraction of the corresponding baseline responses (cluster after subtraction of their expressions in the corresponding untreated groups shown in lane (b)). Lane c clearly shows pour response of post-RASP leukocytes towards SEB exposure compared with pre-RASP leukocytes.

MicroRNA Arrays

Differentially regulated microRNAs (miRs) in pre- and post-Training samples were assayed using Agilent's human microRNA chip containing ~15 000 probes representing 961 unique miRs. Comparison of 535 miRs (that passed normalization and flag filters) using Welch's t-test at $p < 0.1$ with a 1.3 fold change cutoff gave 57 miRs (Fig. 5C). MicroRNA target scan was used to identify high-prediction and experimentally proven targets of these differentially regulated miRs. Among up-regulated miRs, hsa-miR-155 ($p < 0.08$) and hsa-let-7f ($p < 0.1$), were shown to target many suppressed transcripts, including transcription regulators of genes important for dendritic cell maturation and glucocorticoid receptor signaling. Expression of miR-155 was suppressed in pre-Training samples exposed to SEB, but it was induced in post-Training samples treated with SEB (Fig 6). Other stress-induced miRs were predicted to have regulatory connection with stress-affected inflammatory cytokines, antigen-presenting molecules, and transcription regulators of genes involved in immuneresponse (Fig. 5D). Stress-suppressed miRs—miR-662, miR-647, miR-876-5P, miR-631, miR-1296, miR-615-3P, and miR-605—have a number of regulation targets among stress-regulated genes involved in NFkB activation pathways (Fig. 5E). In Fig. 5E enriched pathways: IL-7 and IL-8 signalings, and NFkB activation pathways are shown. No targets were identified for two highly suppressed miRs, miR-1910 and 1909*.

Figure 6 shows predicted targets of miR-155 and hsa-let 7f families. In Fig. 6, expression levels of hsa-miR-155 and hsa-let-7f in pre-RASP (control), post-RASP

(stressed) and pre-RASP exposed to SEB, and post-RASP exposed to SEB groups. Sequences of mature miR-155 and let-7f are also shown.

See also Fig. 5B for predicted and experimentally observed targets of RASP-regulated micro RNAs. 57 microRNAs passed Welch's T-test ($P < 0.1$) and 1.3 fold change. Most (46 of 57) miRs were downregulated, and 11 miRs were upregulated in post-RASP leukocytes.

Expression Data based Prediction of Transcription Factors and Target Genes

Computational & data analyses tools, and databases (see Materials and Methods) were used for empirical and predictive association of transcription factors (TFs) and their regulatory targets among stress-altered genes. Activated or inhibited TFs, common regulatory sites of target genes, and prediction z-scores of identified TFs were computed based on 1369 differentially regulated genes obtained from cDNA array data (Table 2). TFs at the top of stress-inhibited list (IRF7, RELA, NFkB1, RELB, CREB1, IRF1, HMGB1 & CIITA) and their differentially expressed targets (**Table 2**) were found to be involved in inflammation, priming of adaptive immune response, and glucocorticoid receptor signaling (Fig. 7A and Fig. 7B). Fig. 7B shows transcription factors targeting RT-PCR assayed and differentially regulated genes. Both MYC and NR3C1 were predicted to be activated (according to prediction z-score value, which were > 2.5). The top function associated with these targets were apoptosis of leukocytes, hematopoiesis, proliferation of blood cells, immune response; and top pathways are given in the table immediately below in Table A:

Table A

Network showing MYC and NR3C1 targets among immune response genes

Symbol	EntrezID	FC	Family	Drugs	Entrez Gene Name
ACTB	60	-1.73	other		actin, beta
AKT1	207	-3.13	kinase	enzastaurin	v-akt murine thymoma viral oncogene homolog 1
CASP1	834	-1.58	peptidase		caspase 1, apoptosis-related cysteine peptidase
CD44	960	-2.33	other		CD44 molecule (Indian blood group)
CDKN1A	1026	-2.92	kinase		cyclin-dependent kinase inhibitor 1A (p21, Cip1)
HLA-A	3105	-2.63	other		major histocompatibility complex, class I, A

ICAM1	3383	-2.09	transmembrane receptor		intercellular adhesion molecule 1
IL8	3576	-1.53	cytokine		interleukin 8
ITGAM	3684	-2.02	other		integrin, alpha M (complement component 3 receptor 3 subunit)
ITGB2	3689	-1.29	other		integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
MYC	4609		transcription regulator		v-myc myelocytomatosis viral oncogene homolog (avian)
NFKB1	4790	-1.56	transcription regulator		nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKB2	4791	-1.44	transcription regulator		nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NR3C1	2908		ligand-dependent nuclear receptor transcription regulator	rimexolone, NF-kappaB decoy	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
RELA	5970	-1.72			v-rel reticuloendotheliosis viral oncogene homolog A (avian)
TLR2	7097	-3.14	transmembrane receptor		toll-like receptor 2
TNF	7124	-3.74	cytokine	adalimumab	tumor necrosis factor
TNFAIP3	7128	-3.74	enzyme transmembrane receptor		tumor necrosis factor, alpha-induced protein 3
TNFRSF10B	8795	-1.71		tigatuzumab	tumor necrosis factor receptor superfamily, member 10b

Regulatory sites for a number of transcription factors including SP1, CREB1, ATF6, cEBP, and binding sites for the defense critical – NFkB transcription factors complex, and stress response sites (STRE) were among common regulatory motifs identified for some of stress-suppressed genes, STRE site being predicted to be regulated by MAZ and MZF1. Stress activated factors included GFI1, MYC, FOXM1, GLI2, MAX and HNF1A (Table 2), and these factors induced genes important for hormone biosynthesis and suppressed immune related genes.

Fig. 7A shows transcription factors predicted to be inhibited by battlefield stressors and their targets among stress modulated genes. Shown here are transcription factors predicted to be inhibited by battlefield stressors (Table2) and their targets among 288 stress-affected transcripts (filtered using Welch's t-test and FDR, $q < 0.001$, and > 1.5 fold change). Enriched function and pathways of these transcripts include activation and proliferation of leukocytes, maturation of dendritic cells (DCs), communication

between innate and adaptive immunity, glucocorticoid receptor signaling and antigen presentation pathway.

Table 2: Predicted transcription factors and targets identified among 1396 genes that passed Welch's t-test, FDR correction ($q \leq 0.05$) and 1.5 fold change cutoff.

TF	z-score	p-value	target molecules in dataset
activated transcription factors and targets			
GF11	3.1	4.1E-04	CASP1,CDKN1A,CEBPA,GUSB,ICAM1,IL1A,IL1B,IL8,IRF1,MMP7,NFKB1,NFKB2,RELA,RELB,TRAF3
MYC	3	1.6E-17	ACAT1,ACTB,ACTN1,AFP,AHCY,ALB,BCAT1,BCL6,BIN1,BIRC2,BIRC5,CAPN2,CASP1,CASP10,CAV1,CCND1,CCND3,CD44,CD48,CDC20,CDH2,CDK1,CDK11A/CDK11B,CDKN1A,CEBPA,COL14A1,COL1A1,CSPG4,CYFIP2,DDX11/DDX12,DDX3X,DDX5,DUSP6,EDN1,EGR2,EIF2S2,F2,F3,FBN1
FOXM1	2.8	4.8E-05	BIRC5,CCND1,CDC20,CDK1,CDKN1A,CENPA,CENPF,FOXM1,KDR,KIF20A,MMP2,PLK4,TGFB2
GLI2	2.7	3.2E-02	CCL5,CCND1,CDK1,CDKN1A,IL1B,ITGB1,KRT1,KRT17,PTCH1,SFRP1
MAX	2.4	1.4E-03	BCL6,CDKN1A,EDN1,FTH1,ID1,KLF6,LAMP2,MTHFD1,PDGFRB,SERINC3,TSC2,UBE2C
HNF1A	2.1	3.6E-02	ABCC2,AFP,AKR1C4,ALB,ANPEP,APOB,AQP9,BCL6,C2,CCND1,DPP4,DUSP6,FAM107B,FBXO8,FGA,FGB,G0S2,GNB2L1,HNF4A,IGFBP1,KIF20A,KIR3DL1,LCAT,MTHFD1,NAPA,PKK1,PFKP,PIH1D1,PRLR,PZP,SERPINA7,SLC26A1,SLCO1A2,SSTR4,TRA@,UQCRC2,UROD
inhibited transcription factors and targets			
CEBPB	-2.2	1.3E-11	ACTG2,ALB,C3,CCL5,CCND1,CD14,CDKN1A,CEBPA,CEBPB,COL1A1,CP,CSF1R,CTSC,CXCL5,CYP19A1,DDX5,DEGS1,FTL,HLA-C,HP,HSPD1,ICAM1,ID1,IGFBP1,IL1B,IL1RN,IL8,INMT,IRF9,LAMC1,LCP2,LYN,MGP,MIA,PCTP,PDGFRA,PEA15,PLAUR,PPARD,PRKCD,PR
JUNB	-2.3	2.8E-03	ACLY,CAV1,CCND1,CD68,CDC20,COL1A1,CYP19A1,FTH1,MMP2,MVD,NCF2,PTBP2,RELB,SCD
CIITA	-2.4	1.4E-07	B2M,CCND1,CD74,COL1A1,HLA-B,HLA-DOB,HLA-DPA1,HLA-DQA1,HLA-DQB1,HLA-DRA,HLA-DRB1
POU2AF1	-2.6	3.4E-03	BCL6,CCND3,CD79A,CD79B,IGHA1,IGHG1,LCK,TRAF3
STAT1	-2.8	8.2E-12	A2M,B2M,BIRC5,BTG1,C3,CASP1,CASP2,CASP4,CCL5,CCND1,CCND3,CCR7,CD14,

			CDKN1A,DPP4,FCER1G,GATA3,GBP1,GZMB,HLADRB1,ICAM1,IFIT3,IL1B,IL8,IRF1,IR5,IRF7,IRF9,LY96,NFE2,PDGFRB,PF4,PRL,PSMB10,PTGS2,SMAD7,SOCS3,STAT2,TLR4,TN
FOXO3	-2.8	1.8E-04	BIRC5,CCND1,CDKN1A,CTGF,CYR61,FOXM1,FOXO1,GPX1,IER3,IGFBP1,IL8,NAMPT,NOS3,SATB1,SOD2,TNFRSF1B,TXNIP,UBC,UBE2C
SPI1	-2.9	1.5E-10	ACTB,CCR7,CD14,CD68,CD79A,CD79B,CEBPA,CSF1R,CYBB,DUSP6,FCER1G,FLI1,FTH1,GNB2L1,GPX1,IGL@,IL1B,IL1RN,IRF9,ITGA5,ITGAM,ITGB2,MCL1,MM P2,NCF2,P2RY1,PIK3CG,PTGS2,PTPRC,RELA,TK1,TLR2,TLR4
IFI16	-3	1.8E-04	CCL5,CCND1,CDKN1A,EDN1,GPX1,ICAM1,IFI16,IL1B,IL1RN,IL2RB,IL8,RPA3,STAT2
HMGB1	-3.1	1.6E-06	CD83,CDKN1A,CXCL5,HLADRB1,ICAM1,IL1A,IL1B,IL8,MIA,PTGS2,RELB,SIRT1,TLR2,TLR4
IRF1	-3.2	1.0E-06	B2M,CASP1,CASP2,CCL5,CCND1,CDKN1A,CYBB,EIF4A3,HLA-G,IFIT3,IL1B,IL8,IRF1,IRF5,IRF7,IRF9,LTB,NFE2,PF4,PSMB10,PTGS2,SOCS7,STAT2,TRIM22
CREB1	-3.4	1.5E-08	ARPC3,ATP6V0B,BTG2,CCND1,CD3D,CD4,CD68,CD79A,CDH2,CEBPB,CYP19A1,CYP51A1,CYR61,DIO2,EDN1,EGR2,FN1,FOSB,GALNT1,HERPUD1,HLA-DRA,HLA-G,HMGCS1,HSPA4,IL1B,INHA,IRF7,MCL1,PDE3B,PDGFRA,PER1,PRL,PTGS2,SCD,SLC16A1,SLC2A4,SOD2,TF,TFAP2A,UPP1
NFKB1	-3.4	1.9E-08	A2M,ADORA1,AKR1B1,B2M,BTG2,CCL5,CCND1,CDKN1A,COL2A1,CYBB,FANCD2,GATA3,GNB2L1,ICAM1,IER3,IFNGR2,IGHG1,IL1B,IL1RN,IL8,IRF1,LTB,MICA,NFKB1,NFKB2,PLK3,POU2F2,PRKACA,PTGS2,RELA,RELB,SOD2,TK1,TLR2,TNFAIP3
RELA	-3.7	3.1E-17	A2M,ABCG2,ACTA2,AFP,B2M,BIRC2,BTG2,CAV1,CCL5,CCND1,CCR7,CD44,CDKN1A,COL2A1,CXCL1,CYBB,CYP19A1,DIO2,EDN1,EWSR1,F3,GDF15,HLA-B,ICAM1,IER2,IER3,IFNGR2,IGHG1,IL1A,IL1B,IL1RN,IL8,INPP5D,IRF1,IRF7,L
IRF7	-3.9	3.0E-03	CASP4,CCL5,GBP1,IFI16,IFIT3,IRF1,IRF9,ISG20,ITGAM,MCL1,NAMPT,PSMB10,STAT2,TLR4,TMPO,TRIM21,TRIM22

Abbreviation: TF, transcription factor/regulator. Regulation z-score; P-value overlap.

Summary

Most immune response genes were down-regulated in post-Training leukocytes compared to pre-Training leukocytes. Functional enrichment of these down-regulated genes revealed their involvement in microbial pattern recognition, cytokine production and reception, chemotaxis, intercellular adhesion, immunological synapse formation, regulation of immune response, and activation and proliferation of immune cells (Fig.8).

Figure 8 demonstrates a functional network of differentially expressed genes connected by their sub-functions in the immune system. The network shows enriched functions of genes involved in immune responses: activation of immune cells, differentiation, proliferation, antigen presentation, and infection directed migrations. Genes involved in all these functions were down regulated by the Ranger Training stressors. Each node represents a category of gene ontology of the pathways of the immune system. Node sizes are proportional to the number of genes belong to each category according to gene ontology, and intensity of node indicate significance of hypergeometric test after Bonferroni correction ($q \leq 0.05$). The pattern circles show more significant the enrichment than the solid white circles.

Our data suggest that stress induced suppression of microbial patterns of innate immunity (Fig.9A) may impair infection-directed maturation, activation, inflammatory response, motility, and proliferation of myeloid cells (Fig.9B & 9C) These impaired innate cells may also fail in priming the adaptive arm of immune response (Fig.10A).

In Figure 9A, shows altered immune response genes involved in pattern recognition, viral, antibacterial effector (humoral) responses.

In Fig. 9B, roles of stress down regulated genes in the cellular pathways of immune response are shown. Flat-ended arrows represent suppression of the corresponding pathway (biological process). Microbial recognition receptors, inflammatory cytokines (IL1, IL1R, TNF α , CD40), chemotaxis (IL8, IL8R, RANTES, CCR5, CCR7), lymphocyte recruitment (IL4, IL12), and production of effector molecules (INF γ , IL2, IL2RB) were down regulated after Ranger Training

In Fig. 9C, actions of secreted cytokines on other leukocytes are shown. Impaired activity of suppressed IL-1 other myeloid cells to secrete antimicrobial effector molecules; depleted concentration gradient of IL-8 providing curtailed guidance to neutrophils and NK cells to sites of infection, and suppressed IL-8 and RANTES unable to recruit and induce maturation of dendritic cells (for antigen presentation); suppressed transcripts important for T-cell polarization (cellular or humoral) may mean deprivation of the host under stress from having protective immunity.

Figs. 10A and 10B show stress-suppressed genes involved in antigen presentation and synapse formation. Fig. 10A shows antigen presentation pathways: This KEGG

pathway taken via IPA was colored for the 288 stress-regulated genes that passed Welch's t-test, FDR correction ($q \leq 0.001$) and changed by ≥ 1.5 fold (between pre- and post-Training groups).

Fig. 10B shows expression of genes important for immunological synapse formation; suppression of transcripts important in antigen preparation, presentation, chemotaxis, intercellular binding, antigen reception, and downstream signaling (the gene labeled solid nodes) may have impaired formation of productive immunological synapse, and hence the poor response of post-Training leukocytes to SEB challenge although SEB toxin is presented without undergoing intracellular preparation, antigen presenting molecules of the synapse were suppressed.

Adaptive cells' antigen receptors, co-receptors, signal transducers, intercellular adhesion molecules, and chemokine receptors were highly suppressed (Fig.10B). It is less likely that these stress-debilitated lymphocytes can be activated, proliferated, differentiated, and clonally expanded to amount defense response against infections as confirmed by impaired response of post-Training leukocytes to SEB exposure.

Discussion

Suppression of transcripts of critical immune response pathways, and regulatory networks are consistent with impaired innate and adaptive immune responses, including cellular and humoral immunity, as a result of battlefield-like stress.

Down-regulation of transcripts involved in Toll-like receptor, and chemokine and chemokine receptor signaling pathways indicate suppressed inflammatory response, impaired maturation of antigen presenting cells (APCs), impaired affinity maturation of integrins, and impaired migration, extravasation & homing of APCs and T-cells to nearby draining lymph nodes or infection sites.

Antigen preparation and presentation was the most suppressed pathway among immune response processes (Fig. 11). Fig. 11 shows canonical pathways significantly associated with stress-regulated genes that passed Welch's t-test and FDR correction ($p \leq 0.001$) and 1.5 fold change. Numbers on the right side indicate total # of genes in the pathway. Suppression of antigen presentation, T-cell receptor and integrin pathways indicate lack of productive immunological synapse formation (poor MHC-restricted

antigen recognition and T-cell activation), leading to impaired adaptive and effector immune responses. Particularly, suppression of transcripts involved in cytoskeleton-dependent processes (chemokine guided migration, integrin-mediated adhesion, immunological-synapse formation, cellular polarization, and actin-microtubule aided receptor sequestration and signaling) curtails the dynamic cellular framework of T-cell activations (Fig.10).

Unlike reports of differential regulations of Th1 and Th2 type responses observed in college students on the day of a stressful examination¹⁵, and in caregivers of chronically sick relatives¹⁶, our data suggest that battlefield-like stressors impair not only Th1 but also Th2 type responses as shown by suppressed transcripts of TLR2 and 4, and the cytokines IL4, IL4R and IL10RA in post-Training leukocytes. Suppression of inflammatory molecules (e.g., IL1A & 1B, and IL1R1, TNF members and TNF receptors, and NFkB class of factors), and Th2 classes of cytokines show features of battlefield-like stress that are distinct from acute and psychological stresses.

Previously miR-155 is reported to be proinflammatory. MiR-155(-/-) mice are highly resistant to experimental autoimmune encephalomyelitis¹⁷, and show suppressed antigen-specific helper cell, and markedly reduced articular inflammation¹⁸. Here, miR-155 transcripts were elevated in post-Training leukocytes (with or without SEB exposure), but its expression was suppressed by SEB in pre-training leukocytes (Fig. 6).

It seems that miR-155 is anti-inflammatory in humans exposed to stress and SEB toxin. Regulatory connection of miR-155 to many of stress-suppressed inflammatory cytokines may indicate its involvement in regulation of these cytokines, and glucocorticoid receptor elements, and modulate maturation of antigen presenting cells under battlefield-like stress.

Poor response of post-Training leukocytes to SEB ex vivo challenge is consistent with suppressed expression of MHCs, T-cell receptors, co-receptors and integrins which are important for activations of APCs and T-cells. Overall, our results clearly demonstrated that battlefield-like stressors suppress a broad spectrum of immune system process. This suppression of broad categories of immune response pathways may explain why chronically stressed individuals show poor vaccine responses and susceptibility to infections.

Figures 12- 15 were generated from nearest shrunken centroid prediction. The Nearest Shrunken Centroid (NSC) classifier (predictor) is a robust ²¹⁻²² way of identifying genes specific to a certain agent in the presence of other infections or conditions ²³. NSC was used successfully to identify cancer biomarkers ²⁴⁻²⁵ and other disease sub-typing ²⁶⁻²⁷.

