Quantitative Analysis of Cellular Proteome Alterations in CDV-Infected Mink Lung Epithelial Cells

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Canine distemper virus (CDV), a paramyxovirus, causes a severe highly contagious lethal disease in carnivores, such as mink. Mink lung epithelial cells (Mv.1.Lu cells) are sensitive to CDV infection and are homologous to the natural host system of mink. The current study analyzed the response of Mv.1.Lu cells to CDV infection by iTRAQ combined with LC–MS/MS. In total, 151 and 369 differentially expressed proteins (DEPs) were markedly up-regulated or down-regulated, respectively. Thirteen DEPs were validated via real-time RT-PCR or western blot analysis. Network and KEGG pathway analyses revealed several regulated proteins associated with the NF-κB signaling pathway. Further validation was performed by western blot analysis and immunofluorescence assay, which demonstrated that different CDV strains induced NF-κB P65 phosphorylation and nuclear translocation. Moreover, the results provided interesting information that some identified DEPs possibly associated with the pathogenesis and the immune response upon CDV infection. This study is the first overview of the responses to CDV infection in Mv.1.Lu cells, and the findings will help to analyze further aspects of the molecular mechanisms involved in viral pathogenesis and the immune responses upon CDV infection.

Keywords: Canine distemper virus (CDV), Mink lung epithelial cells (Mv.1.Lu cells), Isobaric tags for relative and absolute quantitation (iTRAQ), proteomics, NF-κB signaling

INTRODUCTION

Canine distemper virus (CDV), a negative-sense, single-stranded RNA virus, belonging to the genus Morbillivirus, family Paramyxoviridae, causes a severe highly contagious lethal disease in carnivores, such as dogs, lions, ferrets, raccoon dogs, foxes, and minks (Williams et al., 1988; Deem et al., 2000; Martella et al., 2010; Zhao et al., 2010; Viana et al., 2015). The disease is distributed worldwide and is characterized by respiratory and gastrointestinal tract symptoms with generalized immunosuppression (Blancou, 2004; Decaro et al., 2004). The immune system dysfunction of CDV infection favors opportunistic secondary pathogens, resulting in high morbidity and mortality in a wide range of carnivore species (Appel et al., 1982; Kauffman et al., 1982; Blixenkrone-Moller, 1989). Generally, in domestic dogs, CDV establishes a systemic infection, initiating transmission from immune cells, such as alveolar macrophages and/or dendritic cells, of the upper respiratory tract to the local lymphatic tissues by immune-mediated progression, and ultimately propagates to most organs and tissues, including epithelial tissues via cell-associated viremia (Appel et al., 1982). Epithelial cells are susceptible to CDV infection and play a role in transmission during the late stages of CDV pathogenesis (Pratapakiriya et al., 2012; Noyce et al., 2013). The virus is amplified and secreted from the epithelial cells of the respiratory, gastrointestinal, and urinary systems of
the infected host (Ludlow et al., 2014). The infection of various viruses has been demonstrated to interact widely with numerous host cell proteins. Some interactions elicit changes in the host proteome, as illustrated by the capacity of the virus to both induce and evade the host immune response (Kash et al., 2006), effecting autophagy and apoptosis (Ludwig et al., 2006; Gunnae and Munz, 2009). For measles virus (MV), another morbillivirus closely similar to CDV, cell cycle arrest in lymphocytes (Naniche et al., 1999) and apoptosis in T lymphocytes (Fugier-Vivier et al., 1997) have also been reported. Many studies have reported the effects of CDV infections on the host cell proteins, such as inhibiting STAT1 and STAT2 nuclear import (Rothlisberger et al., 2010), inducing cytokine responses in PBMCs (Nielsen et al., 2009), and inducing lymphocytes apoptosis (Kumagai et al., 2004). However, most of these reports have primarily investigated a single host cell protein or partially selected proteins and the mechanisms of CDV pathogenesis and immunomodulation have not been fully elucidated. Thus, a new approach for further understanding the pathogenic mechanism and immunomodulation of CDV infection is needed, and the identification of global host cell proteins that interact with CDV infection represents one option. More details associated with host responses to CDV infection should also shed some light on potential targets for antiviral agents.

