

# Messenger RNA sequencing reveals similar mechanisms between neonatal and acute respiratory distress syndrome

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**Abstract.** Hypoxemia and hypercarbia resulting from a lack of surfactant is considered to be the primary mechanism underlying neonatal respiratory distress syndrome (NRDS). Surfactant replacement therapy may mitigate the symptoms of the disease by decreasing the surface tension of alveoli and facilitating inflation. However, surfactant serves an additional role in immunological processes. Therefore, it may be hypothesized that mechanisms of NRDS involving surfactant exert additional functions to promoting alveolar inflation. Using peripheral blood obtained from mature infants with and without NRDS, in tandem with mRNA sequencing (mRNA-seq) analysis, the present study identified that, while cell cycle regulation and alveolar surfactants serve a role in deterring the further onset of NRDS, innate and pathogen-induced responses of the immune system are among the most important factors in the pathology. The present study illustrated the regulatory importance of these immune pathways in response to alterations in the expression of gene families, particularly in perpetual lung injury leading to NRDS. Notably, data collected from the mRNA-seq analysis revealed similar mechanisms between NRDS and acute respiratory distress syndrome, a clinical phenotype precipitated by the manifestation of a severe form of lung injury due to numerous lung insults, implying that similar therapies may be applied to treat these two diseases.

## Introduction

Neonatal respiratory distress syndrome (NRDS), also termed hyaline membrane disease, is one of the most prevalent causes of morbidity and mortality in newborns (1). The oxygen

diffusion efficiency through the alveoli-capillary exchange barrier is impacted by the dysregulation of numerous factors, leading to the pathophysiology of NRDS (2). Cellular stress at the gas-blood level is associated with alterations in alveolar surfactant proteins (3) and the upregulation of numerous innate immune responses of pro-inflammatory cytokines to foreign challenges (4). A lack of surfactant may drive the pathogenesis towards NRDS, while surfactant replacement therapy may mitigate symptoms of the disease by decreasing the surface tension of alveoli and facilitating their inflation (5,6). However, surfactant serves an additional role in immunological processes. A recent study demonstrated that applying clinical surfactant for patients led to a stronger response to challenges from foreign microbiota (7). Additionally, animal-derived surfactant applied for clinical use in humans has been demonstrated to be of use against lung pathogens and to mediate excess host damage from neutrophils (8). Therefore, it may be hypothesized that mechanisms that mechanisms of NRDS involving surfactant exert additional functions to promoting alveolar inflation.

Acute respiratory distress syndrome (ARDS) is a clinical phenotype precipitated by the manifestation of a severe form of lung injury due to numerous lung insults (9). The pathophysiological manifestation of ARDS is derived from a cascade that is triggered by the complex combination of risk factors, including asthma, sepsis, pneumonia, increased neutrophil presence in the lungs and other variables; accumulation of these numerous systemic factors forms the pathological endpoint of ARDS (10).

The present study used peripheral blood obtained from infants with (n=4) or without (n=2) NRDS in tandem with mRNA-sequencing (mRNA-seq) analysis to reveal various factors involved in the mechanisms of NRDS, and to compare onset mechanisms between NRDS and ARDS.

## Materials and methods

**Samples.** The Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University (Huhehaote, China) approved the present study. All study participants provided written informed consent and were recruited at the Affiliated Hospital of Inner Mongolia Medical University between January 1 and June 30, 2016.

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A total of four infants with severe NRDS and two healthy infants were recruited for the present study. Subject characteristics are presented in Table I. The subjects were not related, and all of their relatives in the three most recent generations were residents in Inner Mongolia province and self-identified as Chinese Inner Mongolia race. NRDS was diagnosed according to the European Consensus Guidelines on the Diagnosis of NRDS in Preterm Infants 2010 (11). The criteria were as follows: Progressive deterioration of shortness of breath commencing at 12 h post-birth, with arterial partial pressure of oxygen (PaO<sub>2</sub>) <50 mmHg during inspiration accompanied by central cyanosis. Oxygen therapy was required to maintain PaO<sub>2</sub> >50 mmHg. Chest X-ray (CXR) indicated NRDS specific signs, including ground glass opacity and air-filled bronchi. The control groups were defined by CXR demonstrating clear lung field without pulmonary infection signs. White blood cell counts and C-reactive protein values were normal, to exclude the possibility of any infection. Subjects with any of the following were excluded from the present study: i) Any serious congenital diseases, including complex congenital cardiac anomaly or diaphragm hernia; ii) inherited metabolic disorders, including phenylketonuria, congenital hypothyroidism or diabetes mellitus; iii) confirmed maternal infection history during the third trimester of pregnancy; iv) multigestation; v) maternal diabetes mellitus; or vi) neonatal asphyxia.

*mRNA extraction and sequencing.* Total RNAs from 4 NRDS and 2 control subjects were isolated and quality controlled using protocols from Illumina, Inc. (San Diego, CA, USA). A total amount of 3 µg RNA/sample was used as the input material for library construction. Ribosomal RNA (rRNA) was removed using the Epicentre Ribo-zero rRNA Removal kit (Illumina, Inc.), according to the manufacturer's protocol. Strand-specific sequencing libraries were generated via the dUTP method using the resulting RNA with the NEB Next Ultra Directional RNA Library Prep kit for Illumina (New England BioLabs, Inc., Ipswich, MA, USA), according to the manufacturer's protocol. RNA-seq was performed on an Illumina Hiseq 2000 platform (Illumina, Inc.) and 100-bp paired-end reads were generated, according to the manufacturer's protocol.

*RNA-seq data analysis.* The adapter sequences were removed from the raw sequencing data and the individual libraries were converted to the FASTQ format. Sequence reads were aligned to the human genome (hg19) using TopHat2 (v2.0.9; [ccb.jhu.edu/software/tophat/index.shtml](http://ccb.jhu.edu/software/tophat/index.shtml)) and the resulting alignment files were reconstructed using Cufflinks (v2.1.1; [cole-trapnell-lab.github.io/cufflinks](http://cole-trapnell-lab.github.io/cufflinks)) (12) and Scripture (beta2; [software.broadinstitute.org/software/scripture](http://software.broadinstitute.org/software/scripture)). The transcripts assembled by Cufflinks and Scripture were used for the identification of differentially expressed genes (DEGs). For mRNA analyses, the RefSeq database (build 37.3; [www.ncbi.nlm.nih.gov/refseq](http://www.ncbi.nlm.nih.gov/refseq)) was selected for the annotation references. The read counts of each transcript were normalized to the length of the individual transcript and to the total mapped fragment counts in each sample, and expressed as fragments per kilobase of exon per million fragments mapped of mRNAs in each sample. mRNA differential expression analyses were applied to the NRDS and control groups. The P-value was calculated using R3.2.5 software ([www.r-project.org](http://www.r-project.org)).

Table I. Subject information.

Sample ID	Sex	Mother	Gestational age, weeks
Control 1	Male	G2P2	38+3
Control 2	Female	G3P3	38+3
NRDS1	Male	G2P1	37+6
NRDS2	Male	G3P1	37+4
NRDS3	Female	G3P2	37
NRDS4	Female	G2P1	37+3

G, gravida (the total number of pregnancies); P, para (the total number of deliveries).