Figure 12 is a graphical representation of Nearest shrunken centroid (NSC) ranked genes when stressed and control groups compared. The length of the horizontal bars indicate the absolute value of the score (the bigger the absolute value of the score the longer the horizontal bar, and the direction indicate the gene expression direction (left oriented bar indicate down-regulated and right oriented bar up-regulated genes). Here only two groups are compared and the opposite orientations of the horizontal bars indicate that these genes discriminate between the two compared groups.

Figures 13A and 13B are graphical representations of NSC algorithm identified genes which can discriminate stress and other conditions (dengue virus exposure, *Yersinia pestis* or plague infection and SEB toxin exposure; and also unexposed control group). The direction and length of the horizontal bars is given in Fig 12. As shown in Figs. 13 A&B there are 69 genes including 10 specific to the other pathogens that are shown by the corresponding horizontal bars.

Figure 14 shows misclassification error versus threshold (cut-off) values, each line representing each condition. Here the stress (black line) has the lowest misclassification error beyond the threshold value of around 2.6. That means, genes ranked from one to about 260 can discriminate stress from other conditions (shown here). But in our case we took the top ranked genes (even though many more can also be potential stress biomarkers).

Figure 15 is a graph showing that identified genes were cross-validated to ascertain that they were not included by mere chance. The more open circles (under stress) being separated from other shapes indicate that these genes discriminate stressed individuals from other patients (samples collected from patients exposed to other pathogens or control group). Though there is shown in Fig. 15 only 114 samples, the total number of samples used for prediction were 141.

Conclusion

Suppressed expression of genes critical to innate, humoral and cellular immunity is an indicator of compromised protective immunity as confirmed by impaired response of post-Training leukocytes to SEB challenge. Numbers and ratios of different subpopulations of leukocytes being within normal ranges, our observation (of anergic leukocytes of severely stressed individuals) draws some caution on current diagnostic practice of counting immune cells to ascertain integrity of the immune system, and its ability of protection against infection.

On the basis of suppressed inflammatory molecules and pathways, we hypothesized that exposure to battlefield-like and similar stresses may make exposed individuals less susceptible to autoimmune diseases, and sepsis; yet they may easily succumb to toxin or infection since their protective immunity already depleted.

Characterization of molecular signatures of stress pathologies can potentially reveal biomarkers and new pharmacologic targets for improving adaptation to stress and preventing stress-induced pathogenesis. Results such as ours together with proteomic analyses may yield novel preventative, prognostic and therapeutic opportunities to intervene the negative consequences of stress on health.

Materials and Methods

Blood sample collection

Whole blood (from each subject) was drawn in Leucopack tubes (BRT Laboratories Inc., Baltimore, MD) before and after the eight-week Training, and immediately spun at $200 \times g$ for 10 minutes. The concentrated leukocyte layer (buffy coats) was collected and treated with TRIzolTM reagent (Invitrogen, Carlsbad, CA) for RNA isolation and then stored at -80°C . Differential and complete blood counts (CBC) were obtained immediately after blood collection using a hemocytometer, and subsequently using an ABX PENTRA C+ 60 flow cytometer (Horiba ABX, Irvine, California). Blood samples were also collected in PAXgeneTM Blood RNA Tubes (VWR Scientific, Buffalo Grove, IL) for direct RNA isolation.

RNA Isolation

For cDNA microarray analysis, total RNA was isolated using the TRIzol™ reagent according to the manufacturer's instructions. The RNA samples were treated with DNase-1 (Invitrogen, Carlsbad, CA) to remove genomic DNA and were re-precipitated by isopropanol. The TRIzol™ isolated RNA was used in cDNA microarrays analysis¹⁹. For oligonucleotide microarrays, total RNA was isolated using PAXgene tubes following the manufacturer's protocol. The PAXgene tube contains a proprietary reagent that immediately stabilizes RNA at room temperature (18-25°C) without freezing. Isolated RNA samples were stored at -80°C until they were used for microarray and real time PCR analyses. The concentration and integrity of RNA were determined using an Agilent 2000 BioAnalyzer (Palo Alto, CA) according to manufacturer's instructions. The ArrayControl RNA Spikes from Ambion (Austin, TX) were used to monitor RNA integrity in hybridization, reverse transcription and RNA labeling.

cDNA synthesis, labeling, hybridization and image processing

RNA was reverse transcribed and labeled using Micromax Tyramide Signal Amplification (TSA) Labeling and Detection Kit (Perkin Elmer, Inc., Waltham, MA) following the manufacturer's protocol. The slides were hybridized at 60°C for 16 h (for cDNA microarrays and Trizol isolated RNA) and at 55°C for 16 h (for oligonucleotide microarrays and PAXgen isolated RNA). Hybridized slides were scanned and recorded using a GenePix Pro 4000B (Axon Instruments Inc., Union City, CA) optical scanner, and the data were documented using Gene Pix 6.0 (Axon Instruments Inc, Union City, CA).

Preparation of cDNA microarrays

Human cDNA microarrays were prepared using sequence-verified PCR elements produced from ~10,000 well-characterized human genes of The Easy to Spot Human UniGEM V2.0 cDNA Library (Incyte Genomics Inc., Wilmington, DE). The PCR products, ranging from 500 to 700 base pairs, were deposited in 3x saline sodium citrate (SSC) at an average concentration of 165 µg/ml on CMT-GAPS™ II (γ-

aminopropylsilane) coated slides (Corning Inc., Corning, NY), using a Bio-Rad VersArray MicroArrayer (Hercules, CA). The cDNAs were UV-cross-linked at 120 mJ/cm² using UV Stratalinker® 2400 from Stratagene (La Jolla, CA). The microarrays were baked at 80°C for 4 h. The slides were treated with succinic anhydride and N-methyl-2-pyrrolidinone to remove excess amines.

Oligonucleotide microarrays

The Human Genome Array Ready Oligo Set Version 3.0 Set from Operon Biotechnologies (Huntsville, AL) includes 34,580 oligonucleotide probes representing 24,650 genes and 37,123 RNA transcripts from the human genome. The oligonucleotide targets were deposited in 3X saline sodium citrate (SSC) at an average concentration of 165 µg/ml onto CMT-GAPS II aminopropylsilane-coated slides (Corning, Corning, NY) using a VersArray Microarrayer. Microarrays were UV-crosslinked at 120 mJ/cm² using UV Stratalinker® 2400. Then slides were baked at 80°C for 4 hours, and were treated with succinic anhydride and N-methyl-2-pyrrolidinone to remove excess amines on the slide surface. Slides were stored in boxes with slide racks and the boxes were kept in desiccators.

Real time QPCR

Quantitative real time PCR arrays of one hundred genes associated with inflammation, transcription factors, and antigen preparation and presentation pathways were carried out using Dendritic & Antigen Presenting Cell Pathway (PAHS 406) and NFκB Pathway (PAHS 25) RT² Profiler™ PCR Arrays (SABiosciences, Frederick, MD) according to manufacturer's instructions. Four replicates of RNA samples isolated using PAXgene™ from Trainees before and after Training were assayed. The data were analyzed using ABiosciences' web-based software.

Reverse transcriptase reagent (iScript) and real time PCR master mix (QuantiTect™ SYBR® Green PCR Kit) were obtained from BioRad Inc., CA and QIAGEN Inc., Valencia, CA, respectively. Real time polymerase chain reactions (PCR) were carried out in i-Cycler Real-time PCR apparatus (BioRad Inc, Milpitas, CA), using three to five biological replicates for each primer pair (based on sample availability). The

custom oligonucleotide primers were designed using Primer3 software (www.basic.nwu.edu/biotools/Primer3.html), or based on those from UniSTS (<http://www.ncbi.nlm.nih.gov/genome/UniSTS>) and Universal Probe Library for Human (Roche Applied Science). Their specificities were verified in the BLAST domain at NCBI. Parallel amplification reaction using 18S rRNA primers was carried out as a control. Threshold cycle (Ct) for every run was recorded and then converted to fold change using the equation: $[(1+E)^{\Delta Ct}]_{GOI}/[(1+E)^{\Delta Ct}]_{HKG}$, where ΔCt stands for the difference between Ct of control and treated samples of a given gene, which is either gene of interest (GOI) or housekeeping genes (HKG), and E stands for primer efficiency, calculated from slope of best fitting standard curve of each primer pair.

ELISA

Plasma concentrations of prolactin (PRL), insulin-like growth factors I and II (IGF-I & II), tumor necrosis factor alpha (TNF α), and enzymatic activity of superoxide dismutase were determined using ELISA kits from Calbiotech, Inc. (Spring Valley, CA, Catalog # PR063F), Diagnostic Systems Laboratories, Inc. (Webster, TX, Catalog #s DSL-10-2800 and DSL-10-2600), Quantikine® of R&D Systems, Inc. (Minneapolis, MN, Catalog # DTA00C) and Dojindo Molecular Technologies, Inc (Gaithersburg, MD, Catalog # S311), respectively, following manufacturers' protocols.

Microarray Data Analyses

Background and foreground pixels of the fluorescence intensity of each spot on the microarrays were segmented using ImaGene (BioDiscovery Inc., El Segundo, CA) and the spots with the highest 20% of the background and the lowest 20% of the signal were discarded. Local background correction was applied. Genes that passed this filter in all experiments were selected for further study. Then, sub-grid based Lowess normalization was performed for each chip independently. Additional per spot (dividing by control channel) and per gene (to specific samples) normalization were also performed under the Genespring GX platform (Agilent Technologies Inc, Santa Clara, CA). Statistical analysis was computed using Welch's t-test ($p < 0.05$) with Benjamini and Hochberg False Discovery Rate (FDR) Multiple Correction to select the genes with high altered expression (for cDNA microarray data, but oligonucleotide microarray data were

analyzed without FDR Correction). Two-dimensional clustering was carried out based on samples and genes for visualization and assessment of reproducibility in the profile of the significant genes across biological replicates.

Interaction networks and gene ontology enrichment

Bingo 2.3 was used for gene ontology enrichment with hypergeometric distribution with FDR (false discover rate) or Bonferroni corrections ($p < 0.05$). Biological processes, molecular functions, and cellular components of each cluster of genes were compared to the global annotations and over-represented categories after corrections were analyzed and visualized. Functional analysis and pathways associated with stress and pathogen-regulated genes were analyzed using Ingenuity Pathway Analysis (Ingenuity Systems Inc.; Redwood City, CA). Cytoscape Version 2.6.1 (<http://www.cytoscape.org>) was used for visualizing and analyzing enriched gene ontologies, and molecular interaction network constructions.

MicroRNA Analysis

Expression profiles of MicroRNAs were assayed using Agilent's human miRNA v3 microarray (Agilent Technologies Inc) consisting of 15k targets representing 961 microRNAs. Differentially expressed microRNAs were analyzed using Qlucore Omices Explorer 2.2 (Qlucore AB) and GeneSpring GX 11.5 (Agilent Technologies Inc.). Target transcripts of profiled microRNAs were identified using target scan of Genespring, and Ingenuity Pathway Analysis (IPA) (Ingenuity Systems Inc.). Interaction networks of differentially expressed microRNAs and their target mRNAs were constructed using IPA.

Treatment of leukocytes with Staphylococcal enterotoxin B (SEB)

Leukocytes isolated from leucopack blood samples were plated in six well tissue culture plates ($\sim 10^6$ cells/ml in RPMI 1640 and 10% human AB serum) and treated with SEB (Toxin Technology Inc., Sarasota, FL) at a final concentration of 100 ng/ml SEB. Cells were incubated for 6 h at 37°C and 5% CO₂. At the end of the incubation period, treated leukocytes were collected by centrifugation at 350 x g for 15 minutes. Cell pellets were treated with 2 ml TRIzol™ and kept at -80°C for RNA isolation.

cDNA microarray (expression) data based prediction of transcription factors, regulatory binding sites and downstream target identification

Potential regulatory sites of differentially regulated genes were identified using HumanGenome9999 (Agilent Technologies Inc., CA) containing partial human genome sequences (9999 bp upstream region for 21787 genes). Statistically significant ($p < 0.05$) common regulatory motifs of 5 to 12 nucleotides long were identified. The searching region was set to range 1 to 500 nucleotides upstream of transcription start sites. Other tools used for this purpose include MATCH and TFSEARCH. Cognate transcription factors of identified (common regulatory) sites were searched from different prediction and repository databases: DBD (www.transcriptionfactor.org), JASPAR (<http://jaspar.cgb.ki.se>), TRANSFAC® 7.0 - Public (<http://www.gene-regulation.com/pub/databases.html>) using ChipMAPPER²⁰, ConTra, Pscan and Ingenuity Pathway Analysis (IPA, ingenuity inc). Expression databased prediction Z-scores and regulatory targets were analyzed using IPA. Regulator-target interaction networks and pathways were generated using Cytoscape (Cytoscape.org) and IPA.

Table 3A

Transcripts that have passed Welch's T-TEST (& Bonferroni correction at $q < 0.01$), and selected from battlefield-like condition that have Normalized Data values greater or less than those in baseline condition by a factor of 3 fold (59 transcripts)

ID	q-value	Fold change	Symbol	UniGene	Description
AU119825	0.000726	3.29	A2M	Hs.212838	Alpha-2-macroglobulin
BE889785	0.00932	-3.28	ACSL1	Hs.406678	Acyl-CoA synthetase long-chain family member 1
AL558086	0.000818	9.06	ALB	Hs.418167	Albumin
NM_001150	1.86E-05	-5.52	ANPEP	Hs.1239	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150) [up-regulated in late adenovirus type-12 infection (Journal of Virology 2005, 79:4, 2404)]
BG541130	0.000667	-3.52	ANXA1	Hs.494173	Annexin A1

NM_020980	5.62E-05	-8.06	AQP9	Hs.104624	Aquaporin 9 [Dehydration/osmotic adaptation in yeast (JBC 2005; 280:8, 7186); specialized leukocyte functions such as immunological response and bactericidal activity (PUBMED)]
BF432072	0.00212	-3.68	ATP2B1	Hs.506276	ATPase, Ca ⁺⁺ transporting, plasma membrane 1
AV710740	4.47E-07	-3.91	B2M	Hs.534255	Beta-2-microglobulin
NM_012342	0.00103	3.36	BAMB1	Hs.533336	BMP and activin membrane-bound inhibitor homolog (<i>Xenopus laevis</i>)
AI348005	0.00671	-3.42	BTG1L	Hs.710041	Similar to B-cell translocation gene 1,
XM_008651	4.30E-07	-16.98	CCR7		chemokine (C-C motif) receptor 7 [suppression lead to impaired lymphocyte migration, delayed adaptive immune response (cell 1999), CCR7 is key mediator in balancing immunity and tolerance, abnormalities contribute to immune dysregulation (clinical and experimental immunology, 2009)]
AL549182	0.00137	-3.46	CD14	Hs.163867	CD14 molecule
M24915	0.000223	-4.9	CD44	Hs.502328	CD44 molecule (Indian blood group)
BG333618	0.00854	-12.3	CD74	Hs.436568	CD74 molecule, major histocompatibility complex, class II invariant chain
L26165	0.00869	-3.8	CDKN1A	Hs.370771	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
NM_005196	0.00289	3.08	CENPF		synonyms: CENF, PRO1779; centromere protein F (400kD); centromere protein F (350/400kD, mitosin); CENP-F kinetochore protein; AH antigen; cell-cycle-dependent 350K nuclear protein; Homo sapiens centromere protein F, 350/400ka (mitosin) (CENPF), mRNA.
AL570594	5.07E-05	4.15	COL6A1	Hs.474053	Collagen, type VI, alpha 1
BE252062	0.000478	-3.92	CORO1A	Hs.474053	Coronin, actin binding protein, 1A
NM_005211	6.28E-06	-3.25	CSF1R	Hs.586219	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
AU118073	0.00469	-4.52	CSPG2	Hs.64380	Chondroitin sulfate proteoglycan 2

			/VCAN	1	(versican)
BG491425	0.00093 3	-15.22	CXCL1	Hs.789	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) [involved in neutrophil recruitment (Shock 35:6, 604)]
NM_00536 6	0.00015 3	-3.34	MAGE A11	Hs.67025 2	Melanoma antigen family A, 11
AL583593	0.0035	-7.3	FCN1	Hs.44089 8	Ficolin (collagen/fibrinogen domain containing) 1 [expressed at the cell surface of monocytes and granulocytes and its receptor is found at activated but not resting T lymphocytes (journal of leukocyte biology 2010; 88; 1:145); it is part of the innate immune system and function as recognition molecules in the complement system (Journal of innate immunity 2010; 2:1,3)]
NM_01340 9	0.002	3.01	FST	Hs.9914	Follistatin
Z97989	0.00897	-3.82	FYN		FYN oncogene related to SRC, FGR, YES
NM_00147 2	9.99E- 06	3.82	GAGE 7	Hs.46064 1	G antigen 7
AL551154 1	0.00013 1	-6.99	HCLS1	Hs.14601	Hematopoietic cell-specific Lyn substrate 1 [induces G-CSF-Triggered Granulopoiesis Via LEF-1 Transcription Factor (blood 2010 114:22, 229); mutation defects at HCLS1 with Kostmann disease. recombinant human granulocyte colony-stimulating factor (G-CSF), the prognosis and quality of life improved dramatically (European Journal of Pediatrics 2010, 169:6, 659)]
BG327758	0.00021	-15.13	HLA-B	Hs.77961	Major histocompatibility complex, class I, B
BE168491	0.00123	-7.63	HLA-C	Hs.65440 4	Major histocompatibility complex, class I, C
AW407113	2.66E- 05	-5.29	IGKV @,	Hs.66076 6	Immunoglobulin kappa variable group
AV759427	0.00020 5	-6.8	HLA- DPA1	Hs.34727 0	Major histocompatibility complex, class II, DP alpha 1
BF795929	0.00253	-8.33	HLA- DRA	Hs.52004 8	Major histocompatibility complex, class II, DR alpha
M20503	0.00057 5	-11.82	HLA- DRB1/ HLA-	Hs.69621 1/	Major histocompatibility complex, class II, DR beta 1/5

			DRB5		
BF974114	0.00046	-5.24	HLA-DRB1	Hs.69621 1	Major histocompatibility complex, class II, DR beta 1
BF732822	0.00035	-4.98	HLA-DRB1	Hs.69621 1	Major histocompatibility complex, class II, DR beta 1
AW411300	0.00267	4.36	IGF2	Hs.27225 9	Insulin-like growth factor 2 (somatomedin A)
AL542262	0.00121	5.48	IGFBP1	Hs.64293 8	Insulin-like growth factor binding protein 1
AI634950	9.18E-07	-11.82	IGHG1	Hs.51063 5	Immunoglobulin heavy constant gamma 1 (G1m marker)
AA490743	0.001	-4.61	IGHG1	Hs.51063 5	Immunoglobulin heavy constant gamma 1 (G1m marker)
NM_000575	0.00594	-5.15	IL1A	Hs.1722	Interleukin 1, alpha
W38319	6.35E-06	-6.29	IL1B	Hs.12625 6	Interleukin 1, beta
AU122160	0.00081	-4.17	LAIR1	Hs.57253 5	Leukocyte-associated immunoglobulin-like receptor 1
NM_006762	5.04E-07	-16.13	LAPTM5	Hs.37102 1	Lysosomal associated multispanning membrane protein 5 [negative regulation of cell surface BCR levels and B cell activation (The Journal of Immunology, 2010, 185: 294-301); LAPTM5 negatively regulated surface TCR expression by specifically interacting with the invariant signal-transducing CD3 zeta chain and promoting its degradation without affecting other CD3 proteins, CD3 epsilon, CD3 delta, or CD3 gamma (IMMUNITY 29 : 1 Pages: 33-43)]
BF035921	0.00040	-4.65	LCP1	Hs.38109 9	Lymphocyte cytosolic protein 1 (L-plastin)
NM_024318	0.00083	-3.65	LILRA6	Hs.68833 5	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6
AL560682	0.00115	-8.2	IG heavy chain/L	Hs.70393 8	Immunoglobulin Heavy Chain Variable region
NM_004811	0.0021	-4.12	OC652128		
			LPXN	Hs.12547 4	Leupaxin
BF792356	1.21E-05	4.04	MAGEA6	Hs.44111 3	Melanoma antigen family A, 6

