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Gene expression profiles of peripheral blood leukocytes after endotoxin challenge in humans

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Abstract

To define gene expression profiles that occur during the initial activation of human innate immunity, we administered intravenous endotoxin ($n = 8$) or saline ($n = 4$) to healthy subjects and hybridized RNA from blood mononuclear cells (0, 0.5, 6, 24, 168 h) or whole blood (0, 3, 6, 24, 168 h) to oligonucleotide probe arrays. The greatest change in mononuclear cell gene expression occurred at 6 h (439 induced and 428 repressed genes, 1% false discovery rate, and 50% fold change) including increased expression of genes associated with pathogen recognition molecules and signaling cascades linked to receptors associated with cell mobility and activation. Induced defense response genes included cytokines, chemokines, and their respective receptors, acute-phase transcription factors, proteases, arachidonate metabolites, and oxidases. Repressed defense response genes included those associated with co-stimulatory molecules, T and cytotoxic lymphocytes, natural killer (NK) cells, and protein synthesis. Gene expression profiles of whole blood had similar biological themes. Over 100 genes not typically associated with acute inflammation were differentially regulated after endotoxin. By 24 h, gene expression had returned to baseline values. Thus the inflammatory response of circulating leukocytes to endotoxin in humans is characterized by a rapid amplification and subsidence of gene expression. These results indicate that a single intravascular exposure to endotoxin produces a large but temporally short perturbation of the blood transcriptome.

Keywords

innate immunity; inflammation; leukocyte transcriptome

Sepsis is a systemic inflammatory disorder induced by infection and, when severe, is accompanied by shock with organ failure. It is a major cause of morbidity and death in critically ill patients (32). While the host inflammatory response is necessary to localize and resolve an infection, it has a central pathogenic role in the development and severity of

sepsis syndromes (3). Therapy of septic shock is based on infection control, hemodynamic resuscitation, and attempts to modify the inflammatory response. Yet in the latter case, treatment directed at a single mediator or inflammatory pathway has met with only limited success in improving survival in sepsis (3, 4, 17, 35). Major challenges remain in understanding the mechanisms that contribute to these processes.

The pathogenesis of sepsis is directly related to innate immunity, which encompasses the immediate host inflammatory responses that result from exposure to microbial components (5). The rapid immune responses are initiated by conserved microbial structures that activate pattern recognition receptors on cell surfaces and in the circulation (e.g., endotoxin, Toll-like receptors, and lipopolysaccharide-binding protein, respectively) (6, 33). These interactions result in cell activation, the release of inflammatory molecules, and the recruitment of inflammatory cells to sites of infection (37).

Investigators have administered the gram-negative bacterial wall component endotoxin to healthy human subjects to learn more about the mechanisms associated with sepsis (48). This model results in physiological responses that are archetypal of innate immunity, cell activation and inflammatory mediator release that cause fever, leukocytosis, tachycardia, tachypnea, and decreased blood pressure, findings similar to the early signs of infection in patients (1, 41, 46). To further characterize the complex phenomena associated with this common bacterial product, we studied differential gene expression of peripheral blood from healthy subjects challenged with intravenous endotoxin using oligonucleotide gene arrays. Oligonucleotide arrays have been used to profile the complex gene networks associated with normal physiology and disease (11, 44, 45). We have previously shown (42) that some acute-phase proteins persist in the blood for 7–10 days after intravenous endotoxin challenge, and we hypothesized that alterations in the transcriptome may persist for several days mirroring this acute inflammatory response.

We show that the administration of a single dose of intravenous endotoxin to humans leads to a rich profile of gene expression changes in blood. These include the induction of genes associated with pattern recognition molecules, intracellular signaling and transcription, cell mobility, and defense function. T lymphocyte-associated genes were repressed, and many genes not previously associated with endotoxin-induced inflammation were differentially regulated during this response. Notably, these alterations in gene expression were rapidly extinguished within 24 h, and scant residua of this response were detected at 7 days.

METHODS

Materials

Vacutainer CPT cell preparation tubes for isolation of peripheral blood mononuclear cells, mouse anti-human CD14-PE, CD45-PerCP, CD4-FITC, CD8-FITC, CD69-PE, CD25-PE, CD3-PerCP, tricolor CD3-FITC/19-PE/45-PerCP, tricolor CD3-FITC/CD16&56-PE/ CD45-PerCP, tricolor CD3-PerCP/4-FITC/8-PE, IgG-PE, and IgG-FITC antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ). RLT buffer, RNeasy Midi and Mini kits, and DNase 1 were purchased from Qiagen (Valencia, CA). PAXgene blood RNA tubes and PAXgene Blood RNA Kit were purchased from PreAnalytiX, Qiagen. T7-d(T)₂₄ primer was

obtained from Genset (La Jolla, CA). SuperScript Double Stranded cDNA Synthesis Kit was from Invitrogen (Rockville, MD). Phase Lock Gel was obtained from Eppendorf (Westbury, NY), and phenol-chloroform-isoamyl alcohol (25:24:1) was purchased from Invitrogen (Carlsbad, CA). HighYield RNA Transcript Labeling Kit was purchased from Enzo Diagnostic (Farmingdale, NY). GeneChip Sample Cleanup Module was obtained from Affymetrix (Santa Clara, CA). Human Multiplex Antibody Bead Kits for Luminex were purchased from BioSource International (Camarillo, CA).

Human Subjects

Six men and four women (20–45 yr; mean $\pm$ SE, 30 ± 2 yr) in good health participated in the study. None used tobacco products, and all had normal screening blood tests, electrocardiograms, and chest radiographs. Informed written consent was obtained from each subject. Human experimental guidelines of the United States Department of Health and Human Services were followed, and the study was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (Bethesda, MD). Eight subjects were given intravenous endotoxin [4 ng/kg, *Escheria coli* O:113, Clinical Center Reference Endotoxin, National Institutes of Health (NIH), Bethesda, MD], and four subjects were given intravenous saline alone. This dose of endotoxin is safe and elicits a broad variety of physiological responses that are similar to the syndrome of sepsis (41, 46, 48). Two of the four subjects given saline participated in the endotoxin portions of the study, separated by at least 6 wk. Blood was drawn from either radial arterial catheters or by venipuncture after the endotoxin or saline challenge. Temperature was monitored with tympanic membrane thermometers, heart rate by continuous electrocardiogram, and arterial pressure by arterial catheter or cuff pressure. Automated total and differential blood cell counts were determined (Coulter). The effects of endotoxin were studied in two different cell populations: peripheral blood mononuclear cells (PBMC) and whole blood.

Blood Cell Preparation and Total RNA Isolation

PBMC—After endotoxin or saline challenge, blood was drawn at 0, 0.5, 6, 24, and 168 h. These time points were chosen to assess responses before and immediately after the lymphopenia and monocytopenia that occur with intravenous endotoxin (47). To minimize in vitro manipulations that might introduce bias into expression profiles (12, 19), blood was collected directly in cell preparation tubes containing sodium citrate and Ficoll Hypaque density fluid (Vacutainer CPT), placed on ice, and centrifuged within 30 min (2,200 *g*, 8°C for 30 min). The PBMC layer was removed, washed with cold PBS, and centrifuged (900 *g*, 8°C for 10 min). Contaminating red blood cells were lysed with ammonium chloride lysing buffer. Sample quality was confirmed by a differential blood smear, and an aliquot of cells was placed aside for surface marker analysis using flow cytometry. After a washing in PBS, the cells were lysed with RLT buffer, homogenized using a syringe and 20-gauge needle, and then stored at -80°C . The process from sample acquisition to cell lysate was performed within 90 min for each sample to minimize the affects of processing on the in vivo expression profile. After all time points per subject were acquired, total RNA was extracted as per RNeasy Midi protocol (Qiagen). The quality of the total RNA was assessed by visualization of intact 18S and 28S ribosomal RNA bands using a 1.2% formaldehyde agarose gel (Ambion, Austin, TX) stained with Sybr Green II (Molecular Probes, Eugene,

OR). The 260/280 ratio of all samples was between 1.8 and 2.0. Baseline samples (0 h) revealed a negligible number of neutrophils. At 6 h, neutrophil contamination of the PBMC preparations was < 10% of the total cells. Our previous work has shown that, because of the low amount of RNA present in neutrophils, this amount of contamination would contribute minimally to the gene profiles of the PBMC (28).

