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The Peripheral Whole Blood Transcriptome of Acute Pyelonephritis in Human Pregnancy

Ichchha Madan, MD[1,2], **Nandor Gabor Than, MD, PhD**[1,2], **Roberto Romero, MD, DMedSci**[1,2,3], **Piya Chaemsaithong, MD**[1,2], **Jezid Miranda, MD**[1,2], **Adi L. Tarca, PhD**[1,5], **Gaurav Bhatti**[1,5], **Sorin Draghici, PhD**[5], **Lami Yeo, MD**[1,2], **Moshe Mazor, MD**[6], **Sonia S. Hassan, MD**[1,2], and **Tinnakorn Chaiworapongsa, MD**[1,2]

[1]Perinatology Research Branch, NICHD/NIH/DHHS, Bethesda, MD, and Detroit, MI, USA

[2]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, USA

[3]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, USA

[4]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI, USA

[5]Department of Computer Science, Wayne State University, Detroit, MI, USA

[6]Department of Obstetrics and Gynecology, Soroka University Medical Center, School of Medicine, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Abstract

Objective—Human pregnancy is characterized by activation of the innate immune response and suppression of adaptive immunity. The former is thought to provide protection against infection to the mother, and the latter, tolerance against paternal antigens expressed in fetal cells. Acute pyelonephritis is associated with an increased risk of acute respiratory distress syndrome and sepsis in pregnant (vs. nonpregnant) women. The objective of this study was to describe the gene expression profile (transcriptome) of maternal whole blood in acute pyelonephritis.

Method—A case-control study was conducted to include pregnant women with acute pyelonephritis (n=15) and women with a normal pregnancy (n=34). Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were used for gene expression profiling. A linear model was used to test the association between the presence of pyelonephritis and gene expression levels while controlling for white blood cell count and gestational age. A fold change of 1.5 was considered significant at a false discovery rate of 0.1. A subset of differentially expressed genes (n=56) was tested with real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (cases, n=19; controls, n=59). Gene ontology and pathway analysis were applied.

Address correspondence to: Roberto Romero, MD, D. Med. Sci., Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University/Hutzel Women's Hospital, 3990 John R, Box 4, Detroit, MI 48201, USA, Telephone (313) 993-2700, Fax: (313) 993-2694, prbchiefstaff@med.wayne.edu.

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Results—A total of 983 genes were differentially expressed in acute pyelonephritis: 457 were up-regulated and 526 were down-regulated. Significant enrichment of 300 biological processes and 63 molecular functions was found in pyelonephritis. Significantly impacted pathways in pyelonephritis included a) cytokine-cytokine receptor interaction; b) T-cell receptor signaling; c) Jak-STAT signaling; and d) complement and coagulation cascades. Of 56 genes tested by qRT-PCR, 48 (85.7%) had confirmation of differential expression.

Conclusion—This is the first study of the transcriptomic signature of whole blood in pregnant women with acute pyelonephritis. Acute infection during pregnancy is associated with the increased expression of genes involved in innate immunity and the decreased expression of genes involved in lymphocyte function.

Keywords

adaptive immunity; high-dimensional biology; infection during pregnancy; innate immunity; mRNA; PAX gene; urinary tract infection

Introduction

Pregnancy is characterized by the activation of the innate immune response and suppression of adaptive immunity [1, 4, 10, 20, 21, 54, 57, 60, 73, 77–79, 88, 90, 101, 106, 107, 129–131, 133, 134, 137, 144]. This is thought to provide protection against infection for the mother and to promote tolerance of the fetal semi-allograft [1, 4, 10, 13, 20, 21, 54, 57, 60, 73, 77–80, 88, 90, 101, 104–108, 110, 120, 129–134, 137, 144].

The changes in the innate immune response in pregnancy include an increase in the numbers of neutrophils as well as phenotypic and metabolic alterations consistent with leukocyte activation [88, 106, 107]. Despite these physiological changes, pregnant women are more susceptible to the deleterious effects of microbial products than non-pregnant women [46, 81, 84, 101]. Moreover, pregnant animals develop a generalized Schwartzman reaction after a single injection of endotoxin, whereas non-pregnant animals require a priming dose of endotoxin and then a second injection [81–83]. We have attributed this to the physiological activation of innate immunity.

Acute pyelonephritis is a frequent complication of pregnancy [29, 49, 56, 75, 95, 111] and accounts for 12% of all antepartum admissions to an intensive care unit for sepsis [111]. Moreover, acute pyelonephritis during pregnancy can lead to preterm delivery [29, 43,56,58, 63, 74, 102, 109] and is more likely to be complicated by acute respiratory distress syndrome (ARDS) [5, 16, 17, 23–27, 33, 45, 49, 56,66, 97, 116, 136, 147], sepsis [12, 49, 56], septic shock [28, 115], anemia [49, 56], and transient renal dysfunction [40, 49] than acute pyelonephritis in non-pregnant patients. The reasons for this increased susceptibility to microbial products remain unknown. However, acute pyelonephritis during pregnancy has been associated with changes in maternal blood concentrations of soluble cluster of differentiation (CD) 30 (an index of Th2 immune response) [61], adipocytokines (retinol binding protein-4 [141], adiponectin [70], visfatin [71], and resistin [72]), T-cell chemokines (C-X-C motif chemokine 10, CXCL-10) [41], complement products (C5a [117]and fragment

Bb [118]), protein Z [92], and soluble tumor necrosis factor-related apoptosis inducing ligand (TRAIL) [18].

Transcriptome analysis has been used to gain insight into the pathophysiology of disease states and the identification of biomarkers in many disciplines, including obstetrics [37, 42, 50, 56, 67, 76, 93, 99, 100, 113, 121, 135, 142, 145, 148]. The gene expression profiles of peripheral blood have shown promising results in elucidating the mechanisms of other disorders such as multiple sclerosis [2], rheumatoid arthritis [7,139], sepsis [126, 127], and cancer [9, 62, 69, 138]. The transcriptomic profile of peripheral blood leukocytes after intravenous administration of bacterial endotoxin to healthy human subjects has been characterized [14, 123]. However, the peripheral whole-blood transcriptome of acute infection in human pregnancy has not been studied.

The objective of this study was to characterize the transcriptome of whole blood in pregnant women with acute pyelonephritis.

Material and Methods

Study design and sample collection

A cross-sectional study was conducted by searching our clinical database and bank of biological samples, including patients in the following groups: (1) normal pregnancy (n=34); and (2) acute pyelonephritis (n=15). Patients with multiple gestations and fetal anomalies were excluded. The normal pregnant control group consisted of women who were not in labor, without obstetrical, medical, or surgical complications of pregnancy, and had blood samples collected within the same gestational age window as patients with pyelonephritis. Pyelonephritis was diagnosed in the presence of fever (temperature ≥ 38º C), clinical signs of an upper urinary tract infection (e.g. flank pain, costovertebral angle tenderness), pyuria, and a positive urine culture for microorganisms.

All patients provided written informed consent for the collection and use of samples for research purposes under the protocols approved by the Institutional Review Boards of Wayne State University and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services.

RNA preparation

Maternal peripheral venous blood was collected at the time of routine clinical blood draw into PAXgene blood RNA tubes (PreAnalytiX GmbH, distributed by Becton Dickinson Company, Franklin Lakes, NJ, NY). Blood tubes were maintained initially at room temperature for 24 hours and then frozen at −70° C until further processing. Blood lysates were reduced to pellets by centrifugation, washed, and resuspended in buffer. Proteins were removed by Proteinase K digestion, and cellular debris was removed by centrifugation through a PAXgene Shredder spin column (PreAnalytiX GmbH). RNA was semi-precipitated with ethanol and selectively bound to the silica membrane of a PAXgene spin column (PreAnalytiX GmbH). The membrane was treated with DNase I to remove any residual DNA and washed, and the purified total RNA was eluted in nuclease-free water.

Purified total RNA was quantified by UV spectrophotometry using the DropSense96 Microplate Spectrophotometer (Trinean, Micronic North America LLC, McMurray, PA, USA) and RNA purity was assessed based on the A260/A280 and A260/A230 ratios. An aliquot of the RNA was assessed using the RNA 6000 Nano Assay for the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The electrophoretogram, RNA integrity number, and the ratio of the 28S:18S RNA bands were examined to determine overall quality of the RNA.

Microarray analysis

Peripheral blood samples were profiled using Affymetrix GeneChip HG-U133 PLUS 2.0 arrays. Briefly, RNA was amplified using the Ovation RNA Amplification System V2 (NuGEN Technologies, San Carlos, CA, USA). cDNA was synthesized using the Ovation buffer mix, first-strand enzyme mix, and first-strand primer mix with 5μL (~20 ng) of total RNA in specified thermal cycler protocols, according to the manufacturer's instructions. Amplification and purification of the generated cDNA was performed by combining SPIA Buffer Mix, Enzyme Mix, and nuclease-free water with the products of the second-strand cDNA synthesis reactions in pre-specified thermal cycler programs. The optical densities of the amplified cDNA products were obtained to demonstrate product yield and verified purity. Fragmentation and labeling were done using the FL-Ovation cDNA Biotin Module V2 (NuGEN Technologies). In the primary step, a combined chemical and enzymatic fragmentation process was used to produce cDNA products in the 50- to 100-base pair range. Fragmented cDNA products were then biotin-labeled using the Encore Biotin Module (NuGEN Technologies). All reactions were carried out according to the manufacturer's protocols. Amplified, fragmented and biotin-labeled cDNAs were used for hybridization cocktail assembly, and then hybridized to the Affymetrix GeneChip HG-U133 PLUS 2.0 arrays, according to the Affymetrix standard protocol.