Table II. Primers used for RT-qPCR.

Gene	Primer
PIP4K2B	Forward: ATGCACTGGAGACCAGCAA Reverse: GTACGCACAAAAGACTGGCG
FCAR	Forward: GACCACCCTCCTGTGTCTTG Reverse: GGATGGAGTCGTAGCACCTG
MLLT6	Forward: GTGGGCCATGGCAGAAGTAG Reverse: CCCTGATTCAAAGCCCCGAA
DDX52	Forward: TGCATCAACTACTTCGGCA Reverse: ATCGCAGCCATGGACGTTTA
AC010970	Forward: TGTCAGCACTCCCAACAGAC Reverse: GTCCCTCATGGCCACAAGTT
APBA2	Forward: ACTGGGACCGCTACAGTACA Reverse: ATCCAGACTGTCAGCATCGC
BRD2	Forward: GTCTACCGATTCCCACCTCG Reverse: GCCAAGATGGCTGTAGGTGT
HLA-DQA1	Forward: CTGACAAACATCGCTGTGCT Reverse: GAAGCACCAACTGAACGCAG
MDGA1	Forward: CTCCGAGTACCCACAGCTA Reverse: CATGGATCCCCAAAGTTGCAG
WASF3	Forward: CTTTTAGGGAACCCGCTGGA Reverse: TAGAGCGAACATGGACGACAG
GAPDH	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG

AC010970, AC010970.1; APBA2, amyloid β precursor protein binding family A member 2; BRD2, bromodomain containing 2; DDX52, DEXD-box helicase 52; FCAR, Fc fragment of IgA receptor; HLA-DQA1, major histocompatibility complex class II DQ α 1; MDGA1, MAM domain containing glycosylphosphatidylinositol anchor 1; MMLT6, MLLT6 PHD finger containing; PIP4K2B, phosphatidylinositol-5-phosphate 4-kinase type 2 β; WASF3, WAS protein family member 3.

Adjusted P<0.05 (Student's t-test with Benjamini-Hochberg false discovery rate adjustment) was used as the threshold for significant DEGs. DEGs were analyzed by enrichment analyses to detect over-represented functional terms present in the genomic background. Gene ontology (GO) analysis was performed using the GO-seq package ([bioconductor.org/packages/release/bioc/html/goseq.html](http://bioconductor.org/packages/release/bioc/html/goseq.html)) in R3.2.5 software, in

Table III. Results of alignment from RNA sequencing libraries.

Sample ID	Total reads	Uniquely mapped paired read (%)	Uniquely mapped unpaired read (%)	GC rate, %
Control 1	45805780	24193674 (52.8180)	551484 (1.2040)	55.8
Control 2	53239568	36388990 (68.3495)	995799 (1.8704)	48.2
NRDS1	49799542	19819046 (39.7977)	479564 (0.9630)	55.7
NRDS2	53005504	26210282 (49.4482)	573098 (1.0812)	56.2
NRDS3	53700026	23724822 (44.1803)	514729 (0.9585)	56.3
NRDS4	50728662	24525002 (48.3455)	580637 (1.1446)	50.1

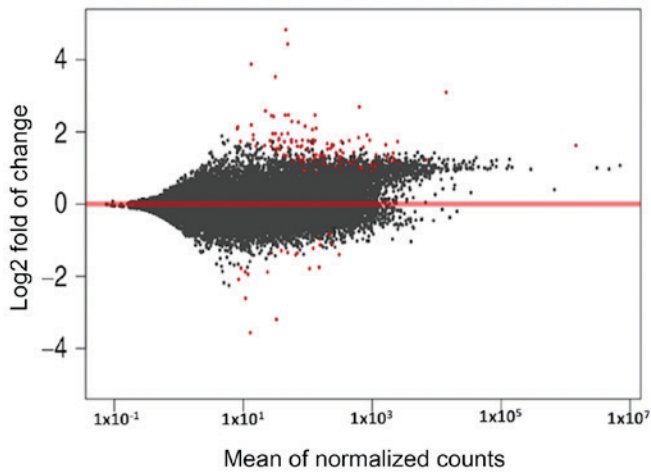


Figure 1. MA plot of all significantly differentially expressed genes between the neonatal respiratory distress syndrome and control groups, marked as red dots ( $P < 0.05$ ;  $\log_2FC > 1$ ).

which gene length bias was corrected. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using KOBAS 3.0 software (kobas.cbi.pku.edu.cn).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNAs were isolated and quality controlled using protocols from Illumina, Inc. All cDNAs were synthesized using Superscript III (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The top five over- and underexpressed genes were selected for the RT-qPCR assay. All RT-qPCR primers are presented in Table II. All qPCR reactions were performed on a Roche Lightcycler 480 PCR system (Roche Applied Science, Penzberg, Germany) using Toyobo Thunderbird SYBR RT-qPCR Mix (Toyobo Life Science, Osaka, Japan), with three technical repeats. The amplification procedure was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 20 sec. Relative quantification of target genes was performed using the  $2^{-\Delta\Delta C_q}$  method with GAPDH as a reference gene (13). Pearson correlation was used to calculate the association between RNA sequence and RT-qPCR (14).

## Results

**RNA-seq libraries.** To replicate the results, control samples were obtained from two patients and NRDS samples from four patients. In total, >300 million reads were acquired from

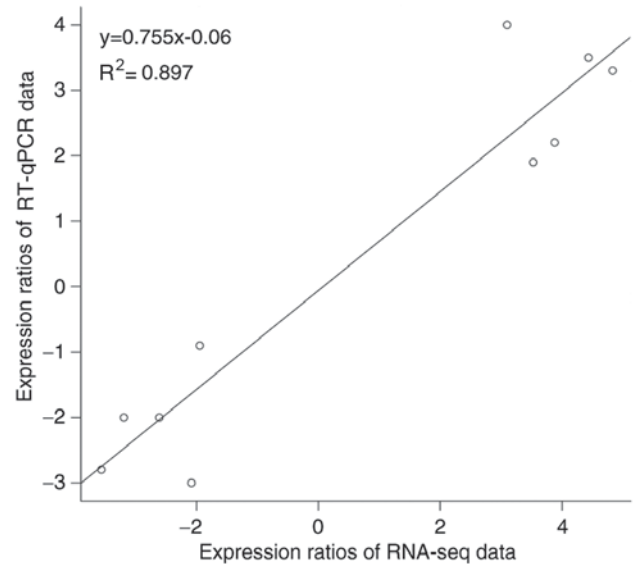


Figure 2. Correlation between RNA-seq and RT-qPCR data. RNA-seq, RNA sequencing; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

the six samples. The average percentage of uniquely mapped paired reads was 50.45% and the average GC percentage was 53.7%. Detailed alignment data are presented in Table III.

**Identification of DEGs.** Compared with the control group, 80 genes were differentially expressed in the NRDS group. Among them, 69 genes were upregulated and 11 were down-regulated. Fig. 1 illustrates all significant DEGs in an MA plot. The top 10 upregulated and downregulated genes (in terms of  $\log_2FC$ ) are presented in Table IV.