For decades, proteomic assays have been applied as significant tools to analyze the interaction of host responses to viral infection. Investigation of the changes in the proteome upon virus infection is becoming an effective instrument for providing potential targets for antiviral research. This approach has revealed the specific insights into the cellular mechanisms involved in viral pathogenesis for several viral pathogens, including transmissible gastroenteritis virus (TGEV) (An et al., 2014), human influenza A (Vester et al., 2009), canine parvovirus (CPV) (Zhou et al., 2016), and canine distemper virus strain CDV-PS (GenBank accession no. JN896331), a low passage isolate (<7 passages) from a morbid dog in 2013 (Yi et al., 2013), was preserved in our laboratory. The virus was propagated in Vero cells. In the study, three additional passages of the virus were performed in Mv.1.Lu cells, resulting in the virus suspension with a titer of 10^3.1 TCID_{50}/mL determined by a 50% tissue culture infectious dose (TCID_{50}) assay (Yamaguchi et al., 1988). Briefly, monolayers of Mv.1.Lu cells in 96-well plates were infected with a 10-fold serial dilution of the supernatant fluids and further incubated for up to 120 h. The wells were assessed for cytopathic effects (CPE) after 3–5 days, and the TCID_{50} was calculated using the Reed–Muench formula. Because of the low virus titer and the impurity of the virus suspension, virus concentration and purification were performed to improve the virus titer and avoid the effect of non-viral components. The clarified suspension was concentrated by polyethylene glycol 6,000 precipitation and purified by ultracentrifugation in a gradient of sucrose according to standard procedures. Sucrose-purified viruses were then titrated using the TCID_{50} assay as described above, and the titer of the virus stocks increased to 10^6.9 TCID_{50}/mL. The attenuated CDV vaccine CDV3 strain was treated as the same as PS. The virus stocks were aliquoted and stored at −80°C until further use in the following experiments.

For the establishment of viral kinetics, Mv.1.Lu cells were grown in 6-well plates and subsequently challenged by the virus (PS) at a multiplicity of infection (MOI) of 2, calculated based on the infectious virus particle concentration determined as TCID_{50}. At 6, 12, 24, 36, 48, 60, and 72 hpi, viral propagation was confirmed by observation of the CPE and viral replication and production of PS nucleoprotein for the different time points analyzed was tested by anti-CDV NP antibody. The one-step growth curve, indicating the viral load with the time, was generated according to Chuzushimi with slight modifications (Ushimi et al., 1972). Briefly, 200 µL of culture medium was collected at indicated time, followed by the extraction of total RNA from all samples. qRT-PCR was then applied to detect the viral RNA at each indicated time. For iTRAQ labeling, Mv.1.Lu cells were grown in T75 flasks to 70–80% confluence and subsequently infected with the virus (PS) at an MOI of 2. As an uninfected control, a mock-infection was performed. The cells were collected at 24 hpi for the protein extraction. Three biological replicates were prepared for all samples. All experiments were performed under Biosafety Level 2 conditions.

**Protein Isolation, Digestion, and Labeling with iTRAQ Reagents**

The collected cells were lysed in lysis buffer containing a protease inhibitor cocktail. The lysate was sonicated and centrifuged at 14,000 g for 40 min, and the supernatant was quantified with the BCA Protein Assay Kit (Bio-Rad, U.S.A.). Subsequently, 200 µg of protein for each sample was digested with 4 µg of trypsin (Promega, WI) overnight at 37°C. According to the protocol of the iTRAQ reagents (8 plex, Applied Biosystem), 100 µg of peptide mixture from each sample was labeled follows: the