AW966037	0.000159	3.1	MDK	Hs.82045	Midkine (neurite growth-promoting factor 2)
BE742106	9.14E-06	-4.03	MGAT1	Hs.519818	Mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
NM_002473	0.00506	-3.39	MYH9	Hs.474751	Myosin, heavy chain 9, non-muscle
AU142621	0.00726	-4.46	PNP	Hs.75514	Nucleoside phosphorylase
XM_007374	0.00795	-3.25	PRKCH		protein kinase C, eta
BE266904	7.79E-05	-4.15	SATB1	Hs.517717	Special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's)
AL550163	0.00157	-28.25	SERP1NB2	Hs.594481	Serpin peptidase inhibitor, clade B (ovalbumin), member 2 [upregulated under different inflammatory conditions, null mice showed increased TH1 response, secreted by macrophages, hemotpoeitic and nonhematopoeitic cells]
BG035651	0.00108	-10.34	SOD2	Hs.487046	Superoxide dismutase 2, mitochondrial [Conditional loss of SOD2 led to increased superoxide, apoptosis, and developmental defects in the T cell population, resulting in immunodeficiency and susceptibility to the influenza A virus H1N1 (Free radical biology and medicine, 201; 50:3, 448); manipulation of SOD2 affects drosophila survival under stress (PLoS One 2011; 6: 5, e19866)]
AL548113	4.31E-05	-3.28	ST14	Hs.504315	Suppression of tumorigenicity 14 (colon carcinoma)
D86980	3.55E-07	-3.57	TTC9	Hs.79170	Tetratricopeptide repeat domain 9
NM_003387	2.52E-05	-4.05	WIPF1	Hs.128067	WAS/WASL interacting protein family, member 1

Table 3B: Top 59 of stress specific genes ranked in order:

Rank	Gene Accession	Gene Name	Control	Dengue Virus	SEB	Stress	Yersinia Pestis	Description
1	XM_008651	CCR7	0.0943	0	0	-0.2854	0	chemokine (C-C motif) receptor 7
2	AI634950	IGHG1	0.1285	0	0	-0.2723	0	Immunoglobulin heavy constant gamma 1 (G1m marker)
3	AU118073	CSPG2	0	0	0	-0.2638	0.0673	Chondroitin sulfate proteoglycan 2
4	NM_006762	LAPTM5	0.1751	0	0	-0.2592	0	Lysosomal associated multispinning membrane protein 5
5	NM_005211	CSF1R	0	0	0	-0.2147	0	Colony stimulating factor 1 receptor,
6	AL558086	ALB	-0.0559	0	0	0.2136	0	Albumin
7	AW407113	HLA-C	0	0	0	-0.2119	0	Major histocompatibility complex, class I, C
8	BF795929	HLA-DRA	0	0	0	-0.193	0	Major histocompatibility complex, class II, DR alpha
9	AV759427	HLA-DPA1	0	0	0	-0.1885	0	Major histocompatibility complex, class II, DP alpha 1
10	AL549182	CD14	0	0	0	-0.187	0.0541	CD14 molecule
11	AL560682	LOC652128	0	0	0	-0.183	0	Similar to Ig heavy chain V-II region ARH-77 precursor
12	BE742106	MGAT1	0	0	0	-0.1764	0	Mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
13	AL551154	HCLS1	0.0306	0	0	-0.1738	0	Hematopoietic cell-specific Lyn substrate 1
14	NM_001150	ANPEP	0.0331	0	0	-0.1713	0	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal

15	W38319	IL1B	0.0424	0	0	-0.1624	0	aminopeptidase, CD13, p150)
16	BG327758	IL1B	0.0702	0	0	-0.1618	0	Interleukin 1, beta
17	BE266904	SATB1	0	0	0	-0.1566	0	Major histocompatibility complex, class I, B
18	BF035921	LCP1	0	0	0	-0.1546	0	Special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's)
19	NM_020980	AQP9	0.0815	0	0	-0.1491	0	Lymphocyte cytosolic protein 1 (L-plastin)
20	M20503	HLA-DRB1	0.0071	0	0	-0.147	0	Aquaporin 9
21	AU142621	NP	0	0	0	-0.1463	0	Major histocompatibility complex, class II, DR beta 1
22	AA334424	AFP	0	0	0	0.1439	0	Nucleoside phosphorylase
23	NM_001946	DUSP6	0	0	0	-0.1433	0.0044	Alpha-fetoprotein
24	AV710740	B2M	0.0427	0	0	-0.1403	0	Dual specificity phosphatase 6
25	XM_003507	SCYB5	0	0	0	-0.1371	0	Beta-2-microglobulin
26	AL583593	FCN1	0.0203	0	0	-0.1359	0	small inducible cytokine subfamily B (Cys-X-Cys), Ficolin (collagen/fibrinogen domain containing) 1
27	BE878314	FTH1	0	0	0	-0.1346	0	Ferritin, heavy polypeptide 1
28	BF732822	HLA-DRB1	0	0	0	-0.1318	0	Major histocompatibility complex, class II, DR beta 1
29	XM_003506	PPBP	0	0	0	-0.1312	0	pro-platelet basic protein (includes platelet basic
30	J04162	FCGR3A	0	0	0	-0.1308	0.0905	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
31	AA490743	IGHG1	0	0	0	-0.1254	0	Immunoglobulin heavy constant

32	AL542262	IGFBP1	0	0	0	0.1214	0	gamma 1 (G1m marker)
33	NM_003387	WIPF1	0	0	0	-0.1193	0	Insulin-like growth factor binding protein 1
34	BF792356	MAGEA6	-0.0079	0	0	0.1181	0	WAS/WASL interacting protein family, member 1
35	NM_004811	LPXN	0	0	0	-0.1162	0	Melanoma antigen family A, 6
36	BG491425	CXCL1	0	0	0	-0.1138	0	Leupaxin
37	NM_001472	GAGE2	-0.0189	0	0	0.1127	0	Chemokine (C-X-C motif) ligand 1
38	L26165	CDKN1A	0	0	0	-0.1121	0	G antigen 2
39	NM_000569	FCGR3A	0	0	0	-0.1107	0	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
40	D86980	TTC9	0.0306	0	0	-0.0992	0	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
41	Z97989	FYN	0	0	0	-0.0989	0	Tetratricopeptide repeat domain 9
42	AL550163	SERPINB2	0.1069	0	0	-0.0971	0	FYN oncogene related to SRC, FGR, YES
43	NM_005196	CENPF	0	0	0	0.095	0	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
44	NM_004987	LIMS1	0	0	0	-0.0887	0	Homo sapiens centromere protein F, 350/400ka (mitosin) (CENPF), mRNA.
45	AW966037	MDK	0	0	0	0.0877	0	LIM and senescent cell antigen-like domains 1
46	AX025098	AX025098	0	0	0	-0.0871	0	Midkine (neurite growth-promoting factor 2)
47	AU119825	A2M	0	0	0	0.0867	0	unnamed protein product; Sequence 22 from Patent WO0031532.
								Alpha-2-macroglobulin

48	BG333618	CD74	0	0	0	-0.0847	0	CD74 molecule, major histocompatibility complex, class II invariant chain
49	N32077	IER3	0	0	0	-0.082	0	Immediate early response 3
50	BE168491	HLA-B	0.0089	0	0	-0.0816	0	Major histocompatibility complex, class I, B
51	BG481840	ACTB	0	0	0	-0.0773	0	Actin, beta
52	BG541130	ANXA1	0	0	0	-0.074	0	Annexin A1
53	AU122160	LAIR1	0.0158	0	0	-0.0709	0	Leukocyte-associated immunoglobulin-like receptor 1
54	M24915	CD44	0.0216	0	0	-0.0704	0	CD44 molecule
55	AL570594	COL6A1	0	0	0	0.0678	0	Collagen, type VI, alpha 1
56	XM_007374	PRKCH	0	0	0	-0.0676	0	protein kinase C, eta
57	AA583143	MAFB	0	0	0	-0.0638	0	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
58	XM_008466	EVI2A	0	0	0	-0.063	0	ecotropic viral integration site 2A
59	AA309971	LAT	0	0	0	-0.0619	0	Linker for activation of T cells

Table 4: After 8 weeks: Transcripts profiled using quantitative real time QPCR arrays (116 transcripts were down-regulated, and 3 transcripts were up-regulated)

Symbol	Fold	StdevRTPCR
	-	
IKBKG	12.6188	0.339657363
	-	
RELB	12.2737	0.284777655
IRAK1	-9.2375	0.360943649
HGDC	-6.8685	0.390462704
JUN	-5.9484	0.27944997
TNFSF14	-4.7158	0.433281621
RELA	-3.9724	0.63019443
CD40	-3.7974	0.189280078
FADD	-3.6364	0.367498363
PPM1A	-3.5988	0.27174874

INHBA	-3.5247	0.104573154
CSF1R	-3.1821	0.758689626
CXCL10	-3.1766	0.277406814
AKT1	-3.1059	0.367849974
TNFRSF1A	-2.9079	0.687128349
ACTB	-2.8481	0.351350801
TRADD	-2.8432	0.506924503
TLR9	-2.8382	0.289278965
TNFRSF10B	-2.8284	0.267218757
LTBR	-2.5847	0.570855834
CXCL1	-2.5403	0.579529817
FCER2	-2.5184	0.414730623
SLC44A2	-2.4967	-0.822611762
HMOX1	-2.4368	0.155559063
CCL4	-2.4116	0.533964145
CD209	-2.4074	0.197647764
IKBKE	-2.3784	0.555233712
ICAM1	-2.3335	0.437161543
HLA-A	-2.3295	1.214034504
ELK1	-2.3254	0.269089688
CCL3L1	-2.2462	0.27090657
TNFAIP3	-2.2346	0.389174835
TLR6	-2.2191	0.872877926
HLA-DOA	-2.2153	0.607988424
MAP3K1	-2.2115	0.61339209
IKBKB	-2.1962	0.538167096
NFKBIA	-2.1772	0.152911234
F2R	-2.1473	0.243094984
CDKN1A	-2.1287	0.707160113
CFB	-2.1287	0.164433367
CD28	-2.114	0.214883087
IL16	-2.0958	-6.38481053
ERBB2	-2.0777	0.192737356
IRAK2	-2.0669	0.234239489
CD1D	-2.035	0.200278319
TLR2	-2.0279	-2.201882954
CCL8	-2.0139	0.148872434
CD4	-2	0.616064291
HLA-DMA	-1.9793	1.430015754
FASLG	-1.9725	0.132549302
CCL11	-1.9252	0.137200432
CCL13	-1.9252	0.137200432
CCL16	-1.9252	0.137200432
CCL7	-1.9252	0.137200432
CXCL12	-1.9252	0.137200432
CXCL2	-1.9252	0.137200432

FCAR	-1.9252	0.137200432
IL2	-1.9252	0.137200432
MDK	-1.9252	0.137200432
TNFSF11	-1.9252	0.137200432
IL12B	-1.8823	0.139095667
CD40	-1.8693	0.396668136
HLA-DPA1	-1.8693	-92.22884305
RELB	-1.8661	0.207774407
REL	-1.8628	0.585844588
TLR1	-1.8628	0.602086965
CD2	-1.8468	0.857944585
ICAM1	-1.8182	0.63392797
TAPBP	-1.8119	0.419814619
RELA	-1.7932	0.273669806
CASP8	-1.7777	0.21122336
IL1R1	-1.7685	0.524613114
TICAM2	-1.7623	0.216623278
CD1B	-1.7381	0.132080179
CEBPA	-1.7112	0.784622441
CASP1	-1.7082	0.934618998
STAT1	-1.7082	0.964130752
TLR4	-1.7082	0.580800815
RAF1	-1.7023	1.180672752
CCR2	-1.6935	0.305351506
IFIT3	-1.6615	0.677571172
TNFRSF10A	-1.6615	0.230520538
IFNGR1	-1.6558	1.492757528
ITGB2	-1.6558	21.10639135
LYN	-1.6558	230.7481187
CCL19	-1.6358	0.131657942
CCL5	-1.6217	1.745561311
RAC1	-1.5938	0.515059945
MALT1	-1.5883	0.281116286
CCL3	-1.5692	0.165855825
CD80	-1.5665	0.132742476
TAP2	-1.5502	0.393041048
ACTB	-1.5369	0.382445363
IL8	-1.5157	0.483025481
CCL2	-1.5105	0.134605272
TLR3	-1.5	0.165275956
IL12A	-1.4974	0.198792804
FCGR1A	-1.4923	0.878361699
NFKB2	-1.4923	0.403365512
EDARADD	-1.4794	0.143070569
NOD1	-1.4768	0.308099861
TRAP1	-1.4439	0.483257919

NLRP12	-1.4439	0.363870333
PDIA3	-1.434	0.406179958
IL8	-1.4216	0.354085621
HLA-DQA1	-1.4167	1.178062108
MIF	-1.402	1.497615941
RPL13A	-1.3899	1.4788335
ITGAM	-1.3779	0.600004582
ATF1	-1.3519	0.183064879
CDC42	-1.3496	3.310458234
ICAM2	-1.3426	0.973584543
CCR5	-1.3333	0.145884175
CD44	-1.3036	1.754787134
IL8RA	-1.3013	1.145515093
RIPK1	-1.3013	0.462210307
CCR3	1.402	0.384641887
TLR8	1.7471	2.50546893
TLR7	1.7654	0.64730932

Table 5: Average fold change: Stress-Regulated Genes Involved in Immune System Processes, oxidative stress response and steroid biosynthesis. Functions were enriched using hypergeometric statistical analysis along with Bonferroni correction ($p < 0.05$). The significance level and fold change for each gene (obtained from microarray statistical analysis) are shown in the last two columns respectively.

Gene ID	Name	Description	fold	p-value
T-cell activation				
AW950965	CD3E	CD3e, epsilon (CD3-TCR complex)	-1.5	9.80E-03
BG333618	CD74	CD74, MHC, class II invariant chain	-12.3	2.90E-05
AA309971	LAT	Linker for activation of T cells	-2.9	3.10E-04
NM_000887	ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)	-1.4	2.10E-02
NM_001767	CD2	CD2 molecule	-1.3	3.40E-02
AA766638	PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1	-1.5	3.10E-02
XM_001772	LCK	lymphocyte-specific protein tyrosine kinase	-2	1.50E-04
NM_000616	CD4	CD4 molecule	-2.3	1.00E-03
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_002838	PTPRC	Protein tyrosine	-3.2	2.50E-03
BG391140	CSK	C-src tyrosine kinase	-1.5	5.00E-03
XM_006041	CD5	CD5 antigen (p56-62)	-2.6	3.10E-04
M12824	CD8A	CD8a molecule	-3.9	1.20E-04
BC001257	GLMN	Glomulin, FKBP associated protein	-1.5	1.80E-02
AA310902	CD3D	CD3d molecule, delta (CD3-TCR complex)	-2.1	2.90E-03
AI803460	CCND3	Cyclin D3	-1.5	8.80E-03
AC002310	ITGAL	integrin, alpha L (antigen CD11A (P180), lymphocyte function-associated antigen 1; alpha polypeptide)	-1.4	7.30E-02

NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03
NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	-2.3	6.10E-04
U81504	AP3B1	Adaptor-related protein complex 3, beta 1 subunit	-1.6	1.00E-02
AW780437	PRKCQ	Protein kinase C, theta	-1.7	9.10E-03
AL136450	BCORL1	BCL6 co-repressor-like 1	-1.7	3.90E-04
NM_004931	CD8B	CD8b molecule	-1.5	2.50E-03
B cell activation				
XM_003106	PRKCD	protein kinase C, delta	-1.9	8.80E-04
AU118181	KLF6	Kruppel-like factor 6	-2.6	3.70E-04
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	-1.4	1.80E-02
L26165	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	-3.8	2.90E-05
NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	-3.2	2.50E-03
Natural killer cell activation				
NM_001767	CD2	CD2 molecule	-1.3	3.40E-02
AI948861	SLAMF7	SLAM family member 7 Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	-1.7	2.50E-02
AF285436	KIR3DL1		-1.8	3.90E-04
AL136450	BCORL1	BCL6 co-repressor-like 1	-1.7	3.90E-04
Myeloid dendritic cell activation				
NM_001767	CD2	CD2 molecule	-1.3	3.40E-02
NM_006509	RELB	V-rel reticuloendotheliosis viral oncogene homolog	-1.9	1.30E-04

		B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)		
Mast cell activation				
AA309971	LAT	Linker for activation of T cells	-2.9	3.10E-04
AF177765	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03
NM_005565	LCP2	Lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	-2.5	9.30E-04
NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03
Macrophage activation				
BG333618	CD74	CD74; MHC, class II invariant chain	-12.3	2.90E-05
AI937452	CD93	CD93 molecule	-1.6	5.60E-04
AF177765	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03
Platelete activation				
AI739539	PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-3.3	1.10E-04
NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	-1.4	1.80E-02
T-cell differentiation				
BG333618	CD74	CD74; MHC, class II invariant chain	-12.3	2.90E-05
AW950965	CD3E	CD3e; epsilon (CD3-TCR complex)	-1.5	9.80E-03
M12824	CD8A	CD8a molecule	-3.9	1.20E-04
NM_001767	CD2	CD2 molecule	-1.3	3.40E-02
AA310902	CD3D	CD3d; delta (CD3-TCR complex)	-2.1	2.90E-03
XM_001772	LCK	lymphocyte-specific protein tyrosine kinase	-2	1.50E-04
NM_000616	CD4	CD4 molecule	-2.3	1.00E-03
NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03

U81504	AP3B1	Adaptor-related protein complex 3, beta 1 subunit	-1.6	1.00E-02
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	-3.2	2.50E-03
B cell differentiation				
AU118181	KLF6	Kruppel-like factor 6	-2.6	3.70E-04
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03
NK T cell differentiation				
U81504	AP3B1	Adaptor-related protein complex 3, beta 1 subunit	-1.6	1.00E-02
Monocyte differentiation				
BG434340	IFI16	Interferon, gamma-inducible protein 16	-1.7	2.70E-03
NM_002473	MYH9	Myosin, heavy chain 9, non-muscle	-3.4	2.00E-05
Myeloid cell differentiation				
AA777633	MYST3	MYST histone acetyltransferase (monocytic leukemia) 3	-1.6	3.30E-03
AL551154	HCLS1	Hematopoietic cell-specific Lyn substrate 1	-7	2.20E-06
AI739539	PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-3.3	1.10E-04
Y14768	TNFA	TNF-alpha	-1.3	9.90E-03
BG108304	LYN	V-src-1 Yamaguchi sarcoma viral related oncogene homolog	-3.2	4.50E-05
XM_008993	SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	-1.5	1.40E-03
AF177765	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02

AA583143	MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	-2.7	1.00E-04
NM_006509	RELB	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	-1.9	1.30E-04
AA253017	MYST1	MYST histone acetyltransferase 1	-1.5	5.70E-02
		T cell proliferation		
AW950965	CD3E	CD3e molecule, epsilon (CD3-TCR complex)	-1.5	9.80E-03
NM_000887	ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)	-1.4	2.10E-02
BC001257	GLMN	Glomulin, FKBP associated protein	-1.5	1.80E-02
AI803460	CCND3	Cyclin D3	-1.5	8.80E-03
AC002310	ITGAL	integrin, alpha I (antigen CD11A (P180), lymphocyte function- associated antigen 1; alpha polypeptide)	-1.4	7.30E-02
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_003177	SYK	Spleen tyrosine kinase	-1.8	7.60E-03
NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	-2.3	6.10E-04
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	-3.2	2.50E-03
AW780437	PRKCQ	Protein kinase C, theta	-1.7	9.10E-03