Quantitative reverse transcription-polymerase chain reaction

A subset of differentially expressed genes (n=56) were selected for validation in an extended set of samples (pyelonephritis cases, n=19; controls, n=59) using the Biomark™ high-throughput real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) system (Fluidigm, San Francisco, CA, USA) based on their rank in the list of all differentially expressed genes as well as biological plausibility. Briefly, the Invitrogen Superscript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used to generate complementary DNA. Pre-amplification procedures included combining 1.25 μL cDNA with 2.5 μL TaqMan PreAMP Mastermix and 1.25 μL pooled assay mix. The reaction was performed with a thermal cycler for one cycle at 95° C for 10 minutes and 14 cycles at 95° C for 15 seconds and 60° C for 4 minutes. After cycling, the reaction was diluted 1:5 by ddH_2O to a final volume of 25 μl. The Fluidigm 96.96 Dynamic Array chip was used to perform the next step qRT-PCR assays. The 96.96 array chip was primed in an integrated fluidic circuit (IFC) controller with control fluid. After priming, 2.5 μl 20X TaqMan gene expression assays (Applied Biosystems) were mixed with a 2.5 μl 2X assay loading reagent (Fluidigm) and loaded into the assay inlet on the 96.96 array chip. A total of 2.25 μl preamplified cDNA was mixed with 2.5 μl TaqMan Universal PCR master mix (Applied Biosystems) and 0.25 μl 20X sample loading reagent (Fluidigm) and loaded

into the sample inlet on the chip. The chip was returned to the IFC controller for loading. After loading, the chip was placed in the Biomark System to run the reactions. The cycle threshold (Ct) value of each reaction was obtained with the Fluidigm RT-PCR analysis software.

Statistical analysis

Analysis for microarray and real-time quantitative polymerase chain reaction data

A linear model was used to test the association between pyelonephritis and gene expression levels determined by microarray analysis while controlling for white blood cell (WBC) count [32, 35, 146] and gestational age. Moderated t-tests [114] were used to assess the significance of the coefficients in the linear model. Probe sets with false discovery rate-adjusted p-values (q-value) of <0.1 and a fold change >1.5 were considered significant. Pathway analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database with an overrepresentation analysis [30] and the signal pathway impact analysis (SPIA) [31, 128]. The SPIA is a systems biology approach that takes into account the gene-gene signaling interactions as well as the magnitude and direction of gene expression changes to determine significantly impacted pathways [128]. Gene ontology analysis was performed using the GOstats package of Bioconductor [34].

The same statistical model was used for qRT-PCR data, and the –ΔCt values were used as a surrogate for $\log_2$ gene expression levels. qRT-PCR results were considered significant when P <0.05 with a one-tailed t-test, using the direction of expression change obtained from the microarray data.

Mann-Whitney U and Chi-square tests were used to compare the differences in demographics and clinical characteristics between patients with acute pyelonephritis and the control group. SPSS (version 15.0; SPSS Inc, Chicago, IL, USA) was used for the analysis of demographic and clinical characteristic data. A probability value of <0.05 was considered significant.

Results

The demographic and clinical characteristics of the study population are displayed in Table 1. Patients with acute pyelonephritis had a significantly lower median gestational age at venipuncture but higher WBC count in both the microarray and qRT-PCR study ($p < 0.0001$ for each). Therefore, we adjusted gene expression data for these two covariates in the microarray and qRT-PCR data analyses. Patients with pyelonephritis had a significantly higher neutrophil count (P<0.001 for each study) and lower lymphocyte count (microarray, P<0.05; qRT-PCR, P=0.001) than controls. Among patients with acute pyelonephritis (in the qRT-PCR experiment), 12 (63.2%) had a positive urine culture for *Escherichia coli,* 4 (21.1%) for *Klebsiella pneumonia*, and the rest were positive for *Enterococcus faecalis* (n=1), *Enterobacter* (n=1), and lactose-fermenting Gram-negative bacilli (n=1).

Microarray analysis

A total of 1309 probe sets corresponding to 983 unique genes demonstrated a differential expression between the two groups (q-value <0.1; fold change >1.5). A total of 457 genes were upregulated and 526 genes were downregulated in pyelonephritis. Table 2 shows the top 100 probe sets with differential expression between the study groups ranked by P-value.

A volcano plot (Figure 1A) displays the differential expression of all the annotated probe sets on the microarray as effect size vs. significance of expression change. An unsupervised principal component analysis (PCA)-based visualization of the microarray data (using all probes on the array, Figure 1B) revealed the between-group differences and no outlier samples.

To gain further insight into the biology of the differences in the transcriptome of whole blood between pregnant women with acute pyelonephritis and controls, gene ontology analysis was used. Significant enrichment of 300 biological processes (Table 3) was found in acute pyelonephritis, including the innate immune response, signal transduction, regulation of cytokine production, regulation of adaptive immune response, immunoglobulin (Ig)-mediated immune response, T-cell immunity, B- and T- cell differentiation, positive regulation of leukocyte activation and proliferation, positive regulation of T-cell receptor signaling pathway, and blood coagulation. Moreover, gene ontology analysis revealed that 63 molecular functions were associated with differentially expressed genes in acute pyelonephritis (Table 4).

Pathway analysis of differentially expressed genes was undertaken with an overrepresentation method and the SPIA method. Using the overrepresentation method, three KEGG pathways were significant (q-value <0.1) in the comparison between the study groups (Table 5): (1) “primary immunodeficiency,” (2) “hematopoietic cell lineage,” and (3) “T-cell receptor signaling pathway.” SPIA identified four pathways that were significantly impacted (Table 6). Three of these pathways had not been identified by the overrepresentation method (the “Jak-STAT signaling pathway,” “cytokine-cytokine receptor interaction,” and “complement and coagulation cascade”) (Table 6, Figure 2).

Quantitative real-time reverse transcription-polymerase chain reaction

qRT-PCR was performed on an extended set of samples (normal controls, n=59; acute pyelonephritis, n=19) to validate microarray results. Of the 56 genes selected for testing from the differentially expressed gene list in the microarray, we confirmed differential expression (in terms of both direction and significance) for 48 (85.7%) of the tested genes by qRT-PCR (Table 7).

Discussion

Principal findings of the study

We report for the first time the transcriptome of maternal whole blood in acute pyelonephritis in pregnancy. The main findings are the following: (1) There was a gene expression signature consistent with a systemic maternal inflammatory response. (2) The

transcriptome of peripheral WBCs in pyelonephritis was similar to that reported after intravenous endotoxin administration to nonpregnant individuals [14]. In both conditions, there was up-regulation of genes involved in innate immune responses and down-regulation of those involved in lymphocyte function. (3) We observed an up-regulated expression of genes involved in the induction of apoptosis and down-regulation of those with anti-apoptotic properties.

Local and systemic immune responses in infection of the urinary tract during pregnancy

The innate limb of the immune response represents the first line of defense against bacterial invasion of the urinary tract [91, 119]. Urinary epithelial cells are the first to enter into contact with microorganisms. Bacterial attachment can trigger exfoliation of bacteria-laden epithelial cells, reconstitution of the urothelium, and an inflammatory response [47, 85–87, 143]. Microorganisms and their products are recognized by pattern recognition receptors, and this leads to the production of chemokines, cytokines, and antimicrobial peptides, and the generation of an acute inflammatory response [47, 85–87, 91, 119, 143]. Neutrophils play an important role in host defense in the urinary tract, and they appear in the bladder and kidney within hours of transurethral inoculation with uropathogenic *E. coli* [38, 48]. Disruption of neutrophil chemotaxis in animals with a gene deletion for the interleukin (IL)-8 receptor homologue [38] or depletion of neutrophils with a granulocyte-specific antibody [48] can lead to an increased bacterial burden in the bladder and kidney [38, 48] as well as bacteremia [38].

In addition to the local host defense in the urinary tract, acute pyelonephritis is also associated with a systemic inflammatory response that is characterized by fever, increased serum concentrations of cytokines (IL-6, IL-8) and acute phase reactant proteins, and an elevated WBC and neutrophil count [68]. Consistent with this, we found in the current study that the median WBC count of patients with pyelonephritis was higher than that of pregnant women with a normal pregnancy outcome (Table 1). We had previously reported that the maternal serum concentrations of a panel of chemokines and cytokines are also higher in this condition than in normal pregnant women [19]. Indeed, the median maternal serum concentrations of IL-8, TNF-α, IL-6, IL-7, IL-10, and interferon (IFN)-γ were higher in pregnant women with acute pyelonephritis than in gestational age-matched normal pregnant women [19]. Moreover, acute pyelonephritis in pregnancy was found to be associated with higher median maternal serum concentrations of the pro-inflammatory chemokine CXCL-10 (also known as IP-10) [41] and higher median maternal plasma concentrations of the pro-inflammatory adipokine resistin [72] than in normal pregnant women. Resistin concentrations are considered an index of the severity of sepsis and a prognostic factor for survival in critically ill nonpregnant patients [55, 72, 122]. Of interest, median maternal plasma concentrations of the anti-inflammatory adipokine adiponectin [70] were lower in patients with pyelonephritis than in normal pregnant women. We have also reported the activation of the complement system, a component of the innate immune system, in acute pyelonephritis in pregnancy, as the median plasma concentrations of complement fragment Bb [118] and complement C5a [117] are higher in pregnant patients with acute pyelonephritis than in normal pregnant women. Complement C5a is a potent chemoattractant for neutrophils and can up-regulate the activating IgG Fc receptors and

down-regulate the inhibitory IgG Fc receptors on leukocytes, linking the complement system and IgG Fc receptor effector pathways [112]. This is consistent with the observations made in the present study, in which we observed an up-regulation of *FCGR1A* and *FCGR1B* expression in pregnant women with acute pyelonephritis. These genes encode for the high-affinity IgG Fc receptor (CD64), which is expressed on neutrophils and other myeloid cells and is involved in the binding of IgG1 and IgG3 [94]. Consistent with this finding, Naccasha et al. [88] reported higher expression of CD64 on granulocyte and monocyte surfaces (determined by flow cytometry as median mean channel brightness) in pregnant women with pyelonephritis than in normal pregnant women. Neutrophil CD64 has emerged as a biomarker for the diagnosis of bacterial infection [22, 64, 94] because resting neutrophils express very low levels of CD64, whereas the expression of CD64 is upregulated in the context of acute bacterial infections [94]. Using flow cytometry analysis of whole blood, a CD64 index (ratio of mean fluorescent intensity of the cell to the beads) of > 1.66 in hospitalized patients had a 100% sensitivity and a 95% specificity in the identification of sepsis, defined as the combination of bacteremia and clinical signs of infection [39]. Meta-analysis of 13 studies showed a pooled sensitivity of 79% [95% confidence interval (CI), 70%–86%)] and a pooled specificity of 91% (95% CI, 85%–95%) in the diagnosis of bacterial infection [22]. No studies have addressed the value of this marker in the assessment of pyelonephritis during pregnancy.

