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Gene expression profiling in peripheral blood mononuclear cells from patients with sporadic amyotrophic lateral sclerosis (sALS)

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Abstract

The aim of this study was to identify gene expression profiles in peripheral blood mononuclear cells (PBMCs) from sporadic amyotrophic lateral sclerosis (sALS) patients to gain insights into the pathogenesis of ALS. We found that upregulation of LPS/TLR4-signaling associated genes was observed in the PMBCs from sALS patients after short-term cultivation, and that elevated levels of gene expression correlated with degree of peripheral blood monocyte activation and plasma LPS levels in sALS. Similar patterns of gene expression were reproduced in LPS stimulated PBMCs from healthy controls. These data suggest that chronic monocyte/macrophage activation may be through LPS/TLR4-signaling pathways in ALS.

Keywords

Amyotrophic Lateral Sclerosis (ALS); Monocyte/macrophage activation; Lipopolysaccharide (LPS); Toll-like receptor 4 (TLR4)

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a debilitating neurological disorder in which neurodegeneration occurs in concert with an ongoing inflammatory process (McGeer and McGeer, 2002). ALS manifests as muscle weakness and paralysis with death ensuing for most patients within 5 years of symptom onset. Almost 90% of ALS patients are characterized as having sporadic ALS (sALS) with 10% having a familial form, a subset of whom have point mutations in their superoxide dismutase (SOD) gene. Evidence accumulating over the past decade has indicated that inflammation and immune activation may be commonly involved in the pathogenesis of ALS (Alexianu et al., 2001; Graves et al., 2004; Henkel et al., 2004; McGeer and McGeer, 2002; Simpson et al., 2004; Zhang et al., 2005). Neuropathologically, ALS is characterized by motor neuron degeneration with ubiquinated inclusions in the spinal cord and motor cortex. The neuropathologic lesions are

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surrounded by activated microglia in both the familial and sporadic forms of ALS disease (Kawamata et al., 1992; McGeer and McGeer, 2002), which may play an active role in ALS disease progression (Beers et al., 2006; Boillee et al., 2006).

Along with activated resident microglia, large numbers of infiltrating macrophages are present in sALS spinal cord lesions. Abnormal secretion of macrophage activation products and proinflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1) (Baron et al., 2005; Henkel et al., 2004; Simpson et al., 2004; Wilms et al., 2003; Zhang et al., 2006), and interleukin-6 (IL-6) (Ono et al., 2001; Sekizawa et al., 1998) have been reported in cerebral spinal fluid (CSF) and sera in patients with ALS. Enhanced levels of tumor necrosis factor-α (TNF-α) also have been seen in the blood of ALS patients (Babu et al., 2008; Cereda et al., 2008; Poloni et al., 2000). Patients with sALS exhibit elevated levels of CD16+ monocytes in peripheral blood (Zhang et al., 2005). These CD16+ ALS associated monocytes are characterized by high expression of monocyte activation marker HLA-DR and low expression of the MCP-1 receptor, CCR2 (Zhang et al., 2006). In addition, our more recent study found significantly increased levels of plasma endotoxin/ lipopolysaccharide (LPS), a systemic macrophage activator, in sALS patients, and that increased LPS levels correlated with degree of abnormally activated monocyte/macrophages in the peripheral blood. Finally, chronic stimulation of innate immunity with LPS has been shown to accelerate the disease course in SOD1 transgenic mice (Nguyen et al., 2004).

Endotoxin/lipopolysaccharide, is a potent inflammatory stimulus and immunostimulatory product (Takeda et al., 2003) and induces its effects through stimulation of CD14-bearing inflammatory cells (Flo et al., 2000; Tobias et al., 1999). LPS associated toxicity is mediated through systemic monocyte/macrophage and endothelial cell activation with release of inflammatory cytokines such as TNF-α and IL-6 (Beutler et al., 1985; Danner et al., 1991; Okusawa et al., 1988; Tracey et al., 1986). The effects of LPS are mediated primarily through Toll-Like Receptor 4 (TLR4). LPS/TLR4 signal transduction can be separated into MyD88-dependent and MyD88-independent pathways; the MyD88-dependent pathway was shown to be responsible for proinflammatory cytokine expression, while MyD88-independent pathway mediates the induction of type I interferons and interferon-inducible genes (Lu et al., 2008; Takeda and Akira, 2007; Tanimura et al., 2008).

Thus, sALS is characterized by evidence of systemic monocyte activation, in addition to local activation within disease affected spinal cords. In an effort to better characterize the systemic immune activation present in ALS patients we undertook a microarray-based gene expression analysis of peripheral blood mononuclear cells from patients with sALS. After short-term culture, peripheral blood mononuclear cells from sALS patients upregulated a different set of genes relative to cells from equivalently-aged healthy individuals, encoding type I interferons, interferon-inducible genes, and genes associated with the secreted activation proteins. Quantitative RT-PCR (qRT-PCR) analysis of gene transcription found that signals obtained from patients positively correlated with degree of abnormally activated monocyte/macrophages and plasma levels of LPS in sALS. This study shows for the first time a linkage between systemically activated monocyte/macrophages in sALS patients and the LPS/TLR4 signaling pathways.

2. Materials and Methods

2.1. Subjects

Patients with sALS, diagnosed using the El Escorial criteria (Brooks et al., 2000), provided informed consent in accordance with guidelines established by the California Pacific Medical Center and University of California San Francisco (UCSF) committees on human research, coordinated by the UCSF AIDS and Cancer Specimen Resource (ACSR) program.

The Revised ALS Functional Rating Scale (ALSFRS-R), scored 0-48, was used to evaluate overall patient functional status (Cedarbaum et al., 1999). All scores were recorded within a month of blood testing. Healthy controls consisted of individuals who had provided informed consent and blood samples to the ACSR. All healthy controls were from the San Francisco bay area and met criteria similar to that required for standard blood donation.