In addition, the  $\log_2$  fold change of 10 genes obtained by RNA-seq and RT-qPCR analysis was compared to validate the DEGs. There was a statistically significant Pearson correlation ( $R^2=0.897$ ) between the expression levels measured by RNA-seq and RT-qPCR (Figs. 2 and 3).

**GO and KEGG analysis.** The mRNA-seq data set comparison to KEGG (12 pathways; Fig. 4) and GO (105 gene sets; top 20 presented in Table V) pathways highlighted the up- and down-regulation of three groupings of genes associated with biological function: Cellular processes; innate immune response; and pathogen recognition response. Of the 12 pathways, five of them were associated with immunological mechanisms: The tumor necrosis factor (TNF) signaling pathway (Fig. 5); influenza A

Table IV. Top 10 differentially expressed genes in the neonatal respiratory distress syndrome group compared with the control group.

Gene	Log <sub>2</sub> FC	P-value	Description
Upregulated			
PIP4K2B	4.83	1.96x10 <sup>-24</sup>	Phosphatidylinositol-5-phosphate 4-kinase type 2β
FCAR	4.43	5.43x10 <sup>-19</sup>	Fc fragment of IgA receptor
MLLT6	3.87	1.19x10 <sup>-13</sup>	PHD finger domain containing
DDX52	3.52	2.82x10 <sup>-11</sup>	DEdD-box helicase 52
AC010970.2	3.09	4.95x10 <sup>-10</sup>	AC010970.2
HBD	2.69	1.49x10 <sup>-08</sup>	Hemoglobin subunit δ
GPR84	2.58	2.65x10 <sup>-07</sup>	G protein-coupled receptor 84
MTRNR2L12	2.47	1.05x10 <sup>-07</sup>	MT-RNR2-like 12
LRRC37A2	2.46	4.07x10 <sup>-07</sup>	Leucine rich repeat containing 37 member A2
Downregulated			
APBA2	-3.56	4.91x10 <sup>-11</sup>	Amyloid β precursor protein binding family A member 2
BRD2	-3.19	2.45x10 <sup>-13</sup>	Bromodomain containing 2
HLA-DQA1	-2.61	1.04x10 <sup>-06</sup>	Major histocompatibility complex, class II, DQ α 1
MDGA1	-2.08	1.10x10 <sup>-04</sup>	MAM domain containing glycosylphosphatidylinositol anchor 1
WASF3	-1.94	1.89x10 <sup>-04</sup>	WAS protein family member 3
COL10A1	-1.88	1.84x10 <sup>-05</sup>	Collagen type X α 1 chain
GAN	-1.78	8.67x10 <sup>-11</sup>	Gigaxonin
LRRN3	-1.39	2.48x10 <sup>-06</sup>	Leucine rich repeat neuronal 3
GATA3	-1.36	9.47x10 <sup>-05</sup>	GATA binding protein 3
CSF1R	-1.13	1.4x10 <sup>-04</sup>	Colony stimulating factor 1 receptor

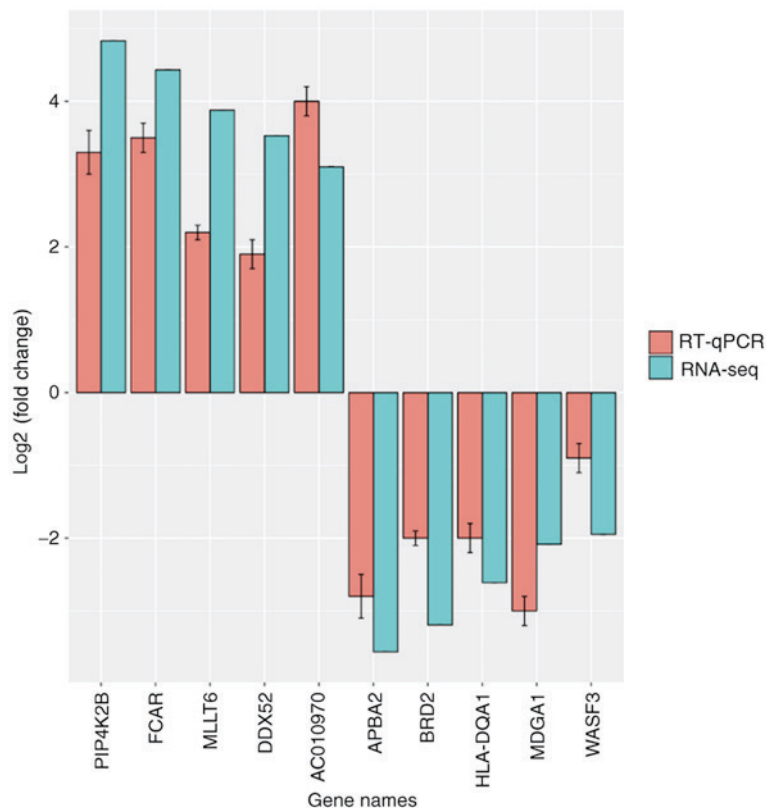


Figure 3. Expression of the top five over- and underexpressed genes, detected by RNA-seq (blue) and RT-qPCR (red). RNA-seq, RNA sequencing; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PIP4K2B, phosphatidylinositol-5-phosphate 4-kinase type 2 β; FCAR, Fc fragment of IgA receptor; MLLT6, MLLT6 PHD finger containing; DDX52, DEdD-box helicase 52; AC070970, AC010970.1; APBA2, amyloid β precursor protein binding family A member 2; BRD2, bromodomain containing 2; HLA-DQA1, major histocompatibility complex class II DQ α 1; MDGA1, MAM domain containing glycosylphosphatidylinositol anchor 1; WASF3, WAS protein family member 3.

Table V. Top 20 enriched GO terms among significantly differentially expressed genes.