Whole blood cells—In four subjects challenged with intravenous endotoxin, samples of whole blood cells were collected at 0, 3, 6, 24, and 168 h in PAXgene blood RNA tubes. These time points were chosen to sample whole blood immediately after the release of cytokines and after the onset of monocytopenia. In addition, PBMC were collected in these same subjects at 0, 6, and 24 h. Whole blood cell RNA was isolated. Total RNA was extracted and treated with DNase 1 as per PAXgene Blood RNA Kit protocol with the following modifications: after 2 h of incubation at room temperature, blood samples were kept for a minimum of 24 h at 4°C, and total RNA was precipitated overnight with 3 M sodium acetate and 100% ethanol. All samples were processed within 72 h from time of collection.

PBMC and Whole Blood Cell Total RNA Hybridization to Oligonucleotide Probe Arrays

Double-stranded cDNA was synthesized from total RNA (5–20 µg) using 7-d(T)₂₄ primer and SuperScript Double-Stranded cDNA Synthesis Kit. cDNA was purified by Phase Lock Gel-phenol-chloroform extraction followed by ethanol precipitation. Biotin-labeled cRNA was prepared by in vitro transcription using HighYield RNA Transcript Labeling Kit followed by fragmentation with 5 × fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate) at 94°C for 35 min. Fragmented cRNA (10 µg) was hybridized to Affymetrix Hu95Av2 oligonucleotide probe arrays for 16 h at 45°C. After removal of hybridization fluid, the arrays were washed and stained with streptavidin-phycoerythrin (SAPE), and signal was amplified by anti-streptavidin antibody using Affymetrix Fluidics Station 400. Probe set signals were measured using Agilent GeneArray Scanner (Affymetrix).

Validation of Gene Expression Using Real-Time PCR

Real-time PCR (RT-PCR) was used to validate the microarray results for PBMC data sets (TaqMan PCR detection; Applied Biosystems, Rockville, MD). Probes and primers for 12 target sequences were designed using Primer Express computer software (Applied Biosystems) or purchased (Applied Biosystems) (see Supplemental Table S1; available at the *Physiological Genomics* web site).¹ Total RNA from the PBMC of four subjects was extracted as above, treated with DNase 1, and reverse transcribed to cDNA using random hexamers and High Capacity cDNA Archive Kit as per the manufacturer's instructions (Applied Biosystems). The gene of interest was then amplified and quantified using TaqMan Universal PCR master mix and TaqMan probe and primers on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). RNase P was used as internal control (Applied Biosystems). The standard curve method was used to quantify the gene of interest

¹The Supplemental Material for this article (Supplemental Tables S1–S5) is available online at <http://physiolgenomics.physiology.org/cgi/content/full/00192.2005/DC1>.

and then normalized to RNase P. Final results were expressed as fold change, comparing 6 with 0 h after intravenous endotoxin.

Flow Cytometry of Isolated PBMC

Cell surface markers for identifying different cell populations were analyzed at times of 0 and 6 h after endotoxin challenge. The PBMC preparations were evaluated for percentage of T cells, B cells, natural killer (NK) cells, and monocytes. T lymphocytes were analyzed for CD4 and CD8 subsets and for markers of early and late activation. Cells were resuspended in PBS, incubated with mouse anti-human antibody for 30 min at 4°C, washed with PBS, and fixed with 1% formaldehyde. Monocyte and lymphocyte differential counts were determined by staining cells with CD14-PE and CD45-PerCP. CD14-PE bright population was back-gated to light scatter to quantify monocyte percentage, and the remaining cells were considered lymphocytes. The percentage of T cells, B cells, and NK cells was determined by staining cells with tricolor CD3-FITC/19-PE/45-PerCP or tricolor CD3-FITC/CD16&56-PE/CD45-PerCP, gating on CD45-PerCP. T lymphocyte subset (CD4 and CD8) percentages were identified using tricolor CD3-PerCP/CD4-FITC/CD8-PE stain, gating on CD3-PerCP. Finally, labeling with CD3-PerCP/CD4-FITC/CD69-PE, CD3-PerCP/CD8-FITC/CD69-PE, CD3-PerCP/CD4-FITC/CD25-PE, or CD3-PerCP/CD4-FITC/CD25-PE identified early and late activation of CD4 and CD8 cells. Gated events (2,000) were collected using FACScan system and analyzed with CELLQuest software (Becton Dickinson).

Measurement of Blood Inflammatory Protein Markers

Serum samples for tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, interferon (IFN)- γ , regulated upon activation, normal T cell expressed, and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , monocyte chemotactic protein (MCP)-1, eotaxin, granulocyte macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor (VEGF) were measured at 0, 3, 6, and 24 h using a multiplex bead-based assay (Human Multiplex Antibody Bead Kits for Luminex) according to the manufacturer's recommendations. The samples were measured using the antibody bead mix in duplicate with a biotinylated detection antibody followed by streptavidin-phycoerythrin. The plate was read using the Luminex XYP platform (Luminex, Austin, TX), and data were collected for 100 beads per cytokine from each well. The raw data (mean fluorescent intensity) were processed on Masterplex Quantitation software (MiraiBio, Alameda, CA) to obtain concentration values.

Statistical Analysis: Data Transformation

The average difference (AD) values (Affymetrix Microarray Suite 4.0) for the oligonucleotide arrays were stored in the NIH Laboratory Information Management System (LIMS) database and retrieved, transformed, and analyzed with the use of the Mathematical and Statistical Computing Laboratory (MSCL) Analyst's Toolbox (<http://abs.cit.nih.gov/MSCLtoolbox/>) written by P. J. Munson and J. Barb in the JMP scripting language (SAS Institute, Cary, NC). Values (AD) were standardized and transformed using the Symmetric Adaptive Transform, which yields quantile-normalized, homogeneous variance scale intensity values, termed S-AD (16, 28). A consistency test was applied to identify subjects

with a consistent change in the S-AD across all treated subjects. The analysis specified a 1% false discovery rate for each set of detected genes found in the PBMC and whole blood (43). The list was further refined by requiring that the relative change between late time points compared with time baseline (fold change) be at least 50%. To eliminate genes that were at or below the stated detection limits for this assay, an average AD value > 20 was required in at least one of the two compared groups.

The transformed data matrix [samples (columns) by genes (rows)] was subjected to principal components analysis to visualize the relative location of chips in a low-dimensional space allowing for detection of outliers or other relevant patterns. Genes were annotated using the Gene Ontology (GO) database (<http://www.geneontology.org>) and the GO-Significant Collection of Annotations (SCAN) program (<http://abs.cit.nih.gov/goscan/>) for assessing significantly overrepresented annotation terms. GO-SCAN presents an interactive table of terms and permits drill-down into the supporting expression data. Overrepresentation of the annotation category was determined using a one-sided Fishers exact test. The annotation terms were then analyzed using the GO-MAP program (<http://abs.cit.nih.gov/goscan/>), which performs a 2-D hierarchical clustering of terms and genes to group possibly redundant annotations and corresponding genes into blocks that can be presented graphically. Groups of similarly annotated genes may then be studied together in relation to their expression results. One hundred twelve genes of the final gene list have been reported in part as a comparison population describing gene expression in patients with stable sickle cell anemia (28). The entire data set of the above investigation has been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; <http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>), accession number GSE3026.

RESULTS

Systemic Responses to Endotoxin

All subjects given endotoxin developed mild to moderate degrees of malaise, headache, and fever (0 h, $37.1 \pm 0.1^\circ\text{C}$, and 3 h, $39.03 \pm 0.2^\circ\text{C}$) tachycardia (0 h, 62 ± 5 beats/min, and 3 h, 96 ± 4 beats/min) and a decrease in mean arterial pressure (0 h, 90 ± 3 mmHg, and 5 h, 74 ± 3 mmHg) (maximum change from baseline, all $P < 0.01$, paired t -test). Saline-challenged subjects had similar baseline vital signs with no significant changes during the study period. Within 1 h, total leukocyte counts reached their nadir due to margination of cells to activated vascular endothelium (31, 39, 47, 51). This was followed by a rapid rise in total leukocyte counts composed primarily of neutrophils, while mononuclear cells were decreased in number compared with baseline. At 6 h, the number of mononuclear cells began to rise and returned toward normal at 24 h (Table 1). Blood cytokines (TNF- α , IL-1 β , IL-6, IL-8, IL-10, MCP-1, MIP-1 α and - β) rose at 3 h and returned to baseline values by 6–24 h (Table 2). These eight cytokines and chemokines have been described in normal subjects given endotoxin compared with saline in previous publications (reviewed in Ref. 48). In these previous studies, no changes occur from baseline in the saline-challenged subjects.