AL136450	BCORL1	BCL6 co-repressor-like 1	-1.7	3.90E-04
B cell proliferation				
XM_003106	PRKCD	protein kinase C, delta	-1.9	8.80E-04
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	-1.4	1.80E-02
L26165	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	-3.8	2.90E-05
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	-3.2	2.50E-03
activated T cell proliferation				
NM_000887	ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)	-1.4	2.10E-02
AC002310	ITGAL	integrin, alpha I (antigen CD11A (P180), lymphocyte function-associated antigen 1; alpha polypeptide)	-1.4	7.30E-02
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02
NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	-2.3	6.10E-04
NK cell proliferation				
AL136450	BCORL1	BCL6 co-repressor-like 1	-1.7	3.90E-04
microbial pattern recognition and binding				
AI739539	PF4	Platelet factor 4 (CXCL4)	-3.3	1.10E-04
AI097512	CHIT1	Chitinase 1 (chitotriosidase)	-1.5	2.00E-02
NM_003264	TLR2	Toll-like receptor 2	-2.6	1.00E-03
AF177765	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03

		Small inducible cytokine A7 (monocyte chemotactic		
XM_012649	SCYA7		-1.5	2.80E-02
AL549182	CD14	CD14 molecule	-3.5	8.20E-06
		Platelet factor 4 variant 1		
NM_002620	PF4V1		-2.7	1.90E-03
		CLP1, cleavage and polyadenylation factor I subunit, homolog (S. cerevisiae)		
AA188236	CLP1		-1.5	1.60E-02
		Toll-like receptor adaptor molecule 1		
AI087056	TICAM1		-1.5	3.30E-03
		Formyl peptide receptor-like 1		
AF054013	FPRL1		-1.9	2.40E-03
		Human N-formyl peptide receptor (FPR1)		
L10820	FPR1		-1.8	3.10E-05
		antigen processing and presentation		
		CD74; MHC, class II invariant chain		
BG333618	CD74		-12.3	2.90E-05
		MHC, class II, DR alpha		
BF795929	HLA-DRA		-8.3	1.20E-05
		MHC, class II, DQ beta 1		
U83582	HLA-DQB1		-2	5.20E-05
		Ig heavy constant gamma1 (G1m marker)		
AI634950	IGHG1		-11.8	6.20E-08
		Fc fragment of IgG, receptor, transporter, alpha		
AL571972	FCGRT		-1.6	5.10E-02
		MHC, class II, DP alpha 1		
AV759427	HLA-DPA1		-6.8	2.70E-06
		MHC, class II, DP beta 1		
M83664	HLA-DPB1		-2.8	6.30E-06
		Interferon, gamma-inducible protein 30		
AL561631	IFI30		-2.6	2.80E-03
		MHC class I polypeptide-related sequence A		
NM_006674	MICA		-2.2	2.50E-03
BG327758	HLA-B	MHC, class I, B	-15.1	2.70E-06
		HLA-G histocompatibility antigen, class I, G		
AF071019	HLA-G		-2.4	2.60E-06

BF663123	IGHA1	Ig heavy constant alpha 1	-2.5	2.80E-03
AW407113	HLA-C	MHC, class I, C	-5.3	6.50E-07
BG176768	HLA-DOB	MHC, class II, DO beta	-2.4	1.60E-04
NM_006509	RELB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 3	-1.9	1.30E-04
M20503	HLA-DRB1	MHC, class II, DR beta 1	-11.8	5.30E-06
U81504	AP3B1	Adaptor-related protein complex 3, beta 1 subunit	-1.6	1.00E-02
AV710740	B2M	Beta-2-microglobulin	-3.9	4.30E-08
		cytokine activity		
XM_003506	PPBP	pro-platelet basic protein (includes platelet basic	-4.1	8.10E-05
AI739539	PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-3.3	1.10E-04
Y14768	TNFA	TNF-alpha	-1.3	9.90E-03
XM_003507	SCYB5	Small inducible cytokine subfamily B (Cys-X-Cys),	-5.2	4.90E-05
XM_005349	TNFSF8	tumor necrosis factor (ligand) superfamily, member 8	-1.9	1.50E-03
W38319	IL1B	Interleukin 1, beta	-6.3	2.70E-07
NM_002988	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	-1.6	1.20E-03
AV717082	IL8	Interleukin 8	-23.8	3.20E-04
BG108304	LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	-3.2	4.50E-05
XM_012649	SCYA7	small inducible cytokine A7 (monocyte chemotactic	-1.5	2.80E-02
NM_000589	IL4	Interleukin 4	-1.6	6.60E-02

NM_000575	IL1A	Interleukin 1, alpha	-5.2	2.30E-05
XM_003508	GRO3	GRO3 oncogene	-1.5	2.10E-02
AA569974	CCL5	Chemokine (C-C motif) ligand 5	-1.6	4.30E-03
NM_005408	CCL13	Chemokine (C-C motif) ligand 13	-1.6	1.90E-02
BG288796	IL1RN	Interleukin 1 receptor antagonist	-3.6	3.90E-04
AW188005	LTB	Lymphotoxin beta (TNF superfamily, member 3)	-3.2	1.00E-03
BC001257	GLMN	Glomulin, FKBP associated protein	-1.5	1.80E-02
AW965098	CCL20	Chemokine (C-C motif) ligand 20	-1.5	4.30E-03
BG393056	PRL	Prolactin	-1.5	1.40E-02
BG491425	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-15.2	6.90E-06
NM_002620	PF4V1	Platelet factor 4 variant 1	-2.7	1.90E-03
		cytokine binding (receptors)		
AF009962	CCR-5	CC-chemokine receptor (CCR-5)	-1.5	1.00E-02
NM_000877	IL1R1	Interleukin 1 receptor, type I	-1.5	3.40E-02
NM_000418	IL4R	Interleukin 4 receptor	-1.6	9.40E-03
XM_008651	CCR7	Chemokine (C-C motif) receptor 7	-17	4.30E-08
NM_001558	IL10RA	Interleukin 10 receptor, alpha	-1.6	1.20E-02
NM_000878	IL2RB	Interleukin 2 receptor, beta	-2.7	6.30E-06
AF012629	TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	-1.7	2.30E-03

XM_001743	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	-2.4	1.80E-03
BC001281	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	-1.5	3.60E-03
NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	-1.4	1.80E-02
AL050337	IFNGR1	interferon gamma receptor 1	-1.6	6.20E-03
AL550285 IL-12 biosynthesis	IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	-1.8	6.50E-03
NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	-3.7	5.20E-05
NM_002198	IRF1	Interferon regulatory factor 1	-2.2	4.50E-04
AF177765 IL-6 biosynthesis	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03
W39546	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	-1.9	5.30E-03
W38319	IL1B	Interleukin 1, beta	-6.3	2.70E-07
AF177765 IL-2 biosynthesis	TLR4	toll-like receptor 4 (TLR4)	-1.8	9.60E-03
BC001257	GLMN	Glomulin, FKBP associated protein	-1.5	1.80E-02
NM_000616	CD4	CD4 molecule	-2.3	1.00E-03
AW780437 IL-3 biosynthesis	PRKCQ	Protein kinase C, theta	-1.7	9.10E-03
NM_003177 IL-1 biosynthesis	SYK	Spleen tyrosine kinase	-1.8	7.60E-03

AF177765	TLR4	toll-like receptor 4 (TLR4) gene, '1	-1.8	9.60E-03
inflammatory response				
AL570708	CD180	CD180 molecule	-1.3	1.50E-02
AL549182	CD14	CD14 molecule	-3.5	8.20E-06
Human complement C8 gamma subunit precursor (C8G) gene, complete cds.				
U08198	C8G		-1.5	4.00E-03
NM_003264	TLR2	Toll-like receptor 2	-2.6	1.00E-03
keratin 1 (epidermolytic hyperkeratosis)				
XM_006848	KRT1		-2	1.70E-03
CD40 molecule, TNF receptor superfamily member 5				
NM_001250	CD40		-1.4	1.80E-02
Interferon regulatory factor 7				
NM_004029	IRF7		-2.2	5.60E-04
CCAAT/enhancer binding protein (C/EBP), beta				
W39546	CEBPB		-1.9	5.30E-03
Cytochrome b-245, beta polypeptide (chronic granulomatous disease)				
X04011	CYBB		-1.6	3.20E-03
toll-like receptor 4 (TLR4)				
AF177765	TLR4		-1.8	9.60E-03
AI090294	CD97	CD97 molecule	-1.7	1.90E-04
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)				
NM_003998	NFKB1		-3.7	5.20E-05
Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)				
NM_000211	ITGB2		-2.2	6.30E-06
integrin, alpha I (antigen CD11A (P180), lymphocyte function- associated antigen 1; alpha polypeptide)				
AC002310	ITGAL		-1.4	7.30E-02

ID	Name	Description	Fold	P-value
Cholesterol and other steroids biosynthesis				
AL558223	ACBD3	Acyl-Coenzyme A binding domain containing 3	1.6	4.10E-03
BE253839	DHCR24	24-dehydrocholesterol reductase	2.1	1.60E-02
AW271546	HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1	1.6	2.50E-03
AF078850	HSD17B12	Hydroxysteroid (17-beta) dehydrogenase 12	1.4	1.70E-02
AK001889	PRLR	Prolactin receptor	1.9	5.00E-03
NM_000786	CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	1.9	3.90E-04
NM_004110	FDXR	Ferredoxin reductase	1.8	5.00E-03
NM_000103	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	1.9	1.60E-02
BE378962	DHCR7	7-dehydrocholesterol reductase	1.8	2.60E-03
J05158	CPN2	Carboxypeptidase N, polypeptide 2, 83kD	1.9	2.10E-03
AL521605	OPRS1	Opioid receptor, sigma 1	2.2	4.70E-04
AW117731	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	2.2	2.00E-03
BG324529	MVD	Mevalonate (diphospho) decarboxylase	2.3	5.20E-03
Ergosterol biosynthesis				
AL521605	OPRS1	Opioid receptor, sigma 1	2.2	4.70E-04
Dopamine biosynthesis				
AW156890	SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	1.5	1.20E-02

Fatty acid biosynthesis

AL359403	MCAT	Malonyl CoA:ACP acyltransferase (mitochondrial)	1.6	5.40E-03
AF097514	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	5.2	4.40E-04
transcription Transcription factors				
BE266904	SATB1	Special AT-rich sequence binding protein 1	-4.2	1.70E-06
NM_006763	BTG2	BTG family, member 2	-3.8	7.70E-04
NM_003998	NFKB1	NFk light polypeptide gene enhancer in B-cells 1 (p105)	-3.7	7.00E-05
AI348005	BTG1	B-cell translocation gene 1, anti-proliferative	-3.4	3.70E-05
NM_006060	IKZF1	IKAROS family zinc finger 1 (Ikaros)	-2.6	7.00E-05
AL555297	SF1	Splicing factor 1	-2.4	1.70E-06
NM_014795	ZFHX1B	Zinc finger homeobox 1b	-2.3	6.10E-04
AL561046	TSC22D3	TSC22 domain family, member 3	-2.2	5.00E-04
NM_002198	IRF1	Interferon regulatory factor 1	-2.2	5.00E-04
NM_004029	IRF7	Interferon regulatory factor 7	-2.2	5.80E-04
AV708340	UBA52	Ubiquitin A-52 residue ribosomal protein fusion product 1	-2.1	6.80E-04
AI631717	HNF4A	Hepatocyte nuclear factor 4, alpha	2	3.90E-03
BG529476	HMGB2	High-mobility group box 2	2.1	2.50E-03
BG340581	SREBF2	Sterol regulatory element binding transcription factor 2	2.3	1.50E-03
AL525810	FOXM1	Forkhead box M1	2.3	2.40E-04

M95585	HLF	Hepatic leukemia factor	2.4	5.00E-04
NM_003220	TFAP2A	Transcription factor AP-2 alpha	2.4	2.00E-03
AL575644	NFKBIL1	NFk light polypeptide enhancer in B-cells inhibitor-like 1	3.3	4.60E-03
Ssuperoxide metabolism				
BG035651	SOD2	Superoxide dismutase 2, mitochondrial	-10.3	1.20E-07
BG421245	CYBA	Cytochrome b-245, alpha polypeptide	-2.1	1.00E-06
XM_002200	NCF2	neutrophil cytosolic factor 2 (65kD, chronic	-2	2.90E-04
heat Heat shock proteins				
BG327949	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1	1.6	4.50E-02
AB007877	HSPA12A	Heat shock 70kDa protein 12A	1.7	2.10E-03
BE742483	HSPA4	Heat shock 70kDa protein 4	1.9	1.00E-05
AI640615	BAG4	BCL2-associated athanogene 4	1.9	1.10E-03
BG032173	HSPD1	Heat shock 60kDa protein 1 (chaperonin)	2.5	5.90E-04

EXAMPLE 1:

The biomarker findings are presented which were identified from gene expression changes in leukocytes collected from (informed and consented) US Army Ranger Cadets who underwent eight-weeks of Army Ranger Training (RASP, Ranger Assessment and Selection Program). Our subjects were exposed to extreme physical and psychological stressors of Ranger Training, which is designed to emulate extreme battlefield scenarios such as strenuous physical activity, sleep deprivation, calorie restriction, and survival emotional stresses – pushing cadets to their physical and psychological limits. Though these men were among the best of the best, many trainees dropped out in the first phase

of the three-phased RASP Training. The Army Ranger population provides a rare opportunity to study extreme stress, and to contribute to the understanding of intense chronic stress in general. Particularly, the ability to collect pre-training samples for comparison with post-training samples is rarely practical in any other chronically and extremely stressed patients.

Our studies focus in identifying molecular mediators of compromised protective immunity caused by social and battlefield-like stresses, and in identifying pathogen-induced biomarkers under severe stress background. Social and physiological stresses, particularly, which are frequent or chronic are major contributors of stress-induced immune dysfunction. In this study, we employed experimental and computational approaches to identify molecules and signaling pathways involved in the host's response towards battlefield-like stress, and in assessing protective immunity status of the stressed host towards infection.

In the first approach, we used genome-wide transcriptome, and microRNA profiling and *in-vitro* pathogen exposure of leukocytes (isolated from Army Ranger Trainees) to identify stress-suppressed transcripts and pathways critical in protective immune response. We have identified a number of stress response biomarkers (transcripts and pathways) that have potential implication in compromising the immune function. The most compromised pathways include antigen preparation and presentation, and T-cell activation pathways. Suppressed immune response genes remained suppressed even after *ex-vivo* exposure of post-RASP leukocytes to the mitogenic toxin, *Staphylococcal enterotoxin B* (SEB). On the other hand, complete and differential counts of post-training WBCs were within normal ranges. This impaired activation is an indicator of anergy, and compromised protective immunity.

EXAMPLE 2

In the second approach, we used rigorous computational analyses in identifying up-stream regulatory modules (and molecular networks) of stress-suppressed genes. We identified up-stream regulators of differentially altered transcripts, which include immune related and steroid hormone inducible transcription factors, stress response factors, and microRNAs. Some stress induced microRNAs, and a number of stress-inhibited

transcription factors were found to regulate or be modulated by many compromised immune response transcripts.

The identification of exceptionally enriched suppression of antigen presentation and lymphocyte activation pathways (in spite of normal blood cell counts) are remarkable since these findings are consistent with prior observations of poor vaccine responses, impaired wound healing and infection susceptibility associated with chronic intense stress.

Some of the transcripts were unique to RASP stressors (severe and chronic stress), even in the presence of other pathogens, to which we briefly refer in this manuscript. These specific transcripts may have potential use as diagnostic markers to distinguish debilitating chronic stress from that of infection.

Conclusion:

The subject matter of the present invention (biomarkers) solves the drawbacks of other routinely used assays that check the status of the immune system process. Many clinical laboratories do differential and complete white blood cell counting to ascertain integrity of the immune system. Some advanced clinical laboratories do challenge assays (proliferation assays) to check the viability of immune cells (in addition to cell counting). In our case, even though the cells are within their normal ranges (cell counting would have indicated normal), we still see no measurable response to SEB challenge (and we have the molecular indicators of the why). Our molecular markers can be used to check the protective or compromised nature of the immune system regardless of whether the cells are anergic (within normal range in terms of their numbers but not protective) or otherwise.

Definitions:

Welch's t-test: Statistical comparative analysis whereby the means and variance of compared groups are not assumed to be the equal.

Transcriptome: Genome-wide transcripts of human or any other living thing.

Transcript: Messenger RNA (ribonucleic acid) or any other small RNA molecule.

Pathway: regulatory hierarchy of bio-molecules (proteins, transcripts, or metabolites) forming a specific biological process (function).

Normal Control: A person or sample from a person, or genes or transcripts from a person, or expression profile from a person or persons that has not been subjected to stress.

Diagnostic biomarkers: stress effected genes, transcripts, cDNAs, mRNA, miRNAs, rRNA, tRNA, peptides and proteins.

****Gene names and accession numbers** presented herein are standard gene names and accession numbers for genes that are found in the NCBI GenBank ®. GenBank ® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (*Nucleic Acids Research*, 2013 Jan;41(D1):D36-42). GenBank is part of the International Nucleotide Sequence Database Collaboration , which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis.

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WHAT IS CLAIMED IS:

1. A set of isolated diagnostic biomarkers for diagnosing immune suppression/dysfunction, wherein said diagnostic biomarkers are genes or transcripts that have expression that are up-regulated or down-regulated upon stress when compared to a normal control.
2. The set of isolated diagnostic biomarkers of claim 1, wherein said biomarkers comprise the at least 5 or more of the genes: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, HLA-DRB1, PPBP, FCGR3A, IGHG1, IGFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, FCGR3A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT.
3. The set of isolated diagnostic biomarkers of claim 1, wherein said suppressed immune response includes suppressed inflammatory response, suppressed leukocyte activations and proliferations, and/or suppressed response to pathogens.
4. The set of isolated diagnostic biomarkers of claim 1, wherein said diagnostic biomarkers comprises at least five of differentially regulated genes or transcripts comprising: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R.
5. The set of isolated diagnostic biomarkers of claim 1, wherein said diagnostic biomarker comprises at least seven differentially regulated genes or transcripts comprising: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C.
6. The diagnostic biomarkers of claim 1, wherein said diagnostic biomarkers comprise at least 10 differentially regulated genes or transcripts comprising: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14.
7. The set of isolated diagnostic biomarkers of claim 1, wherein said diagnostic biomarkers comprise at least 20 differentially regulated genes or transcripts comprising: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA,

HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1.

8. A library of differentially regulated transcripts or genes from their corresponding pathway, wherein said differently regulated transcripts or genes from their corresponding pathway are suitable for use as diagnostic biomarkers for diagnosing suppressed immune response in a subject, wherein said suppressed immune response is due to stress.
9. The library of claim 8, wherein said suppressed immune response includes suppressed inflammatory response, suppressed leukocyte activation and proliferations, and/or suppressed response to pathogens.
10. A micro- or nano-chip, or PCR for observing differentially regulated genes or transcripts wherein said micro- or nano-chip comprises the genes: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, HLA-DRB1, PPBP, FCGR3A, IGHG1, IGFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, FCGR3A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT, and said PCR uses cDNAs of said differentially regulated genes or transcripts.
11. The micro- or nano-chip of claim 10, wherein said cDNAs are electrochemically tethered in the wells of said micro-or nano-chip.
12. A diagnostic kit for use in screening integrity of the immune function of a subject, wherein said diagnostic kit comprises a set of isolated diagnostic biomarkers of a claim 1.
13. The diagnostic kit of claim 12, wherein said diagnostic biomarkers are present in/on a micro- or nano-chip.
14. The diagnostic kit of claim 13, wherein said diagnostic biomarkers are electrochemically tethered in the wells of said micro- or nono-chip.
15. A method of evaluating immune function or dysfunction in a patient, comprising the steps of a) creating a library of leukocyte diagnostic biomarkers that are up-

regulated or down-regulated in response to stress; comparing a set of diagnostic markers in said patient to said diagnostic markers in said library; and determining whether said patient is under stress.

16. The method of claim 15, wherein said diagnostic biomarkers are selected from the following genes:

CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, HLA-DRB1, PPBP, FCGR3A, IGHG1, IGFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, FCGR3A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT.

17. The method of claim 15, wherein said diagnostic biomarkers are at least 5 of the following genes:

CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, HLA-DRB1, PPBP, FCGR3A, IGHG1, IGFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, FCGR3A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT.

18. A method of screening a subject for immune suppression or dysfunction, comprising: taking a sample of diagnostic biomarkers from said subject; comparing said patient's diagnostic markers to normal diagnostic markers in a control library; and determining whether said patient has immune suppression or dysfunction.