2.2. Preparation of peripheral blood mononuclear cells

Blood was drawn into tubes containing heparin anticoagulant and mixed with an equal volume of sterile isotonic phosphate-buffered solution (PBS, Ca++, Mg++ free) (UCSF cell culture facility, San Francisco, CA). The diluted blood was layered over Percoll (Amersham Biosciences, Piscataway, NJ) at 1.087g/ml, centrifuged, and the peripheral blood mononuclear cell (PBMC) layer was collected. After isolation, PBMCs were washed once with PBS and resuspended at a density of ~10^6 cells/ml in RPMI-1640 (UCSF cell culture facility, San Francisco, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 110 μg/ml sodium pyruvate (UCSF cell culture facility, San Francisco, CA). Subsequently PBMCs were cultured overnight (20-24 hours), unless otherwise stated, at 37°C in a humidified, 5% CO_2 incubator, and then pelleted by centrifugation and resuspended in TRIZOL (Invitrogen Corp, Carlsbad, CA). Total RNA was then prepared according to manufacturer's instructions.

2.3. Microarray analysis

Total RNA prepared isolated using TRIZOL was further purified using the PureLink™ Micro-to-Midi™ Total RNA Purification kit (Invitrogen, Carlsbad, CA). Quality of RNA was determined using the 2100 Bioanalyzer RNA LabChip (Agilent Technologies, Santa Clara, CA). Individual genechips were used for every subject. 100ng of high-quality total RNA was subjected to Affymetrix 2-cycle synthesis amplification, fluorescent labeling and hybridization to Affymetrix HG-U133_Plus_2 human genome array according to manufacturers' protocols (Affymetrix, Santa Clara CA). Expression data was obtained using an Affymetrix GSC3000 scanner and Affymetrix CEL file data processed and normalized using the robust multi-chip average (RMA) algorithm as implemented by Partek Genomics Suite (Partek Inc, St Louis, MO). Mean signal comparisons for each probe set were performed using 3 factor ANOVA including age, sex, and sample class (ALS, Healthy) also in Partek Genomics Suite. A probe set was declared to exhibit significantly changed expression if it exhibited a 4 fold change in mean signal between sALS patients and healthy controls with a p value of less than or equal to 10^{-4}. This would limit the number of probe sets included via a Type I error to less than 5.

2.4. Quantitative RT-PCR (qRT-PCR)

Approximately 150ng of total RNA from each sample was converted to cDNA using the First Strand cDNA Synthesis Kit for RT-PCR [AMV] kit (Roche Applied Diagnostics, Indianapolis IN) according to manufacturer's instructions. After first-strand synthesis the reverse transcriptase was denatured by incubation at 99°C for 5 minutes followed by quick cooling. DNA was stored at −20°C until use. PCR was performed on a LightCycler (Roche Applied Diagnostics, Indianapolis IN) using the LightCycler FastStart DNA Master SYBR Green I kit and ~ 4 ng cDNA sample. Amplifications included one cycle of template denaturation at 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, 68°C for 10 seconds, and 72°C for 16 seconds. The presence of a single amplified product was confirmed by DNA melting point analysis. Threshold cycles (Ct) for each amplification reaction were determined using LightCycler Software version 3.5 (Roche Applied Diagnostics, Indianapolis IN). All samples were also amplified with the human β-actin LightCycler – Primer Set (Roche Applied Diagnostics, Indianapolis IN). The sequences of gene-specific primers employed are provided in supplemental table 1. All primers had

amplification efficiencies approaching 2.0. Results with gene-specific primers for individual samples were normalized to signals obtained with β-actin from the same sample. Both "raw" normalizations ($Ct_{\beta\text{-actin}} - Ct_{gene}$) and "processed" normalizations ($2^{(Ct\ \beta\text{-actin} - Ct\ gene)}$) were employed.

2.5. Flow cytometry

Flow cytometric analysis of monocyte phenotype was performed on whole blood as described (Zhang et al., 2005). Briefly, whole blood was stained with surface antigens of anti-CD14-fluorescein isothiocyanate (FITC) and anti-CD16-phycoerytherin (PE) (DAKO, Carpinteria, California, USA) and anti-HLA-DR-PE (Becton-Dickinson, San Jose, California, USA) for 20 minutes at room temperature. Negative controls consisted of aliquots stained with isotype IgG-FITC and IgG-PE. Samples were then lysed with FACS Lysing Solution (Becton-Dickinson) for 10 minutes at room temperature followed by PBS $Ca^{++}Mg^{++}$ free wash. The stained cells were then resuspended in 1 ml of fixing solution (1% paraformaldehyde in PBS, with 0.1% sodium azide). Analysis was accomplished by acquisition of data on a FACScan flow cytometer (Becton-Dickinson) with Cellquest Pro software where at least 20,000 cells were counted per analysis.

2.6. Endotoxin/Lipopolysaccharide Detection

Plasma from sALS patients' blood was obtained by Percoll gradient centrifugation, and was frozen at −70°C until assayed. Duplicate plasma LPS levels in all plasma specimens were quantified by the LAL (Limulus Amebocyte Lysate) Chromogenic Endpoint Assay (Cell Sciences Inc., Canton, MA, USA) according to the manufacture's instructions. The sensitivity of the assay is 1.4 pg/ml with a measurable concentration range of 1 to 1,000 pg/ml.

2.7. Stimulation of LPS/TLR4 signaling pathways

Lipopolysaccharide (LPS, from *Escherichia coli*) was purchased from Sigma-Aldrich, and dissolved into solution according to manufacturer's recommendation. Percoll-isolated PBMCs from healthy individuals were cultured with/without 10ng/ml of LPS at the same conditions as described above. After 3-hour short-term incubations, total RNAs were extracted from PBMCs using PureLink™ Micro-to-Midi™ Total RNA Purification kit (In-Vitrogen, Carlsbad, CA). The RNAs were then quantitated via qRT-PCR as described above.

2.8. Statistical analysis

The results from the experiments are expressed as the mean ± SD. Statistical analysis of group differences, linear regression, and Pearson correlations were performed by GraphPad Prism 4.0 program (GraphPad Software, San Diego, Cal ifornia, USA). Distribution of groups was analyzed by Kolmogorov-Smirnov test. For all analysis, a value of $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of study populations

Patients with sALS and healthy controls were recruited for this study over a period of 21 months. Basic demographic and clinical information about the patients and healthy control samples are provided in Table 1. Twenty sALS patients were examined in the microarray analysis of gene expression and the PBMCs of an additional 42 sALS patients were analyzed in subsequent experiments. The average time since the participating individuals were diagnosed with probable or definite ALS according to the El Escorial criteria was

approximately two years. Revised ALS functional rating scale (ALSFRS-R) scores of the patients ranged from 19 to 47 with the mean value of 32. The majority of the ALS patients (~85%) were taking riluzole. As seen previously (Zhang et al., 2005), while the percentage of cells that expressed CD14 in each group was comparable (3.86% vs 4.33%, p = ns), the mean fraction of monocytes expressing CD16 (43.9% vs 25.8%, $p < 0.0001$) and the HLA-DR expression of monocytes (843 vs 577, $p < 0.0001$) were significantly elevated in patients with sALS relative to healthy controls.