Cell Components of Mononuclear Cell Preparations

The proportion of lymphocytes and monocytes present after density centrifugation separation at 0 and 6 h is summarized in Fig. 1. Compared with baseline, endotoxin administration resulted in the percentage of lymphocytes falling by almost 50% and the percentage of monocytes increasing by threefold at 6 h. The percentage of T lymphocytes and NK cells remained the same, while the proportion of B cells increased at 6 h (Fig. 1, *inset*). The percentage of CD3+ lymphocytes at 6 h expressing CD4 and CD8 surface markers did not change from baseline (0 h: CD4 $58.0 \pm 2.8\%$; CD8, $35.0 \pm 3.2\%$). CD4+ and CD8+ cells expressing an early activation marker (CD69) increased at 6 h (CD4+ 69+ 0 h, $0.8 \pm 0.3\%$, and 6 h, $5.0 \pm 1.7\%$, $P = 0.035$; and CD8+ 69+ 0 h, $1.9 \pm 0.7\%$, and 6 h, $7.6 \pm 3.1\%$, $P = 0.07$, paired *t*-test). No changes in CD4+69+ or CD8+ 69+ cells occurred in the control subjects, suggesting that cell processing was not associated with induction of this early marker of lymphocyte activation. The percentage of CD4+ or CD8+ cells expressing a late marker of activation (CD25) was unchanged at 6 h (0 h: CD4+25+, $18.1 \pm 4.4\%$; CD8+ CD25+, $1.4 \pm 0.4\%$).

Differential Gene Expression in PBMC After Endotoxin

Endotoxin is cleared from the blood within 30 min after intravenous administration, suggesting that many of its downstream effects are due to secondary mediators that amplify the initial host response to this bacterial component (50). A major source of TNF, an important secondary mediator that enhances the host response to endotoxin, is from the liver (23, 34). The resultant inflammatory milieu in blood has profound effects on genes associated with both the afferent and efferent arms of innate immunity. At 6 h, > 800 genes were differentially regulated; 439 genes were induced, and 428 genes were repressed compared with baseline (Fig. 2A and Supplemental Table S2A). GO categories that were significantly ($P < 0.01$) represented among these genes are depicted by GO-MAPs found in Supplemental Table S2, B (induced genes) and C (repressed genes). Twelve genes were differentially regulated at other time points: 30 min (4 induced), 24 h (5 induced and 2 repressed), and 168 h (1 induced). Eight genes were altered in control subjects, of which only two genes (Hgb- β and AP1G2) overlapped with the endotoxin-associated gene list and were removed from further analysis.

Afferent Limb of Innate Immunity

The afferent limb of innate immunity encompasses those responses necessary for the host to recognize and to activate inflammatory responses to a pathogen (Table 3). Endotoxin administration was associated with the induction of cell surface receptor genes whose products enhance cell recognition of microbial signals [TLR1 and -2, CD14, FPRL1, and scavenger receptors (MARCO)]. Soluble pathogen-recognition molecules and their respective receptors or receptor components were induced [i.e., bactericidal permeability-increasing protein, properdin P factor, C1q receptor 1, C3a receptor 1, and C (3b/4b) receptor 1]. The mRNA of immune complex receptors (Fc- γ receptor I and II) and leukocyte immunoglobulin-like receptors (LILRA3, LILRB2, LILRB3, and LILRB4) was upregulated. The latter are associated with major histocompatibility complex (MHC) class I antigens and may result in a stimulatory (LILRA3) or inhibitory cascade (LILRB2, -3, -4). These

responses suggest that the mononuclear cells are primed to respond to further microbial component exposure.

Simultaneously, major changes occur in the induction of genes associated with intracellular signaling cascades: G protein-linked receptors (protein kinase C or PRKCD), MAPKs (MAPK14 or p38), p21 activated kinase 2 (PAK2), receptor tyrosine kinases [phosphatidylinositol 3-kinase (PI3K), GT-Pase], and receptor tyrosine kinase-associated genes (Src, JAK, Ras, Rho). These pathways are linked to receptor families that affect cell mobility, proliferation, activation, and effector function.

Efferent Limb of Innate Immunity

The activation of host inflammatory cells initiates the efferent limb of innate immunity (Table 3). This results in the release of inflammatory molecules and changes in cell structural components to enhance association with activated endothelium and migration to a nidus of infection. The activation of the mononuclear cells results in changes in cytoskeleton (MARCKS, actin), membrane and ion channel genes (aquaporin 9), and genes that enhance cell motility (uPAR, L-selectin or CD63, CD11b or complement receptor type 3, α -subunit). Anti-proteases (SERPINA1 and -B1) and disintegrins with domains that have potential for adhesion and metalloprotease activity are upregulated (ADAM8 and -9). Immediate anti-pathogen responses include the induction of microbicidal oxidases [CYBA (p22-phox), CYBB (gp91-phox)] and their regulators [superoxide dismutase (SOD)]. These efferent responses are modulated by the induction of cytokines (IL-1Ra), cytokine and chemokine receptors (INFR2, IL-10R- β , IL-17R, CCR1, CCR2), growth factors (VEGF, ECGF1), and growth factor receptors (CSF2RA and -B, BST1 and -2). Arachidonate metabolism is potentiated by the upregulation of platelet-activating factor acetylhydrolase (PLA2G7) and leukotriene B4 receptor and the induction of arachidonate 5-lipoxygenase-activating protein (ALOX5AP), which, with 5-lipoxygenase (ALOX5), is required for leukotriene synthesis. Genes associated with cell stress are upregulated [heat shock (HS) 90-kDa protein 1 β ; HS 70-kDa protein 1A, 1B, and 5; HS27-kDa protein 1; HS-binding protein 1, a negative regulator of HS response].

Transcriptional activator genes responsive to cytokine induction increase in expression including STAT3 (acute-phase response factor-binding IL-6 response elements), STAT1 (transcription factor that binds IFN-stimulated response elements), and enhancer of STAT responses (NMI or N-myc). Genes encoding proteins that bind to regulatory regions involved in inflammatory responses (especially the IL-1 response element of IL-6 gene), CCAAT/enhancer-binding protein (CEBP)B and CEBPD, as well as members of the Fos and JunB family [which dimerize to regulate AP-1/activating transcription factor (ATF) transcription sites] are induced. Negative regulators of transcription appeared as well: BCL6; calreticulin (CALR), which modulates nuclear hormone receptor activity; BATF, a regulator of AP-1/ATF transcriptional events; and HSBP1, a regulator of the HS response.

Repression of Genes After Endotoxin Administration

Signals initiated by the induction of innate immunity (i.e., cytokine, chemokine, and co-stimulatory molecules) control the activation of adaptive immune responses (Table 3).

Activated antigen-presenting cells process proteins from microorganisms into antigenic peptides that are complexed with MHC class II molecules on the cell surface and, in conjunction with co-stimulatory molecules (CD80, CD86), activate T cells. Within hours of the sterile inflammation induced by endotoxin, we observed suppression of genes associated with antigen presenting cell function (CD86, MHC complex class II antigens) and T and B lymphocyte genes (adenosine deaminase; T cell receptor complex components; CD2-, CD3-, and CD4-related genes; IL-2 receptor). Genes associated with T and B cell receptor activation (TNFRSF7) and one of its binding proteins [CD27-binding (siva) protein] as well as CD160, which activates NK, and cytolytic T cells are repressed.

Genes associated with NK cells were likewise suppressed (killer cell lectin-like receptors, or KLRC), as were killer inhibitory receptors (KIR2DL3, KIR3DL1). The latter bind human leukocyte antigen (HLA) class I molecules and are associated with inhibitory cell signals. Genes associated with cell products from cytolytic T cells and NK cells that have a direct lytic potential for target cells were repressed (granulysin; perforin; granzyme A, M, and K; cathepsin).