19. A method of identifying the capability of immune cells to respond to pathogenic agents without exposing the subjects or their cells to any pathogens, comprising obtaining a sample from said subject and comparing diagnostic biomarkers in said sample to a biomarker stress profile, said biomarker stress profile comprising a pre-determined set of transcripts that indicate stress.

20. The method of claim 19, wherein said set includes 5 or more of the following transcripts: CCR7, IGHG1, CSPG2, LAPTM5, CSF1R, ALB, HLA-C, HLA-DRA, HLA-DPA1, CD14, LOC652128, MGAT1, HCLS1, ANPEP, IL1B, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, HLA-DRB1, PPBP, FCGR3A, IGHG1, IGFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, FCGR3A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT.

21. The set of isolated diagnostic biomarkers for diagnosing immune suppression/dysfunction of claim 1, wherein said diagnostic biomarkers are cDNAs of said differentially regulated genes or transcripts.

22. The set of isolated diagnostic biomarkers for diagnosing immune suppression/dysfunction of claim 1, wherein said diagnostic biomarkers are RNAs of said differentially regulated genes or transcripts.

23. The set of isolated diagnostic biomarkers for diagnosing immune suppression/dysfunction of claim 1, wherein said biomarkers are associated with microbial pattern recognition, inflammation, cytokine production and reception, , intracellular adhesion, immunological synapse formation, regulation of immune response, chemotaxis, antigen presentation and activations of lymphocytes, activation of myloid lymphocytes, activation of mast cells and activation of macrophages.

24. The set of isolated diagnostic biomarkers for diagnosing immune suppression/dysfunction of claim 1, wherein said set includes transcription factors and stress response factors.

25. The peptides or proteins encoded by the genes of claim 1.

26. A method of diagnosing exposure to SEB, dengue virus and/or yersinia pestis in a patient, comprising:

a) creating a library of stress specific genes or transcripts differentiating stress in leukocytes from subjects exposed to SEB, dengue virus and Yersinia pestis;

b) taking a sample from said patient;

- c) comparing genes or transcripts from said sample to said library;
- c) determining whether said patient has been exposed to SEB, dengue virus, or yersinia pestis based on the comparison of said genes from said sample to said library.

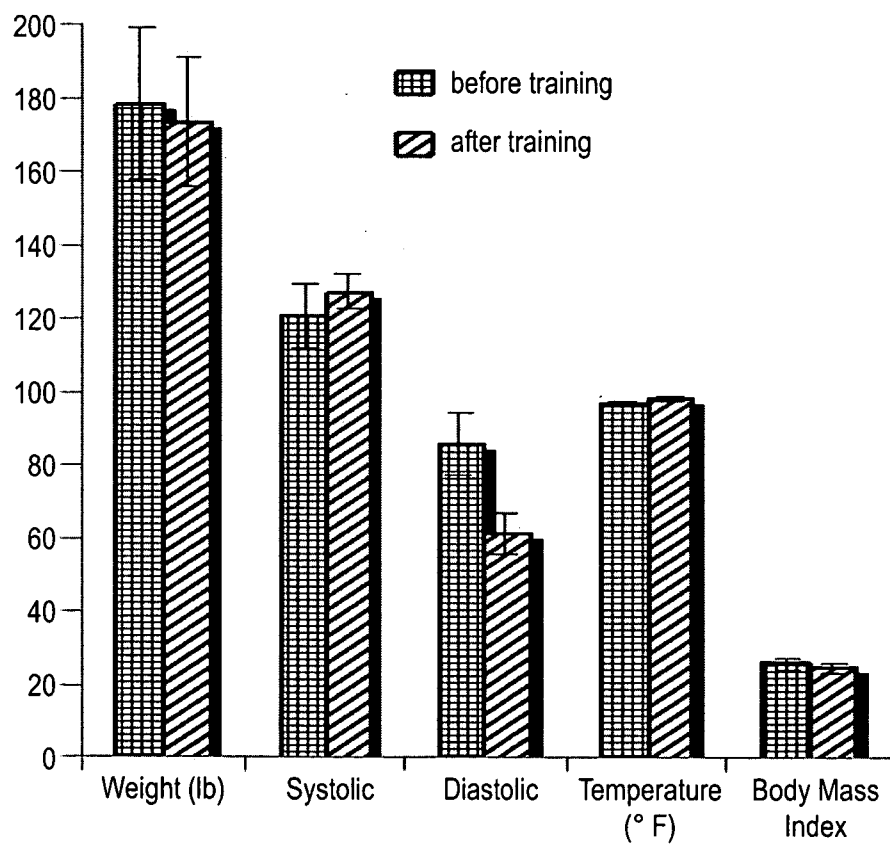


FIG. 1A

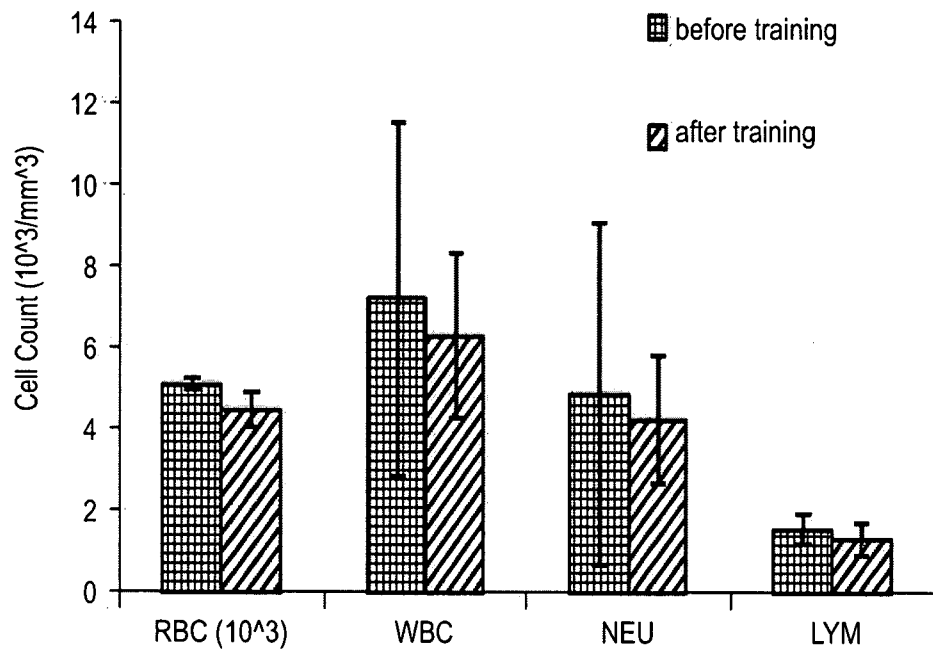


FIG. 1B

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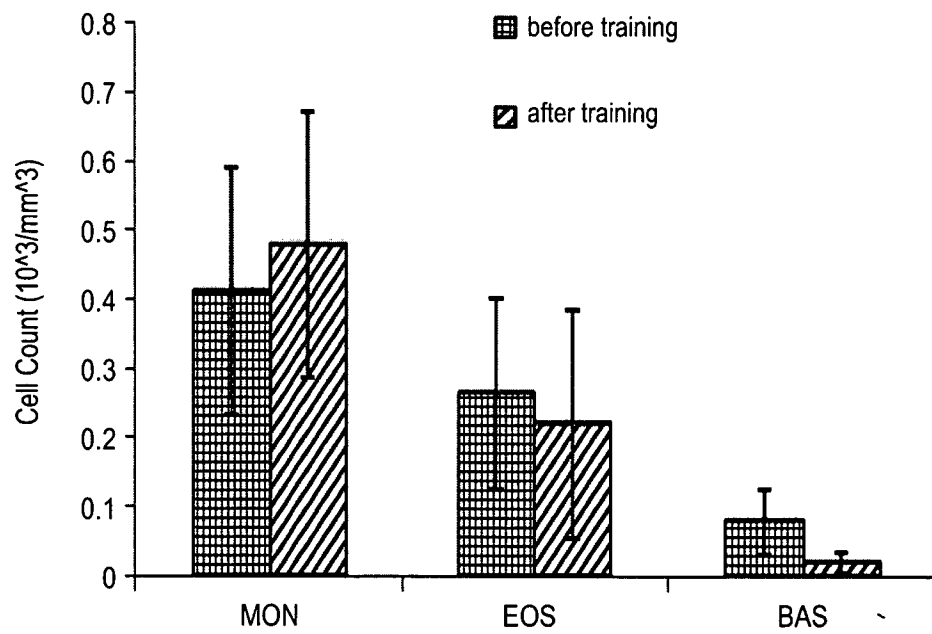


FIG. 1C

Displaying 1983 entities out of 8968 satisfying corrected p-value (Benjamini Hochberg FDR) cut-off 0.05.

Differential Expression Analysis Report						
Test Description						
Selected Test: TTest unpaired unequal variance (Welch)						
p-value computation: Asymptotic						
Multiple Testing Correction: Benjamini-Hochberg						
Result Summary						
	p all	p < 0.05	p < 0.02	p < 0.01	p < 0.0050	p < 0.0010
FC all	8968	1983	1181	820	575	288
FC > 1.1	7120	1983	1181	820	575	288
FC > 1.5	1725	1396	1011	759	557	288
FC > 2.0	434	423	402	369	332	225
FC > 3.0	109	109	107	102	98	87
Expected by chance		99	23	8	2	0

FC = Fold Change

FIG.2A

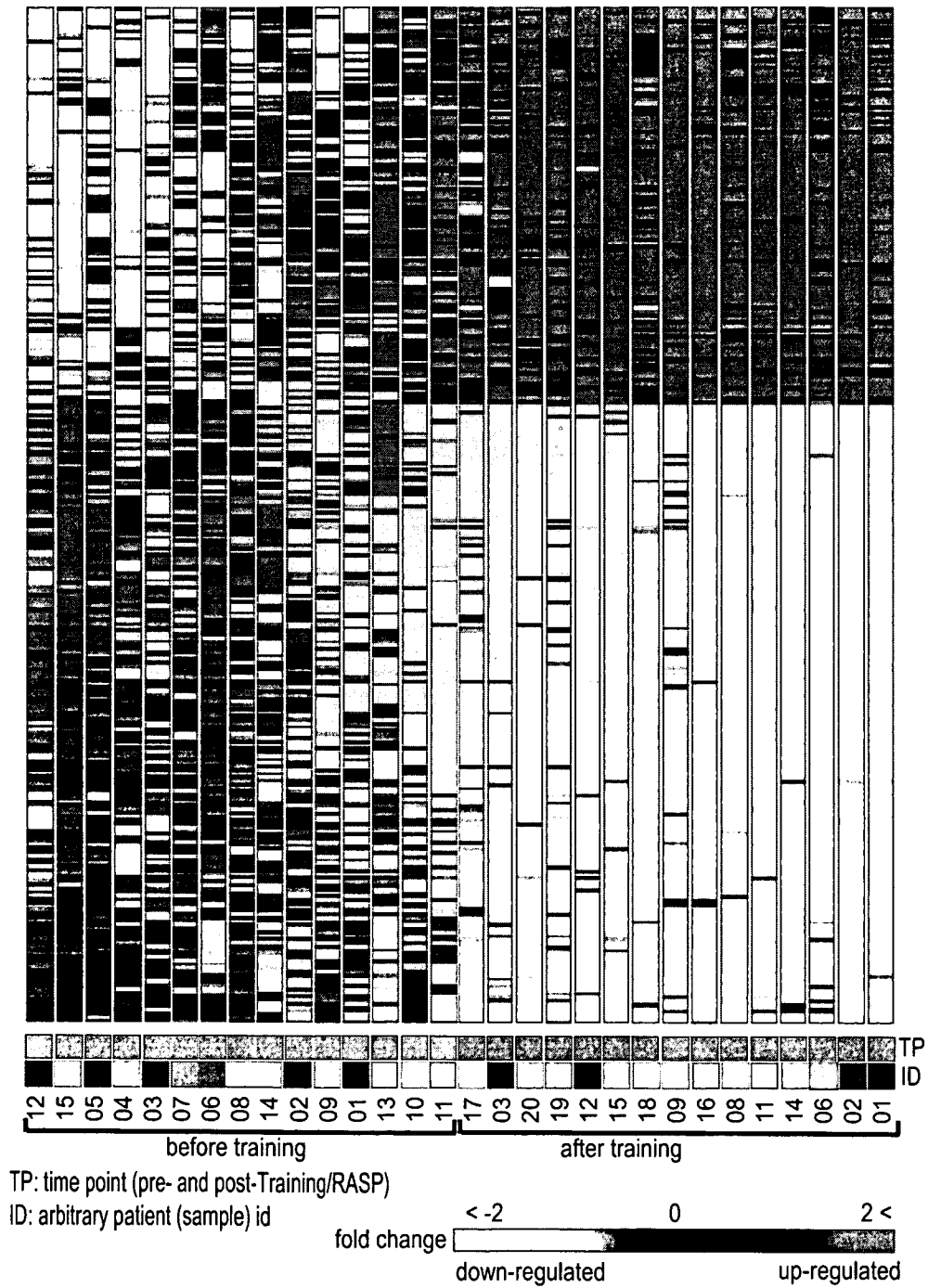


FIG.2B

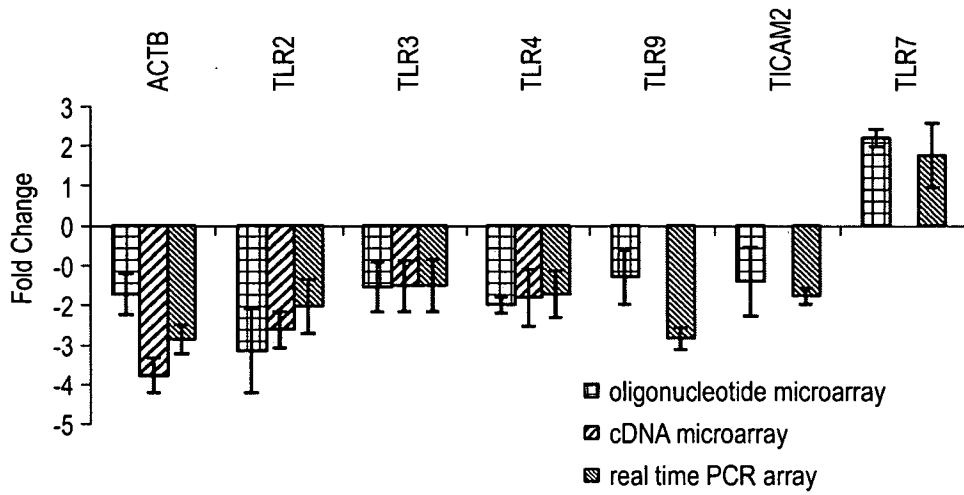


FIG.3A

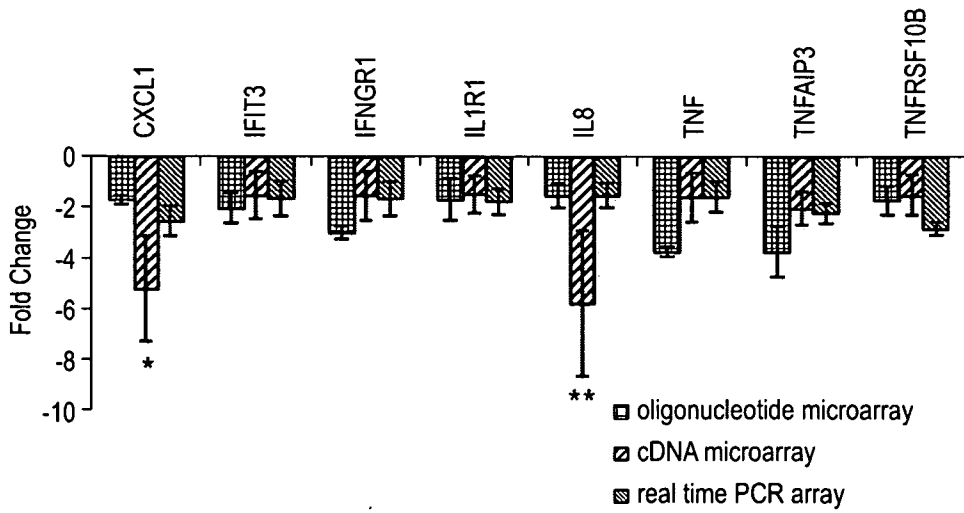


FIG.3B

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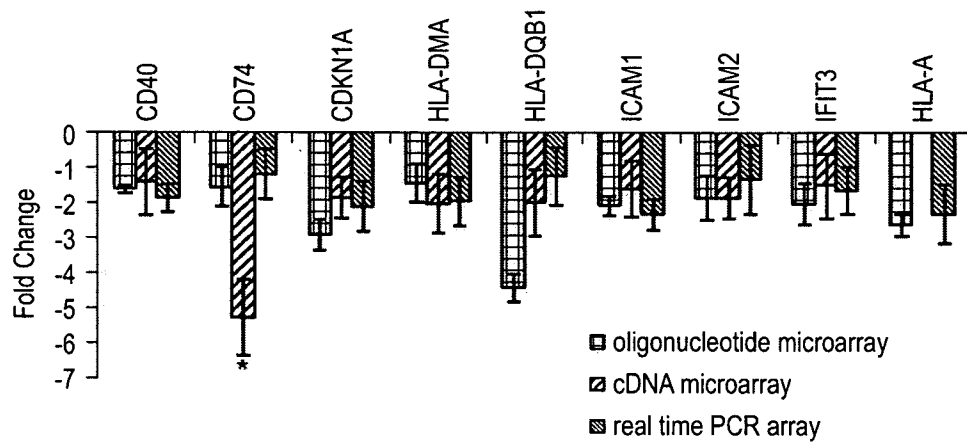