Changes in the transcriptome of whole-blood leukocytes in acute pyelonephritis in pregnancy

This is the first report of the transcriptome of whole-blood cells in pregnant women with pyelonephritis. This snapshot of the global mRNA expression of peripheral blood leukocytes and subsequent pathway analyses revealed interesting features of the systemic inflammatory response to bacterial infection in human pregnancy. We found 1309 probe sets corresponding to 983 unique genes differentially expressed in pregnant women with pyelonephritis, of which 457 genes were upregulated and 526 were downregulated. Gene ontology analysis indicated that these findings were associated with 63 molecular functions enriched in leukocytes, many of them strongly related to the innate and adaptive immune responses (e.g., “C-C chemokine receptor activity,” “complement receptor activity,” MHC class I protein binding,” “immunoglobulin receptor activity,” “T-cell receptor binding,” “chemokine binding,” “scavenger receptor activity,” “peptide antigen binding,” “cytokine receptor binding”) (Table 4). In accordance, several of the most enriched biological processes in pyelonephritis during pregnancy are related to immune responses (e.g., “positive regulation of leukocyte activation,” “innate immune response,” “activation of immune response,” “regulation of lymphocyte activation,” “inflammatory response”). Of interest, biological processes related to apoptosis (i.e., “positive regulation of apoptosis,” “positive regulation of cell death”) are also among the most enriched processes (Table 3).

To identify pathways significantly impacted in pyelonephritis during pregnancy, we applied two pathway analysis methods. The overrepresentation analysis method identified three KEGG pathways significantly impacted in pyelonephritis (“primary immunodeficiency,” “hematopoietic cell lineage,” “T-cell receptor signaling pathway”) (Table 5). The SPIA method, which also takes into account the gene-gene signaling interactions as well as the

magnitude and direction of gene expression changes besides differential expression [31, 128], identified one pathway in common with the overrepresentation method ("T-cell receptor signaling pathway") and three pathways that the overrepresentation method could not identify (the "Jak-STAT signaling pathway," "cytokine-cytokine receptor interaction," and "complement and coagulation cascade") (Table 6). Importantly, all of these impacted pathways are related to immune responses, suggesting that the systemic inflammatory response elicited in pyelonephritis in pregnancy has a characteristic gene expression signature in peripheral blood leukocytes.

To get further insight into the cellular pathways of systemic inflammation in pyelonephritis in pregnancy, we compared transcriptomic changes in our study to those documented in systemic inflammation in response to bacterial endotoxin in nonpregnant individuals (see below).

Changes in the transcriptome of whole-blood leukocytes from nonpregnant individuals after treatment with bacterial endotoxin

The transcriptome of peripheral blood leukocytes of non-pregnant volunteers has been studied after the administration of a single dose of bacterial endotoxin [14, 123]. Calvano et al. [14] reported dysregulation of 3714 genes in whole-blood cells at 2, 4, and 9 hours after endotoxin administration and noted that gene expression returned to baseline by 24 hours after endotoxin injection. The endotoxin quickly and transiently activated genes involved in the innate immune response, and after an initial pro-inflammatory phase, a self-limiting counter-regulatory response followed, with eventual resolution of gene expression changes within a day of endotoxin administration. Specifically, there was an increased expression of pro-inflammatory cytokines and chemokines (e.g., *IL1A*, *IL1B*, *IL8*, *TNF*) and NFκB family transcription factors within 2–4 hours of endotoxin treatment [14]. There was upregulation of transcription factors critical in both the initiation and the containment of an innate immune response [e.g., signal transducer and activators of transcription (*STAT*) genes, suppressor of cytokine signaling 3, *SOCS3*], which was observed within 4–6 hours of endotoxin administration. There was also increased expression of genes encoding membrane-bound and secreted proteins that limit the inflammatory response (e.g., *IL1R2*, *IL1RAP*, *IL10*) [14].

Similar observations were made by Talwar et al. [123], who investigated temporal gene expression changes in peripheral blood mononuclear cells and whole-blood cells from nonpregnant volunteers after a single dose of endotoxin. An upregulation of genes associated with pattern recognition receptors, intra-cellular signaling, cell mobility, and defense function was reported. The largest change in gene expression occurred 6 hours after endotoxin treatment, with changes returning to baseline within 24 hours [123]. Collectively, these results suggest that leukocyte response to bacterial products include a short pro-inflammatory phase followed by a counter-regulatory phase and resolution of inflammation [14, 123].

Similarities in the expression of innate immune genes in pregnant women with acute pyelonephritis and nonpregnant individuals after endotoxin administration

As the microarray data set of whole-blood leukocytes after endotoxin administration was available online from the study of Calvano et al. [14], we compared such data set with our findings (this comparison included only those genes from the study of Calvano et al. [14] that were differentially expressed at three to five time points after endotoxin administration). We found that 296 of the 983 genes in our study changed in the same direction as that of the Calvano et al. study [14] (Table 1).

Differentially expressed genes involved in the innate immune response in both studies were mainly upregulated (Table 8). Among the functions of the proteins encoded by these genes, the following groups emerged: (a) cell adhesion and cell-cell signaling (*CD44*, *CLEC4D*, *CLEC4E*, *CLEC5A*, *ICAM1*), (b) activation and/or differentiation of macrophages (*CEBPD*), (c) inflammasome priming (*CASP1*, *CASP4*, *CASP5*), (d) activation of the nuclear factor (NF) κB and mitogen-activated protein kinase (MAPK) pathways (*IL18R1*, *IL18RAP*, *IRAK2*, *IRAK3*), (e) cellular binding to particles and immune complexes that have activated complement (*CR1*), (f) phagocytosis and antibody-dependent cell-mediated cytotoxicity (*FCAR*, *FCGR1A*, *FCGR1B*, *MARCO*), and (g) breakdown of extracellular matrix and type IV and V collagens (*MMP9*). These results suggest that acute pyelonephritis during pregnancy elicits a host response similar to that induced by intravenous bacterial endotoxin in non-pregnant volunteers.

Similarities in the expression of genes involved in lymphocyte functions in pregnant women with acute pyelonephritis and nonpregnant individuals after endotoxin administration

SPIA pathway analysis of the commonly differentially expressed genes in pyelonephritis and in the endotoxin-induced model of acute bacterial infection revealed five impacted pathways in both conditions: (1) “T-cell receptor signaling pathway,” (2) “natural killer cell-mediated cytotoxicity,” (3) “cytokine-cytokine receptor interaction,” (4) “RIG-I-like receptor signaling pathway,” and (5) “complement and coagulation cascades.” We observed that the pathways inhibited in pyelonephritis included those generally implicated in adaptive immune responses. This is consistent with the findings of Talwar et al. [123], who described the downregulation of T lymphocyte-associated genes after endotoxin administration. Moreover, differentially expressed genes involved in lymphocyte functions common in our study and in the study reported by Calvano et al. [14] were mainly downregulated (Table 9).

The decreased expression of these genes and the encoded proteins may result in impairment of (a) T-cell recognition of antigens displayed by antigen-presenting cells (*CD3D*, *CD3E*, *CD8A*, *CD8B*, *CD247*), (b) T-cell chemotaxis and migration to inflamed tissues (*CXCR3*), (c) T-helper lineage development (*GATA3*, *STAT4*, *TBX21*), (d) T-cell activation, proliferation, development, signal transduction, survival, and cytokine production (*CCR7*, *CD6*, *CD28*, *DPP4*, *IL23A*, *IL23R*, *IL2RB*, *IL5RA*, *IL9R*, *ITK*, *LCK*, *PRKCQ*, *TCF7*, *ZAP70*), (e) generation and long-term maintenance of T-cell immunity (*CD27*), (f) T-cell-mediated cytotoxicity (*GNLY*, *GZMA*), and (g) regulation of B-cell activation, V(D)J recombination, and Ig synthesis (*CCR7*, *IL7R*).

These observations are consistent with the findings derived from transcriptome analysis of patients with sepsis [65, 125]. By analyzing multiple microarray data sets, Lindig et al. [65] found that the upregulated gene ontology categories in patients with sepsis include those related to innate immune responses, whereas the down-regulated categories include those related to adaptive immune responses. The systematic review of the transcriptomic data of 12 studies by Tang et al. [125] revealed reduced expression of genes associated with immune response in lymphocytes (e.g., *CCR7, CD28, CXCR3, IL2RB*, *IL7R*) and upregulation of genes limiting inflammatory responses (e.g., *SOCS1, SOCS3*), which also changed in the same direction in our study.

Immunosuppression in sepsis

Contrary to what was originally believed, sepsis does not represent a steady and uncontrolled systemic pro-inflammatory response [6, 51]. Some patients have a state consistent with immune suppression [51, 53, 140], which has been termed "immunoparalysis" [3, 53], characterized by a decreased Th1-like response [3, 51]. The initial pro-inflammatory state in sepsis is followed by a compensatory anti-inflammatory state or, in some cases, both pro-inflammatory and anti-inflammatory responses can occur at the onset of sepsis [51, 140]. The composition and direction of this complex systemic host response to infection depends on the load and virulence of the pathogen, the genetic characteristics of the host, and coexisting illnesses [6, 140].

Of importance, the majority of deaths occur in patients with sepsis who are immunosuppressed [53], and the prevention of immunosuppression improves the survival rate in animal models of sepsis [51]. The occurrence of the immunosuppression state has been attributed to the following [3, 11, 44, 51, 53, 98, 103, 140]: (1) an adaptive compensatory response characterized by increased expression of anti-inflammatory mediators (e.g., *IL1RN, SOCS1, SOCS3*) and the activation of T regulatory cells and myeloid-derived suppressor cells, (2) apoptosis of T and B lymphocytes due to activation of Fas-Fas-ligand system and caspases as well as decreased expression and/or function of anti-apoptotic molecules (e.g., Bcl-2), (3) anti-inflammatory responses in phagocytic cells induced by the uptake of apoptotic immune cells, and (4) activation of a neuroinflammatory reflex through vagal nerve stimulation, which leads to acetylcholine secretion by a subset of T-helper lymphocytes and the subsequent suppression of proinflammatory cytokine release from acetylcholine receptor-expressing macrophages.