Cytokines, chemokines, and chemokine receptors that regulate lymphocyte activation (lymphotoxin LTB) and trafficking (CCL5-RANTES, XCL1 lymphotactin) are downregulated, as are G protein-coupled chemokine receptors (CCR7 and CXCR3). Suppression of multiple genes associated with protein synthesis (ribosomal genes), translation, and processing was observed.

Forty-eight genes whose absence or mutation has been associated with overt immunodeficiency (i.e., JAK3, NCF1, CYBA, LRBA), metabolic defects (i.e., CTNS, PYGL, GM2A), neurological syndromes (i.e., TBL2, HIP1, FAHL4), tumorigenesis (i.e., RB1, NBS1, CD63), and eye diseases (i.e., VMD2, RP2) were differentially regulated by endotoxin. Many of these genes have not typically been associated with the acute-phase response or innate immunity (Table 4).

Gene Expression in Whole Blood Samples

To assess changes in a complementary tissue, we measured gene expression in whole blood from four subjects (Fig. 2B and Supplemental Table S3A). Whole blood gene expression was altered at 3 h (40 up- and 9 downregulated genes), 6 h (100 up- and 92 downregulated genes), and 24 h (1 upregulated gene). No genes in whole blood were differentially regulated at 168 h. At 6 h, we measured simultaneous changes in gene expression in the PBMC of the same four subjects; 134 genes were induced and 129 genes were repressed compared with baseline. Twenty-nine induced and 32 repressed genes were common between the PBMC and whole blood samples. GO categories that were significantly ($P < 0.01$) represented among these genes are depicted by GO-MAPs found in Supplemental Table S3, B (induced genes) and C (repressed genes).

The defense response was an overrepresented gene category that included genes from the afferent arm of innate immunity (peptidoglycan recognition protein, TLR5, CD14, C3aR1, LILRA3), intracellular signaling proteins (MAPK14, MAP4K4, FGR, JUNB), transcription factors (NFkB2, BATF, ETV6), cell mobility (VASP, LIMK2, MARCKS, aquaporin 9), and

cytokine and cytokine receptors (IL-1 β , IL-1RAP, IL-1RN, IL-1RII, IL-4R, IL-18R, IL-18RAP) (Supplemental Table S3B).

Downregulated defense response included T cell- and B cell-associated genes [CD2 antigen (p50); CD3D antigen; T cell receptor- α , - β , and - γ ; CD79A; CD79B], cytolytic and NK cell-associated genes (perforin 1, granulysin, granzyme M), MHC molecules (MHC II DP- α 1 and - β 1, MHC II DQ- α 1), and cytokine receptors (IL-2R β , IL-7R) as well as genes for multiple ribosomal proteins (Supplemental Table S3C).

Thus similar biological themes emerge from analysis of whole blood gene expression compared with mononuclear cells: increased gene expression associated with the defense response and response to pathogens, and decreased expression of genes associated with lymphocytes and ribosomal proteins (Supplemental Table S4).

Quantitative PCR and Array Expression

Confirmatory assays of relative gene expression across a range of array expression were performed on 12 genes using real-time quantitative PCR (TaqMan). Results are represented in fold change, comparing 6 with 0 h. TaqMan fold changes were comparable with the array results (Fig. 3).

DISCUSSION

To model gene profiles in the early phases of a systemic infection, we administered endotoxin to normal volunteers and measured serial changes in gene expression in PBMC and whole blood. The greatest changes occurred by 6 h and resolved by 24 h. Exposure to a single microbial component resulted in a rich variety of changes in gene expression including the induction of genes associated with pattern recognition molecules, signal transduction, transcription factors, cell mobility, cell proliferation, and microbial killing, the downregulation of lymphocyte-associated genes, and expression of genes not previously associated with endotoxemia or acute inflammation. The endotoxin phenotype did not persist in the blood transcriptome at 24 h. This broad and rapid on-off response of gene expression is in stark contrast to the sustained inflammatory phenotype that occurs during clinical sepsis, due in part to continued exposure to microbial components with recruitment of pathways that promote a sustained inflammatory response (3, 25). Several different counterregulatory mechanisms may have contributed to the extinction of the endotoxin phenotype: anti-inflammatory cytokines (i.e., IL-1 receptor antagonist, IL-10), inflammatory mediator clearance, removal of circulating inflammatory cells by adherence to endothelium or by apoptosis, and marrow replenishment of cells to the circulation.

Human immune cell responses to pathogens and their components have a shared core pattern of gene expression as well as pathogen-specific responses (26, 38). Monocyte-derived macrophages activate genes encoding receptors, signal transduction molecules, and transcription factors that prime the macrophage for subsequent environmental interactions (38). Gene profiles induced by endotoxin stimulation of monocyte-derived dendritic cells mimic almost the entire core gene expression induced by intact bacteria in vitro (26). While transcript levels of genes associated with phagocytosis and pathogen recognition decline

immediately after contact with pathogens, a wide variety of genes associated with immunity are activated (26). Gene levels of cytokines, chemokines and their receptors, and cytoskeleton, signaling, and transcription factors are increased. Antigen processing and presentation genes as well as genes involved in reactive oxygen species are induced in a sustained fashion (26). In contrast to these studies of in vitro monocyte-derived cells, our expression profiles of PBMC and whole blood in vivo show a strong upregulation of genes associated with pathogen recognition and phagocytosis at 6 h.

Other investigators have evaluated gene expression changes in PBMC in vitro after exposure to endotoxin or killed bacteria using amplified polyA RNA and a cDNA microarray format (7). A stereotypic program of gene expression was found with the preponderance of induced genes associated with cell-cell signaling pathways and proinflammatory mediators and repressed genes that included chemokine and chemokine receptors, pathogen recognition and adhesion molecules, components of the respiratory burst, and genes encoding MHC class II molecules (7). Only a modest overlap was found between our 439 induced genes at 6 h and those described by Boldrick et al. (7) in PBMC stimulated with endotoxin; 35 (7.8%) of our induced genes were found in their gene database. Eleven genes were concordant whereas 24 were discordant with the direction of change. Of our 428 repressed genes observed at 6 h after endotoxin, 50 (12%) were found in their database: 30 were concordant and 20 were discordant with our results. Our gene list differs from these in vitro observations with an enhanced role for genes associated with pathogen recognition, chemokines and their receptors, adhesion molecules, and respiratory burst but is similar regarding the suppression of MHC class II genes.

The lack of similarity of our observations of gene expression in PBMC after endotoxin challenge with in vitro data may be due to culture conditions and different array targets (i.e., arrays enriched for detection of lymphocyte-associated genes) (7). Gene expression profiles obtained from in vitro experiments provide important information regarding gene expression potential, yet several factors must be considered when extrapolating these results to clinical states. Cell isolation and culture conditions, including in vitro differentiation (i.e., monocyte-derived macrophages or dendritic cells), may affect gene expression profiles (14, 30). Cultured cells stimulated with endotoxin are further altered by the accumulation of secreted mediators. The lack of primary and secondary mediator clearance limits the ability to assess physiological levels of inflammatory molecules on gene expression. Furthermore, expression profiling of isolated cells precludes an assessment of cell interactions on differential gene regulation. Thus fundamentally different information is obtained from assessment of gene expression in vivo.

Mononuclear cells undergo changes in number and proportion after endotoxin challenge, and this will affect the relative contribution of RNA associated with a specific cell type (i.e., T lymphocyte) in the array results. Activated T cells contain almost twice as much total RNA as resting cells (49), and analyses based on the proportion of cells present in a mixed cell sample may not account for differences in RNA content per cell. In our study, the percentage of T lymphocytes remained constant within the PBMC preparation (Fig. 1). When a fixed proportion of blood cells (whole blood) are stimulated in vitro with endotoxin, genes for ribosomal proteins, cytotoxic T lymphocyte factors, and T cell receptor and

nuclear activation factors are downregulated, similar to our in vivo observations (22). A functional consequence of ribosomal gene down-regulation in vitro and in vivo is suggested by a decrease in T lymphocyte protein synthesis (~58%) immediately after endotoxin challenge in humans (27).

Animal and human studies of systemic endotoxin challenge further support our observations. Whole blood arrays of rats challenged with endotoxin show that ribosomal and MHC class II genes are repressed as observed in the current study (20). With the use of a cDNA array, qualitative changes in 23 downregulated (i.e., ribosomal genes and HLA antigens) and 31 upregulated genes (i.e., transcription factors, cytokines, CD14, and mitochondrial proteins) were described in humans challenged with either 2 or 3 ng/kg endotoxin (40). The relationship of mRNA and protein expression for cytokines, cytokine receptors, and adhesion molecules was variable, suggesting either non-PBMC sources of protein or the effects of posttranscriptional regulatory events (40).