3.2. Gene expression Studies

Total RNA was prepared from sALS patients (n = 20) and age-matched healthy controls (n = 22), subjected to quality assessment, amplified, and hybridized to HGU 133 plus2.0 human genome microarrays. An RNA signal was detected for approximately 44% of the Probe sets (range 37 to 50%) in both samples from sALS patients and controls. Of these, 76 probe sets representing 63 known genes and 3 transcribed sequences exhibited significantly changed transcription levels in the RNA from sALS PBMCs relative to healthy control RNA. Inspection of the genes (59) and transcripts (3) that had 4-fold or greater increased signal in incubated PBMCs from sALS patients (Table 2) revealed a number of genes known to be stimulated by type I interferons [e.g. interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), 2′-5′-oligoadenylate synthetase 3 (OAS3), and interferon, alpha-inducible protein IFI-6-16 (G1P3)] (Baechler et al., 2003; Der et al., 1998), which is recognized to be induced through the MyD88-independent LPS/TLR4 signal transduction pathway. Another prominent category among the upregulated genes were receptors associated with monocytes and granulocytes including the formyl peptide receptors (FPR1, FPRL1) and G-protein coupled receptor 43 (GRP43). Also notable were a wide range of secreted cytokines (interleukin 1 receptor antagonist or IL1RN), peptidase inhibitor 3 (elafin or PI3), other secreted protein (α-1-acid glycoprotein or ORM1), and B-factor or properdin genes (Pahl, 1999). Upregulation of both secreted cytokine genes and monocyte and granulocyte receptor genes are also associated with LPS/TLR4 signaling transduction pathways.

3.3. Quantitative RT-PCR analyses

To confirm the microarray results, several of the genes with significantly increased signals and LPS/TLR4 signaling transduction-related, were evaluated by qRT-PCR using an expanded sample set (sALS, n = 42; Healthy, n =35). The mean RNA signals for the putative upregulated genes based on microarray analysis, G1P3, GPR43, IFIT2, ORM1, PI3, chitinase 3-like 1 (CHI3L1), IL1RN, and TNF-related apoptosis inducing ligand (TRAIL or TNFSF10), were between 7- to 20-fold increased in sALS patients relative to that seen in healthy individuals (Figure 1a, $p < 0.0001$ for all 8 genes). However, there was no significant difference of mean β-actin RNA signals between sALS patients and healthy individuals. The mean signals for CD14 RNA were also similar between two groups (Figure 1b). Hence, gene upregulation in sALS most likely is related to genetic characteristics of disease rather than associated with changes in overall cell numbers or in the amount of circulating monocytes.

3.4. Effect of cultivation time on ALS specific gene expression profile

Peripheral blood mononuclear cells from 6 sALS patients and 6 healthy individuals were prepared by Percoll separation of whole blood. The PBMCs were then incubated for 0 to 24 hours at 37°C. Total RNA was prepared and analyzed by qRT-PCR with primers specific for G1P3, GPR43, IFIT2, and ORM1. As expected, β-actin RNA levels were similar in PBMCs from sALS patients and healthy individuals and did not change over time (data not shown). PBMCs from healthy individuals expressed relatively constant RNA signals of specified genes at time points from 0 to 24 hours, as showed in Figure 2, though GPR43 showed a

steady decline in RNA signals over time. In contrast, PBMCs isolated from sALS patients exhibited a rapid increase in RNA signal that reached its maximum level in 2 to 3 hours which either remained constant (G1P3, ORM1) or declined slowly (GPR43, IFIT2) over 24 hours. Therefore, PBMC short-term incubation at 37°C leads to a rapid induction of RNA signals for patients with sALS compared to healthy individuals.

3.5. ALS gene expression profile reflects degree of blood monocyte activation

To ascertain if the ALS gene expression response would be associated with the phenotype of the monocytes present in their peripheral blood, RNA signals from G1P3, GPR43, IFIT2, ORM1, PI3, IL1RN, CHI3L1, TNFSF10, and β-actin genes were compared to levels of HLA-DR and CD16 expression on CD14-monocytes. There was no correlation between the percentage of CD14 cells that also expressed CD16 and the RNA signals from β-actin gene and all 8 genes from individual ALS patient's PBMCs (Table 3). However, the degree of monocyte activation as measured by CD14 monocyte HLA-DR expression showed a significantly positive relationship with the RNA signals from GPR43, ORM1, PI3, and CHI3L1 genes in patients with sALS, as shown in Table 3 and Figure 3. Apparently, the degree of gene expression of the above 4 genes reflects the degree of monocyte/macrophage activation but not the absolute level of CD16+ monocytes present in sALS blood.

3.6. ALS gene expression profile corresponds to plasma LPS levels

Our previous studies found significantly elevated levels of plasma LPS in sALS, and that increased LPS levels correlated with levels of abnormally activated monocyte/macrophages (Zhang et al., 2009). As a systemic monocyte/macrophage activator and a potent inflammatory stimulus, LPS induces its effects through stimulation of CD14-bearing inflammatory cells. To confirm the relationship between this gene expression profile and ALS monocyte activation-associated response, the RNA signals from G1P3, GPR43, IFIT2, ORM1, PI3, CHI3L1, IL1RN, TNFSF10, and β-actin genes were compared with the levels of plasma LPS in sALS patient blood. There was a positive correlation between plasma LPS levels and the strength of RNA signals for 5 out of 8 genes (Table 4 and Figure 4). The elevated expression of these LPS/TLR4-signaling associated and monocyte activation genes were directly correlated with elevated levels of plasma LPS in sALS patient blood. The correlation between both systemic immune parameters and expression of LPS/TLR4 signal transduction genes in sALS blood suggests that there is a link between this gene expression profile and the ALS monocyte activation-associated response.