Patients with sepsis may have a complex set of immunologic defects attributable to the cross-talk between specialized cells in the immune system that coordinate microbial eradication. For example, T lymphocytes play a pivotal role in the initial response to microbial infection because they produce IFN-γ, which activates macrophages [52, 53], and reduced Th1 function due to apoptotic cell death of T lymphocytes in sepsis leads to dampened cytokine production [52]. Indeed, the anti-inflammatory responses in sepsis lead to enhanced susceptibility to secondary infections [3, 6].

Differential expression of genes implicated in immunosuppression and apoptosis

We found upregulation of *IL1RN* (IL-1 receptor antagonist) and suppressors of cytokine signaling (*SOCS1, SOCS3*) in women with pyelonephritis in pregnancy – these findings are consistent with a compensatory anti-inflammatory response. Moreover, we found upregulation of *FAS* (CD95/Fas cell surface death receptor), which plays a central role in the apoptosis of lymphocytes in sepsis and in the pathogenesis of ARDS [36], a severe complication of pyelonephritis in pregnancy [5, 16, 17, 23–27, 33, 45, 49, 56, 66, 97, 116, 136, 147].

Consistent with the observation of increased apoptosis of lymphocytes in sepsis [3, 44, 51, 53, 98], we found upregulation of pro-apoptotic genes (*CFLAR*, *PDCD1LG2*) as well as downregulation of anti-apoptotic genes (*BCL2*, *FAIM3*) in cases of acute pyelonephritis (Table 10). Moreover, the expression of *CD36* (thrombospondin receptor), which is involved in the phagocytosis of apoptotic cells, was increased in pyelonephritis during pregnancy. We also found that the median absolute lymphocyte count of patients with pyelonephritis was significantly lower than that of controls in both the microarray ($P < 0.05$) and qRT-PCR ($P = 0.001$) populations in the current report (Table 1).

In contrast, the median absolute neutrophil count was higher in patients with pyelonephritis than in controls ($P < 0.001$, Table 1). This finding is consistent with the well-known phenomenon of delayed apoptosis of neutrophils in sepsis [8, 96, 124]. Previous studies also reported a higher median maternal plasma concentration of visfatin [71], a pro-inflammatory adipokine that promotes delayed neutrophil apoptosis as well as a lower median maternal plasma concentration of TRAIL [18], one of the mediators responsible for neutrophil apoptosis, in patients with pyelonephritis in pregnancy than in controls.

Differences in gene expression patterns between pregnant women with acute pyelonephritis and nonpregnant individuals after endotoxin administration

There were 22 differentially expressed genes in our study that changed in the opposite direction from that reported by Calvano et al. [14]. In addition, there were 665 differentially expressed genes in our study that did not change in expression after bacterial endotoxin administration [14]. Enrichment analyses showed that these 665 genes play a role in “immune response,” “immune effector process,” “inflammatory response,” “lymphocyte activation,” and “response to viruses,” among other biological processes. These results suggest that, although the pathways fundamentally impacted in pyelonephritis during pregnancy and endotoxin challenge in nonpregnant women are similar, the increased susceptibility of pregnant women to microbial products [46, 81, 84, 101] may have a well-defined molecular basis, and such differences (qualitative and quantitative) may be responsible for the difference in nature of the immune response to microbial products or infection in pregnant women. This requires further study of nonpregnant patients with pyelonephritis.

The administration of a single intravenous dose of endotoxin has been used as a model to study the systemic effects of a microbial product (endotoxin) and assumed by many to represent a state similar to systemic infection. However, this experimental approach cannot

be equated with intravenous administration of live bacteria or actual systemic infection in humans. Therefore, the comparison of our results in pregnant women with pyelonephritis to those reported by Calvano et al. [14] should be interpreted with caution. The findings reported herein can be considered to reflect real bacterial infection in pregnant women.

There are technical differences between our study and that of Calvano et al. [14]. (1) Methods used in blood collection, leukocyte lysis, storage, and RNA isolation were not the same. Whole-blood samples were collected into PAX gene tubes in our study, whereas leukocyte separation by centrifugation was undertaken by Calvano et al. before RNA isolation. (2) Our study was cross-sectional, whereas blood was collected at several time points [before (0 hour) and at 2, 4, 6, 9, and 24 hours after endotoxin infusion] in the study of Calvano et al. [14]. (3) Our population had several bacteria identified in urine and/or blood, whereas nonpregnant volunteers in the study of Calvano et al. received a single dose of endotoxin [14]. (4) We adjusted our gene expression results as a function of WBC count, but this was not performed by Calvano et al. [14].

Differential expression of *FAM20A* and *ETV7*

The top three significantly upregulated probe sets in our study correspond to transcripts encoded by the *FAM20A* (family with sequence similarity 20, member A) gene. FAM20A belongs to an evolutionarily conserved family of three proteins (FAM20A, FAM20B, and FAM20C) secreted by hematopoietic cells [89]. FAM20A is a glycoprotein with high expression levels in hematopoietic tissues [89], especially in those cells committed to the granulocytic lineage [89]. Another gene involved in hematopoiesis and upregulated in pyelonephritis was *ETV7* (encoding for ETS variant 7). The expression of *ETV7* in normal and leukemic hematopoietic cells suggests an important role for this gene in normal hematopoietic development as well as in oncogenesis [15, 59]. As gene expression in our data was adjusted for WBC count, the results are not simply the reflection of a higher WBC count in patients with pyelonephritis but probably reflect enhanced hematopoiesis that involves the upregulation of these genes in response to acute microbial infection.

Strengths and Limitations

This is the first study to characterize the transcriptome of whole blood in pregnant women with an acute episode of infection. A limitation of this study was that the differentially expressed genes identified reflect the changes in the total intracellular mRNA in whole blood. However, it is not possible to attribute these changes to a particular population of leukocytes or reticulocytes. Meanwhile, if we had attempted to separate WBCs before RNA isolation, artifacts derived from cell separation procedures may have been introduced.

Conclusions

This is the first report of the transcriptome of whole-blood cells in pregnant women with acute pyelonephritis. We found increased expression of genes involved in innate immunity and decreased expression of genes that participate in lymphocyte function. These findings are similar to the transcriptional changes reported in nonpregnant individuals exposed to bacterial endotoxin. However, we identified a set of differentially expressed genes that were

unique in pyelonephritis during pregnancy. Our study provides necessary information to characterize the nature of a systematic inflammatory response in pregnant women. A major reason for this study is the interest in comparing conditions in which there is acute intravascular inflammation (such as preeclampsia) with that induced by microorganisms (such as pyelonephritis).

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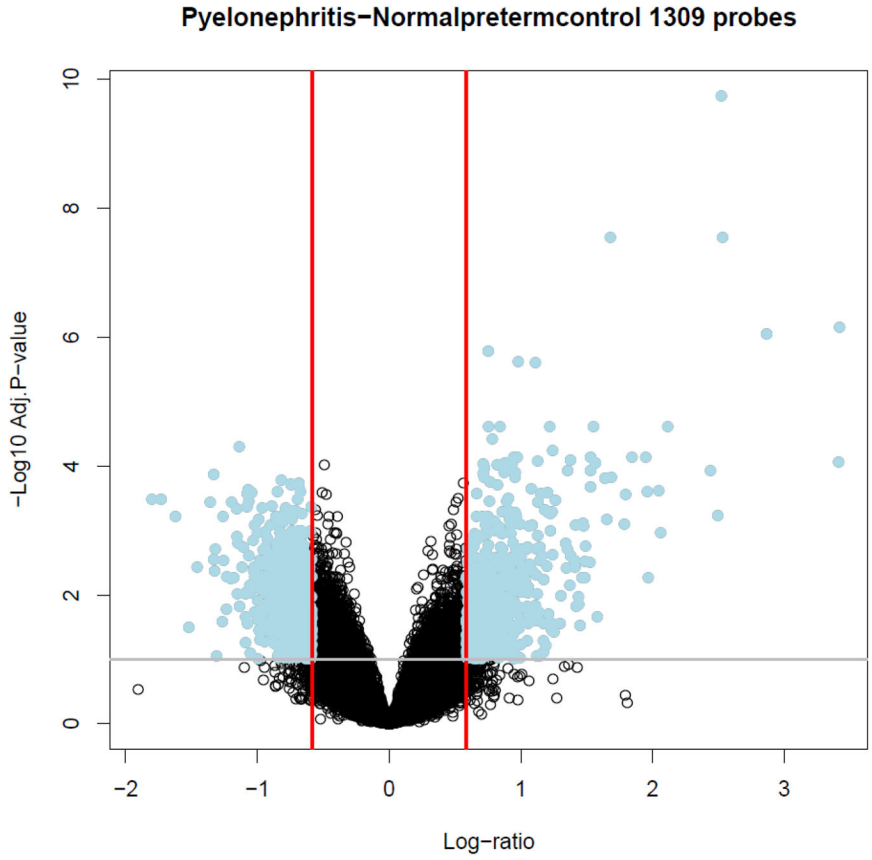

Figure 1A

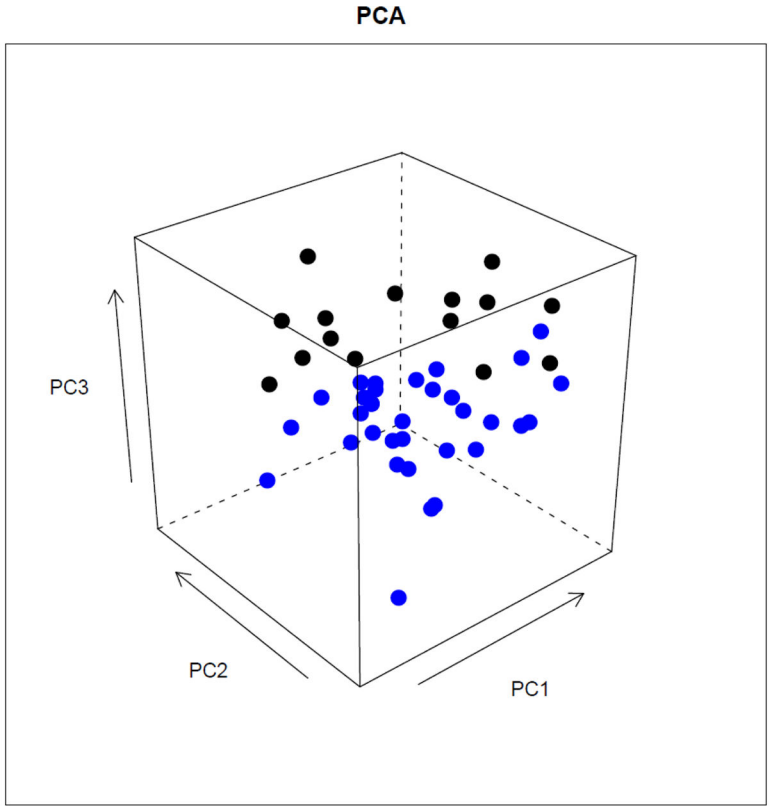

Figure 1B

Figure 1. Volcano plot and three dimensional principal component analysis (PCA) plot