**MATERIALS AND METHODS**

**Cell Culture and Virus Infection**

Mink lung epithelial cells (Mv.1.Lu cells) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and grown in Minimum Essential Medium (Gibco® Invitrogen, U.S.A.), supplemented with 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO₂. The canine distemper virus strain CDV-PS (GenBank accession no. JN896331), a low passage isolate (<7 passages) from a morbid dog in 2013 (Yi et al., 2013), was preserved in our laboratory. The virus was propagated in Vero cells. In the study, three additional passages of the virus were performed in Mv.1.Lu cells, resulting in the virus suspension with a titer of 10^3.1 TCID_{50}/mL determined by a 50% tissue culture infectious dose (TCID_{50}) assay (Yamaguchi et al., 1988). Briefly, monolayers of Mv.1.Lu cells in 96-well plates were infected with a 10-fold serial dilution of the supernatant fluids and further incubated for up to 120 h. The wells were assessed for cytopathic effects (CPE) after 3–5 days, and the TCID_{50} was calculated using the Reed–Muench formula. Because of the low virus titer and the impurity of the virus suspension, virus concentration and purification were performed to improve the virus titer and avoid the effect of non-viral components. The clarified suspension was concentrated by polyethylene glycol 6,000 precipitation and purified by ultracentrifugation in a gradient of sucrose according to standard procedures. Sucrose-purified viruses were then titrated using the TCID_{50} assay as described above, and the titer of the virus stocks increased to 10^6.9 TCID_{50}/mL. The attenuated CDV vaccine CDV3 strain was treated as the same as PS. The virus stocks were aliquoted and stored at −80°C until further use in the following experiments.

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three mock-infected samples were each labeled with iTRAQ 113, 114, or 115, and the three PS-infected samples were labeled with iTRAQ 116, 117, or 118. The labeled samples were then mixed and dried with a rotary vacuum concentrator.

**Peptide Fractionation and LC-MS/MS Analysis**

To reduce the complexity of the peptide mixtures, iTRAQ-labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). Briefly, the dried peptide mixture was reconstituted and acidified with buffer A (10 mM KH₂PO₄ in 25% of ACN, pH 3.0) and loaded onto a PolySULFOETHYL 4.6 × 100 mm column (5 μm, 200 A, PolyLC Inc., U.S.A.). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer B (500 mM KCl, 10 mM KH₂PO₄ in 25% of ACN, pH 3.0). The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. A total of 15 fractions were collected with screening, and then desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL) and concentrated by vacuum centrifugation.

Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18) connected to the C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% Formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology. The LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (ThermoFisher, U.S.A.) coupled to the Easy nLC chromatography system (ThermoFisher, U.S.A.). The mass spectrometer was operated in positive ion mode. MS data was acquired using a data-dependent top 10 method, dynamically selecting the most abundant precursor ions from the survey scan (300–1,800 m/z) for HCD fragmentation. The automatic gain control (AGC) target was set to 3e6, and the maximum inject time was set to 10 ms. Dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200, and the isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with the peptide recognition mode enabled.

**Protein Identification and Quantification**

All MS raw data files were analyzed by Proteome Discoverer software 1.4 (ThermoFisher, U.S.A.) using the Mascot 2.2 search engine against a database of *mustela putorius furo* protein sequences (NCBI, released March 23, 2017, containing 38,992 sequences). For protein identification, a mass tolerance of 0.1 Da was allowed for fragmented ions, with permission of two missed cleavages in the trypsin digest: iTRAQ8-plex (Y), oxidation (M) as the potential variable modifications, and carbamidomethyl (C), iTRAQ8-plex (N-term), and iTRAQ8-plex (K) as fixed modifications. The strict maximum parsimony principle was performed, and only peptide spectra with high or medium confidence were considered for protein grouping. A decoy database search strategy was also used to estimate the false discovery rate (FDR) to ensure the reliability of the proteins identified.