Recently, other investigators (8) have studied the effects of endotoxin (2 ng/kg) in four healthy subjects and, using a higher-density oligonucleotide array, described differential regulation of 3,714 genes in whole blood leukocytes. Their analysis revealed dysregulation of leukocyte bioenergetics and modulation of translational machinery. Genes for cytokines and chemokines and their receptors as well as members of the NF- κ /relA family of transcription factors were activated within the first 2–4 h (8). These data complement our observations by providing data from whole blood leukocytes (buffy coat) with similar differentially regulated genes and biological themes. We compared our 6-h mononuclear cell data (HU95A2 microarray, Affymetrix) with the 6-h whole blood data (HU133A/B, Affymetrix) from Calvano et al. (8). Using a >50% increased or decreased relative change from baseline, we evaluated probe sets in common and different between the two studies (Supplemental Table S5). We found 162 induced and 299 repressed probe sets that were common between the two studies and noted 264 upregulated and 143 downregulated probe sets unique to PBMC and distinct from the 281 (upregulated) and 856 (downregulated) probe sets discovered by Calvano et al. in whole blood (Supplemental Table S5). The unique PBMC probe sets included genes associated with heat shock proteins and chemokine and cytokine receptors (all induced) and an enrichment of T lymphocyte-associated genes (repressed). These data demonstrate that, while a common set of genes are expressed in blood leukocytes after endotoxin challenge, unique gene expression profiles emerge when enriching the RNA pool with specific cell types such as PBMC.

The repression of genes associated with lymphocytes in PBMC and whole blood preparations is consistent with previous observations of a state of immunosuppression that occurs after acute inflammation (3, 25, 36). We found that, while an early marker of lymphocyte activation (CD69 surface marker expression) was increased on lymphocytes after endotoxin challenge, a broad category of genes associated with T and B cell surface receptors, NK cell-related cytolytic granules, and MHC antigens were downregulated. Th1 cytokine (IL-2 and IFN- γ) blood levels did not increase. IL-10 levels increased in blood, whereas other Th2 cytokines (IL-4 and IL-5) were unchanged. Mice stimulated with endotoxin activate T cells, as reflected by increased CD69 and CD25 expression, but have a decreased capacity to produce Th1 cytokines (9). Whole blood obtained from volunteers

after intravenous endotoxin challenge respond to T cell stimuli with reduced IFN- γ and IL-2 production, and this tolerant state was due in part to serum factors (29). Similarly, mononuclear cells from patients with sepsis or traumatic injury or in the postoperative state have decreased responsiveness to secondary stimuli (i.e., endotoxin, anti-CD3/CD28) with impaired Th1 cytokine secretion and T cell proliferative responses and decreased expression of HLA-DR molecules (2, 13, 15, 18, 24). This tolerance phenomenon has been postulated to be an adaptive response to limit tissue damage (10). However, tolerance is not characterized by a global hyporesponsiveness to all bacterial components (2). The latter case is consistent with our observation of increased expression of genes associated with pathogen recognition molecules.

We analyzed whole blood cells with the PAXgene blood collection system, which has the advantage of rapid stabilization of nucleic acids on collection. This ease of sample acquisition facilitates its use in clinical studies. However, this method has limitations because of an abundant globin and reticulocyte message that contributes to increased signal noise and decreased sensitivity compared with gene expression profiles of blood cells isolated by other methods (12, 19, 21). Despite these limitations, we show that similar biological themes of whole blood compared with PBMC emerge from analysis of the activated cells at 6 h.

Thus the interrogation of the transcriptome associated with endotoxin-induced inflammation integrates a broad variety of biological themes that collectively reflect the activation of innate immunity. We conclude that, while some of the observed changes in gene expression on endotoxin administration are due to gene regulation at a single cell level (i.e., changes in the transcriptome), others may be caused by changes in the proportion of each blood cell type (e.g., different subtypes of mononuclear cells) and are not due to gene regulation. Further experiments are needed to determine which of the differentially expressed genes are regulated at the mRNA level. Nevertheless, it is likely that a significant fraction of the observed changes are due to gene regulation, and that the group of regulated genes reveals a broad variety of biological themes that reflect the activation of innate immunity. The pathway redundancy and parallelism of the innate immunity support the usefulness of broadly acting anti-inflammatory agents during severe infections (35). These observations provide a blueprint of expected changes as well as new pathways and molecules that are engaged during the early response to this common bacterial component.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Proportion of lymphocytes and monocytes present after density centrifugation separation at 0 and 6 h. Compared with baseline, endotoxin administration resulted in the percentage of lymphocytes falling by almost 50% and the percentage of monocytes increasing by 3-fold at 6 h. *Inset:* percentage of T lymphocytes and natural killer (NK) cells remained the same while the proportion of B cells increased at 6 h.

Fig. 2.

A: heat map of changes in gene expression over time (0.5 h, $n = 6$; 6 h, $n = 8$; 24 h, $n = 7$; and 168 h, $n = 6$) of 982 differentially expressed probe sets in peripheral blood mononuclear cells (PBMC) after intravenous endotoxin challenge. Each row represents a gene probe set and each column a subject at the respective time point. Change from baseline is determined by the average difference in the probe set (standardized and transformed using the symmetric adaptive transform as per METHODS), with increased gene expression depicted by red and decreased gene expression depicted by green. *B*: heat map of changes in gene

expression over time (3 h, $n = 3$; 6 h, $n = 4$; 24 h, $n = 4$; and 168 h, $n = 4$) of 224 differentially expressed probe sets in whole blood cells after intravenous endotoxin challenge. Each row represents a gene probe set and each column a subject at the respective time point. Change from baseline is determined by the average difference in the probe set, as above.

Fig. 3.

Real-time PCR (RT-PCR) was used to validate the microarray results for PBMC data sets (TaqMan PCR detection, Applied Biosystems). Probes and primers for 12 target sequences were designed using Primer Express computer software (Applied Biosystems). Data are presented as median values [$\pm 95\%$ confidence limit (CL)]; $n = 4$. CD8A, CD8 antigen, α -polypeptide (p32); CD3G, CD3G antigen, γ -polypeptide (TiT3 complex); SH2D1A, SH2 domain protein 1A; TRB, T cell receptor- β locus; IL7R, interleukin (IL)-7 receptor; LAT, linker for activation of T cells; CSFR2, colony-stimulating factor 2 receptor- β , low affinity; FAS, tumor necrosis factor receptor superfamily, member 6; SOD2, superoxide dismutase 2,

mitochondrial; ADM, adrenomedullin; S100A12, S100 calcium-binding protein A12; MMP9, matrix metalloproteinase 9.

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Table 1

Changes in peripheral blood leukocytes after intravenous endotoxin administration

	0 h	6 h	24 h	168 h
<i>Endotoxin</i>				
Total cells, $\times 10^9$ cells/l	4.65 $\pm$ 0.55	11.97 $\pm$ 0.85	7.69 $\pm$ 0.50	7.14 $\pm$ 0.63
Neutrophils, %	55.4 $\pm$ 3.3	92.5 $\pm$ 1.4	68.6 $\pm$ 2.9	63.6 $\pm$ 2.2
Lymphocytes, %	34.05 $\pm$ 3.6	3.7 $\pm$ 0.7	22.41 $\pm$ 2.0	28.6 $\pm$ 2.0
Monocytes, %	6.1 $\pm$ 1.5	3.3 $\pm$ 0.7	6.8 $\pm$ 1.0	5.0 $\pm$ 0.7
<i>Saline</i>				
Total cells, $\times 10^9$ cells/l	5.22 $\pm$ 0.35	5.15 $\pm$ 0.41	5.90 $\pm$ 0.19	5.55 $\pm$ 0.36
Neutrophils, %	52.9 $\pm$ 2.9	49.2 $\pm$ 3.2	54.9 $\pm$ 2.3	52.6 $\pm$ 2.0
Lymphocytes, %	36.4 $\pm$ 1.9	41.5 $\pm$ 2.3	36.1 $\pm$ 2.9	37.7 $\pm$ 2.5
Monocytes, %	7.9 $\pm$ 1.0	7.4 $\pm$ 0.8	7.2 $\pm$ 1.2	7.5 $\pm$ 1.3

Values are means $\pm$ SE.