(A) The volcano plot shows probability values of all probes in the microarray plotted against the fold change. In this figure, the log (base 10) of the false discovery rate-adjusted probability values are plotted against the log (base 2) of fold-change between patients with pyelonephritis and normal controls. On the Y-axis, values higher than the gray-line threshold represent significant probes with an adjusted probability value of <0.1. On the X-axis, values outside the red lines represent fold change of >1.5. **(B)** The three-dimensional PCA plot demonstrates a degree of segregation between women with acute pyelonephritis and normal controls. Blue dots indicate samples from the normal control group, whereas black dots represent individual samples from the acute pyelonephritis group.

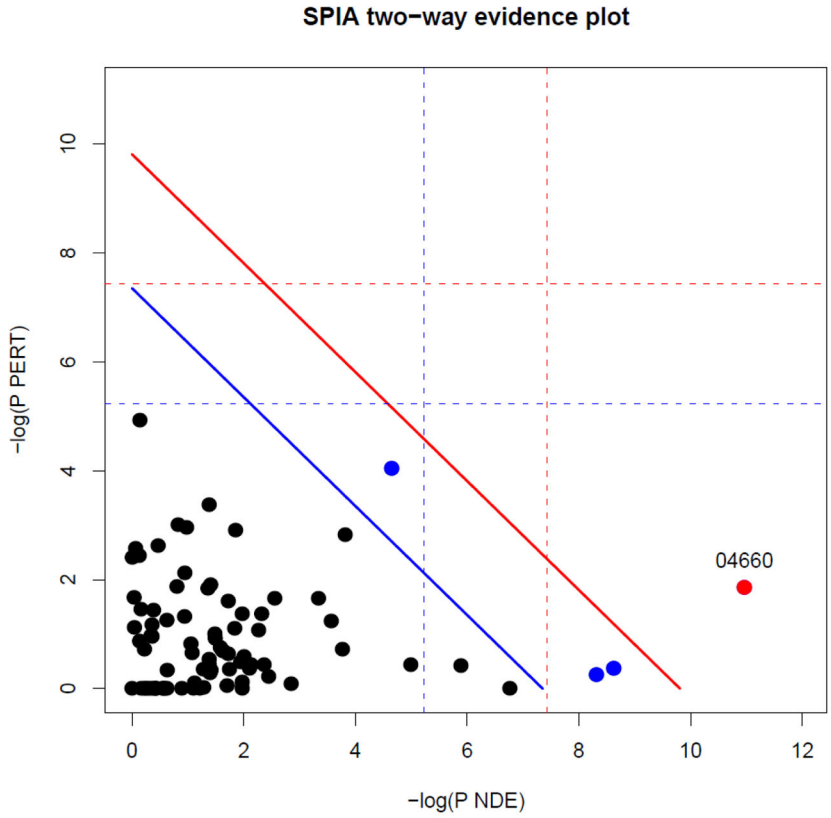

Figure 2. Two-dimensional plot illustrates the relationship between the two types of evidence considered by SPIA

The X-axis shows the overrepresentation evidence (–log [P-value]), whereas the Y-axis shows the perturbation evidence (–log [perturbation P-value]). Each pathway is represented by a point. Pathways above the oblique red line (red dots) are significant at 5% after Bonferroni correction; pathways (blue dots and red dots) above the oblique blue line are significant at 5% after false discovery rate correction. The vertical and horizontal thresholds represent the same corrections for the two types of evidence considered individually.

Table 1

Demographic and clinical characteristics of the study groups

Group	Microarray			qRT-PCR		
	Normal preterm control (n=34)	**Pyelonephritis (n=15)**	**P value**	**Normal preterm control (n=59)**	**Pyelonephritis (n=19)**	**P value**
Maternal age(yrs)	21.7 (16–34)	24.3 (19–29)	0.182	22.9 (16–35)	23.4 (18–29)	0.935
Nulliparity (%)	19 (55.9)	4 (26.7)	0.071	27 (45.8)	6 (31.6)	0.276
African Americans (%)	30 (88.2)	13 (86.7)	1.0	49 (83.1)	17 (89.5)	0.720
Smoking	3 (8.8)	5 (33.3)	0.047	10 (16.9)	6 (31.6)	0.170
Gestational age at venipuncture (wks)	30.9 (20.6–36.0)	25.1 (20.4–36.0)	0.004	31.4 (20–36.9)	25.4 (20.4–36.3)	0.014
WBC count ($\times 10^3/\mu L$)	9 (4.7–13.8)	14.8 (7.3–19.9)	<0.0001	8.9 (2.7–13.8)	14.3 (7.3–19.9)	<0.0001
Neutrophil count ($\times 10^3/\mu L$)	6.7 (4.9–8.1)	12.4 (10.1–13.2)	<0.001	6.3 (4.8–8.1)	12.4 (10.6–13.2)	<0.001
Lymphocyte count ($\times 10^3/\mu L$)	1.6 (1.3–2.1)	1.2 (1.0–1.4)	<0.05	1.7 (1.3–2.0)	1.0 (0.9–1.3)	0.001
Birth weight (gms)	3297.5 (2575–3995)	3067.5 (2135–3985)	0.118[a]	3310 (2575–4005)	3142.5 (2135–3985)	0.093[b]

[a] In the microarray experiments, birth weight data were not available for one case.

[b] In the qRT-PCR experiments, birth weight data were not available for one case.

WBC, white blood cell.

Table 2

Top 100 probe sets differentially expressed between acute pyelonephritis and normal controls

Rank	Name	Entrez ID	Gene Symbol	Fold change	Adjusted P-Value (q-value)	Dysregulated after endotoxin challenge in the same direction [14]
1	Family with sequence similarity 20, member A	54757	*FAM20A*	5.74	1.80E-10	
2	Family with sequence similarity 20, member A	54757	*FAM20A*	3.21	2.83E-08	
3	Family with sequence similarity 20, member A	54757	*FAM20A*	5.78	2.83E-08	
4	Methyltransferase like 7B	196410	*METTL7B*	10.71	6.92E-07	
5	Ankyrin repeat domain 22	118932	*ANKRD22*	7.29	8.80E-07	
6	Kiaa1632	57724	*KIAA1632*	1.69	1.64E-06	
7	Fc fragment of IgG, high-affinity Ia, receptor (CD64)	2209	*FCGR1A*	1.97	2.36E-06	
8	Dual-specificity phosphatase 3	1845	*DUSP3*	2.16	2.47E-06	Yes
9	Proline-serine-threonine phosphatase-interacting protein 2	9050	*PSTPIP2*	1.79	2.44E-05	Yes
10	Glucosamine (*N*-acetyl)-6-sulfatase	2799	*GNS*	1.69	2.44E-05	Yes
11	Guanylate-binding protein 1, IFN-inducible, 67 kDa	2633	*GBP1*	4.33	2.44E-05	Yes
12	Erythrocyte membrane protein band 4.1-like 5	57669	*EPB41L5*	2.93	2.44E-05	
13	Feline leukemia virus subgroup C cellular receptor family, member 2	55640	*FLVCR2*	2.33	2.44E-05	
14	Dual-specificity phosphatase 3	1845	*DUSP3*	1.72	3.82E-05	Yes
15	Kelch-like 3 (*Drosophila*)	26249	*KLHL3*	−2.20	5.00E-05	
16	Lipoma HMGIC fusion partner-like 2	10184	*LHFPL2*	2.36	5.75E-05	
17	Sortilin 1	6272	*SORT1*	1.96	7.22E-05	
18	Membrane-spanning 4-domains, subfamily A, member 4	51338	*MS4A4A*	3.59	7.22E-05	Yes
19	Membrane-spanning 4-domains, subfamily A, member 4	51338	*MS4A4A*	3.86	7.22E-05	Yes
20	MAPK kinase 6	5608	*MAP2K6*	1.93	7.22E-05	Yes
21	Membrane-spanning 4-domains, subfamily A, member 4	51338	*MS4A4A*	2.89	7.22E-05	Yes
22	Apolipoprotein L, 6	80830	*APOL6*	2.60	8.22E-05	
23	Cornichon homolog 4 (*Drosophila*)	29097	*CNIH4*	2.19	8.34E-05	Yes
24	ETS variant 7	51513	*ETV7*	10.65	8.79E-05	
25	TRAF-interacting protein with forkhead-associated domain	92610	*TIFA*	2.96	9.07E-05	
26	Acyl-Coenzyme A oxidase 2, branched chain	8309	*ACOX2*	1.84	9.07E-05	