3.7. ALS monocyte-associated response gene expression profile and disease progression in sALS

To evaluate whether induction of LPS/TLR4 signal transduction would be related to clinical stage of disease, the RNA signals from G1P3, GPR43, IFIT2, ORM1, PI3, IL1RN, CHI3L1, TNFSF10, and β-actin genes were compared with duration or severity of disease in sALS. As seen in the Table 5, all 8 genes showed similar results. There was no statistically significant relationship between the RNA signals observed and disease time-course in sALS. Almost 90% of tested samples had gene expression signals above the average levels observed in healthy individuals, and approximately 90% of sALS patients exhibited the upregulated expression of LPS/TLR4 signal transduction genes at times throughout the symptomatic stages of their disease. A typical example (ORM1) of this relationship is shown in Figure 5a. It appears similar to the status of monocyte/macrophage activation in the peripheral blood from patients with sALS as observed in our previous studies (Zhang et al., 2005) in which monocyte/macrophages were persistently activated throughout the sALS disease process. RNA signals from most genes were also independent of ALS disease severity as defined by ALSFRS-R score (Table 5). However, there was an inverse

correlation between the RNA signals of ORM1 with the disease severity, as showed in Figure 5b.

3.8. LPS/TLR4 signaling pathways are involved in the upregulation of ALS monocyte-associated response genes

As a systemic macrophage activator and one of the best studied immunostimulators, LPS can clearly induce systemic inflammation *in vivo*. The effects of LPS are mediated primarily through TLR4. LPS/TLR4 signaling pathways mediate the activation of proinflammatory cytokine genes, type I interferons and interferon-inducible genes (Lu et al., 2008; Takeda and Akira, 2007; Tanimura et al., 2008). To test the involvement of LPS/TLR4 signaling pathways in monocyte/macrophage activation in ALS, we evaluated RNA signals of the ALS monocyte-associated response genes in Percoll-separated PBMCs from healthy individuals after exposure to LPS. As shown in Figure 6, after a 3-hour incubation, PBMCs without LPS treatment showed similar RNA signals as that seen in PBMCs immediately after isolation (0 hour) in healthy individuals. However, short-term cultivation with LPS resulted in the upregulated expression of RNA signals for the ALS monocyte-associated response genes in PBMCs from healthy individuals, similar to the transcriptional response observed in the short-term cultivation of sALS PBMCs. Therefore, the transcriptional response of PBMCs from ALS patients may use LPS/TLR4 signaling pathways in a manner similar to that seen in PBMCs from LPS stimulated healthy individuals.

4. Discussion

Sporadic ALS is characterized by large numbers of activated microglia/macrophages in the regions of the spinal cord undergoing neurodegeneration (Graves et al., 2004; Henkel et al., 2004). Recent studies in ALS disease models have implicated important roles for activated microglia/macrophages in ALS disease progression *in vivo* (Beers et al., 2006; Boillee et al., 2006). sALS is also characterized by elevated levels of abnormally activated monocyte/macrophages in peripheral blood (Zhang et al., 2005). In the current study we found that short-term cultivation of peripheral blood mononuclear cells from patients with sALS resulted in induction of genes related to the LPS/TLR4 signal transduction pathways. The pattern of gene transcription observed in sALS patients was best replicated by exposure of PBMCs from healthy subjects to the TLR4 ligand LPS, which stimulated expression of all sALS associated RNAs. Therefore, abnormally activated monocytes in sALS patients appear to be primed to become activated via LPS/TLR4 pathways. To our knowledge, this is the first demonstration of a role for LPS-mediated stimulation of the innate immune response in patients with sporadic ALS.

The monocyte ALS gene expression profile was only observed after short-term cultivation, but not in the peripheral blood mononuclear cells immediately after Percoll separation. This activation profile may be related to LPS priming of monocytes which upon cultivation results in LPS/TLR4-mediated signaling. However, if the LPS responsible for this LPS/TLR4 mediated signaling is already bound to the blood cells *in vivo*, why was there not a partial stimulation of the characteristic genes in time zero samples? Certainly there is some level of abnormal monocyte activation in ALS patient blood as the level of CD14/DR expression is elevated and the degree of this elevation is related to plasma LPS levels (Zhang et al., 2009). The LPS/TLR4 stimulation pathway induces potent responses and results in abnormally activated monocyte/macrophages and expressions of proinflammatory cytokines *in vivo* (Beutler et al., 1985; Danner et al., 1991; Okusawa et al., 1988; Tracey et al., 1986); however, the systemic activation and inflammation responses may trigger the negative regulation of the TLR4 signaling pathway (Lu et al., 2008) to down-regulate the activation of LPS/TLR4-mediated signaling which serves as a feedback or protective mechanism in modulation of inflammation-induced damage. Therefore, interferon and

protein secretion associated genes may require more stimulation than is possible in the blood to see elevation of these genes. The priming of abnormally activated monocytes may also be LPS-independent, and driven by other stimuli of TLR4 signaling pathways (Biragyn et al., 2002; Ohashi et al., 2000; Termeer et al., 2002), it may have to do with an unknown, ALS related process inherent to the macrophages. Further studies looking at the persistence of RNA induction in ALS will be required to test this.

Recently, HIV infected individuals with acquired immune deficiency syndrome (AIDS) have been reported to have elevations in plasma LPS levels with concomitant immune activation (Brenchley et al., 2006). The elevated plasma LPS is associated with the gut associated microbial translocation. Increased expression of many of the genes that were significantly upregulated in this study have also been described in a study of frontal-lobe gene expression in simian immunodeficiency virus mediated encephalitis (SIVE) (Roberts et al., 2003) which is used as a model system to understand HIV associated dementia (HAD). Our previous finding of higher levels of plasma LPS, and that increased LPS levels correlated with degree of activated monocyte-associated signaling genes in the current studies in ALS suggests that chronic stimulation of innate immunity via LPS/TLR4 signaling pathways may be a common theme in a subset of neurodegenerative diseases.