Table 2

Acute-phase cytokines in serum after intravenous endotoxin challenge

	0 h	3 h	6 h	24 h	P Value
TNF α	94 $\pm$ 1	454 $\pm$ 100	98 $\pm$ 2	94 $\pm$ 1	<0.0001
IL-1 β	35 $\pm$ 6	197 $\pm$ 32	49 $\pm$ 10	37 $\pm$ 4	<0.0001
IFN- γ	54 $\pm$ 32	55 $\pm$ 28	50 $\pm$ 27	55 $\pm$ 35	NS
IL-2	130 $\pm$ 24	79 $\pm$ 3	130 $\pm$ 23	133 $\pm$ 25	NS
IL-4	82 $\pm$ 2	84 $\pm$ 3	83 $\pm$ 3	82 $\pm$ 3	NS
IL-5	52 $\pm$ 0	52 $\pm$ 1	52 $\pm$ 0	51 $\pm$ 0	NS
IL-6	0	3,155 $\pm$ 1,054	24 $\pm$ 24	0	<0.0001
IL-8	26 $\pm$ 1	1,023 $\pm$ 170	206 $\pm$ 82	36 $\pm$ 4	<0.0001
IL-10	87 $\pm$ 0	326 $\pm$ 41	94 $\pm$ 2	87 $\pm$ 0	<0.0001
MCP-1	303 $\pm$ 74	18,179 $\pm$ 2,078	5,218 $\pm$ 2,794	452 $\pm$ 58	<0.0001
MIP1 α	261 $\pm$ 1	581 $\pm$ 28	300 $\pm$ 15	266 $\pm$ 2	<0.0001
MIP1 β	100 $\pm$ 46	13,696 $\pm$ 1,624	2,118 $\pm$ 570	207 $\pm$ 89	<0.0001
RANTES	1,717 $\pm$ 71	1,997 $\pm$ 175	1,833 $\pm$ 90	1,933 $\pm$ 114	NS
Eotaxin	188 $\pm$ 22	252 $\pm$ 53	291 $\pm$ 81	272 $\pm$ 47	NS
VEGF	306 $\pm$ 30	342 $\pm$ 36	325 $\pm$ 22	300 $\pm$ 23	NS

Serum samples (means $\pm$ SE) for the following were measured at baseline (0 h) and 3, 6, and 24 h using a multiplex bead-based assay and analyzed by ANOVA for time effect: tumor necrosis factor (TNF) α ; interleukin (IL)-1 β , -2, -4, -6, -8, and -10; interferon (IFN) γ ; regulated on activation, normal T cell expressed and secreted (RANTES); macrophage inflammatory protein (MIP)1 α and -1 β ; monocyte chemoattractant protein (MCP)-1; eotaxin; and vascular endothelial growth factor (VEGF). NS, not significant.

Table 3

Defense response genes altered in peripheral blood mononuclear cells 6 h after intravenous endotoxin challenge

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
<i>Pathogen recognition and immune response</i>	
40331_at macrophage receptor with collagenous Structure	13.6 (9.3, 20.0)
37220_at Fc fragment of IgG, high affinity Ia, receptor for (CD64)	9.9 (7.2, 13.6)
31438_s_at CD163 antigen	7.5 (6.1, 9.2)
32068_at complement component 3a receptor 1	4.8 (3.5, 6.6)
37054_at bactericidal/permeability-increasing protein	4.0 (2.5, 6.4)
37095_r_at formyl peptide receptor-like 1	3.9 (2.8, 5.4)
36753_at leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	3.1 (2.4, 4.1)
37148_at leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	3.1 (2.3, 4.3)
40310_at toll-like receptor 2	2.7 (2.2, 3.4)
35094_f_at leukocyte immunoglobulin-like receptor, Subfamily A (without TM domain), member 3	2.7 (2.3, 3.2)
37689_s_at Fc fragment of IgG, low affinity IIa, receptor for (CD32)	2.6 (1.7, 3.8)
36889_at Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.5 (2.1, 3.1)
36661_s_at CD14 antigen	2.5 (1.9, 3.3)
35036_at complement component 1, q subcomponent, receptor 1	2.5 (1.6, 3.8)
38533_s_at integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170))	2.3 (1.7, 3.2)
39221_at leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2	2.2 (1.6, 3.1)
39997_at properdin P factor, complement	2.2 (1.6, 3.2)
34665_g_at Fc fragment of IgG, low-affinity IIb, receptor for (CD32)	2.1 (1.5, 2.8)
36243_at toll-like receptor 1	2.0 (1.5, 2.6)
37799_at asialoglycoprotein receptor 2	2.0 (1.4, 2.7)
34033_s_at leukocyte immunoglobulin-like receptor, Subfamily A (with TM domain), member 2	1.9 (1.4, 2.7)
37470_at leukocyte-associated Ig-like receptor 1	1.9 (1.6, 2.3)
<i>Chemokine/cytokine receptors</i>	
37603_at interleukin 1 receptor antagonist	4.7 (3.1, 7.1)
39994_at chemokine (C-C motif) receptor 1	3.0 (2.1, 4.2)
41140_at interferon gamma receptor 2 (interferon gamma transducer 1)	3.0 (2.3, 3.9)
36229_at interleukin 17 receptor	2.5 (1.9, 3.4)

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
41677_at interleukin 15 receptor, alpha	2.5 (1.8, 3.5)
38969_at interleukin 27	2.4 (1.9, 3.0)
39938_g_at chemokine (C-C motif) receptor 2	2.2 (1.7, 2.9)
33227_at interleukin 10 receptor, beta	2.1 (1.6, 2.7)
39424_at tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	0.7 (0.5, 0.9)
35659_at interleukin 10 receptor, alpha	0.6 (0.4, 0.8)
1097_s_at chemokine (C-C motif) receptor 7	0.5 (0.3, 0.8)
1897_at transforming growth factor, beta receptor III (betaglycan, 300kD)	0.4 (0.3, 0.6)
1404_r_at small inducible cytokine A5 (RANTES)	0.4 (0.2, 0.7)
34077_at G protein-coupled receptor 9	0.4 (0.3, 0.5)
40729_s_at lymphotoxin beta (TNF superfamily, member 3)	0.4 (0.3, 0.6)
38578_at tumor necrosis factor receptor superfamily, member 7	0.3 (0.2, 0.5)
1370_at interleukin 7 receptor	0.3 (0.2, 0.5)
31496_g_at small inducible cytokine subfamily C, member 1 (lymphotactin)	0.3 (0.2, 0.5)
34023_at Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	0.1 (0.1, 0.2)
<i>Growth factors</i>	
37494_at colony-stimulating factor 2 receptor, beta, low affinity (granulocyte-macrophage)	4.1 (2.0, 8.4)
32675_at bone marrow stromal cell antigen 1	3.0 (2.4, 3.7)
36879_at endothelial cell growth factor 1 (platelet-derived)	2.9 (2.1, 4.1)
39061_at bone marrow stromal cell antigen 2	2.7 (2.1, 3.5)
33665_s_at colony-stimulating factor 2 receptor, alpha, low affinity (granulocyte-macrophage)	2.2 (1.5, 3.3)
1953_at vascular endothelial growth factor	1.7 (1.3, 2.2)
<i>Microbicidal oxidase and oxidative stress</i>	
34666_at superoxide dismutase 2, mitochondrial	9.0 (5.4, 15.0)
38893_at neutrophil cytosolic factor 4 (40kDa)	3.1 (2.3, 4.1)
37975_at cytochrome b-245, beta polypeptide (chronic granulomatous disease)	2.4 (1.9, 3.2)
824_at glutathione-S-transferase like; glutathione transferase omega	1.9 (1.3, 2.8)
35807_at cytochrome b-245, alpha polypeptide	1.8 (1.6, 2.1)
39729_at peroxiredoxin 2	0.6 (0.5, 0.7)
<i>Protease and protease inhibitors</i>	
31859_at matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase)	44.1 (18.6, 104.6)
36984_f_at haptoglobin-related protein	5.7 (2.8, 11.8)
34761_r_at a disintegrin and metalloproteinase domain 9 (meltrin gamma)	4.5 (2.9, 7.0)
1693_s_at tissue inhibitor of metalloproteinase 1	4.4 (3.5, 5.6)