GO ID	Title	Description	No. of genes	P-value
GO:0006260	DNA replication	The cellular metabolic process in which a cell duplicates one or more molecules of DNA.	5	0.000278
GO:0001878	Response to yeast	Any process that results in a change in state or activity of a cell or an organism as a result of a stimulus from a yeast species.	2	0.000873
GO:0060045	Positive regulation of cardiac muscle cell proliferation	Any process that activates or increases the frequency, rate or extent of cardiac muscle cell proliferation.	2	0.001500
GO:0032094	Response to food	Any process that results in a change in state or activity of a cell or an organism as a result of a food stimulus.	2	0.001750
GO:0051726	Regulation of cell cycle	Any process that modulates the rate or extent of progression through the cell cycle.	4	0.001760
GO:0042246	Tissue regeneration	The regrowth of lost or destroyed tissues.	2	0.002900
GO:0042742	Defense response to bacterium	Reactions triggered in response to the presence of a bacterium that act to protect the cell or organism.	3	0.003520
GO:0060333	Interferon-gamma-mediated signaling pathway	A series of molecular signals initiated by the binding of interferon-gamma to a receptor on the surface of a cell, and ending with regulation of a downstream cellular process.	3	0.004290
GO:0032201	Telomere maintenance via semi-conservative replication	The process in which telomeric DNA is synthesized semi-conservatively by the conventional replication machinery and telomeric accessory factors as part of cell cycle DNA replication.	2	0.005140
GO:0045003	Double-strand break repair via synthesis-dependent strand annealing	Synthesis-dependent strand annealing is a major mechanism of double-strand break repair in mitosis that allows for the error-free repair of a double-strand break without the exchange of adjacent sequences.	2	0.006020
GO:0000077	DNA damage checkpoint	A cell cycle checkpoint that regulates progression through the cell cycle in response to DNA damage.	2	0.006490
GO:0006513	Protein monoubiquitination	Addition of a single ubiquitin group to a protein.	2	0.006490
GO:0006953	Acute-phase response	An acute inflammatory response that involves non-antibody proteins whose concentrations in the plasma increase in response to infection or injury of homeothermic animals.	2	0.006490
GO:0000722	Telomere maintenance via recombination	Any recombinational process that contributes to the maintenance of proper telomeric length.	2	0.007460
GO:0050829	Defense response to Gram-negative bacterium	Reactions triggered in response to the presence of a Gram-negative bacterium that act to protect the cell or organism.	2	0.007970
GO:0006955	Immune response	Any immune process that functions in the calibrated response of an organism to a potential internal or invasive threat.	5	0.008730
GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	A series of molecular signals in which a signal is conveyed from the cell surface to trigger the apoptotic death of a cell.	2	0.011300
GO:0009408	Response to heat	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a heat stimulus, a temperature stimulus above the optimal temperature for that organism.	2	0.017400



Table V. Continued.

GO ID	Title	Description	No. of genes	P-value
GO:0032508	DNA duplex unwinding	The process in which interchain hydrogen bonds between two strands of DNA are broken or 'melted', generating a region of unpaired single strands.	2	0.018100
GO:0001504	Neurotransmitter uptake	The directed movement of neurotransmitters into neurons or glial cells.	1	0.022300

GO, gene ontology.

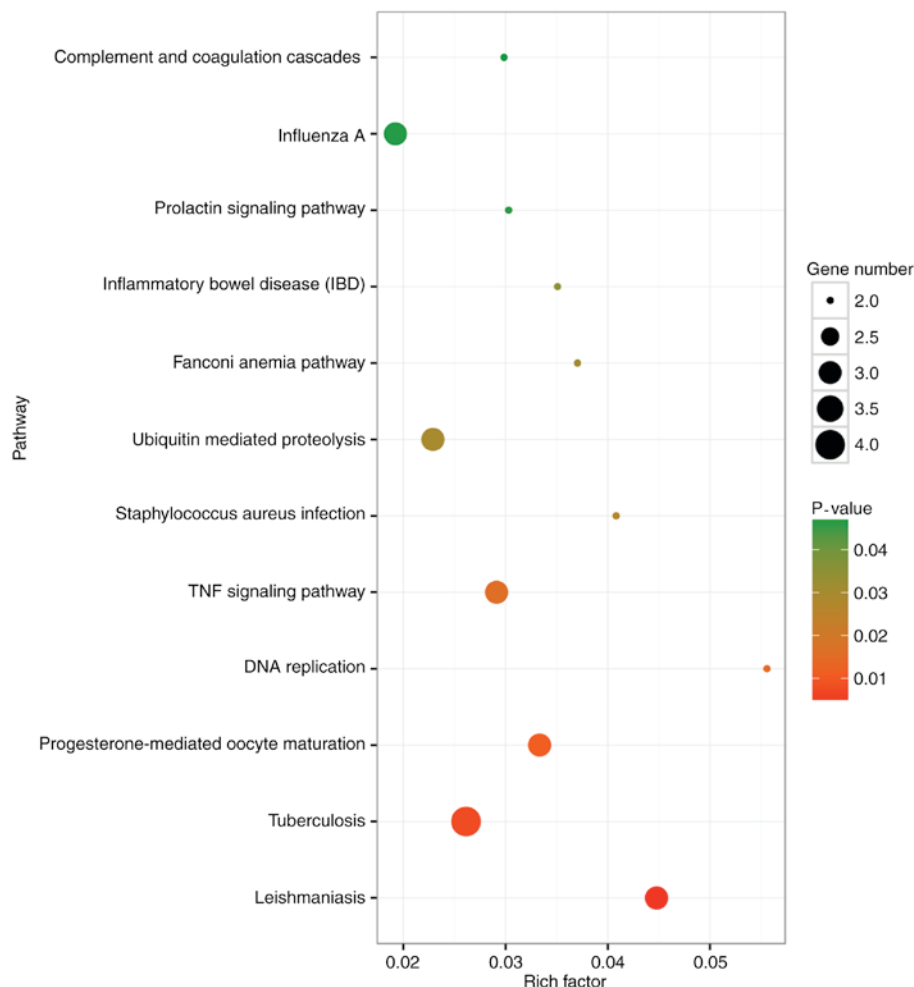


Figure 4. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed genes ( $P < 0.05$ ).

(Fig. 6); leishmaniasis (Fig. 7); *Staphylococcus aureus* infection (Fig. 8); and tuberculosis (Fig. 9). Suppressor of cytokine signaling 3, mitogen-activated protein kinase 14 and major histocompatibility complex II are overexpressed in these pathways; all three of these genes are involved in immune processes.

Within the groupings of cellular processes, the DNA replication and Fanconi anemia pathways were downregulated by 93 and 318 nuclear receptor (NR) genes, respectively, in response to NRDS pathogenesis compared with the control group. By contrast, the cellular proteolysis by ubiquitination and hormone signaling pathways were upregulated by 104 and 82 NR genes, respectively.

## Discussion

The phenotype of NRDS is not concluded from a single symptom; rather it is derived from numerous cumulative factors which precipitate NRDS. RNA-seq is a useful tool to illustrate the physiological responses to multiple factors which contribute to the manifestation of NRDS. The following identifies a number of mechanisms observed in the RNA-seq results, which are consistent with those reported in the process of ARDS.

In a case-control study reported by Molnar *et al* (15), increased levels of ubiquitin specific peptidase 10 (USP10) were observed in the ARDS group. In the present study, USP1 was observed