Activation of LPS/TLR4 related pathways was observed in sALS patient blood cells at all time points and independent of symptomatic phase of their disease course. Overall, approximately 90% of the samples from sALS patients exhibited elevated transcription of the LPS/TLR4 stimulated genes versus less than 5% of those from healthy individuals. In addition, the increase in RNA signals from ORM1 was associated with ALS disease severity as defined by ALSFRS-R score. Our recent studies on blood specimens from patients with sALS found elevated levels of plasma LPS, and plasma LPS levels were found to positively correlate with levels of abnormally activated monocyte/macrophages in sALS (Zhang et al., 2009). Elevated levels of TNF-α and other pro-inflammatory cytokines have been seen in SOD1 transgenic mice (Hensley et al., 2003) and patients with ALS (Babu et al., 2008; Cereda et al., 2008; Ono et al., 2001; Poloni et al., 2000). Chronic stimulation of innate immunity with LPS has also been shown to accelerate the disease course in SOD1 transgenic mice (Nguyen et al., 2004). In the current study, the positive correlations between those two systemic immune parameters, degree of abnormally activated monocyte/ macrophages and plasma LPS levels, and RNA signals of multiple LPS/TLR4 stimulated genes in ALS patients may provide a link between the *in vitro* transcriptional response observed and the systemic immune activation, suggesting that chronic activation of monocyte/macrophages via LPS/TLR4 signaling pathways would have a deleterious effect on disease progression in ALS. Whether this PBMC sALS transcriptional response can be used as a means to identify or monitor disease progression in ALS is still an open question. Protein expression levels of those “ALS monocyte-associated response genes”, for example ORM1 protein, appear definitely worthy of further investigation. Furthermore, agents that inhibit LPS/TLR4 mediated activation would be predicted to interfere with disease progression. These observations may provide a new area of investigation for the development of novel therapeutics for ALS and other neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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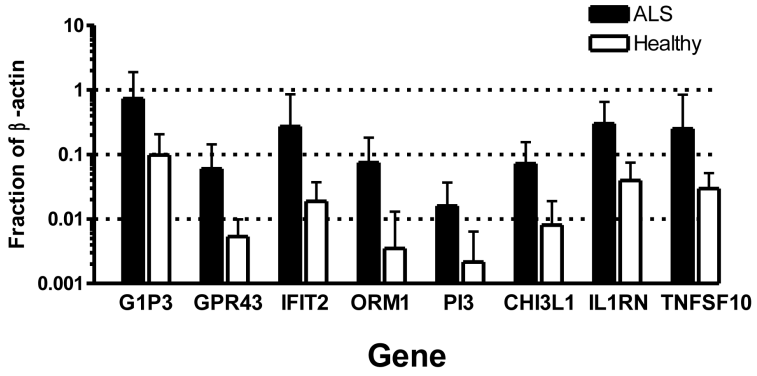

Figure 1a.

qRT-PCR analysis of upregulated genes.

Mean β-actin normalized signals obtained from qRT-PCR of total RNA samples from incubated PBMCs of sALS patients (n = 42, black bars) or age matched healthy controls (n = 35. white bars) for the indicated genes (x-axis). All genes from sALS PBMCs had a p-value < 0.0001 for comparison to mean β-actin normalized signals in PBMCs from healthy individuals.

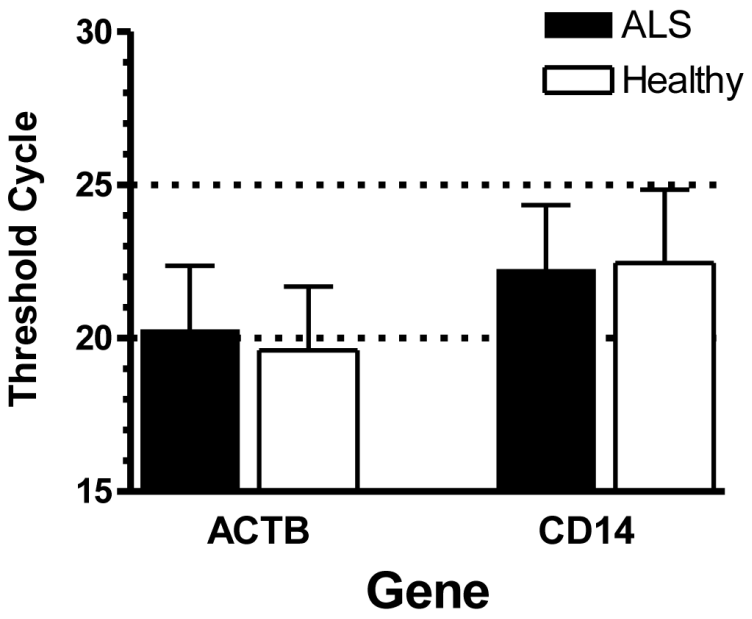

Figure 1b.

qRT-PCR analysis of upregulated genes.

Mean threshold cycles obtained from qRT-PCR of total RNA samples from incubated PBMCs of sALS patients (n = 42, black bars) or age matched healthy controls (n = 35, white bars) for the control genes β-actin (ACTB) and CD14. Differences between signals from ALS or healthy patients were not significant.

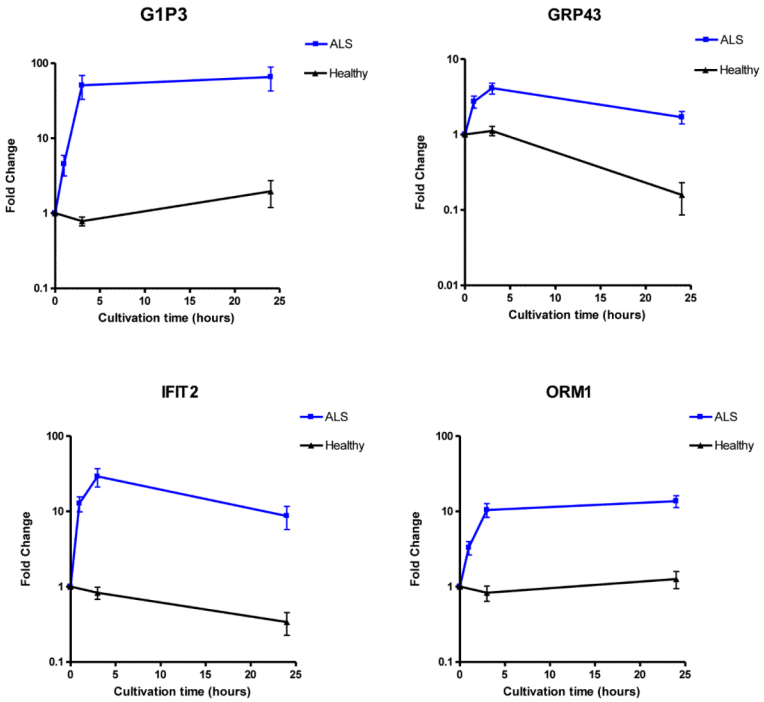

Figure 2.