Rank	Name	Entrez ID	Gene Symbol	Fold change	Adjusted P-Value (q-value)	Dysregulated after endotoxin challenge in the same direction [14]
27	Fc fragment of IgG, high affinity Ib, receptor (CD64)	2210	*FCGR1B*	1.64	9.37E-05	Yes
28	Family with sequence similarity 89, member A	375061	*FAM89A*	1.91	9.37E-05	
29	Tumor protein p53 inducible protein 3	9540	*TP53I3*	1.77	9.48E-05	Yes
30	Pseudo-uridylate synthase 3	83480	*PUS3*	1.65	0.00011	
31	Basic leucine zipper transcription factor, ATF-like 2	116071	*BATF2*	5.42	0.00012	
32	Basic leucine zipper transcription factor, ATF-like	10538	*BATF*	1.84	0.00012	Yes
33	Apolipoprotein L, 6	80830	*APOL6*	2.56	0.00012	
34	Guanylate-binding protein 1, IFN-inducible, 67 kDa	2633	*GBP1*	2.89	0.00012	Yes
35	Dual-specificity phosphatase 3	1845	*DUSP3*	1.83	0.00013	Yes
36	Alkaline ceramidase 3	55331	*ACER3*	1.94	0.00013	
37	Similar to hcg2041270	344887	*LOC344887*	1.64	0.00013	
38	Chromosome 13 open reading frame 15	28984	*C13orf15*	−2.52	0.00014	
39	ER lipid raft-associated 1	10613	*ERLIN1*	1.65	0.00015	Yes
40	Kynureninase (L-kynurenine hydrolase)	8942	*KYNU*	1.94	0.00015	
41	CD274 molecule	29126	*CD274*	3.22	0.00015	
42	Guanylate-binding protein 1, IFN-inducible, 67 kDa	2633	*GBP1*	3.11	0.00016	Yes
43	IL-23, α subunit p19	51561	*IL23A*	−1.76	0.00017	
44	Methionine sulfoxide reductase B2	22921	*MSRB2*	1.70	0.00018	Yes
45	SH3-binding domain kinase 1	388228	*SBK1*	−1.61	0.00018	
46	Pleckstrin homology domain containing, family G (with rhogef domain) member 3	26030	*PLEKHG3*	−1.68	0.00019	
47	Cyclin B1 interacting protein 1	57820	*CCNB1IP1*	−1.67	0.00019	
48	MARCKS-like 1	65108	*MARCKSL1*	−1.61	0.00019	
49	G-protein-coupled receptor 107	57720	*GPR107*	1.77	0.00019	Yes
50	Guanylate-binding protein 1, IFN-inducible, 67 kDa	2633	*GBP1*	2.89	0.00021	Yes
51	Kynureninase (L-kynurenine hydrolase)	8942	*KYNU*	2.11	0.00022	
52	Fc fragment of IgG-binding protein	8857	*FCGBP*	−2.10	0.00024	
53	G-protein-coupled receptor 84	53831	*GPR84*	4.13	0.00024	
54	Ankyrin repeat domain 22	118932	*ANKRD22*	3.89	0.00025	

Rank	Name	Entrez ID	Gene Symbol	Fold change	Adjusted P-Value (q-value)	Dysregulated after endotoxin challenge in the same direction [14]
55	Kiaa0748	9840	*KIAA0748*	−1.80	0.00025	
56	Hypothetical protein LOC284023	284023	*LOC284023*	−1.59	0.00025	
57	Cholesteryl ester transfer protein, plasma	1071	*CETP*	2.30	0.00026	
58	ATP-grasp domain containing 1	57571	*ATPGD1*	−2.06	0.00026	
59	T-cell receptor δ locus	6964	*TRD@*	−2.11	0.00027	
60	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	9861	*PSMD6*	1.59	0.00027	
61	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	634	*CEACAM1*	2.22	0.00027	Yes
62	Oleoyl-ACP hydrolase	55301	*OLAH*	3.47	0.00028	Yes
63	Growth arrest-specific 7	8522	*GAS7*	1.87	0.00031	Yes
64	Leucine-rich repeat neuronal 3	54674	*LRRN3*	−3.49	0.00033	
65	Poly(A)-binding protein interacting protein 2B	400961	*PAIP2B*	−1.62	0.00033	
66	Leucine-rich repeat neuronal 3	54674	*LRRN3*	−3.32	0.00033	
67	Chromosome 6 open reading frame 150	115004	*C6orf150*	2.40	0.00034	
68	Transmembrane protein 204	79652	*TMEM204*	−2.09	0.00036	Yes
69	Multiple C2 domains, transmembrane 1	79772	*MCTP1*	1.70	0.00036	Yes
70	Kynureninase (L-kynurenine hydrolase)	8942	*KYNU*	1.86	0.00036	
71	Plexin domain containing 1	57125	*PLXDC1*	−2.29	0.00036	
72	Phosphatidylinositol glycan anchor biosynthesis, class L	9487	*PIGL*	−2.56	0.00036	
73	Cytochrome P450, family 1, subfamily B, polypeptide 1	1545	*CYP1B1*	2.19	0.00036	Yes
74	CD6 molecule	923	*CD6*	−1.85	0.00042	Yes
75	Phosphodiesterase 9A	5152	*PDE9A*	−2.11	0.00043	
76	Filamin B β	2317	*FLNB*	−1.51	0.00043	
77	MTERF domain containing 3	80298	*MTERFD3*	−1.72	0.00045	
78	*N*-myristoyltransferase 2	9397	*NMT2*	−1.95	0.00045	Yes
79	RCAN family member 3	11123	*RCAN3*	−2.23	0.00046	Yes
80	Macrophage receptor with collagenous structure	8685	*MARCO*	2.27	0.00049	Yes
81	TRAF2- and NCK-interacting kinase	23043	*TNIK*	−1.64	0.00049	
82	Potassium voltage-gated channel, subfamily H (EAG-related), member 7	90134	*KCNH7*	1.87	0.00050	

Rank	Name	Entrez ID	Gene Symbol	Fold change	Adjusted P-Value (q-value)	Dysregulated after endotoxin challenge in the same direction [14]
83	Non-protein coding RNA 219	114915	*NCRNA00219*	−1.65	0.00050	
84	Dehydrogenase/reductase (SDR family) member 9	10170	*DHRS9*	2.36	0.00051	
85	Lactamase β	114294	*LACTB*	1.81	0.00051	
86	KCNE1-like	23630	*KCNE1L*	1.94	0.00052	
87	Dehydrogenase/reductase (SDR family) member 9	10170	*DHRS9*	2.31	0.00054	
88	F-box protein 6	26270	*FBXO6*	2.34	0.00055	
89	Zinc finger protein 638	27332	*ZNF638*	−1.73	0.00055	
90	RAR-related orphan receptor A	6095	*RORA*	−1.58	0.00055	Yes
91	CD247 molecule	919	*CD247*	−1.73	0.00058	Yes
92	ETS variant 7	51513	*ETV7*	5.64	0.00059	
93	Noggin	9241	*NOG*	−3.08	0.00060	
94	Methionine sulfoxide reductase B2	22921	*MSRB2*	1.63	0.00060	Yes
95	TRAF-interacting protein with forkhead-associated domain	92610	*TIFA*	1.68	0.00060	
96	Kiaa1632	57724	*KIAA1632*	1.58	0.00060	
97	DNA damage-regulated autophagy modulator 1	55332	*DRAM1*	1.95	0.00060	Yes
98	Solute carrier family 16, member 10 (aromatic amino acid transporter)	117247	*SLC16A10*	−2.40	0.00060	
99	Lymphocyte-specific protein tyrosine kinase	3932	*LCK*	−1.82	0.00061	Yes
100	WAS protein family, member 1	8936	*WASF1*	1.91	0.00061	

Up-regulation in acute pyelonephritis in pregnancy is shown with positive values, whereas down-regulation is depicted with negative values.

Table 3

Gene ontology analysis: top 100 biological processes with enrichment in acute pyelonephritis

Rank	Biological process	Number of genes in the differentially expressed list	Number of genes in the reference array	P value
1	Regulation of cell activation	28	143	0.00000
2	Positive regulation of leukocyte activation	22	92	0.00000
3	Cell surface receptor-linked signaling pathway	70	606	0.00001
4	Positive regulation of developmental process	33	206	0.00001
5	Positive regulation of calcium-mediated signaling	7	12	0.00001
6	Multi-organism process	66	546	0.00001
7	Innate immune response	19	91	0.00001
8	Response to biotic stimulus	40	289	0.00003
9	Activation of immune response	17	80	0.00004
10	Regulation of lymphocyte activation	22	122	0.00004
11	Regulation of response to stress	31	206	0.00005
12	Inflammatory response	31	208	0.00005
13	Signal transduction	181	1980	0.00007
14	Immune response-regulating signaling pathway	14	63	0.00010
15	Positive regulation of defense response	7	18	0.00013
16	Response to virus	16	81	0.00014
17	Regulation of inflammatory response	12	50	0.00015
18	Lymphocyte activation	18	101	0.00018
19	Response to wounding	22	140	0.00020
20	Cell activation	22	141	0.00025
21	CD8-positive, α-β T-cell differentiation	3	3	0.00035
22	Positive regulation of T-cell receptor signaling pathway	3	3	0.00035
23	Positive regulation of T-cell activation	12	55	0.00036
24	Cellular defense response	11	48	0.00042
25	Immune effector process	15	82	0.00049
26	Positive regulation of apoptosis	44	372	0.00052
27	Immune response	33	269	0.00055
28	Epithelial to mesenchymal transition	7	22	0.00058
29	Positive regulation of cell death	44	375	0.00062
30	Leukocyte differentiation	23	157	0.00067
31	Positive regulation of acute inflammatory response	4	7	0.00074
32	Immune response-activating cell surface receptor signaling pathway	10	44	0.00082
33	Regulation of cell differentiation	41	348	0.00086
34	Regulation of MAP kinase activity	17	103	0.00086
35	Cell recognition	9	37	0.00088
36	Response to hypoxia	16	95	0.00097