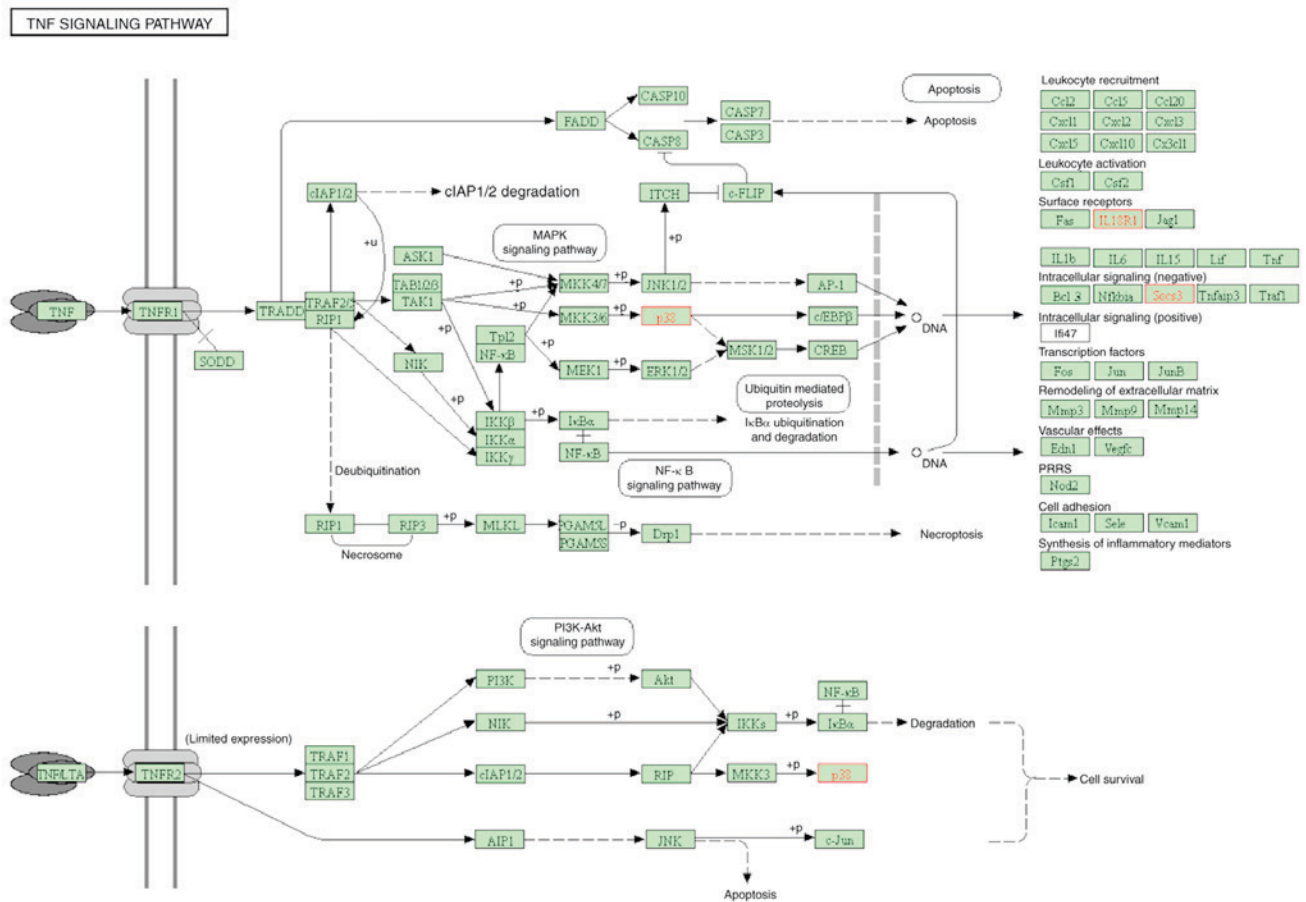


Figure 5. Differentially expressed genes (red) in the neonatal respiratory distress syndrome group compared with the control group in the TNF signaling pathway. TNF, tumor necrosis factor; SOCS3, suppressor of cytokine signaling 3; IL18R1, interleukin 18 receptor 1; p38, mitogen-activated protein kinase 14.

to be increased in the NRDS group (data not shown). As USP proteins are involved in autophagy, it may be hypothesized that ARDS and NRDS exhibit similar processes of autophagy.

A recent study by Qiao *et al* (16) illustrated that the ubiquitin-proteasome pathway (UPP) targeted signal transducer and activator of transcription 6 and further regulated T helper cells 2 and 9; this regulation may serve as an ideal target for mitigating the underlying pathology of allergic diseases, particularly asthma. Dysregulation and chronic overexpression of the UPP ligase systems and other ubiquitinated kinase protein components may cause inflammation of the bronchi and promote the pathophysiology of NRDS. Majetschak *et al* (17) demonstrated that ubiquitin-activated 20S core proteasome particles in bronchoalveolar lavage fluid were markedly increased following lung contusions. The proteolytic cleavage of functional proteasome complexes and additional post-protein processing observed within the alveolar space following lung injury may be associated with the pathogenesis of NRDS. It is unknown how cleavage and post-regulation of proteins involved in lung edema clearance interact with essential surfactant proteins, which may further enhance the NRDS pathological cascade.

Inhibition of the ubiquitinated protein kinase myristoylated alanine rich protein kinase C substrate (MARCKS) serves a role in mitigating excess pro-inflammatory cytokines release from neutrophils following lipopolysaccharide (LPS) from *Escherichia coli* and the activation of Toll-like receptor (TLR)2, TLR4 and TLR9 (18). The results of this previous

study illustrated correct and incorrect post-transcriptional regulation. When organisms were treated with the protein myristoylated N-terminal sequence, an inhibitor of MARCKS, neutrophil migration was mediated; marked alterations were observed in the reduction of mRNA expression associated with LPS-challenged interleukin-8 and TNF- $\alpha$ .

The assessment of various lung cellular responses to numerous environmental factors, by assessing mRNA-seq associated with NRDS, is a viable and sophisticated way to characterize NRDS and ARDS. Assessing the progression of various symptoms towards the onset of diseases may help to further characterize cellular responses associated with disease and to further elucidate the underlying pathogenesis of diseases. A previous study assessed how alterations at the mRNA level affect how transcriptional gene regulation is moderated prior to the production and translation of proteins (15). Gao and Barnes (19) performed a meta-analysis using RNA-seq data of 83 lung-specific genes; only 62 of the genes were required to code for proteins necessary for lung development and pathogenesis. Specifically, in ARDS there are 21 principal genes of interest to target that are associated with the physiological onset of ARDS and similar acute lung injuries.

Due to the hydrophobic nature of surfactants, they are able to keep the alveoli dry and allow for optimal oxygen-blood exchange. Dysregulation of surfactant phospholipids has been previously demonstrated to be associated with alveolar instability and an increased patient risk of developing NRDS (20).