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
31891_at chitinase 3-like 2	3.8 (2.5, 5.5)
33305_at serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	3.0 (2.3, 4.0)
37021_at cathepsin H	2.6 (1.9, 3.4)
34876_at carboxypeptidase D	2.5 (2.1, 3.1)
40712_at a disintegrin and metalloproteinase domain 8	2.2 (1.6, 3.1)
36781_at serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase), member 1	1.9 (1.5, 2.5)
39581_at cystatin A (stefin A)	1.9 (1.4, 2.7)
<i>Cytotoxin</i>	
36766_at ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	5.2 (3.5, 7.8)
33979_at ribonuclease, RNase A family, 3 (eosinophil cationic protein)	3.1 (1.9, 5.0)
<i>Arachidonate metabolism</i>	
40082_at fatty-acid-coenzyme A ligase, long-chain 2	11.5 (7.5, 17.8)
32775_r_at phospholipid scramblase 1	5.2 (3.3, 8.0)
37099_at arachidonate 5-lipoxygenase-activating protein	4.5 (3.4, 6.0)
39799_at fatty acid-binding protein 5 (psoriasis associated)	2.2 (1.4, 3.5)
39624_at leukotriene b4 receptor (chemokine receptor-like 1)	2.0 (1.7, 2.4)
1671_s_at mitogen-activated protein kinase 14	2.0 (1.4, 2.9)
38099_r_at fatty-acid-coenzyme A ligase, long-chain 4	1.8 (1.3, 2.4)
37692_at diazepam-binding inhibitor (GABA receptor modulator, acyl-coenzyme A-binding protein)	1.8 (1.3, 2.5)
<i>Metal-binding proteins</i>	
40456_at upregulated by BCG-CWS	20.6 (11.8, 36.2)
38598_at solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	3.6 (1.6, 8.4)
36130_f_at <i>Homo sapiens</i> , similar to RNA helicase-related protein, clone MGC:9246 IMAGE:3892441	2.2 (1.7, 2.9)
33943_at ferritin, heavy polypeptide 1 Coagulation	1.9 (1.3, 2.7)
35245_at coagulation factor V (proaccelerin, labile factor)	6.2 (3.7, 10.5)
33803_at thrombomodulin	2.2 (1.5, 3.2)
<i>S100 calgranulins</i>	
34319_at S100 calcium-binding protein P	15.3 (8.6, 27.0)
38879_at S100 calcium-binding protein A12 (calgranulin C)	8.4 (5.9, 12.2)
41471_at S100 calcium-binding protein A9 (calgranulin B)	2.4 (1.9, 3.0)
38138_at S100 calcium-binding protein A11 (calgizzarin)	2.2 (1.8, 2.6)
41096_at S100 calcium-binding protein A8 (calgranulin A)	2.0 (1.6, 2.4)
<i>T- and B cell-associated genes</i>	

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
38006_at CD48 antigen (B-cell membrane protein)	0.7 (0.6, 0.9)
36798_g_at sialophorin (gpL115, leukosialin, CD43)	0.6 (0.5, 0.7)
40667_at CD6 antigen	0.5 (0.4, 0.7)
40688_at linker for activation of T cells	0.5 (0.4, 0.6)
771_s_at CD7 antigen (p41)	0.5 (0.3, 0.6)
40738_at CD2 antigen (p50), sheep red blood cell receptor	0.4 (0.3, 0.5)
36277_at CD3E antigen, epsilon polypeptide (TiT3 complex)	0.4 (0.3, 0.5)
931_at Epstein-Barr virus-induced gene 2 (lymphocyte-specific G protein-coupled receptor)	0.4 (0.2, 0.6)
1105_s_at T cell receptor beta locus	0.4 (0.2, 0.5)
32070_at protein tyrosine phosphatase, receptor type, C-associated protein	0.3 (0.2, 0.5)
41654_at adenosine deaminase	0.3 (0.2, 0.5)
38147_at SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	0.3 (0.2, 0.5)
41468_at T cell receptor gamma locus	0.3 (0.2, 0.4)
1498_at zeta-chain (TCR)-associated protein kinase (70 kDa)	0.3 (0.2, 0.4)
38319_at CD3D antigen, delta polypeptide (TiT3 complex)	0.3 (0.2, 0.4)
35517_at CD4 antigen (p55)	0.3 (0.2, 0.5)
32794_g_at T cell receptor beta locus	0.3 (0.1, 0.5)
432_s_at T cell receptor alpha locus	0.3 (0.1, 0.5)
1106_s_at T cell receptor alpha locus	0.3 (0.2, 0.4)
37078_at CD3Z antigen, zeta polypeptide (TiT3 complex)	0.3 (0.2, 0.4)
38917_at T cell receptor delta locus	0.3 (0.2, 0.4)
39226_at CD3G antigen, gamma polypeptide (TiT3 complex)	0.2 (0.1, 0.3)
1365_at interleukin 2 receptor, beta	0.1 (0.1, 0.3)
<i>Cytotoxic and NK cells</i>	
39239_at CD8 antigen, beta polypeptide 1 (p37)	0.5 (0.4, 0.7)
39119_s_at natural killer cell transcript 4	0.4 (0.2, 0.6)
36887_f_at killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	0.3 (0.2, 0.8)
36280_at granzyme K (serine protease, granzyme 3; tryptase II)	0.3 (0.2, 0.5)
36777_at DNA segment on chromosome 12 (unique) 2489 expressed sequence	0.3 (0.2, 0.5)
36886_f_at killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	0.3 (0.2, 0.6)
32297_s_at killer cell lectin-like receptor subfamily C, member 2	0.3 (0.2, 0.6)
32904_at perforin 1 (pore-forming protein)	0.3 (0.2, 0.5)
40699_at CD8 antigen, alpha polypeptide (p32)	0.3 (0.2, 0.5)

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
40718_at cathepsin W (lymphopain)	0.3 (0.2, 0.3)
32370_at similar to granzyme B (granzyme 2, cytotoxic T lymphocyte-associated serine esterase 1)	0.2 (0.1, 0.4)
32287_s_at killer cell lectin-like receptor subfamily C, member 3	0.2 (0.1, 0.4)
35449_at killer cell lectin-like receptor subfamily B, member 1	0.2 (0.1, 0.3)
40757_at granzyme A (granzyme 1, cytotoxic T lymphocyte-associated serine esterase 3)	0.2 (0.1, 0.3)
37145_at granulysin	0.1, (0.1, 0.3)
32264_at granzyme M (lymphocyte met-ase 1)	0.1 (0.0, 0.2)
<i>MHC and APC</i>	
32035_at major histocompatibility complex, class II, DR beta 4	0.7 (0.1, 1.2)
36270_at CD86 antigen (CD28 antigen ligand 2, B7–2 antigen)	0.6 (0.4, 0.7)
36878_f_at major histocompatibility complex, class II, DQ beta 1	0.4 (0.3, 0.7)
38833_at major histocompatibility complex, class II, DP alpha 1	0.4 (0.3, 0.5)
38096_f_at major histocompatibility complex, class II, DP beta 1	0.4 (0.3, 0.5)
41609_at major histocompatibility complex, class II, DM beta	0.3 (0.3, 0.4)
36155_at KIAA0275 gene product	0.3 (0.2, 0.5)
<i>Apoptosis</i>	
33727_r_at tumor necrosis factor receptor superfamily, member 6b, decoy	6.0 (2.9, 12.7)
2002_s_at BCL2-related protein A1	4.8 (3.0, 7.6)
1497_at lymphotoxin beta receptor (TNFR superfamily, member 3)	3.7 (2.0, 6.9)
33412_at lectin, galactoside binding, soluble, 1 (galectin 1)	2.3 (1.8, 2.9)
1563_s_at tumor necrosis factor receptor superfamily, member 1A	1.9 (1.6, 2.3)
32725_at BH3-interacting domain death agonist	1.9 (1.4, 2.5)
2066_at BCL2-associated X protein	1.8 (1.5, 2.2)
31536_at reticulon 4	1.8 (1.5, 2.1)
37643_at tumor necrosis factor receptor superfamily, member 6	1.7 (1.3, 2.3)
41189_at tumor necrosis factor receptor superfamily, member 12	0.6 (0.5, 0.8)
31491_s_at caspase 8, apoptosis-related cysteine protease	0.6 (0.4, 0.8)
849_g_at TNF receptor-associated factor 1	0.6 (0.4, 0.7)
35238_at TNF receptor-associated factor 5	0.5 (0.4, 0.6)
37127_at death effector filament-forming Ced-4-like apoptosis protein	0.5 (0.3, 0.8)
1847_s_at B cell CLL/lymphoma 2	0.5 (0.3, 0.7)

Probe Set and Gene Name	Fold Change 0–6 h (95% LCL, UCL)
32967_at regulator of Fas-induced apoptosis	0.4 (0.3, 0.5)

LCL, lower confidence limit; UCL, upper confidence limit; NK cells, natural killer cells; MHC, major histocompatibility complex; APC, antigen presenting cell.