For relative quantitation, proteins that involved at least one unique peptide were considered a highly confident identification and used for quantification. Additionally, to guarantee the accuracy of quantification, the proteins with coefficient of variation values <20% for three biological repeats were considered DEPs. The quantitative protein ratios were calculated and normalized by the median ratio in Mascot. For comparison, three identical mock samples, labeled with iTRAQ 113, 114, and 115, were used as references. Between samples, the proteins with fold-change ratios ≥1.20 or ≤0.83 and a p < 0.05 were considered DEPs according to the t-test.

**Bioinformatics Analysis**

To further explore the impact of the DEP on cell physiological processes and discover internal relations between DEPs, an enrichment analysis was performed. GO enrichment on three ontologies [biological process (BP), molecular function (MF), and cellular component (CC)] was applied based on the Fisher’s exact test, considering the whole quantified protein annotations as the background dataset. Benjamini–Hochberg correction for multiple testing was further applied to adjust derived p-values. Only functional categories with p-values under a threshold of 0.05 were considered significant. KEGG pathway annotation was extracted from the online KEGG PATHWAY Database (http://www.kegg.jp/kegg/pathway.html).

The protein–protein interaction information involved in the immune response process of the studied proteins was subsequently retrieved from STRING software (http://string-db.org/). Then, the results were imported into Cytoscape5 software (http://www.cytoscape.org/ version 3.2.1) to visualize and further analyze functional protein–protein interaction networks.

**Real-Time RT-PCR**

Total RNA was isolated using TRIzol Reagent (Invitrogen, U.S.A.) from Mv.1.Lu cells infected with 2 MOI PS or mock-infected cells at 12 and 24 hpi. After treatment with gDNA Removal (TransGen Biotech, China), 4 μg of each total RNA was used for cDNA synthesis. Real-Time RT-PCR (qRT-PCR) assays were performed on an Applied Biosystems® QuantStudio® 3 System (Thermo Fisher Scientific, U.S.A.) employing the TransStart Top Green qPCR SuperMix kit (TransGen Biotech, China) according to the manufacturer’s protocol. The primers for amplifying TRAF6, TRAF2, IRAK4, IRAK2, NFκB2, CCL2, TNF-α, IL-6, and GAPDH are presented in Table 1. Each experiment was performed in triplicate. The relative gene expression was calculated using the 2−ΔΔCT model, which is representative of n-fold changes compared with mock-infected samples. The data was analyzed by two-way ANOVA followed by Duncan’s test.

**Western Blot Analysis**

For testing the production of PS nucleoprotein for the different time points analyzed, cell lysates were harvested at
6, 12, 24, 36, 48, and 60 hpi from PS- and mock-infected samples. For confirmation of the iTRAQ-MS data by western blotting, cell lysates were harvested at 12 and 24 hpi from PS-, CDV+-, and mock-infected cultures. After measuring the protein concentrations, equivalent amounts of cellular proteins from the triplicates were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose PVDF membranes (Millipore, U.S.A.). The membranes were blocked with 2% BSA dissolved in TBS, containing 0.05% Tween-20, followed by incubation with the corresponding primary antibodies (see below) at 4°C overnight and incubation with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (Sangong Biotech, China) at room temperature for 2 h. The protein bands were detected using the ECL Detection Kit (Beyotime, China). The GAPDH protein was used as an internal control.

The following primary polyclonal antibodies were used: anti-CDV NP mouse monoclonal antibody (prepared in our laboratory), NF-κB p65 (RelA) rabbit polyclonal antibody (AN365, Beyotime, China), NFκB1 p105 rabbit polyclonal antibody (4717, CST, U.S.A), NFκBIB (IkB-β) rabbit polyclonal antibody (PA5-40909, ThermoFisher, U.S.A), MHC-I mouse monoclonal antibody (ab23755, Abcam, UK), RPS29 rabbit polyclonal antibody (PA5-41744, ThermoFisher, U.S.A), IkB-α rabbit polyclonal antibody (4812, CST, U.S.A), Phospho-NFκB p65 rabbit polyclonal antibody (MA5-15181, ThermoFisher, U.S.A), and GAPDH rabbit polyclonal antibody (CW0101M, CWBIO, China).