Induction of RNA signals in sALS peripheral blood mononuclear cells. Mean RNA signals determined by qRT-PCR with primers specific for G1P3, GPR43, IFIT2, and ORM1 genes in Percoll-purified PBMCs from sALS patients (n = 6) and healthy individuals (n = 6) after increasing amounts of time in culture at 37°C. The data represented the mean of the fold change observed relative to time 0 point (immediately after PBMC separation)

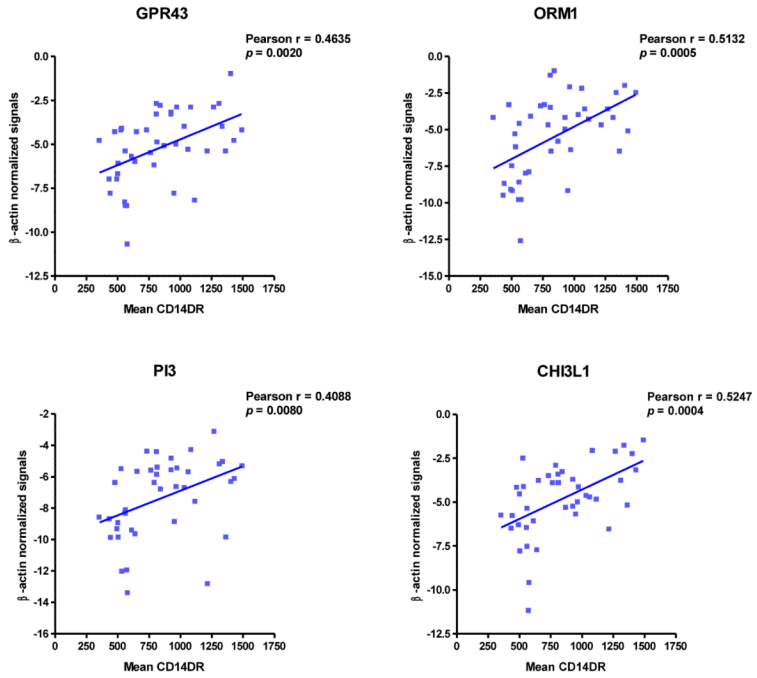

Figure 3.

ALS gene expression response correlates with degree of systemic monocyte activation in the peripheral blood.

The RNA from short-term culture sALS PBMCs was analyzed by qRT-PCR with primers for the indicated genes. The y-axis indicates the raw β-actin normalized signals. The x-axis indicates the degrees of monocyte/macrophage activation (Mean CD14DR) in sALS. Pearson correlation coefficients (r) derived from comparing the β-actin normalized signals with CD14 monocytes HLA-DR expression and its significance are indicated (n = 42).

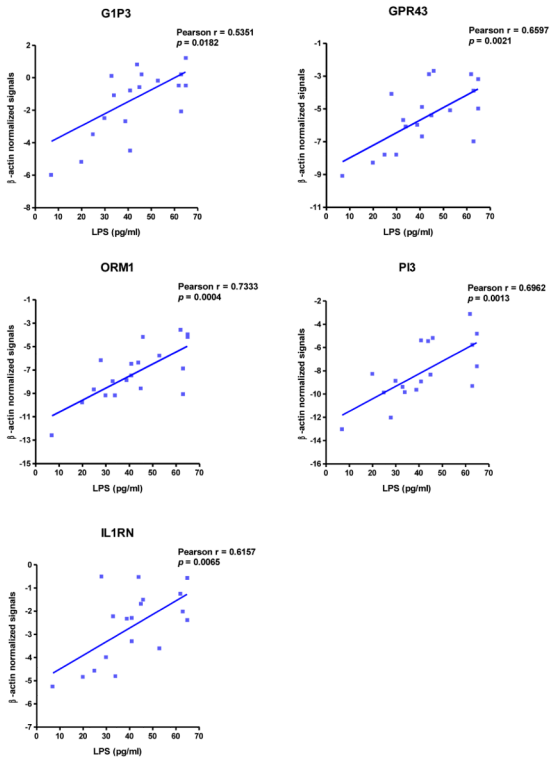

Figure 4.

Gene expression response correlates with plasma levels of LPS in sALS.

The RNA from short-term culture sALS PBMCs was analyzed by qRT-PCR with primers for the indicated genes. The y-axis indicates the raw β-actin normalized signals. The x-axis indicates the levels of plasma LPS in sALS. Pearson correlation coefficients (r) derived from comparing the β-actin normalized signals with plasma LPS levels and its significance are indicated (n = 19).

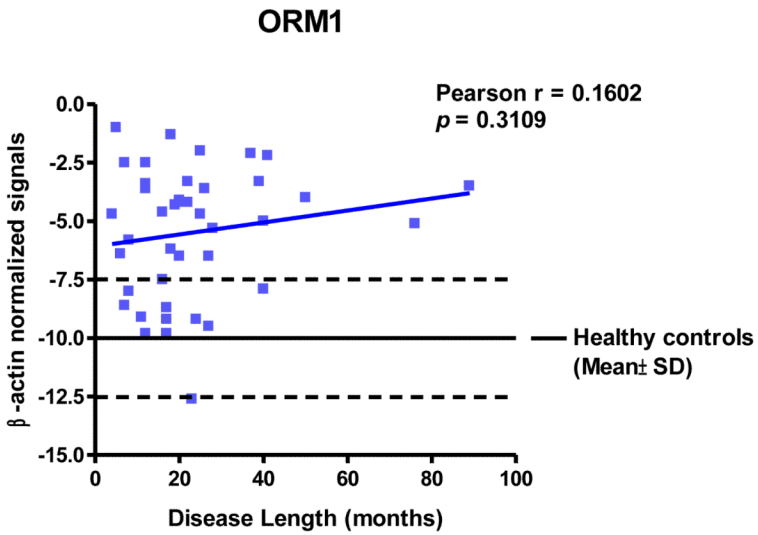

Figure 5a.