Table 4

Atypical acute-phase genes differentially regulated after intravenous endotoxin challenge

Association of Gene Products with Disease						
Probe set	Gene Symbol	Title	Function or Disorder	Fold Change	LCL 95%	UCL 95%
Induced						
35245_at	F5	coagulation factor V (proaccelerin, labile factor)	hemorrhagic diathesis	5.6	3.3	9.2
37003_at	CD63	CD63 antigen (melanoma 1 antigen)	Hermansky-Pudlak syndrome	4.0	2.9	5.5
1786_at	MERTK	c-mer proto-oncogene tyrosine kinase	retinitis pigmentosa	3.2	2.1	4.8
40594_r_at	ACVRL1	activin A receptor type II-like 1	Rendu-Osler-Weber syndrome 2	2.8	1.0	7.6
1547_at	JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	autosomal SCID (severe combined immunodeficiency disease).	2.8	1.3	6.1
40159_r_at	NCF1	neutrophil cytosolic factor 1 (47kD)	chronic granulomatous disease.	2.8	1.7	4.6
41061_at	HIP1	huntingtin interacting protein 1	cytoskeleton disease, Huntingtons disease	2.6	1.8	3.9
37096_at	ELA2	elastase 2, neutrophil	cyclic neutropenia	2.6	1.1	5.9
39075_at	NEU1	sialidase 1 (lysosomal sialidase)	sialidosis	2.4	1.4	4.3
37975_at	CYBB	cytochrome b-245, beta polypeptide	X-linked chronic granulomatous disease	2.2	1.7	2.8
41471_at	S100A9	S100 calcium binding protein A9 (calgranulin B)	altered expression cystic fibrosis	2.2	1.7	2.7
40459_at	ACOX1	acyl-Coenzyme A oxidase 1, palmitoyl	pseudoneonatal adrenoleukodystrophy	2.1	1.4	3.2
39791_at	ATP2A2	ATPase, Ca+++ transporting, cardiac muscle, slow twitch 2	Darier-White disease, keratosis follicularis	2.1	1.6	2.7
2044_s_at	RB1	retinoblastoma 1 (including osteosarcoma)	embryonic malignant neoplasm	2.0	1.3	3.1
38601_at	VMD2	vitelliform macular dystrophy	bestrophin, macular dystrophy	2.0	1.4	2.9
39964_at	RP2	retinitis pigmentosa 2 (X-linked recessive)	retinitis pigmentosa	2.0	1.4	2.9
37215_at	PYGL	phosphorylase, glycogen; liver	Hers disease, glycogen storage disease type VI	2.0	1.6	2.5
35820_at	GM2A	GM2 ganglioside activator protein	glycolipid transport, Tay-Sachs disease	2.0	1.4	2.8
36184_at	PLOD	procollagen-lysine, 2-oxoglutarate 5-dioxygenase	Ehlers-Danlos syndrome type VI	1.9	1.4	2.6
36090_at	TBL2	transducin (beta)-like 2	intracellular signaling, deleted in William-Beuren Syndrome	1.9	1.4	2.4
36636_at	OAT	ornithine aminotransferase (gyrate atrophy)	mitochondrial enzyme, autosomal recessive eye disease, gyrate atrophy	1.9	1.5	2.3

Association of Gene Products with Disease						
Probe set	Gene Symbol	Title	Function or Disorder	Fold Change	LCL 95%	UCL 95%
41131_f_at	HNRPH2	heterogeneous nuclear ribonucleoprotein H2 (H ₂)	Fabry disease and X-linked agammaglobulinemia	1.8	1.5	2.3
40402_at	SLC6A2	solute carrier family 6 member 2	orthostatic intolerance	1.7	1.2	2.6
38350_f_at	TUBA2	tubulin, alpha 2	Clouston hidrotic ectodermal dysplasia and Kabuki syndrome	1.7	1.2	2.4
40639_at	SCO2	SCO cytochrome oxidase deficient homolog 2 (yeast)	fatal infantile cardioencephalomyopathy, hypertrophic cardiomyopathy, lactic acidosis, gliosis	1.7	0.9	3.2
37126_at	SSA1	Sjogren syndrome antigen A1 (52kD)	protein interacts with autoantigens of Sjogrens and systemic lupus	1.7	1.5	1.9
35807_at	CYBA	cytochrome b-245, alpha polypeptide	Autosomal recessive chronic granulomatous disease	1.6	1.5	1.8
32643_at	GBE1	glucan (1,4-alpha-), branching enzyme 1	glycogen storage disease IV (Andersen's disease)	1.6	1.4	2.0
222_at	EXT1	exostoses (multiple) 1	hereditary multiple exostoses	1.6	1.2	2.2
36566_at	CTN5	cystinosis, nephropathic	cystinosis	1.6	1.2	2.2
38099_r_at	FACL4	fatty-acid-Coenzyme A ligase, long-chain 4	mental retardation or Alport syndrome	1.6	1.2	2.2
35153_at	NBS1	Nijmegen breakage syndrome 1 (nibrin)	mental retardation, microcephaly, growth retardation, immunodeficiency, and cancer	1.6	1.2	2.0
38378_at	CD53	CD53 antigen	immunodeficiency associated with recurrent infectious	1.5	1.4	1.8
<i>Repressed</i>						
38789_at	TKT	transketolase	Wernicke-Korsakoff syndrome	0.7	0.5	0.9
31330_at	RPS19	ribosomal protein S19	Diamond-Blackfan anemia (erythroblastopenia)	0.6	0.5	0.7
34355_at	MECP2	methyl CpG binding protein 2	mental retardation, Rett syndrome	0.5	0.4	0.7
37543_at	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	mental retardation	0.5	0.5	0.6
34842_at	SNRPN	small nuclear ribonucleoprotein polypeptide N	Angelman syndrome or Prader-Willi syndrome	0.4	0.3	0.6
36673_at	MPI	mannose phosphate isomerase	carbohydrate deficient glycoprotein synthesis, type 1b	0.4	0.3	0.6
41814_at	FUCA1	fucosidase, alpha-L-1, tissue	autosomal recessive lysosomal storage disease	0.4	0.3	0.6

Association of Gene Products with Disease						
Probe set	Gene Symbol	Title	Function or Disorder	Fold Change	LCL 95%	UCL 95%
2000_at	ATM	ataxia telangiectasia mutated	ataxia telangiectasia	0.4	0.3	0.6
41866_s_at	DTNB	dystrobrevin, beta	defective in Duchenne muscular dystrophy	0.4	0.2	0.7
38063_at	PBXIP1	hematopoietic PBX-interacting protein	Ehler-Danlos syndrome, osteogenesis imperfecta	0.4	0.3	0.5
35371_at	LRBA	vesicle trafficking, beach and anchor containing	Chediak-Higashi syndrome	0.4	0.3	0.5
2047_s_at	JUP	junction plakoglobin	arrhythmic right ventricular cardiomyopathy, palmoplantar keratoderma, woolly hair	0.3	0.2	0.7
1370_at	IL7R	interleukin 7 receptor	severe combined immunodeficiency	0.3	0.1	0.5
33825_at	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A, member 3	mutations in Parkinson disease, chronic obstructive pulmonary disease	0.2	0.1	0.5
37456_at	LGALS2	lectin, galactoside-binding, soluble, 2 (galectin 2) 3	a single-nucleotide polymorphism is associated with susceptibility to myocardial infarction.	0.1	0.0	0.2