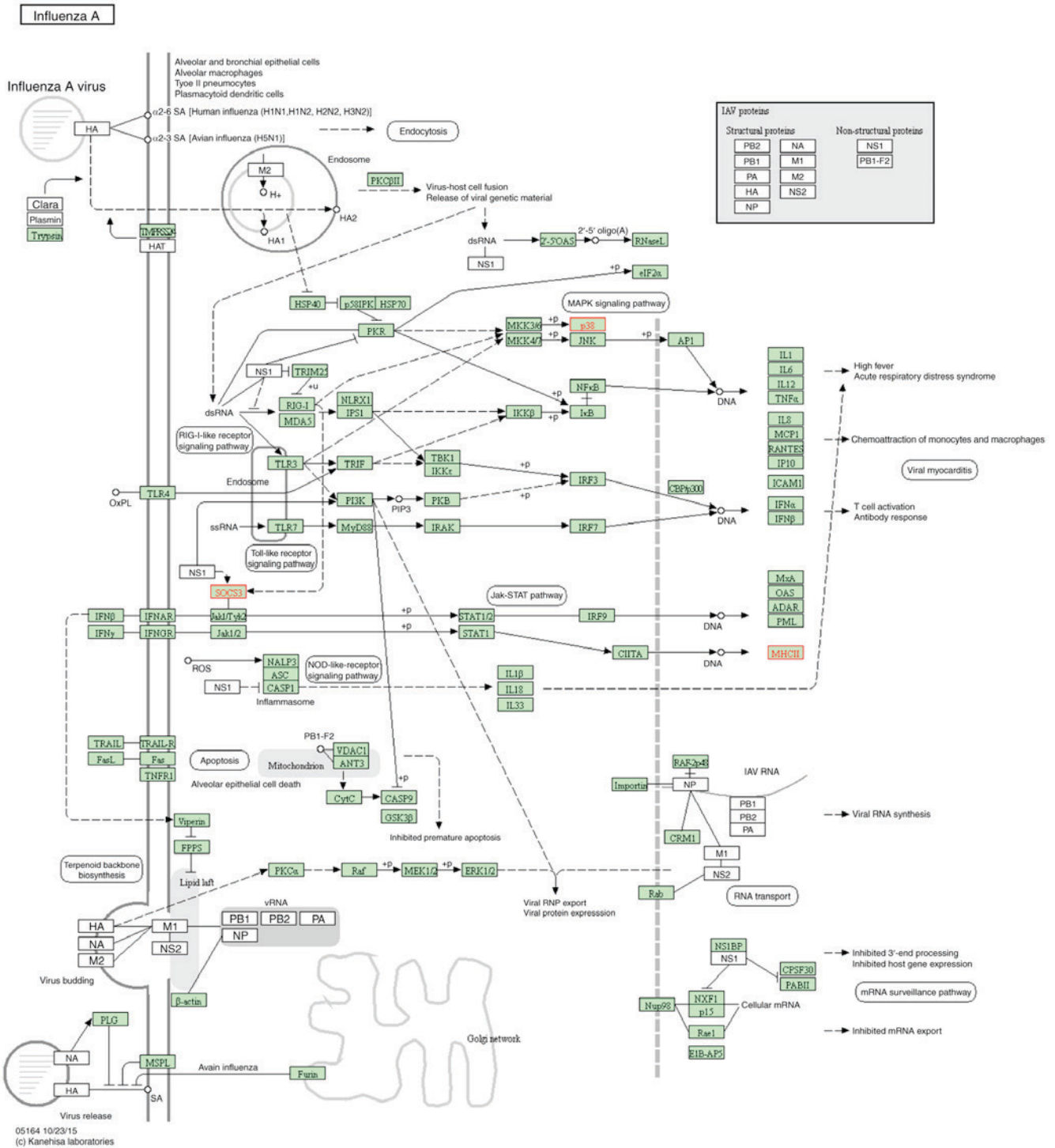


Figure 6. Differentially expressed genes (red) in the neonatal respiratory distress syndrome group compared with the control group in the influenza A pathway. SOCS3, suppressor of cytokine signaling 3; MHCII, major histocompatibility complex II; p38, mitogen-activated protein kinase 14.

Translational regulation of gene families has essential roles in maintaining lung homeostasis, including surfactant-associated proteins (SFTPs; SFTPA1, SFTPA2, SFTPC and SFTPD) which serve a role in understanding the pathology of NRDS (21,22).

In a study by D'Ovidio *et al* (23), clinicians assessed the mortality associated with lung transplants and various mRNA levels among different experimental groups and their surfactant protein polymorphism (SP-A, SP-B, SP-C and SP-D) expression levels. The pilot study demonstrated notable

mortality rates in patients; however, those with increased mRNA expression of SP generally exhibited a decreased incidence of allograft rejection by the innate immune system, thus illustrating a marked difference in lung transplant mortality associated with surfactants. Additionally, Silva *et al* (24) observed that the application of the anti-inflammatory agent LASSBio596 may be applied to ARDS mouse models to increase survival rates by mitigating macrophages, neutrophils and transforming growth factor-β by increasing surfactant yield, which in turn reduced surface tension. By maintaining



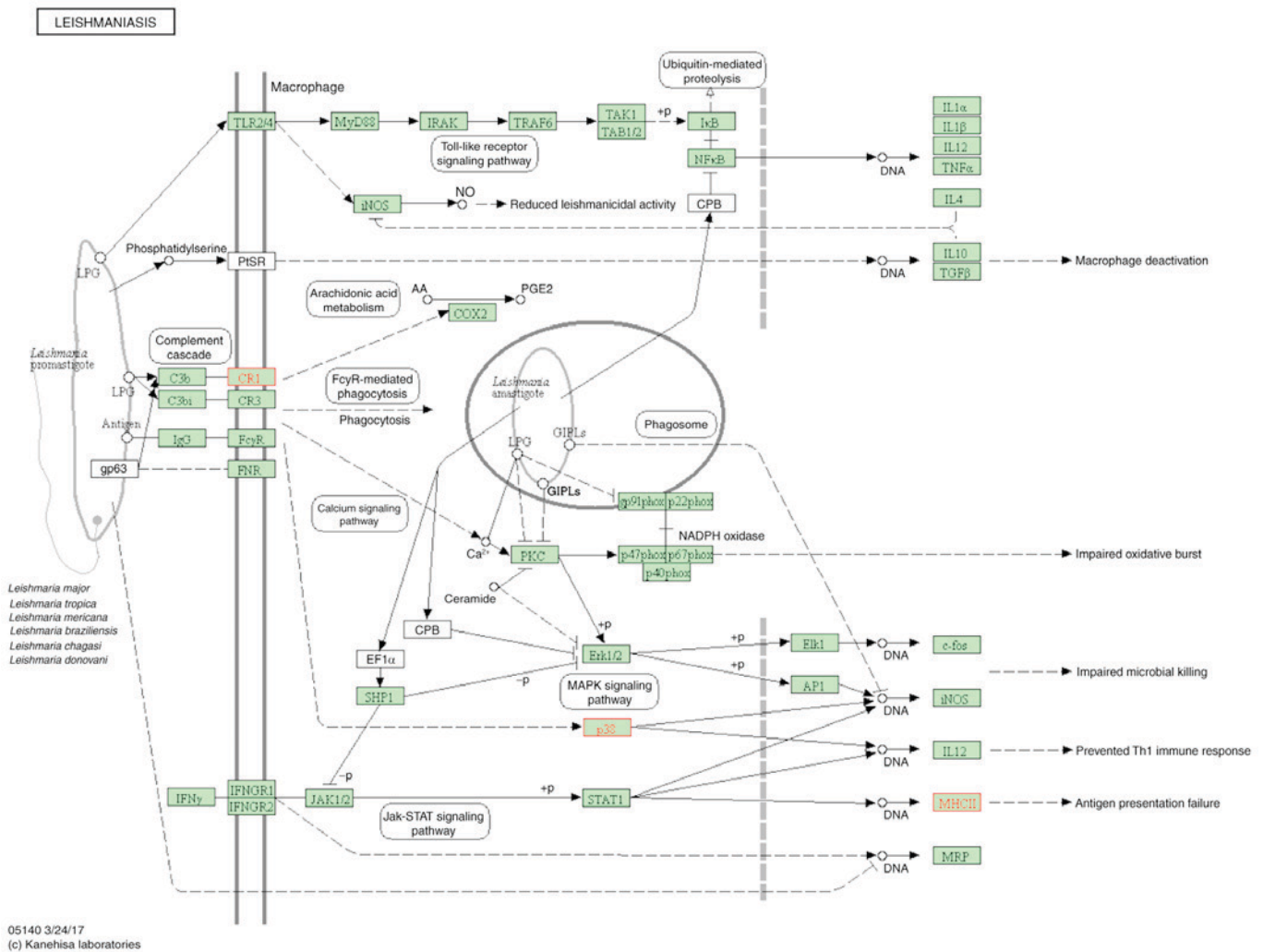


Figure 7. Differentially expressed genes (red) in the neonatal respiratory distress syndrome group compared with the control group in the Leishmaniasis pathway. MHCII, major histocompatibility complex II; CR1, complement C3b/C4b receptor 1; p38, mitogen-activated protein kinase 14.