FIG.3C

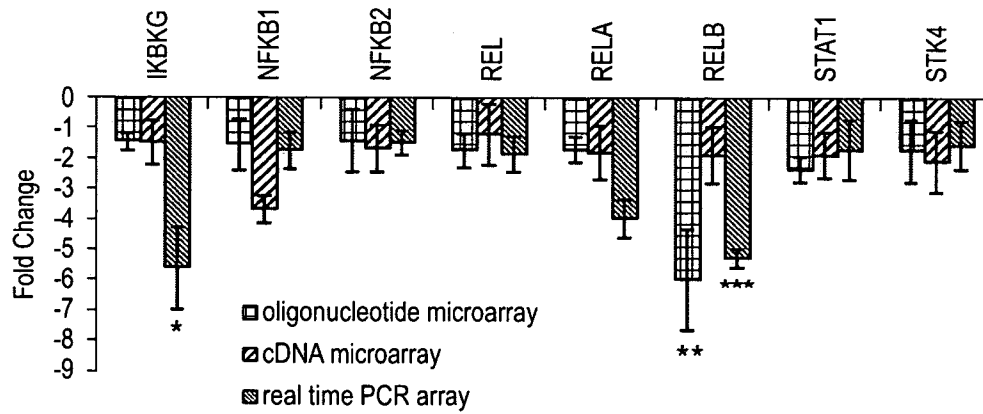


FIG.3D

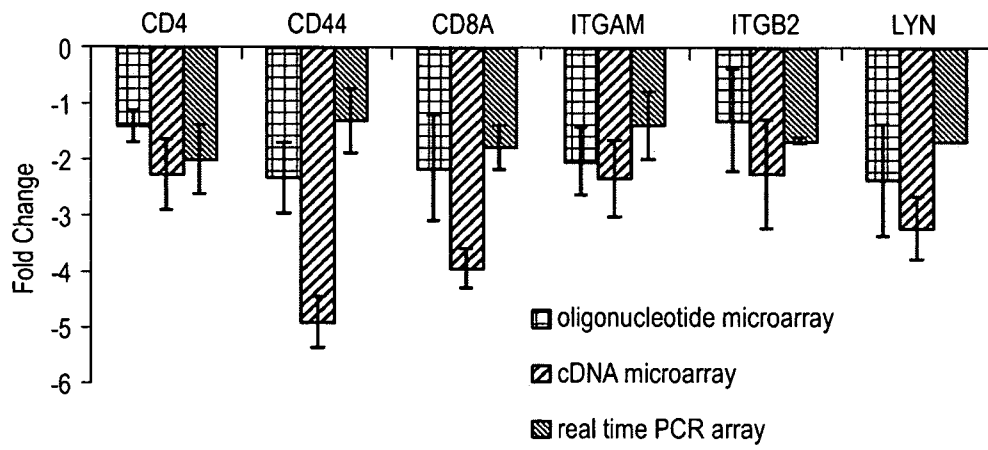


FIG.3E

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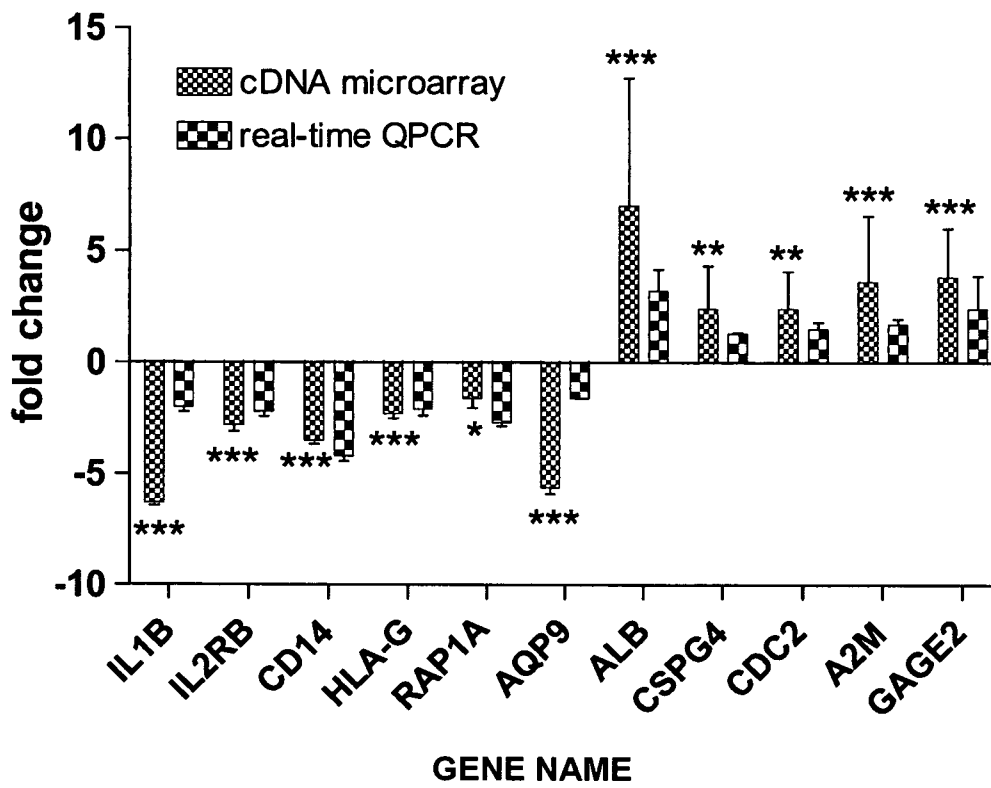


FIG.4A

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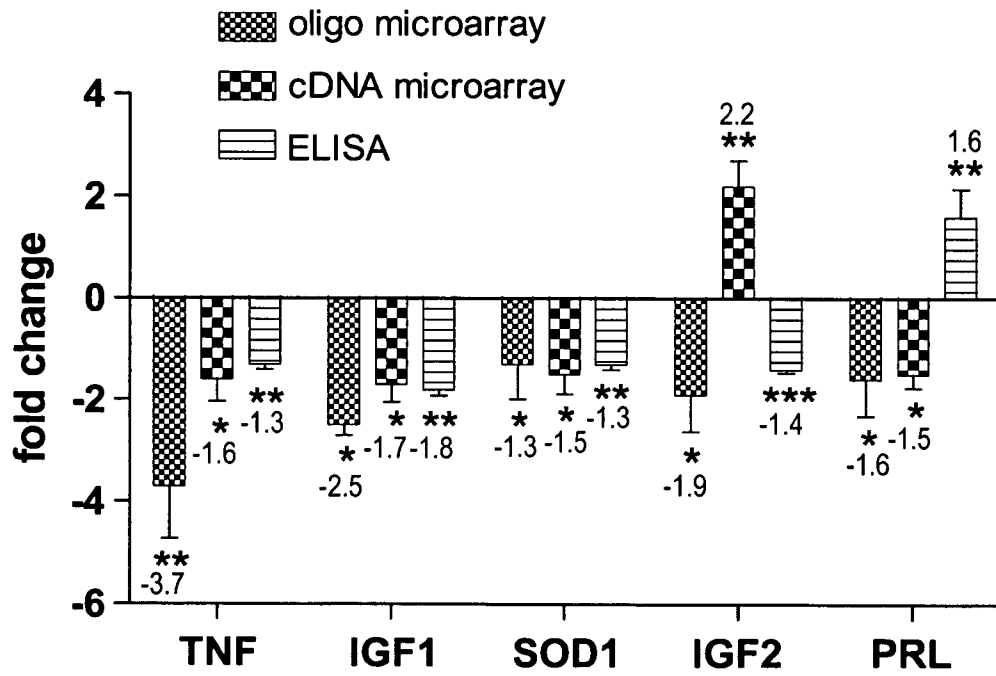


FIG.4B

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FIG.5A

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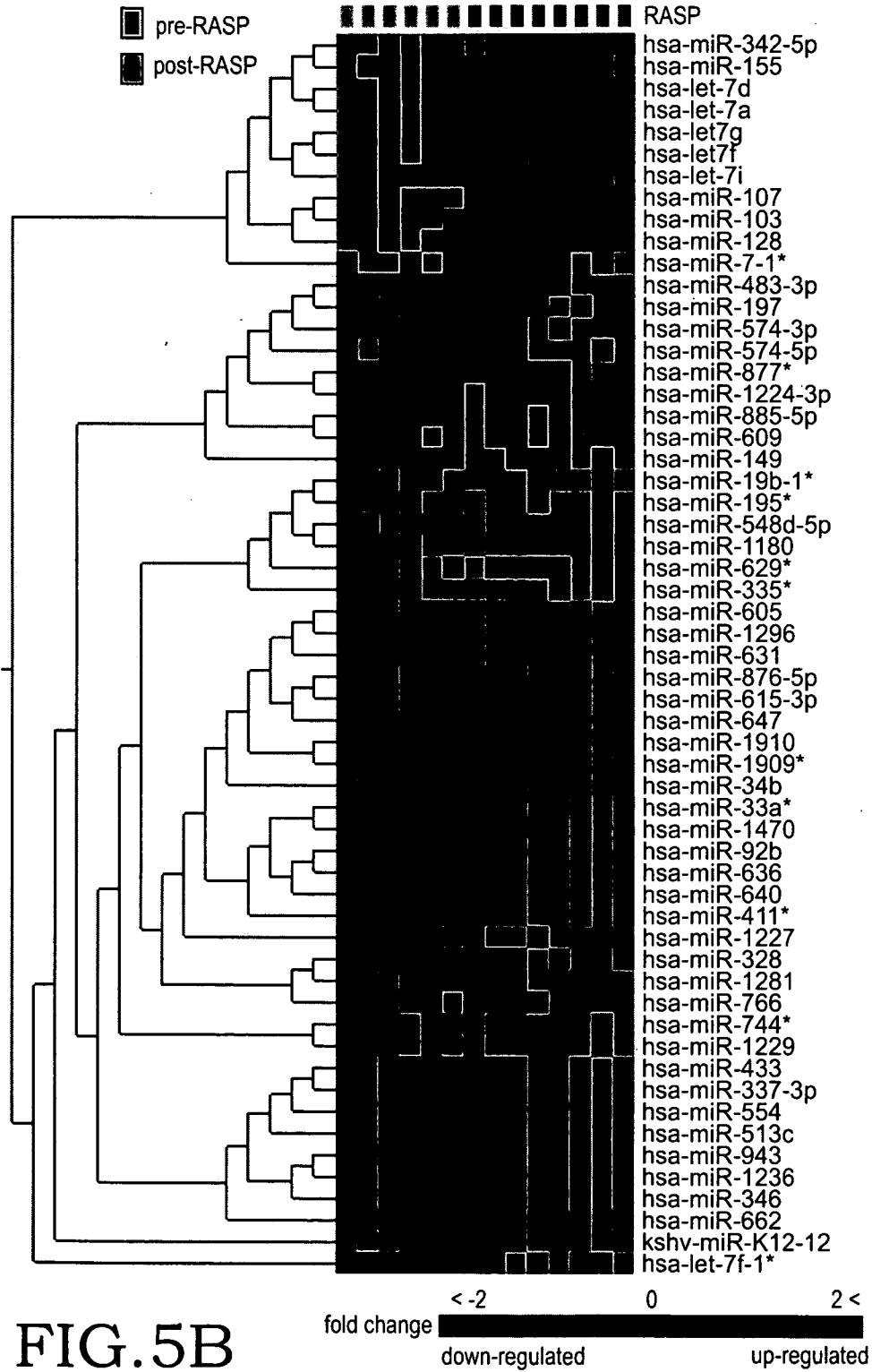