Rank	Biological process	Number of genes in the differentially expressed list	Number of genes in the reference array	P value
37	Positive regulation of immune system process	15	89	0.00108
38	Mechano-sensory behavior	3	4	0.00134
39	Regulation of cell-cell adhesion mediated by integrin	3	4	0.00134
40	Regulation of fibroblast growth factor receptor signaling pathway	3	4	0.00134
41	Regeneration	9	40	0.00160
42	Regulation of anatomical structure morphogenesis	23	168	0.00166
43	Regulation of body fluid levels	16	100	0.00169
44	Blood coagulation	14	82	0.00172
45	System development	135	1513	0.00177
46	Response to heat	9	41	0.00192
47	Peptidyl-tyrosine modification	14	83	0.00194
48	Adaptive immune response based on somatic recombination of immune receptors built from Ig superfamily domains	12	67	0.00228
49	Immunoglobulin-mediated immune response	10	50	0.00231
50	Response to cytokine stimulus	12	67	0.00237
51	Regulation of protein amino acid phosphorylation	19	133	0.00257
52	Regulation of cell-matrix adhesion	6	21	0.00268
53	Regulation of mononuclear cell proliferation	12	68	0.00270
54	Signaling process	132	1541	0.00296
55	Regulation of cytokine production	20	145	0.00306
56	Cell killing	8	36	0.00313
57	Tyrosine phosphorylation of Stat1 protein	3	5	0.00318
58	Regulation of adaptive immune response	9	44	0.00322
59	Positive regulation of lymphocyte proliferation	9	44	0.00322
60	Response to γ radiation	6	22	0.00347
61	Positive regulation of leukocyte proliferation	9	45	0.00378
62	Negative regulation of signaling process	23	179	0.00391
63	Skin development	5	16	0.00399
64	T-cell selection	5	16	0.00399
65	Humoral immune response	10	54	0.00416
66	Cellular component morphogenesis	37	333	0.00416
67	Lymphocyte-mediated immunity	8	38	0.00419
68	Regulation of α-β T-cell activation	7	30	0.00422
69	Positive regulation of peptidyl-tyrosine phosphorylation	7	30	0.00422
70	Signal initiation by diffusible mediator	11	63	0.00435
71	JAK-STAT cascade	9	46	0.00441
72	Regulation of immune response	13	83	0.00453
73	Positive regulation of phosphorylation	12	73	0.00493
74	Regulation of morphogenesis of a branching structure	2	2	0.00502

Rank	Biological process	Number of genes in the differentially expressed list	Number of genes in the reference array	P value
75	Macrophage fusion	2	2	0.00502
76	Axon regeneration in the peripheral nervous system	2	2	0.00502
77	Mesenchymal cell differentiation	8	39	0.00528
78	Calcium-mediated signaling	6	24	0.00532
79	Activation of MAPK activity	10	56	0.00545
80	T-cell co-stimulation	4	11	0.00553
81	T-cell differentiation	9	48	0.00563
82	Regulation of transferase activity	33	294	0.00566
83	G-protein-coupled receptor protein signaling pathway	40	374	0.00572
84	Positive thymic T-cell selection	3	6	0.00603
85	Axonal fasciculation	3	6	0.00603
86	Cgmp-mediated signaling	3	6	0.00603
87	Signaling	41	420	0.00609
88	Circulatory system process	18	134	0.00636
89	Multicellular organismal process	134	1645	0.00648
90	Death	87	949	0.00664
91	Intracellular signaling pathway	56	573	0.00678
92	Regulation of T-cell proliferation	9	49	0.00680
93	Positive regulation of phosphorus metabolic process	12	76	0.00685
94	Lymphocyte activation during immune response	6	25	0.00687
95	Protein kinase cascade	41	393	0.00692
96	Induction of programmed cell death	32	287	0.00703
97	Positive regulation of response to stimulus	14	97	0.00734
98	Cell adhesion	46	455	0.00735
99	α-β T-cell activation	4	12	0.00765
100	Programmed cell death	80	867	0.00780

Table 4

Gene ontology analysis: 63 molecular functions associated with differentially expressed genes in acute pyelonephritis

Rank	Molecular function	Number of genes in the differentially expressed list	Number of genes in the reference array	P-value
1	Molecular transducer activity	134	1144	0.00000
2	Collagen binding	8	21	0.00005
3	Calcium ion binding	63	557	0.00011
4	Serine-type endopeptidase inhibitor activity	10	41	0.00043
5	Hyaluronic acid binding	4	8	0.00137
6	C-C chemokine receptor activity	5	13	0.00138
7	Tropomyosin binding	4	9	0.00233
8	G-protein-coupled receptor activity	27	219	0.00316
9	Peptidase inhibitor activity	12	70	0.00334
10	Receptor activity	31	282	0.00476
11	Hepoxilin-epoxide hydrolase activity	2	2	0.00497
12	Complement receptor activity	2	2	0.00497
13	Methylenetetrahydrofolate dehydrogenase (NADP+) activity	2	2	0.00497
14	MHC class I protein binding	4	11	0.00543
15	SH3/SH2 adaptor activity	9	48	0.00574
16	Phosphotyrosine binding	3	6	0.00595
17	Calmodulin binding	14	95	0.00657
18	Phosphoprotein binding	6	25	0.00672
19	Nucleotide receptor activity	6	27	0.00995
20	G-protein chemoattractant receptor activity	5	20	0.01098
21	Protein dimerization activity	42	415	0.01113
22	Transmembrane receptor activity	24	214	0.01158
23	Non-membrane-spanning protein tyrosine kinase activity	7	36	0.01167
24	Immunoglobulin receptor activity	2	3	0.01422
25	Sodium/amino acid symporter activity	2	3	0.01422
26	Methenyltetrahydrofolate cyclohydrolase activity	2	3	0.01422
27	T-cell receptor binding	2	3	0.01422
28	Chemokine binding	5	22	0.01660
29	Receptor signaling complex scaffold activity	4	15	0.01793
30	Growth factor binding	10	67	0.01800
31	Receptor signaling protein activity	16	129	0.01883
32	SH2 domain binding	5	23	0.02001
33	Hydrolase activity, acting on ether bonds	3	9	0.02131
34	Transforming growth factor β binding	3	9	0.02131
35	Endoribonuclease activity, producing 3′-phosphomonoesters	3	9	0.02131

Rank	Molecular function	Number of genes in the differentially expressed list	Number of genes in the reference array	P-value
36	Dipeptidyl-peptidase activity	3	9	0.02131
37	Low-density lipoprotein receptor activity	3	9	0.02131
38	Co-receptor activity	4	16	0.02260
39	Steroid binding	7	41	0.02319
40	Protein kinase binding	16	133	0.02485
41	ρ-Guanyl-nucleotide exchange factor activity	9	61	0.02631
42	NADPH binding	2	4	0.02711
43	Growth hormone receptor binding	2	4	0.02711
44	Lipoxygenase activity	2	4	0.02711
45	Alcohol dehydrogenase (NAD) activity	2	4	0.02711
46	Adenosine receptor activity, G-protein coupled	2	4	0.02711
47	Scavenger receptor activity	5	25	0.02813
48	Epidermal growth factor receptor binding	3	10	0.02887
49	Peptide antigen binding	3	10	0.02887
50	Cytokine receptor activity	7	43	0.02945
51	Protein kinase inhibitor activity	6	34	0.02978
52	Phospholipid binding	16	136	0.02987
53	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines	5	26	0.03287
54	Receptor binding	50	549	0.03509
55	Racemase and epimerase activity	3	11	0.03766
56	Purinergic nucleotide receptor activity, G-protein-coupled	4	19	0.04043
57	Poly(U) RNA binding	2	5	0.04309
58	RS domain binding	2	5	0.04309
59	Hematopoietin/IFN class (D200-domain) cytokine receptor signal transducer activity	2	5	0.04309
60	Sulfuric ester hydrolase activity	3	12	0.04765
61	Antigen binding	4	20	0.04770
62	Single-stranded RNA binding	4	20	0.04814
63	Small GTPase regulator activity	24	238	0.04841

Table 5

Pathway analysis using the over-representation method

Pathway	KEGG identification number	Number of genes in the differentially expressed list	Number of genes in the reference array	Odds ratio	Adjusted P-value (q-value)
Primary immunodeficiency	5340	11	33	5.79	0.00283
Hematopoietic cell lineage	4640	19	67	4.68	0.00013
T-cell receptor signaling pathway	4660	19	95	2.93	0.0098

Table 6

Pathway analysis using the SPIA method

Pathway	KEGG identification number	Number of genes in the differentially expressed list	Number of genes in the reference array	Adjusted P-value (q-value)
T-cell receptor signaling path	4660	19	95	0.00315
Jak-STAT signaling pathway	4630	18	103	0.03792
Complement and coagulation cascade	4610	7	36	0.03792
Cytokine-cytokine receptor interaction	4060	25	172	0.03792

Table 7

Comparison of microarray gene expression data to qRT-PCR gene expression data of selected genes in an extended sample set

		Microarray analysis			qRT-PCR analysis		
Gene Name	**Gene Symbol**	**Fold-change**	**Adjusted P-value (q-value)**	**Direction of change**	**Fold-change**	**P-value**	**Direction of change**
Leucine-rich repeat neuronal 3	*LRRN3*	3.61	3.22E-05	↓	4.77	2.47E-09	↓
Chemokine (C-C motif) receptor 3	*CCR3*	1.96	0.005192	↓	4.59	2.68E-08	↓
KIAA1671	*KIAA1671*	2.72	0.0017063	↓	4.72	1.49E-07	↓
Hairy/enhancer-of-split related with YRPW motif 1	*HEY1*	2.46	0.0017839	↓	3.95	1.29E-05	↓
Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	*SLC2A5*	1.70	0.0939416	↓	2.60	5.20E-05	↓
Defensin, α4, corticostatin	*DEFA4*	2.24	0.0994301	↓	4.92	0.000112	↓
EF-hand domain (C-terminal) containing 1	*EFHC1*	1.70	0.0070406	↓	1.87	0.000139	↓
Protease, serine, 33	*PRSS33*	3.15	0.0123263	↓	5.33	0.000465	↓
EF-hand domain (C-terminal) containing 2	*EFHC2*	1.74	0.0583685	↓	1.98	0.000869	↓
Carcinoembryonic antigen-related cell adhesion molecule 8	*CEACAM8*	2.10	0.0729948	↓	3.44	0.002103	↓
Cysteine-rich secretory protein 3	*CRISP3*	2.27	0.0999189	↓	2.37	0.002528	↓
Cathepsin G	*CTSG*	2.50	0.0533428	↓	3.51	0.002572	↓
Noggin	*NOG*	3.13	0.0001584	↓	2.50	0.011715	↓
Ribonuclease, RNase A family, 3	*RNASE3*	2.65	0.0460622	↓	2.72	0.013675	↓
Contactin-associated protein-like 3B	*CNTNAP3B*	2.14	0.0547154	↓	7.84	0.042431	↓
CD24 molecule	*CD24*	1.75	0.0276683	↓	2.49	0.047949	↓
Contactin-associated protein-like 3	*CNTNAP3*	2.20	0.0269219	↓	1.76	NS	↓
Chromosome 13 open reading frame 15	*C13orf15*	2.85	1.15E-05	↓	1.40	NS	↓
Methyltransferase-like 7B	*METTL7B*	11.84	1.05E-08	↑	26.06	3.08E-18	↑
Family with sequence similarity 20, member A	*FAM20A*	5.78	3.36E-12	↑	14.57	9.11E-18	↑
Feline leukemia virus subgroup C cellular receptor family, member 2	*FLVCR2*	3.00	8.41E-05	↑	2.98	6.48E-11	↑
Ankyrin repeat domain 22	*ANKRD22*	8.42	7.23E-08	↑	5.19	1.47E-09	↑
Proline-serine-threonine phosphatase-interacting protein 2	*PSTPIP2*	1.81	1.89E-06	↑	3.68	2.04E-09	↑
Oleoyl-ACP hydrolase	*OLAH*	3.05	0.0002631	↑	5.63	1.06E-08	↑