**Immunofluorescence Assay**

Mv.1.Lu cells were cultivated on cover glasses in 24-well plates, followed by infection with PS or CDV at an MOI of 2 when the cells reached ~70% confluence. The mock-infected cells were treated with PBS as a negative control. Next, at 24 hpi, the cells were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.1% Triton X-100. Further, the cells were incubated with an NF-κB P65 rabbit polyclonal antibody (Beyotime, China) and a mouse monoclonal antibody specific to CDV N protein and incubated with Cy3-labeled goat anti-mouse IgG (Beyotime, China) and FITC-conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher, U.S.A) prior to staining with DAPI. The fluorescent images were analyzed under confocal microscopy (Leica, Germany).

**RESULTS**

**Verification of PS Replication in Mv.1.Lu Cells**

A previous study demonstrated the capacity of CDV growth in Mv.1.Lu cells (Lednický et al., 2004), thus, we initially confirmed the ability of PS replication in Mv.1.Lu cells and established the growth kinetics of PS replication. An optimal time point under PS infection for proteomic analysis was then identified.

As shown in Figure 1A, CPEs in the infection groups became visible at 24 hpi and progressed thereafter. Up to 36 hpi, an obvious CPE was observed and nearly 50 percent of the cells were detached at 48 hpi. The one-step growth curve revealed that the virus load reached a plateau of ~4.8 log10 copy numbers/μL between 24 and 60 hpi, followed by a gradual decline (Figure 1B). Collectively, 24 hpi was considered the optimal time-point for further proteomic analysis, at which a high viral load was maintained and most cells showed little CPE. Virus replication at 6 and 48 hpi was additionally ensured through RT-PCR.

The abundance of the CDV-N gene increased as the infection progressed (Figure 1C). Further validation was performed by sequencing analysis of the PCR products (data not shown). Moreover, the production of nucleoprotein for the different time points analyzed was tested by anti-CDV NP antibody, the result showed quite similar tendency of the viral one-step growth curve (Figure 1D).

**Identification of Differentially Expressed Proteins in PS-Infected Mv.1.Lu Cells**

The host response to PS infection at 24 hpi was analyzed by examining differences in protein expression. Based on a combination of three biological replicates from mock-infected and PS-infected samples, the iTRAQ-coupled LC-MS/MS analysis identified and measured a total of 37,145 peptides and 6,184 proteins. The proteins were designated DEPs based on the following criteria: a fold-change ratio of ≥2 or ≤0.5 and fold-change ratios ≥2 or ≤0.833. Among all the DEPs, 151 and 369 proteins were markedly up-regulated or down-regulated, respectively. Partial DEPs are shown in Table 2 and more detailed information for all DEPs is collated in Table S1.

**Functional Characterization of the DEPs**

To characterize the biological functions of the 520 DEPs, canonical Gene Ontology (GO) enrichment were performed using DAVID (Dennis et al., 2003) and UniProt databases to obtain relevant annotations about the cellular components (CC), molecular functions (MF), and biological processes (BP).
First, the putative subcellular localizations of the DEPs were analyzed. As depicted in Figure 2, a majority of the DEPs were mainly distributed in the nucleus (45.64%) and cytoplasm (20.09%), followed by extracellular space (10.50%), mitochondria (9.75%), and plasma membrane (9.72%), and a smaller portion were localized in the chloroplast (2.66%), lysosome (0.59%), Golgi (0.30%), cytoskeleton (0.30%), peroxidase (0.30%), and ER (0.15%) (more detailed information is collated in Table S2). Interestingly, the GO analysis showed that most proteins were assigned to functions involved in similar molecular functions and biological processes. As shown in Figure 3A, most DEPs were closely related to binding and catalytic activity when infected by PS infection (more detailed information is provided in Table S3). The BP annotation showed that DEPs associated with various biological processes, including cellular process, metabolic process, biological regulation, immune system process and process of response to stimulus (Figure 3B) (more detailed information is provided in Table S4). Collectively, these categories consisted of the following proteins: CCL2, IRAK4, UBE2L6, NFkB1, NFkB2, TNF-a, IRAK2, IL-6, TRAF6, APOA1, TNFAIP3, TRAF2, RelA, and VCAM1 (up-regulated proteins) and CCR7, CXCR7, SMURF1, NFkBB, MAPK7, RBM15, IGF2, TSC1, and CD59 (down-regulated proteins). To further investigate the pathways involving the identified DEPs, KEGG pathway analysis was performed. According to the results, DEPs were mainly involved in the NF-κB and NOD-Like receptor (NLR) signaling pathways. In addition, several proteins could be mapped to apoptosis and specific disease associations, consisting of infectious and respiratory diseases (Figure 3C) (more detailed information is shown in Table S5).