elasticity and surfactant alterations in lung mechanics, the authors were able to counteract degradation immediately following lung injury leading to ARDS.

The removal of surfactant proteins leads to substantial alterations in the alveoli-capillary barrier, leading to the serious phenotype of sepsis. Pathogenesis-induced sepsis of the peripheral lung is denoted by inflammation associated with the presence of the microbial metabolite lactate, which is a standard lung biomarker of sepsis (25). In response to a microbial presence in the form of adhesion or byproducts, the innate immune system is the first to mount a response. In order to further assess the biomarkers of NRDS, Ware *et al* (26) demonstrated that SP-D overexpression is an effective marker of sepsis prognosis; increased expression of this protein from alveolar epithelial type II cells is associated with increased mortality.

In the grouping of gene families associated with the innate immune response, it has been reported that all four pathways, complement and coagulation cascades, tumor TNF signaling, inflammatory bowel disease and prolactin signaling, were upregulated by 50-67 genes in response to NRDS. In the case of pneumonia, pro-inflammatory cytokines and systemic inflammation, the host-associated response will lead to increased TNF expression followed by dysregulation of lung-induced

apoptosis by neutrophils (27). Proteinase-activated cascades following lung injury promote the activation of the coagulation response that begins the innate inflammatory response and the additional pro-inflammatory factors, including TNF (28). Macrophage-derived TNF has been demonstrated to further provoke lung injury by stimulating p55 receptor-mediated death signaling; this further exacerbates injury by increasing lung neutrophils and leads to the deleterious phenotype of ARDS (29).

Upstream activation of TNF- $\alpha$  within the lung periphery is an essential mediator of neutrophils, and the pathology of NRDS and ARDS. Plasma TNF- $\alpha$  levels have been observed to be increased in patients with NRDS compared with controls (30). A study performed in mice by Lomas-Neira *et al* (31) demonstrated that the transfer of TNF- $\alpha$  to mice lacking neutrophils did not stimulate the progression of lung injury. By comparison, mice lacking neutrophils expressing TNF- $\alpha$  displayed reduced acute lung injury. These previous results suggested that TNF- $\alpha$  expression in neutrophils is an underlying factor that promotes the progression of the pathogenesis from the initial lung-injury into a shock-primed innate immune response.

In response to colonization by pathogenic organisms, including *Streptococcus pneumoniae*, proteinase-activated receptor 1 serves a role in splicing for the activation of

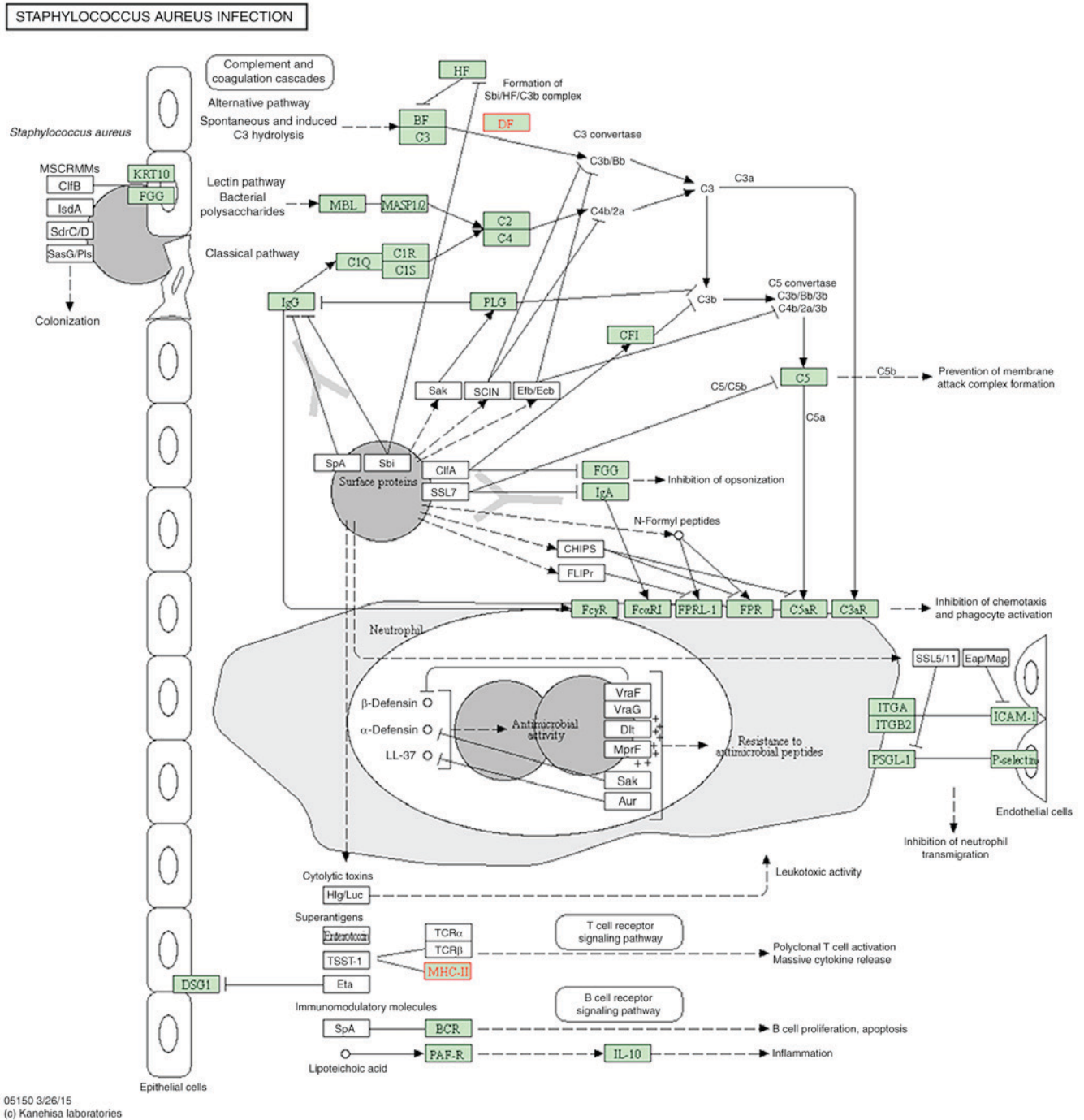


Figure 8. Differentially expressed genes (red) in the neonatal respiratory distress syndrome group compared with the control group in the *Staphylococcus aureus* infection pathway. DF, complement factor D; MHCII, major histocompatibility complex II.

coagulation pathways leading to inflammation; thus, beginning the cascade of neutrophilic pro-inflammatory responses to invasive pathogens, excess neutrophil recruitment contributes to lung injury through alveolar surfactant barrier disruption and alveolar leakage (18).