		Microarray analysis			qRT-PCR analysis		
Gene Name	**Gene Symbol**	**Fold-change**	**Adjusted P-value (q-value)**	**Direction of change**	**Fold-change**	**P-value**	**Direction of change**
TRAF-interacting protein with forkhead-associated domain	*TIFA*	3.21	4.10E-06	↑	2.41	1.32E-08	↑
Dual-specificity phosphatase 3	*DUSP3*	2.28	7.11E-08	↑	1.96	1.09E-06	↑
G-protein-coupled receptor 84	*GPR84*	4.00	0.0001185	↑	3.72	2.08E-06	↑
SLAM family member 8	*SLAMF8*	2.78	0.0004544	↑	2.70	2.71E-06	↑
Carcinoembryonic antigen-related cell adhesion molecule 1	*CEACAM1*	2.71	0.0002781	↑	3.61	3.10E-06	↑
Fc fragment of IgG, high-affinity Ia, receptor (CD64)	*FCGR1A*	2.01	1.53E-07	↑	2.80	3.35E-06	↑
ETS variant 7	*ETV7*	13.07	3.11E-06	↑	6.82	3.83E-06	↑
CD274 molecule	*CD274*	3.41	1.35E-05	↑	3.55	1.18E-05	↑
Sphingomyelin synthase 2	*SGMS2*	1.83	0.0527572	↑	1.78	2.12E-05	↑
BMX non-receptor tyrosine kinase	*BMX*	1.73	0.0138943	↑	1.95	3.22E-05	↑
Guanylate-binding protein 1, IFN-inducible, 67 kDa	*GBP1*	4.62	1.61E-06	↑	3.66	3.51E-05	↑
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	*SERPINB2*	3.01	0.0003419	↑	2.47	3.88E-05	↑
Lipoma HMGIC fusion partner-like 2	*LHFPL2*	2.52	2.34E-06	↑	2.76	5.17E-05	↑
Short stature homeobox 2	*SHOX2*	2.81	0.009752	↑	4.58	6.51E-05	↑
Chromosome 15 open reading frame 48	*C15orf48*	3.86	0.0005621	↑	2.87	8.61E-05	↑
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	*PFKFB2*	2.80	0.0147768	↑	2.15	0.000375	↑
V-set and Ig domain-containing 4	*VSIG4*	1.72	0.0187304	↑	2.11	0.000415	↑
Epithelial stromal interaction 1	*EPSTI1*	3.38	0.0002715	↑	2.79	0.000553	↑
Basic leucine zipper transcription factor, ATF-like 2	*BATF2*	6.35	6.95E-06	↑	3.60	0.000685	↑
Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	*SERPING1*	4.67	0.0007851	↑	4.33	0.000863	↑
KIAA1632	*KIAA1632*	1.74	0.0012914	↑	1.56	0.00178	↑
Membrane-spanning 4-domains, subfamily A, member 4	*MS4A4A*	4.22	5.22E-06	↑	3.46	0.001822	↑
Ribonuclease, RNase A family, 1	*RNASE1*	3.02	0.0010624	↑	2.41	0.002062	↑
Transmembrane protein 176B	*TMEM176B*	2.86	0.0598179	↑	2.78	0.008158	↑
Guanylate-binding protein 5	*GBP5*	3.16	0.0006686	↑	2.70	0.013125	↑
Apolipoprotein L, 6	*APOL6*	2.95	0.0035536	↑	2.32	0.044719	↑
Radical *S*-adenosyl methionine domain containing 2	*RSAD2*	2.76	0.0238295	↑	2.18	NS	↑

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		Microarray analysis			qRT-PCR analysis		
Gene Name	**Gene Symbol**	**Fold-change**	**Adjusted P-value (q-value)**	**Direction of change**	**Fold-change**	**P-value**	**Direction of change**
Cornichon homologue 4	*CNIH4*	2.36	5.41E-06	↑	1.57	NS	↑
Glucosamine (*N*-acetyl)-6-sulfatase	*GNS*	1.68	4.49E-06	↑	1.48	NS	↑
Erythrocyte membrane protein band 4.1-like 5	*EPB41L5*	3.05	1.93E-06	↑	1.22	NS	↓
Purinergic receptor P2Y, G-protein-coupled, 14	*P2RY14*	3.08	0.0004666	↑	1.38	NS	↑
Chromosome 1 open reading frame 192	*C1orf192*	2.46	0.0001388	↑	1.16	NS	↓

Table 8

Differentially expressed innate immune genes common in acute pyelonephritis during pregnancy and in bacterial endotoxin administration in non-pregnant individuals

Gene symbol	ENTREZ ID	Gene name	Direction of change
CASP1	834	caspase 1, apoptosis-related cysteine peptidase	UP
CASP4	837	caspase 4, apoptosis-related cysteine peptidase	UP
CASP5	838	caspase 5, apoptosis-related cysteine peptidase	UP
CD44	960	CD44 molecule (Indian blood group)	UP
CD59	966	CD59 molecule, complement regulatory protein	UP
CEBPD	1052	CCAAT/enhancer-binding protein (C/EBP), δ	UP
CLEC4E	26253	C-type lectin domain family 4, member E	UP
CLEC5A	23601	C-type lectin domain family 5, member A	UP
CR1	1378	complement component (3b/4b) receptor 1 (Knops blood group)	UP
FCAR	2204	Receptor for Fc fragment of IgA	UP
FCGR1B	2210	Fc fragment of IgG, high-affinity Ib, receptor (CD64)	UP
ICAM1	3383	intercellular adhesion molecule 1	UP
IL18R1	8809	IL-18 receptor 1	UP
IL18RAP	8807	IL-18 receptor accessory protein	UP
IRAK3	11213	IL-1 receptor-associated kinase 3	UP
MARCO	8685	macrophage receptor with collagenous structure	UP
MMP9	4318	matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	UP
FCER1A	2205	Fc fragment of IgE, high-affinity I, receptor for; α polypeptide	DOWN
IL11RA	3590	IL-11 receptor α	DOWN
KLRB1	3820	killer cell lectin-like receptor subfamily B, member 1	DOWN
KLRK1	22914	killer cell lectin-like receptor subfamily K, member 1	DOWN
NLRC3	197358	NLR family, CARD domain containing 3	DOWN

The direction of change in expression in acute pyelonephritis in pregnant women and in non-pregnant individuals after bacterial endotoxin administration [14] is depicted in the right column.

Table 9

Differentially expressed genes associated with lymphocyte function common in acute pyelonephritis during pregnancy and in bacterial endotoxin administration in non-pregnant individuals

Gene symbol	ENTREZ ID	Gene name	Direction of change
CCR7	1236	chemokine (C-C motif) receptor 7	DOWN
CD24	100133941	CD24 molecule	DOWN
CD247	919	CD247 molecule	DOWN
CD27	939	CD27 molecule	DOWN
CD28	940	CD28 molecule	DOWN
CD3D	915	CD3d molecule, δ (CD3-TCR complex)	DOWN
CD3E	916	CD3e molecule, ε (CD3-TCR complex)	DOWN
CD5	921	CD5 molecule	DOWN
CD6	923	CD6 molecule	DOWN
CD8A	925	CD8a molecule	DOWN
CD8B	926	CD8b molecule	DOWN
CXCR3	2833	chemokine (C-X-C motif) receptor 3	DOWN
DPP4	1803	dipeptidyl-peptidase 4	DOWN
GATA3	2625	GATA binding protein 3	DOWN
GNLY	10578	granulysin	DOWN
GPR183	1880	G protein-coupled receptor 183	DOWN
GZMA	3001	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	DOWN
IL2RB	3560	IL-2 receptor β	DOWN
IL7R	3575	IL-7 receptor	DOWN
ITK	3702	IL2-inducible T-cell kinase	DOWN
LCK	3932	lymphocyte-specific protein tyrosine kinase	DOWN
PRKCQ	5588	protein kinase C, θ	DOWN
STAT4	6775	signal transducer and activator of transcription 4	DOWN
TBX21	30009	T-box 21	DOWN
TCF7	6932	transcription factor 7 (T-cell specific, HMG-box)	DOWN
TRAC	28755	T-cell receptor α constant	DOWN
ZAP70	7535	ζ-chain (TCR)-associated protein kinase 70 kDa	DOWN
IL1RN	3557	interleukin 1 receptor antagonist	UP
SOCS3	9021	suppressor of cytokine signaling 3	UP

The direction of change in expression in acute pyelonephritis in pregnant women and in non-pregnant individuals after bacterial endotoxin administration [14] is depicted in the right column.

Table 10

Differentially expressed genes involved in apoptosis common in acute pyelonephritis during pregnancy and in bacterial endotoxin administration in non-pregnant individuals

Gene symbol	ENTREZ ID	Gene name	Direction of change
CFLAR	8837	CASP8 and FADD-like apoptosis regulator	UP
FAS	355	Fas (TNF receptor superfamily, member 6)	UP
BCL2	596	B-cell CLL/lymphoma 2	DOWN
FAIM3	9214	Fas apoptotic inhibitory molecule 3	DOWN

The direction of change in expression in acute pyelonephritis in pregnant women and in non-pregnant individuals after bacterial endotoxin administration [14] is depicted in the right column.