**Network Analysis of the DEPs Involved in Immune Response Process**

In the present study, we detected a total of 27 DEPs involved in the immune response process. To further investigate the interaction network associated with the immune response, these 27 proteins were imported into STRING software and further analyzed by Cytoscape5. As shown in Figure 4, 13 strongly interacting proteins were interestingly grouped into a functional set chiefly associated with the NF-κB signaling pathway.
| Accession No. | Gene Description | Log2 ratios (infection/control) |
|---------------|------------------|---------------------------------|
| gi|511835322 | C2orf78 Chromosome 2 open reading frame 78 | 4.41 |
| gi|511926358 | MHC-I MHC class I | 3.40 |
| gi|511879565 | ALDH1A3 Aldehyde dehydrogenase family 1, subfamily A3 | 3.09 |
| gi|511896227 | CBLC Casitas B-lineage lymphoma c | 2.53 |
| gi|470656855 | PPP4R4 Protein phosphatase 4, regulatory subunit 4 | 2.30 |
| gi|511911718 | VCAM1 Vascular cell adhesion molecule 1 | 2.23 |
| gi|41068650 | FMNL2 Formin-like 2 | 2.23 |
| gi|511856886 | IRAK4 Interleukin-1 receptor-associated kinase 4 | 2.08 |
| gi|511851258 | APOA1 Apolipoprotein A-I | 2.03 |
| gi|511858549 | TSPAN8 Tetraspanin 8 | 2.00 |
| gi|545550325 | PKM Pyruvate kinase, muscle | 1.99 |
| gi|511830126 | IGFBP3 Insulin-like growth factor binding protein 3 | 1.98 |
| gi|511841556 | AHSG alpha-2-HS-glycoprotein | 1.96 |
| gi|512014297 | COL4A3 Collagen, type IV, alpha 3 | 1.95 |
| gi|390460231 | GPM6A Glycoprotein m6a | 1.95 |
| gi|511845472 | CCL2 Chemokine (C-C motif) ligand 2 | 1.95 |
| gi|512003405 | CDR2 Cerebellar degeneration-related 2 | 1.82 |
| gi|297291910 | RPS29 Ribosomal protein S29 | 1.80 |
| gi|511836837 | FAM71C Family with sequence similarity 71, member C | 1.73 |
| gi|511894864 | FN1 Fibronectin 1 | 1.73 |
| gi|355716083 | ReIA V-rel reticuloendotheliosis viral oncogene homolog A | 1.71 |
| gi|511888661 | UGDH UDP-glucose 6-dehydrogenase | 1.70 |
| gi|511829546 | HUS1 Hus1 homolog | 1.57 |
| gi|511902668 | DCLK1 Doublecortin-like kinase 1 | 1.56 |
| gi|472388445 | IRGM1 immunity-related GTPase family M 1-like | 1.56 |
| gi|511881226 | ENO3 Enolase 3, beta muscle | 1.56 |
| gi|402961872 | POU3F2 POU domain, class 3, transcription factor 2 | 1.55 |
| gi|472347917 | NINJ1 Ninjurin 1 | 1.52 |
| gi|511875241 | NSUN6 NOP2/Sun domain family, member 6 | 1.52 |
| gi|511910703 | KRT85 Keratin 85 | 1.52 |
| gi|511859527 | STX11 Syntaxin 11 | 1.49 |
| gi|511902630 | S100P S100 calcium binding protein P | 1.48 |
| gi|511846797 | ABCA1 ATP-binding cassette, sub-family A (ABC1), member 1 | 1.48 |
| gi|6841210 | ABRACL costars family ABRACL | 1.47 |
| gi|511844818 | B4GALT5 UDP-Gal:beta GlcNAc beta 1,4-galactosyltransferase, polypeptide 5 | 1.46 |
| gi|511898186 | TACO1 Translational activator of mitochondrial encoded cytochrome c oxidase I | 1.44 |
| gi|511986473 | RER1 RER1 retention in endoplasmic reticulum 1 homolog | 1.43 |
| gi|511932373 | MAN1A2 mannosidase, alpha, class 1A, member 2 | 1.43 |
| gi|511908385 | SH3BP5 SH3-domain binding protein 5 (BTK-associated) | 1.42 |
| gi|511943416 | COMMD9 COMM domain containing 9 | 1.42 |
| gi|511895957 | TMEM68 Transmembrane protein 68 | 1.42 |
| gi|511849632 | APOH Apolipoprotein H | 1.42 |
| gi|514558454 | ARF1 ADP-ribosylation factor 1 | 1.41 |
| gi|511935026 | GBP6 Guanylate binding protein family, member 6 | 1.41 |
| gi|512011829 | EHD1 EH-domain containing 1 | 1.41 |
| gi|533173825 | PCP4 Purkinje cell protein 4 | 1.40 |
| gi|511837380 | GCAT Glycine C-acetyltransferase | 1.40 |
| gi|512004618 | NIT1 Nitrilase 1 | 1.39 |
| gi|511870449 | TRAF6 tumor necrosis factor receptor-associated factor 6 | 1.39 |
| gi|511895854 | SLC8A2 Solute carrier family 8, member 2 | 1.38 |