The pathogen recognition response for NRDS KEGG pathways all had upregulated gene families in the present study; this was the case for all except *Staphylococcus aureus* infection, which was downregulated by 10 NR genes. Upregulated gene families included leishmaniasis, tuberculosis, and influenza A, and the number of genes involved in these three groups were 3, 4 and 3, respectively. Microbial

proliferation and sepsis following acute lung injury is a primary contributor to the cascade and severity of NRDS. Lv *et al* (32) demonstrated through transcriptomic analysis that genes associated with foreign pathogens (NLR family pyrin domain containing 3 and S100 calcium binding protein A8/A9) were markedly upregulated in response to microbial LPS.

In addition to sepsis, other factors not involved with the innate immune response or surfactants may contribute to NRDS and ARDS. For example, Lv *et al* (32) demonstrated, through systematic mRNA-seq of ARDS, a significant upregulation of 122 genes and a downregulation of 91 genes associated with essential protein functions, including the mitotic cell cycle.

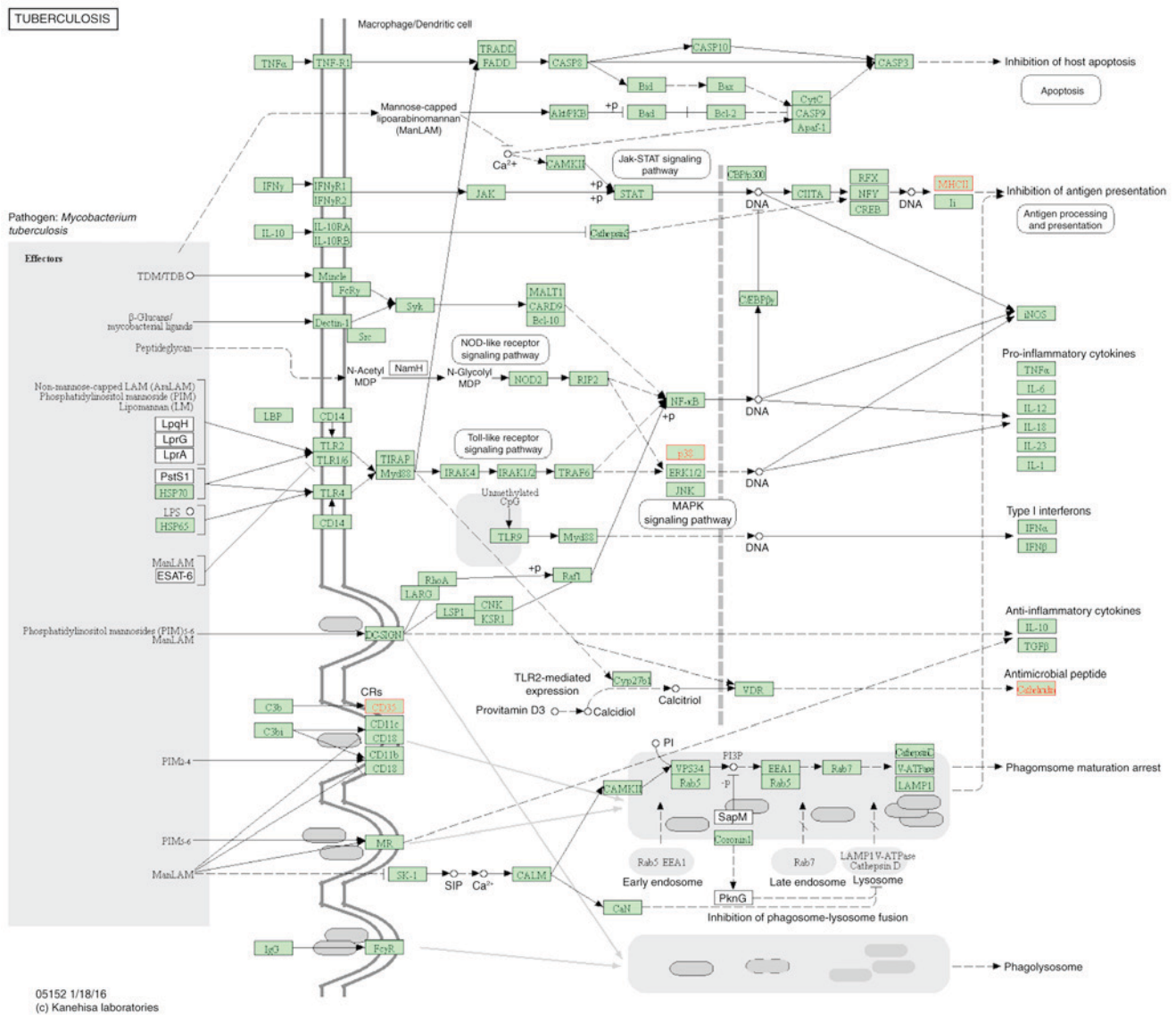


Figure 9. Differentially expressed genes (red) in the neonatal respiratory distress syndrome group compared with the control group in the tuberculosis pathway. p38, mitogen-activated protein kinase 14; MHCII, major histocompatibility complex II; CD35, complement C3b/C4b receptor 1.

Specifically, the investigators noted that cyclin (CCNB)1 and CCNB2, which serve a role in cell cycle regulation, may be associated with the onset of ARDS.

A previous study of the human microbiome indicated dysbiosis within the human system, typically leading to a dysregulation of host responses to various systemic challenges; in particular, these challenges threaten lung homeostasis and provoke the perpetual inflammation associated with invasive pathogenic microbiota (33). Invasion, persistence and colonization of foreign microbiota challenge the host immune system to further provoke ARDS; the proliferation of microbiota and heightened responses to challenge further provoke the accumulated lung injury to develop into sepsis (34). Infections with and the persistence of numerous viral strains and microbiota, including influenza A (35), *Mycobacterium tuberculosis* (36), *Staphylococcus aureus*/H1N1 strain of the influenza virus (37) and multi-drug resistant *Acinetobacter baumannii* (38).

Dysregulation of TNF and the coagulation cascade pathways may be associated with ubiquitin-mediated kinase activation

in the case of proteasome activated lysis, which may activate cascades leading to neutrophil over-stimulation and eventual dysregulated lung damage. In conclusion, the mechanisms leading to a specific NRDS phenotype are complex, although mRNA-seq as a tool may be used to elucidate genomic response to lung-induced stress. Consequently, cascading immune mechanisms associated with the dysregulation of one aspect of the immune response may accumulate progressively towards NRDS.

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