FIG.5B

13/30

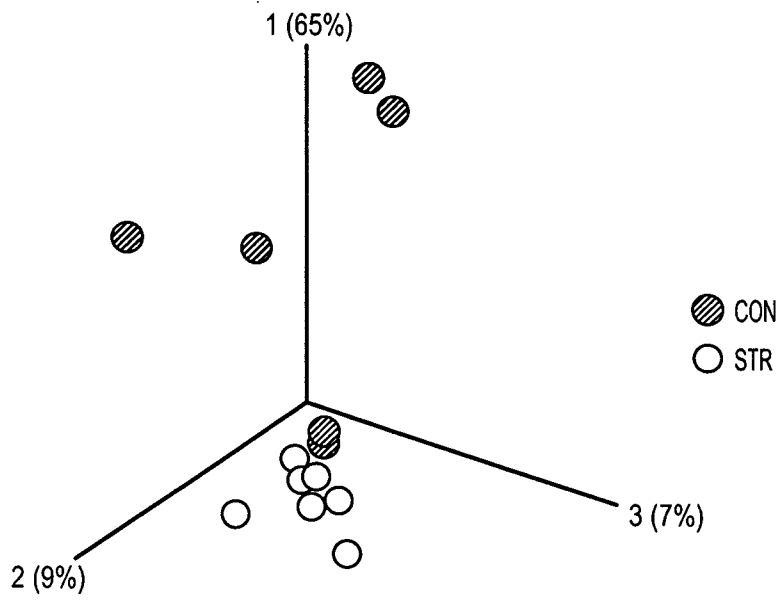


FIG.5C

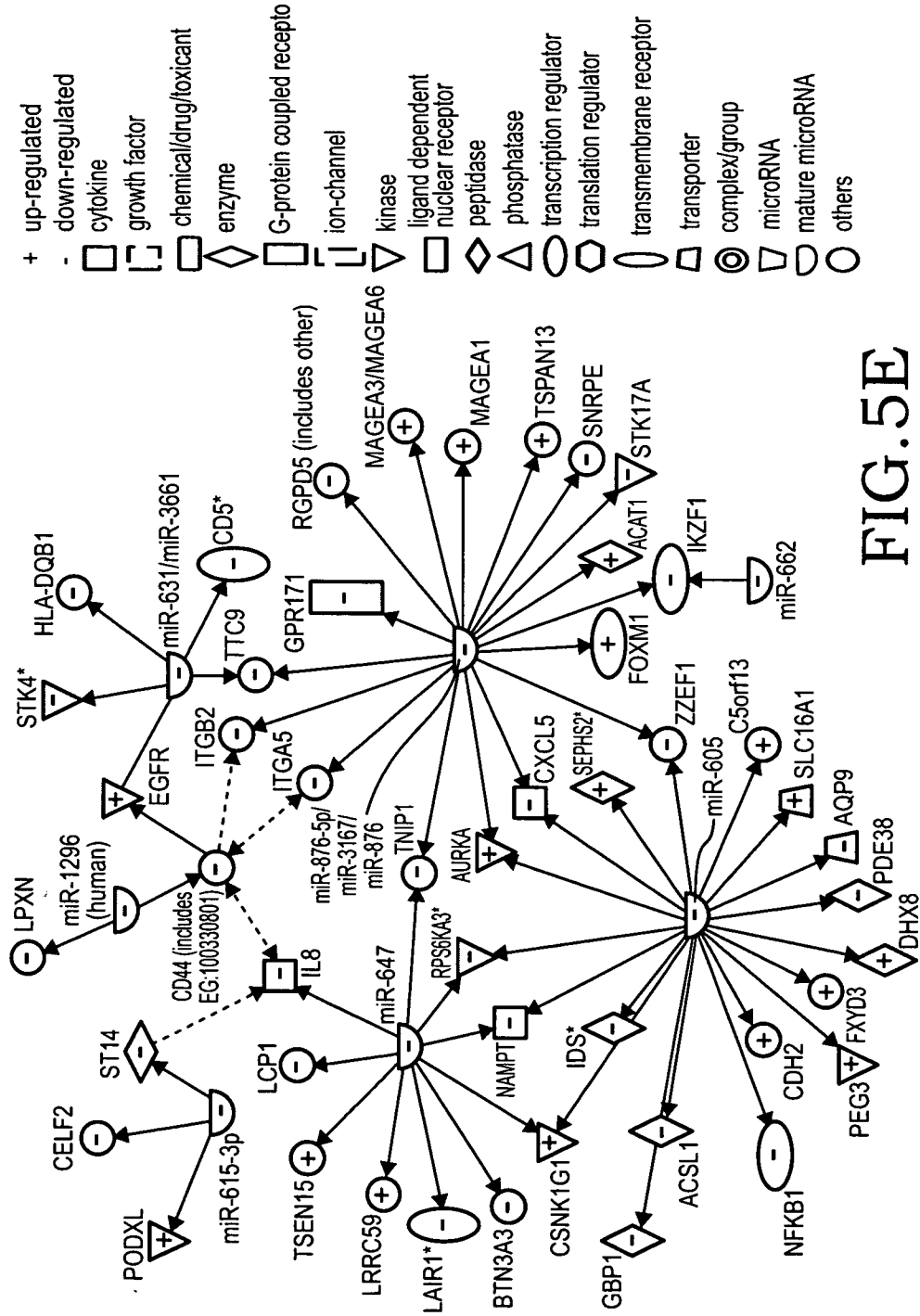


FIG. 5E

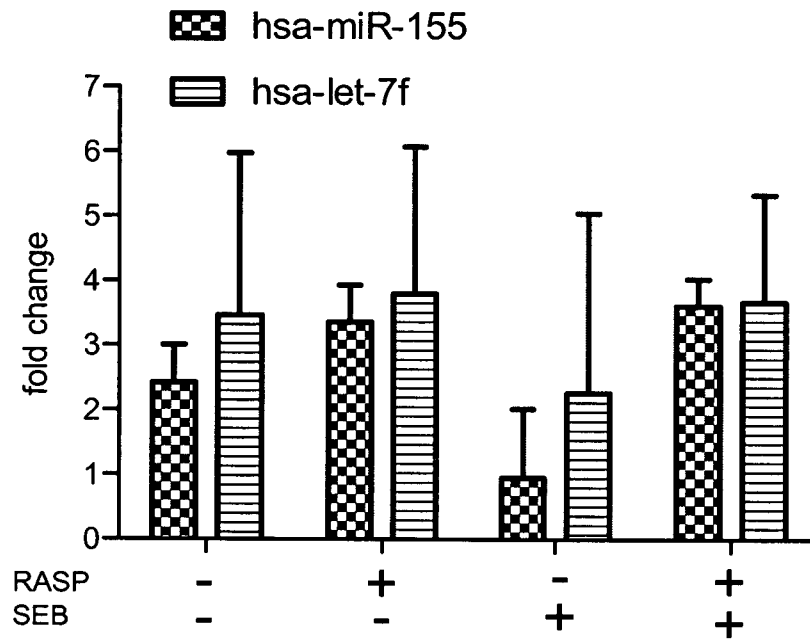


FIG.6

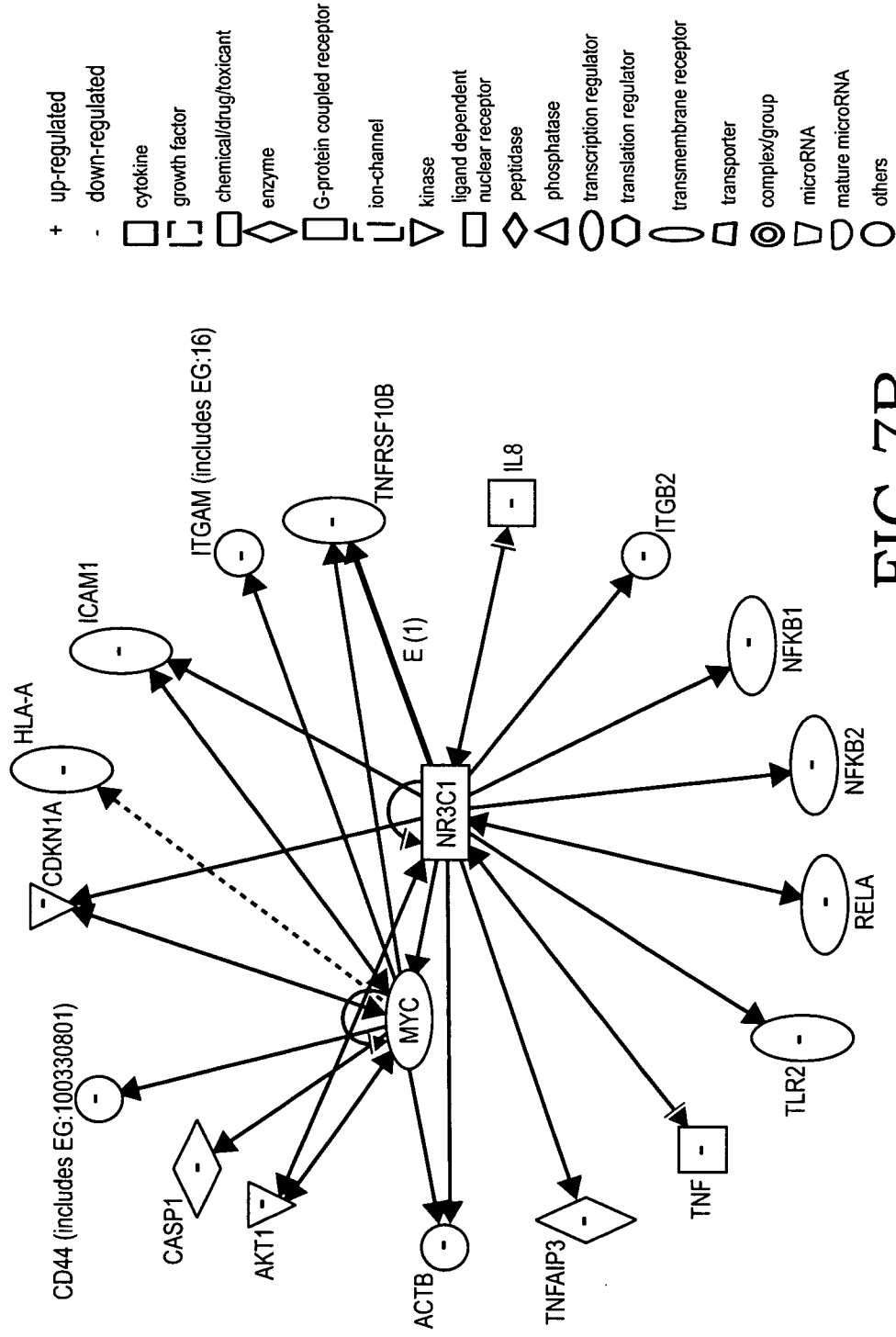


FIG. 7B

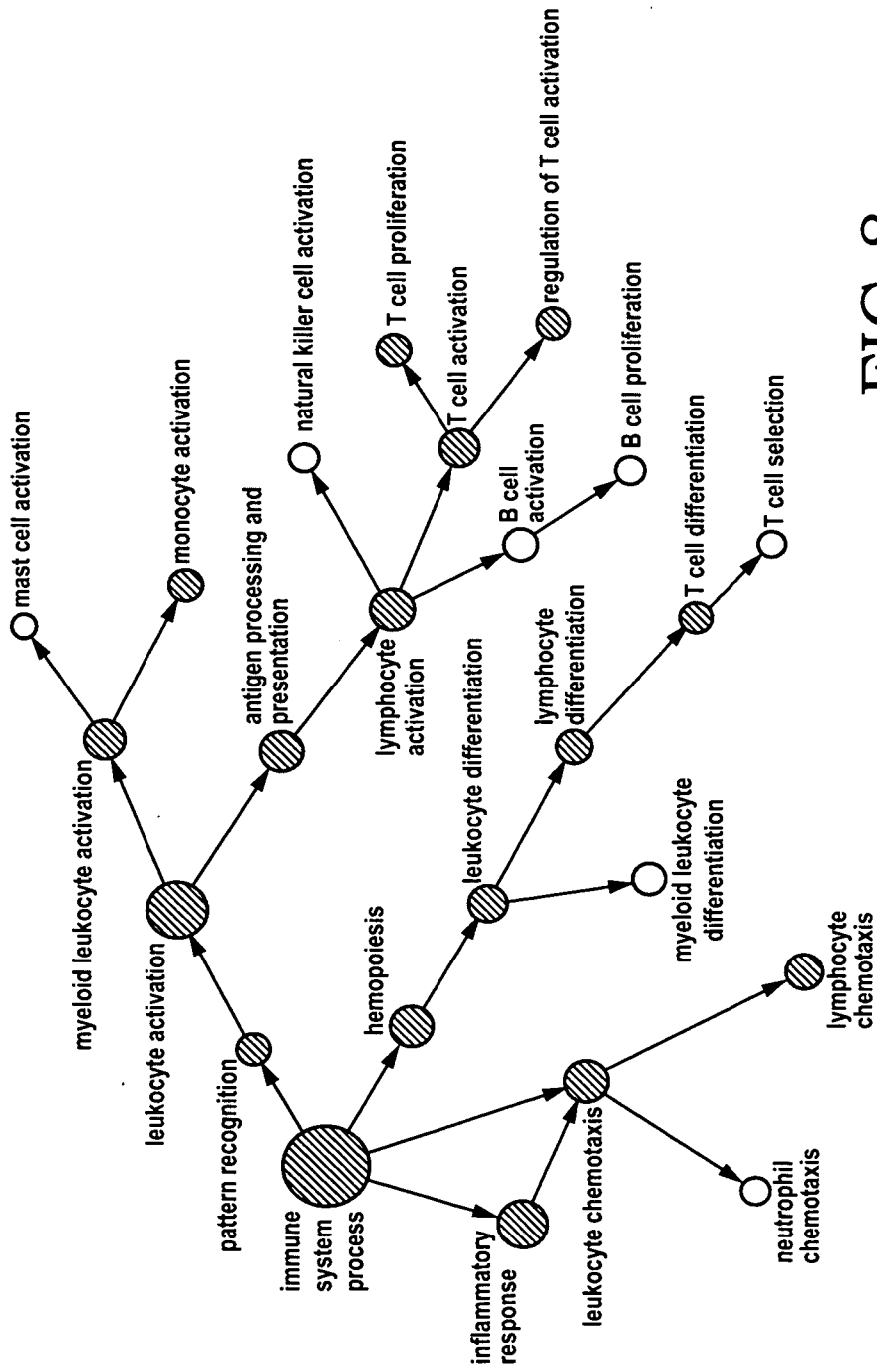


FIG.8

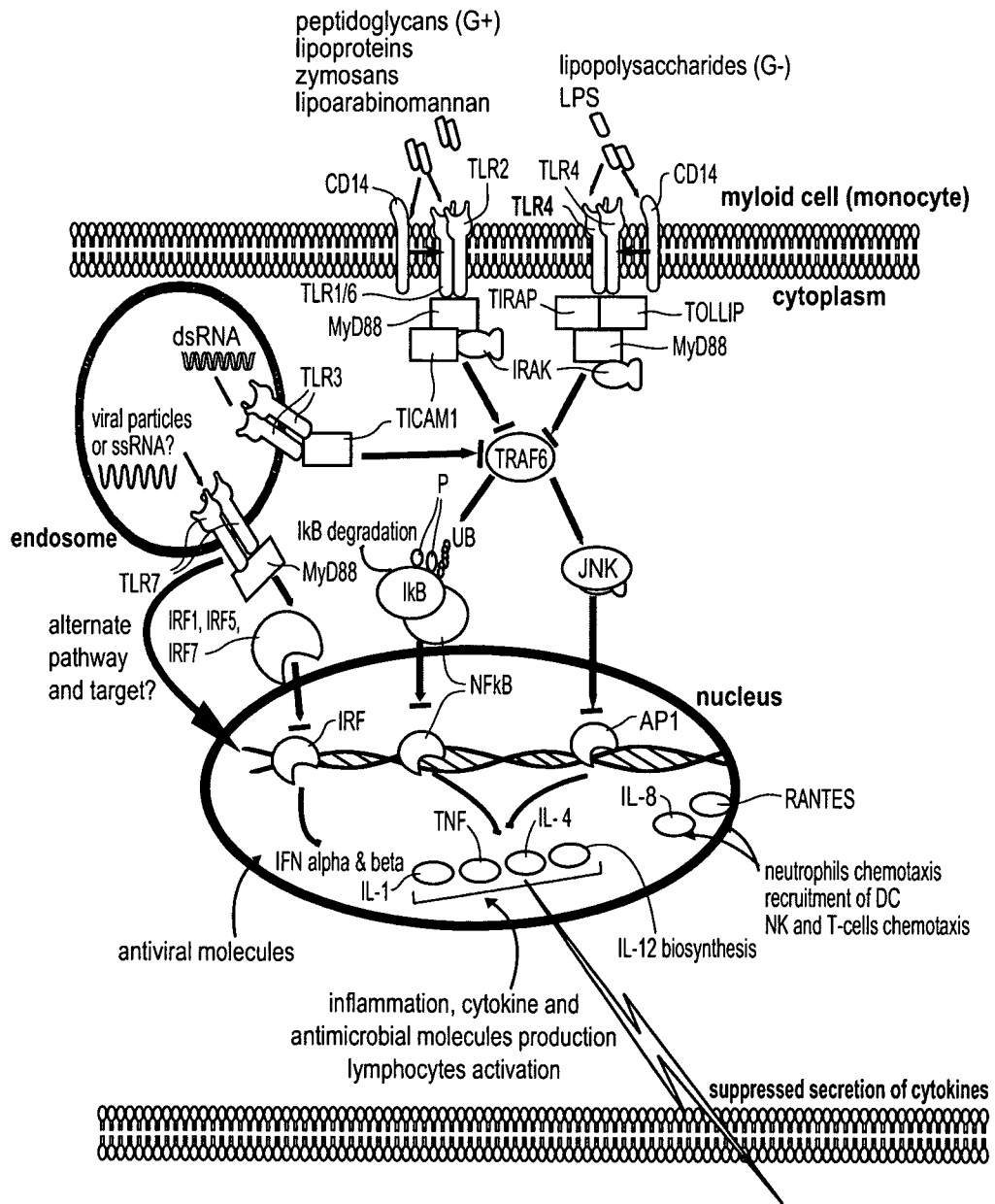


FIG.9B

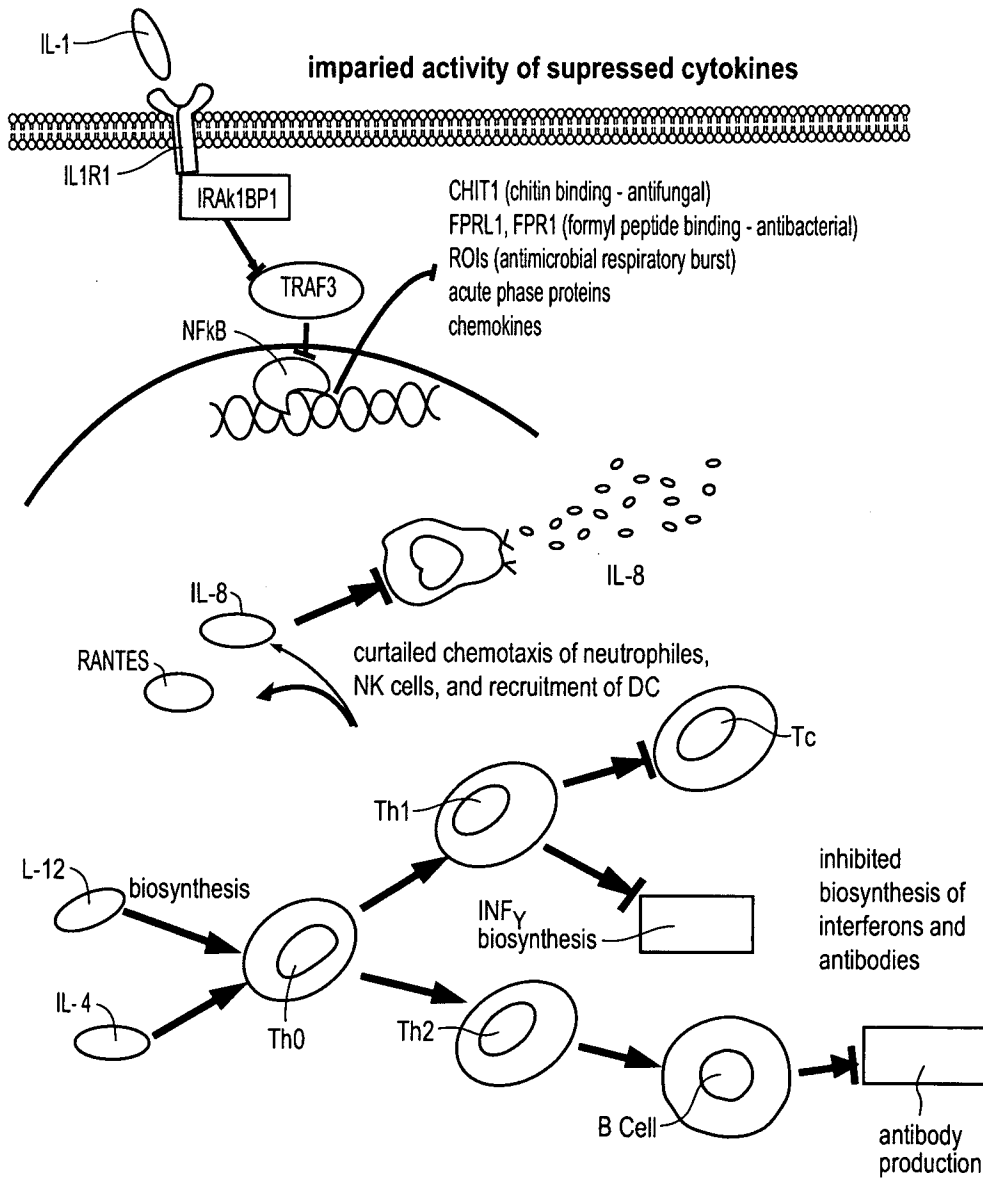


FIG. 9C

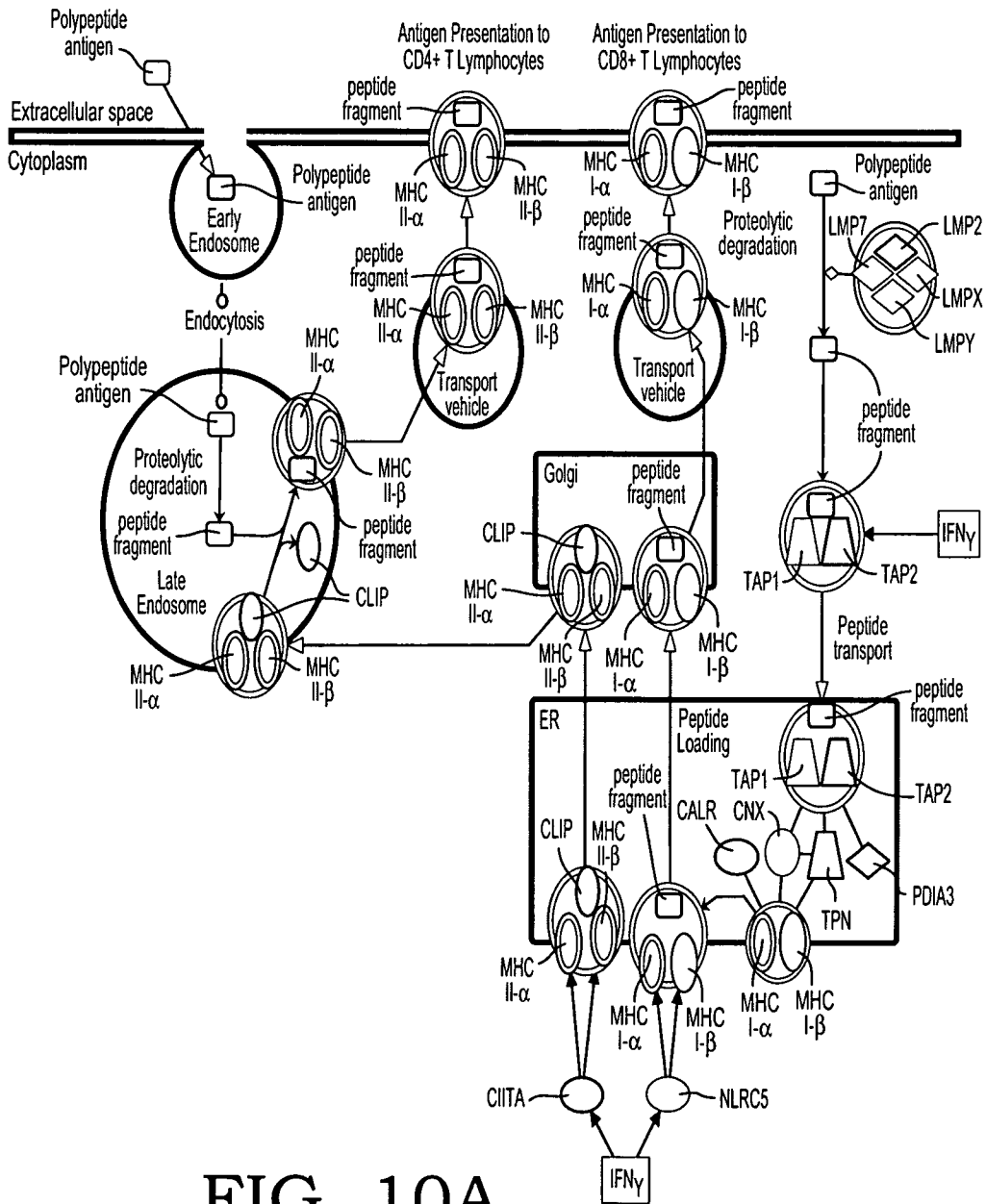
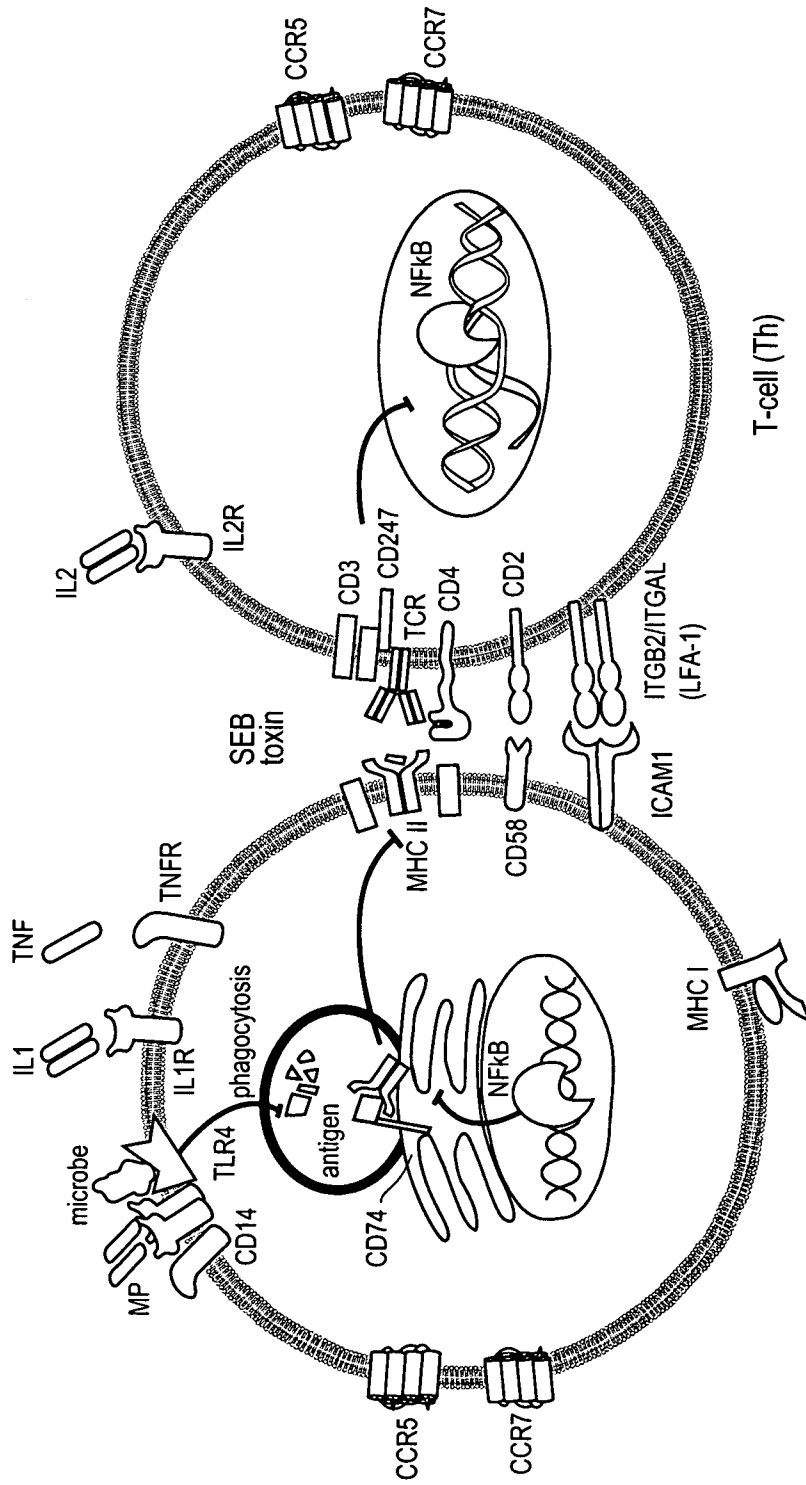


FIG. 10A



antigen presenting cell (DC or MON)

T-cell (Th)