Relationship between monocyte-associated response genes and disease progression in sALS. Induction of monocyte-associated response genes is independent of disease time-course (disease length) in sALS. The y-axis indicates the raw β-actin normalized signals of ORM1 gene. The x-axis indicates the disease length (months) (Pearson r = 0.3109, $p = 0.1602$, n = 42). Horizontal lines showed the range of raw β-actin normalized signals from 35 healthy controls (mean ± SD).

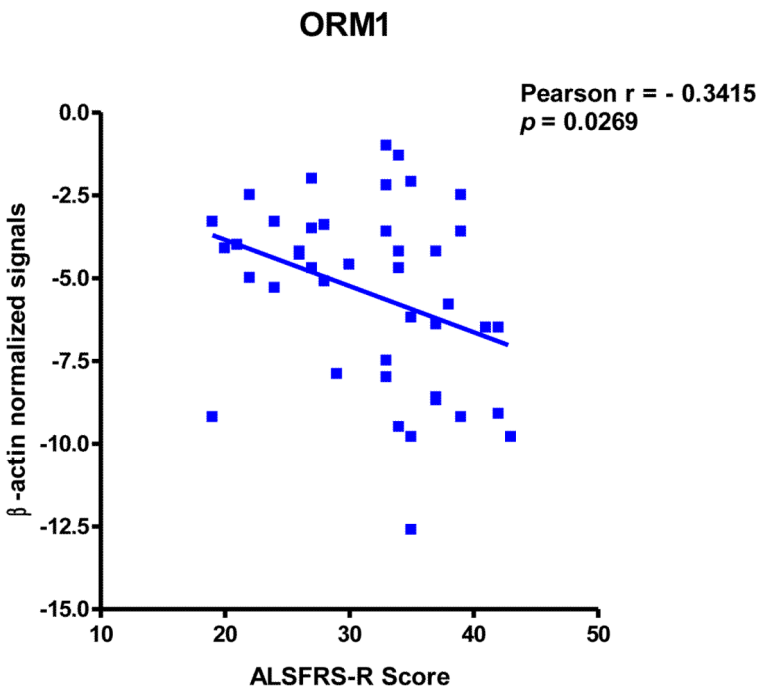

Figure 5b.
Relationship between monocyte-associated response genes and disease progression in sALS. Significantly negatively correlation of ORM1 gene expression with the disease status defined by ALSFRS-R score (Pearson r = −0.3415, $p = 0.0269$, n = 42)

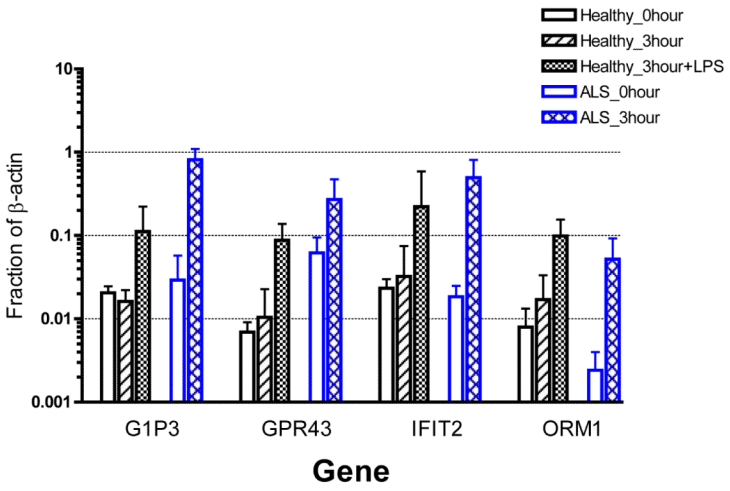

Figure 6.

Involvement of LPS/TLR4 signaling pathways in ALS monocyte-associated response genes. The RNA signals of PBMCs from 6 healthy individuals and 6 sALS patients were isolated and analyzed via qRT-PCR with primers specific for G1P3, GPR43, IFIT2, and ORM1 genes. LPS stimulation induced the upregulated expression of ALS monocyte-associated response genes in the healthy controls after a 3-hour cultivation. Bars indicate the mean β-actin-normalized signals obtained.

Table 1

Characteristics of study populations

	Microarray Samples			*Additional Samples*		
	ALS	Healthy	*p* Value	ALS	Healthy	*p Value*
N	20	22		42	35	
Age	60.1 ± 11.4	57.5 ± 21.0	NS[c]	60.0 ± 10.2	56.0 ± 20.6	NS
Male / Female	13 / 7	10 / 12		26 / 16	18 / 17	
Disease Length (months)	31.2 ± 22.0 (5-89)	N/A [b]		23.4 ± 17.2 (4-89)	N/A	
ALSFRS-R	31.0 ± 7.4 (20-47)	N/A		31.7 ± 6.8 (19-43)	N/A	
% CD14	3.92 ± 1.20	4.36 ± 1.25	NS	3.86 ± 1.21	4.33 ± 1.18	NS
% CD14CD16	48.3 ± 12.7	22.7 ± 12.6	<0.0001	43.9 ± 14.0	25.8 ± 11.9	<0.0001
Mean CD14DR [a]	987 ± 259	559 ± 236	<0.0001	843 ± 320	577 ± 218	<0.0001

[a]Mean HLA-DR fluorescence expressed on CD14 monocyte.

[b]N/A = not applicable.

[c]NS = not significant.

Table 2

Upregulated RNAs in short-term culture PBMCs from sALS patient [a]

N	*Gene*	*Genbank ID*	*Fold Change*	*p-value*
		Secreted Proteins		
1	ORM1	NM_000607	17.47	9.19E-09
		NM_000607	11.47	4.97E-09
2	CHI3L1	M80927	12.66	1.21E-08
		M80927	8.66	1.09E-07
3	PI3	L10343	12.42	2.52E-09
		NM_002638	9.72	1.75E-08
4	BF	NM_001710	7.94	3.12E-05
5	SLPI	NM_003064	6.44	8.98E-08
6	SERPINA1	NM_000295	5.19	1.93E-06
		AF119873	4.51	1.13E-06
7	ORM2	NM_000608	5.14	3.31E-08
8	TNFSF10	U57059	4.80	4.30E-08
		NM_003810	4.39	1.35E-07
9	IL1RN	BE563442	4.47	6.41E-05
10	SERPING1	NM_000062	4.44	1.14E-05
11	TNFAIP6	AW188198	4.43	9.08E-06
12	PBEF1	BC020691	4.42	9.35E-05
13	LRG1	AA622495	4.34	2.33E-06
14	FAS	X83493	4.18	8.48E-06
		Interferon-induced Proteins		
1	IFIT2	BE888744	9.87	1.27E-08
2	IFIT1	NM_001548	7.08	2.35E-05
3	IFIT3	NM_001549	6.07	3.49E-07
	IFIT3	AI075407	5.48	2.87E-07
4	G1P3	NM_022873	5.21	5.53E-05
5	OAS3	NM_006187	4.52	8.38E-06
6	HERC5	NM_016323	4.08	1.19E-05
		Miscallaneous		
1	ETV7	AF218365	6.87	2.47E-07
2	NBN	AK001017	5.55	2.05E-06
		AI796269	5.20	2.67E-09
		AF049895	4.21	8.50E-06
3	APOBEC3A	U03891	5.37	1.56E-05
4	KRT23	NM_015515	5.20	2.57E-07
5	DGAT2	AW469523	4.31	3.96E-05
6	MXD1	NM_002357	4.28	5.03E-06
7	BCL2A1	NM_004049	4.16	8.54E-05
8	PIK3AP1	BC029917	4.00	4.90E-05

N	*Gene*	*Genbank ID*	*Fold Change*	*p-value*
		Receptors		
1	P2RY13	NM_023914	8.88	2.43E-05
2	GPR43	NM_005306	7.81	7.24E-09
3	CLEC4E	NM_014358	6.75	4.58E-08
		BC000715	4.15	5.44E-08
4	SIGLEC5	NM_003830	6.03	3.08E-07
5	KCNJ2	AF153820	5.76	7.65E-07
		BF111326	4.72	9.26E-06
6	AQP9	NM_020980	5.19	1.97E-07
7	CEACAM1	X16354	5.14	1.21E-07
8	FCAR	NM_002000	5.03	1.18E-05
9	GPR109B	NM_006018	4.89	2.41E-05
10	TREM1	NM_018643	4.70	3.06E-06
11	SLAMF7	AJ271869	4.53	1.01E-05
12	PLXNC1	NM_005761	4.44	2.41E-05
13	CD274	AF233516	4.29	1.61E-05
14	OR52K3P	AF143328	4.27	4.17E-08
15	CR1 /// CR1L	BE552138	4.24	7.24E-08
16	FPRL1	U81501	4.22	3.38E-05
		Kinase / Phosphatase		
1	LIMK2	AL117466	4.72	4.32E-09
2	SGPP2	AW779536	4.59	5.77E-05
3	IRAK3	BC029493	4.04	3.14E-08
		Enzyme		
1	CHST7	NM_019886	4.49	1.59E-08
2	UPB1	NM_016327	4.36	6.37E-07
3	QPCT	NM_012413	4.14	1.44E-06
		Function Unknown		
1	–	AK096134	8.36	9.02E-07
2	DTNBP1	AW026356	6.74	4.76E-07
3	FXYD6	NM_022003	6.24	1.43E-07
4	MGC39372	AI347139	4.63	8.46E-06
5	LINCR	AL389981	4.37	9.98E-06
6	IBRDC2	AL575512	4.36	6.95E-07
7	–	AA149736	4.22	1.46E-07
8	HSPC159	AK025603	4.17	9.89E-07
9	FLJ31978	AI041543	4.08	6.80E-06
10	BTNL8	NM_024850	4.06	2.28E-05
11	–	H22954	4.05	8.07E-05
12	ZCCHC2	BE676543	4.01	1.07E-07

[a]The Gene ID and Genbank ID for all Probe sets of genes with a 4-fold or greater increase in mean signal in sALS patient PBMCs (n = 20) relative to healthy PBMCs (n = 22) are provided as the associated p-value (unpaired t-test with Welch's correction for unequal variance). A Genbank ID is listed multiple times (e.g ORM1, CHI3L1) if two or more Probe sets corresponding to that sequence were identified.

Table 3

Relationship of raw β-actin normalized signals to blood monocyte phenotypes in sALS

Genes (n =42)	%CD14CD16		Mean CD14DR	
	Pearson *r*	*p* Value	Pearson *r*	*p* Value
G1P3	0.1481	0.3492	0.3026	0.0514
GPR43	0.2187	0.1641	0.4635	0.0020 **
IFIT2	0.1492	0.3457	0.1986	0.2074
ORM1	0.1044	0.5106	0.5132	0.0005 ***
PI3	0.1262	0.4318	0.4088	0.0080 **
IL1RN	0.0562	0.7236	0.1454	0.3582
CHI3L1	0.1741	0.2701	0.5247	0.0004 ***
TNFSF10	0.0060	0.9701	0.0778	0.6244
ACTB	0.0569	0.7204	0.0749	0.6375

*** = $p \leq 0.0005$,

** = $p < 0.01$.

Table 4

Correlation between raw β-actin normalized signals with plasma levels of LPS in sALS

Genes (n =19)	Plasma LPS (pg/ml)	
	Pearson *r*	*p* Value
G1P3	0.5351	0.0182 *
GPR43	0.6597	0.0021 **
IFIT2	0.1048	0.6694
ORM1	0.7333	0.0004 ***
PI3	0.6962	0.0013 **
IL1RN	0.6157	0.0065 **
CHI3L1	0.4353	0.0710
TNFSF10	0.1837	0.4516
ACTB	−0.0307	0.8979

*** = $p < 0.001$,

** = $p < 0.01$,

* = $p < 0.02$.

Table 5

Relationship of raw β-actin normalized signals to clinical disease status in sALS

Genes (n =42)	Disease Length (months)		ALSFRS-R score	
	Pearson *r*	*p* Value	Pearson *r*	*p* Value
G1P3	0.0780	0.6235	−0.1548	0.3278
GPR43	0.1715	0.2776	−0.2431	0.1208
IFIT2	0.1212	0.4444	−0.3017	0.0521
ORM1	0.1602	0.3109	−0.3415	0.0269 *
PI3	0.1964	0.2184	−0.2787	0.0777
IL1RN	0.1587	0.3153	−0.1448	0.6303
CHI3L1	0.0819	0.6061	−0.2106	0.1807
TNFSF10	0.0425	0.7891	−0.1783	0.2585
ACTB	0.0078	0.9611	−0.1511	0.3394

* = $p < 0.05$.