FIG. 10B

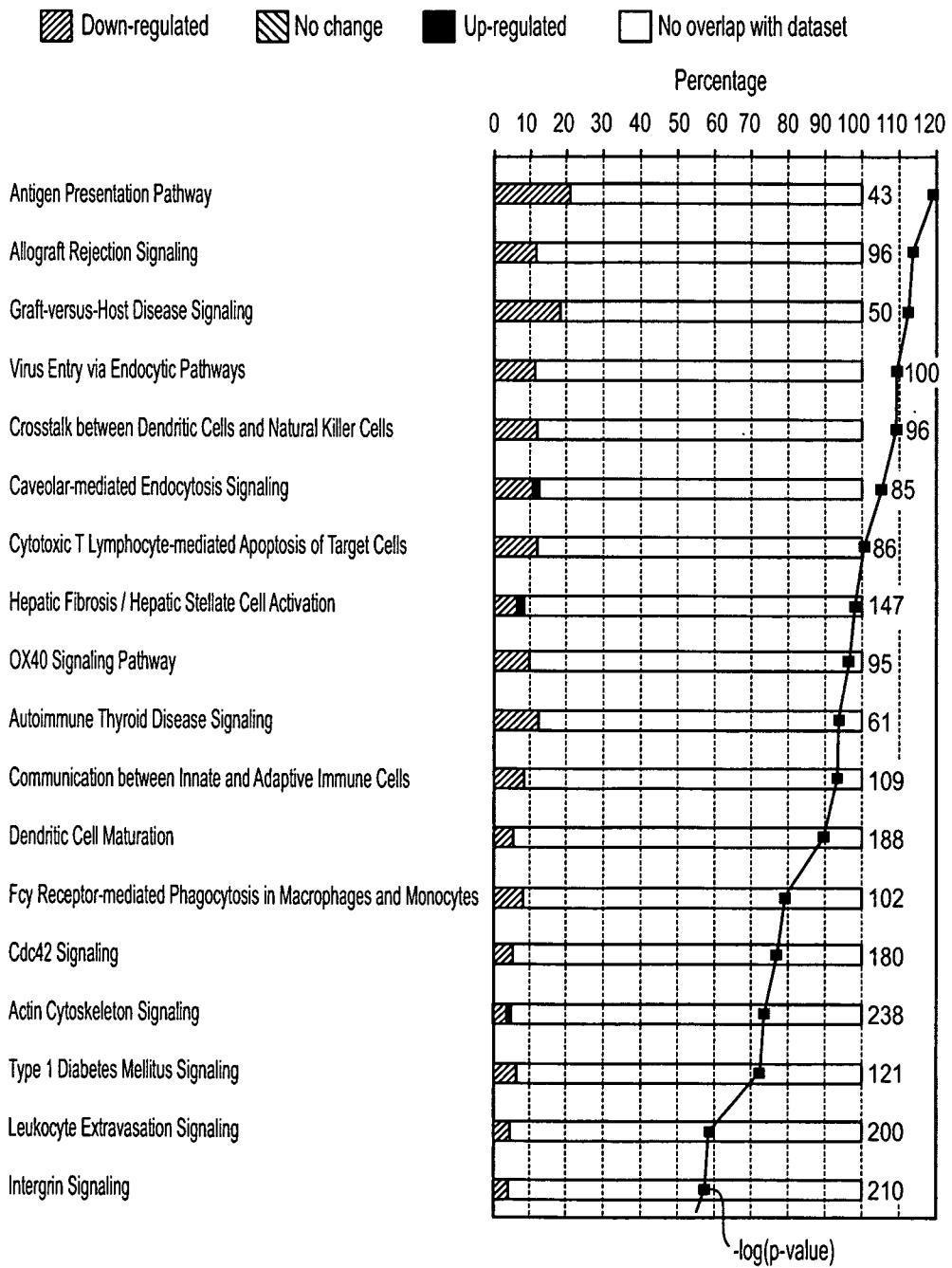


FIG. 11

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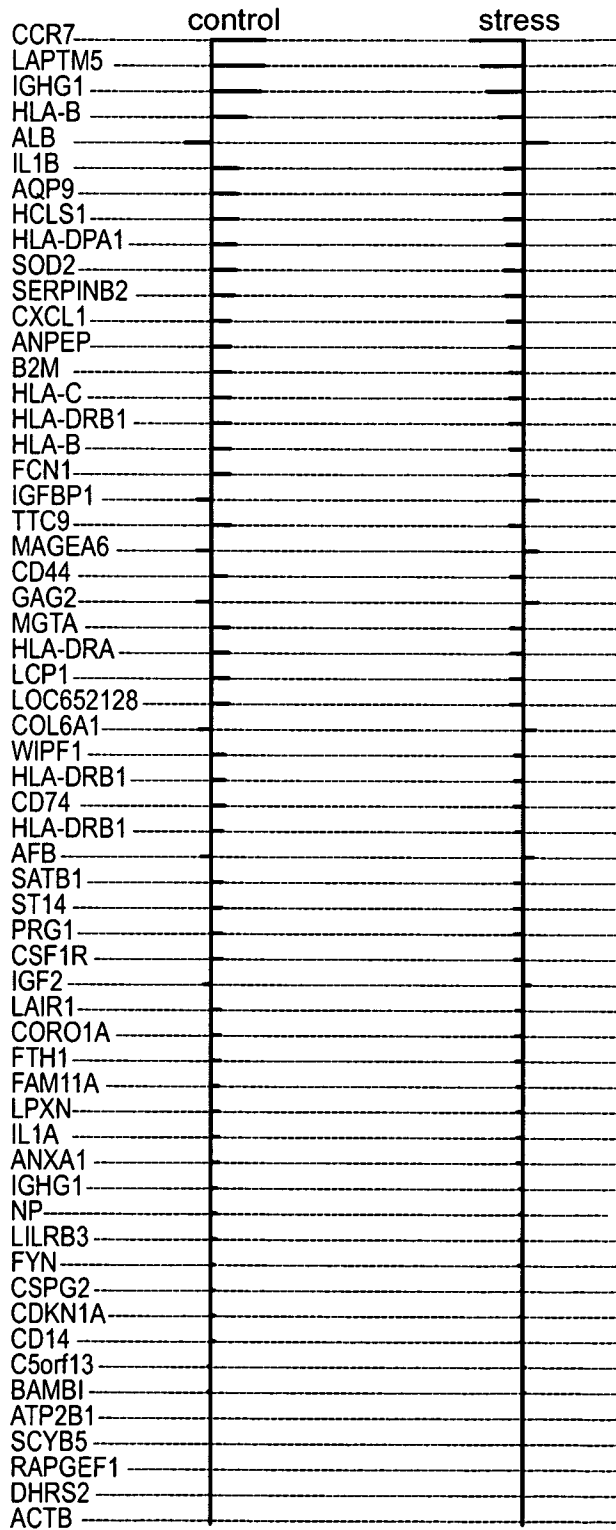
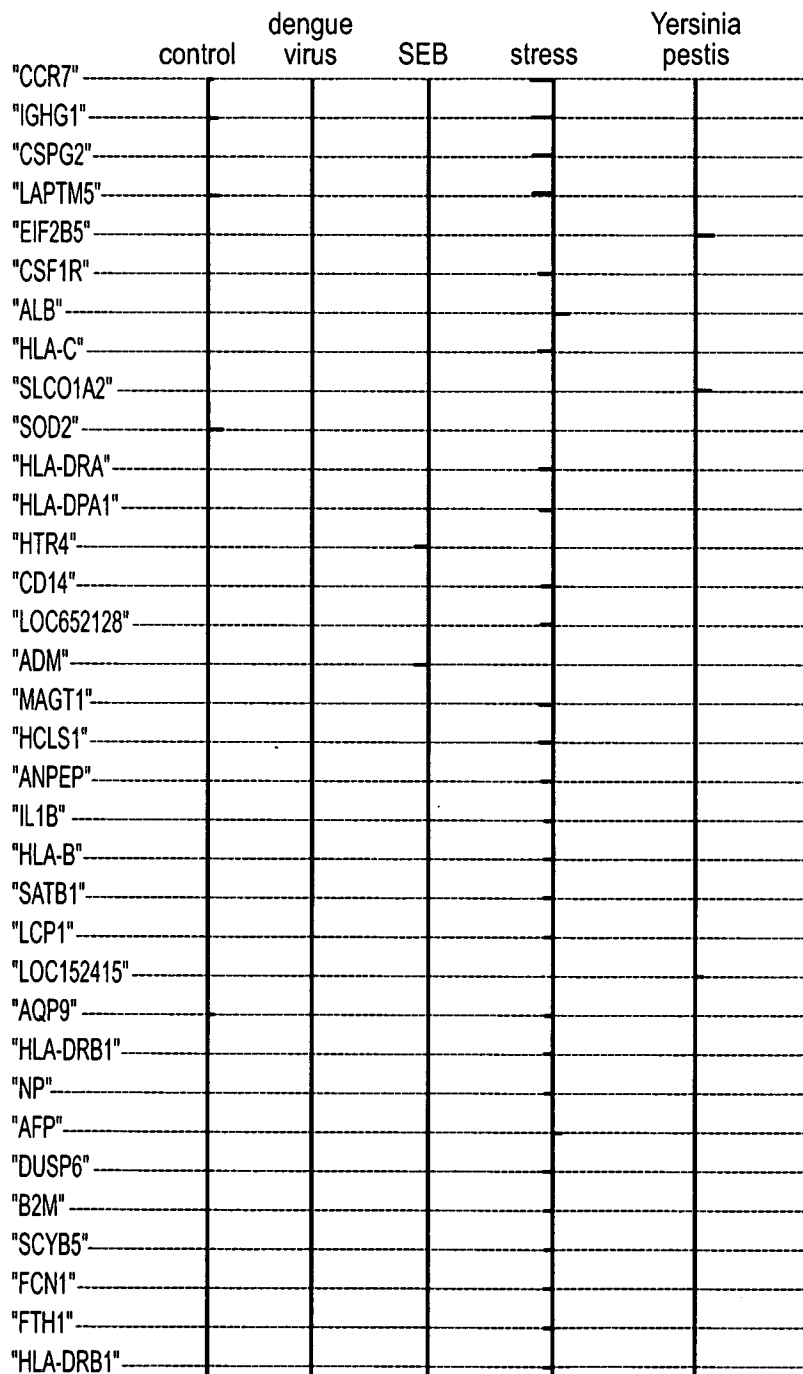


FIG. 12

FIG. 13A



	control	dengue virus	SEB	stress	Yersinia pestis
"PPBP"					
"FCGR3A"					
"IGHG1"					
"CRYAA"					
"IGFBP1"					
"WIPF1"					
"MAGEA6"					
"LPXN"					
"ITPKB"					
"LST1"					
"CXCL1"					
"GAGE2"					
"CDKN1A"					
"FCGR3A"					
"KCMF1"					
"SERPINB2"					
"TTC9"					
"FYN"					
"ZNF609"					
"CENPF"					
"LIMS1"					
"MDK"					
"AX025098"					
"A2M"					
"CD74"					
"IER3"					
"HLA-B"					
"ACTB"					
"ANXA1"					
"GGTL3"					
"FAM11A"					
"LAIR1"					
"CD44"					
"COL6A1"					
"PRKCH"					

FIG. 13B

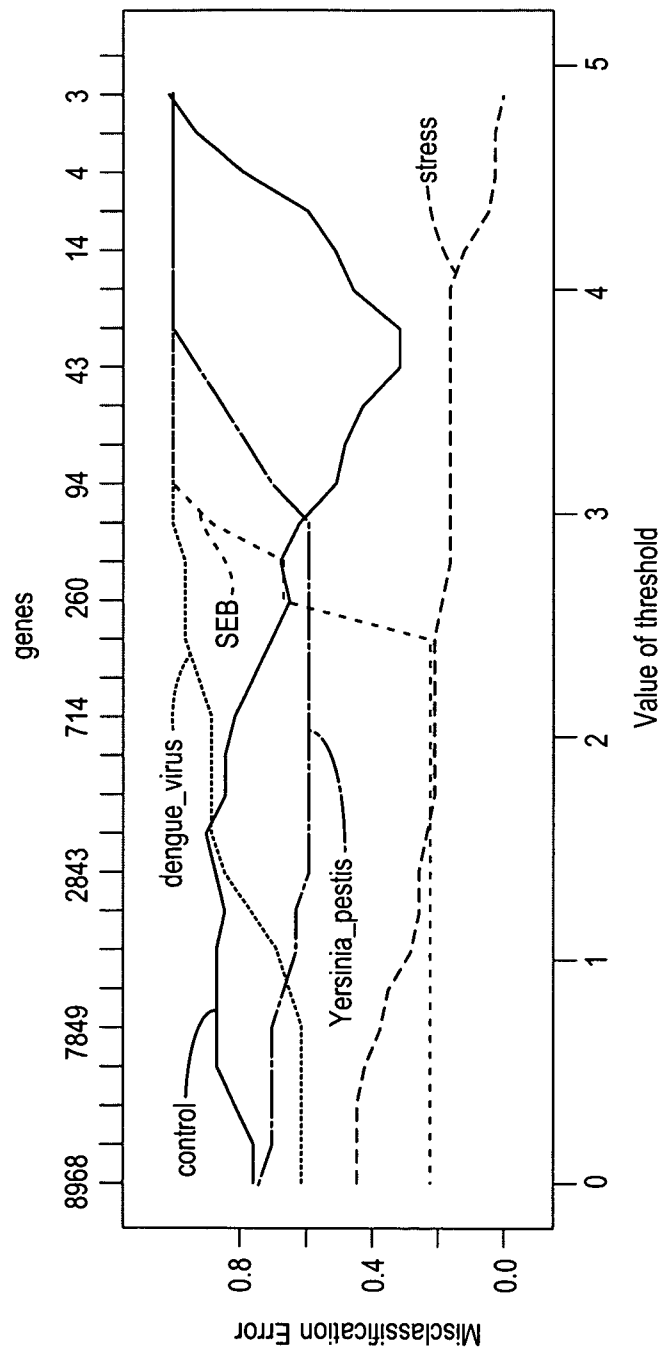


FIG. 14

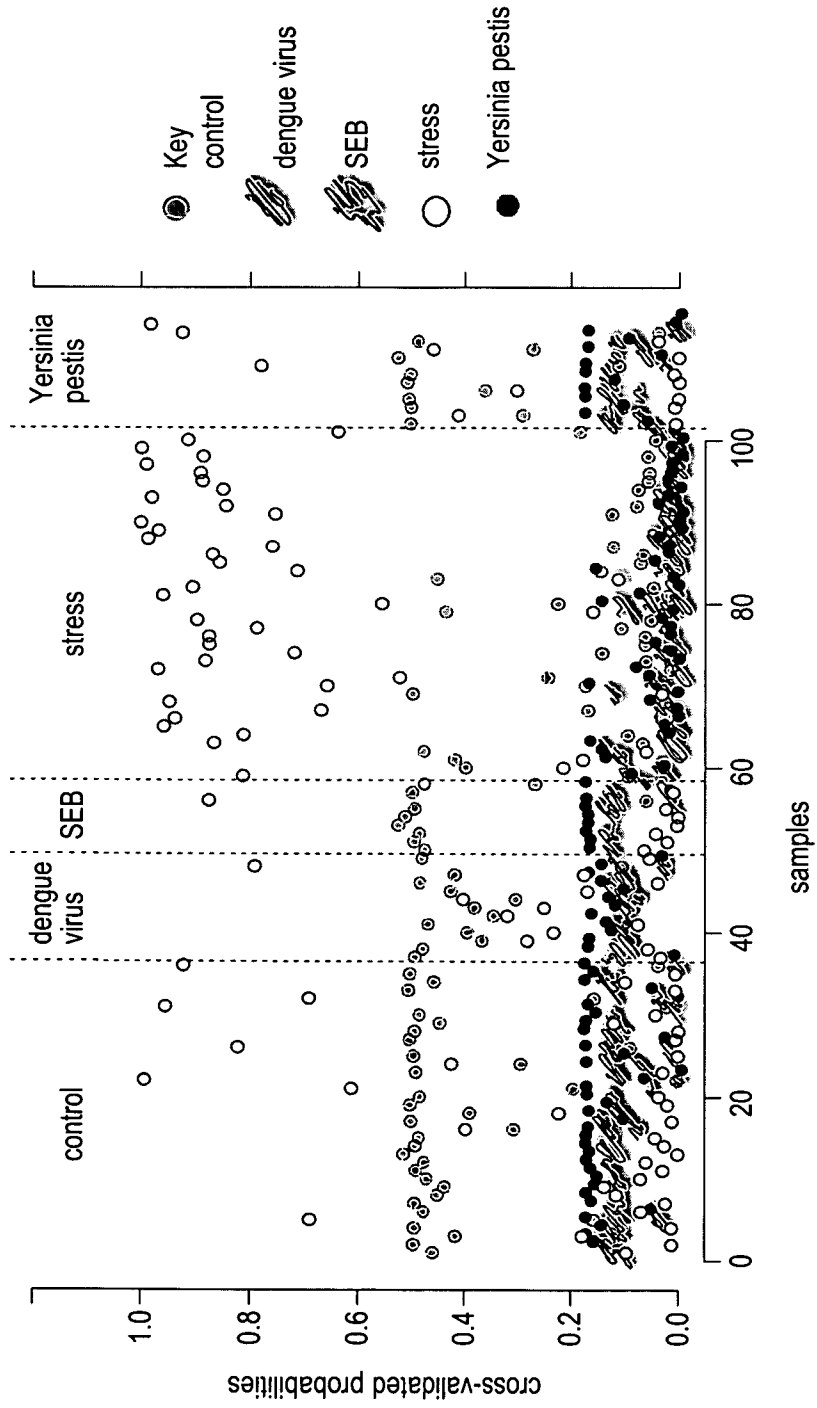


FIG. 15

A. CLASSIFICATION OF SUBJECT MATTER**C12Q 1/68(2006.01)i, C12N 15/11(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68; C12N 15/09; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: stress, immune suppression/dysfunction, diagnostic biomarker, transcripts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZIEKER et al., 'Differential gene expression in peripheral blood of patients suffering from post-traumatic stress disorder' Molecular Psychiatry, Vol.12, No.2, pp.116-118 (2007) See pages 116-117.	1,3,8-9,12-14 ,21-25
A	UDDIN et al., 'Epigenetic and immune function profiles associated with post traumatic stress disorder' PNAS, Vol.107, No.20, pp.9470-9475 (2010) See the whole document.	1,3,8-9,12-14 ,21-25
A	SEGMAN et al., 'Peripheral blood mononuclear cell gene expression profiles identify emergent post-traumatic stress disorder among trauma survivors' Molecular Psychiatry, Vol.10, No.5, pp.500-513 (2005) See the whole document.	1,3,8-9,12-14 ,21-25
A	KAWAI et al., 'Gene expression signature in peripheral blood cells from medical students exposed to chronic psychological stress' Biological Psychology, Vol.76, No.3, pp.147-155 (2007) See the whole document.	1,3,8-9,12-14 ,21-25
A	JP 2008-054590 A (HITACHI HIGH-TECHNOLOGIES CORP et al.) 13 March 2008 See the whole document.	1,3,8-9,12-14 ,21-25

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family


Date of the actual completion of the international search

29 August 2013 (29.08.2013)

Date of mailing of the international search report

02 September 2013 (02.09.2013)

Name and mailing address of the ISA/KR



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INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2013/000097

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	MUHIE et al., 'Transcriptome characterization of immune suppression from battlefield-like stress' Genes and Immunity, Vol.4, pp.19-34 (January 2013) See the whole document.	1,3,8-9,12-14 ,21-25

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 15-20, 26
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 15-20 and 26 pertain to diagnostic methods of human body, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(See extra sheet.)

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1, 3, 8-9, 12-14, 21-25

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

(Continuation of Box No. III)

Invention 1 (claims 1, 3, 8-9, 12-14, 21-25) relates to a set, a library or a kit of diagnostic biomarkers for diagnosing suppressed immune response which is due to stress and peptides or proteins encoded by said biomarkers.

Inventions 2-6 (claims 2, 4-7, partially) relate to a set comprising a stress-regulated gene comprising CCR7, IGHG1, CSPG2, LPTM5, CSF1R, respectively.

Inventions 7-8 (claims 2, 5-7, partially) relate to a set comprising a stress-regulated gene comprising ALB, HLA-C, respectively.

Inventions 9-11 (claims 2, 6-7, partially) relate to a set comprising a stress-regulated gene comprising HLA-DRA, HLA-DPA1, CD14, respectively.

Inventions 12-20 (claims 2, 7, partially) relate to a set comprising a stress-regulated gene comprising LOC652128, MGAT1, HCLS1, ANPEP, IL1B, SATB1, LCP1, AQP9, HLA-DRB1, respectively.

Inventions 21-56 (claim 2, partially) relate to a set comprising a stress-regulated gene comprising NP, AFP, DUSP6, B2M, SCYB5, FCN1, FTH1, PPBP, FCGR3A, IGHFBP1, WIPF1, MAGEA6, LPXN, CXCL1, GAGE2, CDKN1A, TTC9, FYN, SERPINB2, CENPF, LIMS1, MDK, AX025098, A2M, CD74, IER3, HLA-B, ACTB, ANXA1, LAIR1, CD44, COL6A1, PRKCH, MAFB, EVI2A, LAT, respectively.

Invention 57 (claims 10-11) relates to a micro- or nano-chip comprising 55 genes listed in claim 10.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/000097

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2008-054590 A	13/03/2008	None	