(Continued)
| Accession No. | Gene          | Description                                      | Log2 ratios (infection/control) |
|--------------|--------------|--------------------------------------------------|--------------------------------|
| gi|512006423    | REEP6     | Receptor accessory protein 6                     | 1.38                           |
| gi|511916720    | UBE2L6    | Ubiquitin ISG15-conjugating enzyme E2L 6         | 1.37                           |
| gi|511833334    | USP48     | Ubiquitin specific peptidase 48                  | 1.37                           |
| gi|13775200     | SF3B5     | Splicing factor 3b, subunit 5                     | 1.36                           |
| gi|511848426    | TMCC3     | Transmembrane and coiled coil domains 3          | 1.36                           |
| gi|511983423    | ATP5D     | ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit | 1.35                           |
| gi|511921987    | RBM15D    | RNA binding motif protein 15B                    | 1.35                           |
| gi|511686041    | AP1G2     | Adaptor protein complex AP-1, gamma 2 subunit    | 1.35                           |
| gi|511842841    | FAM49A    | Family with sequence similarity 49, member A     | 1.35                           |
| gi|511829942    | SEMA3C    | Semia domain, immunoglobulin domain              |                                |
| gi|511915046    | CTSSK     | Cathepsin K                                     | 1.34                           |
| gi|511991226    | SMS       | Spermine synthase                                | 1.34                           |
| gi|511869470    | AEBP2     | AE binding protein 2                             | 1.34                           |
| gi|511832998    | SFN       | Stratifin                                        | 1.33                           |
| gi|30584771     | TUBA4A    | Tubulin, alpha 4a                                | 1.33                           |
| gi|511882364    | UNC13A    | Unc-13 homolog A                                 | 1.33                           |
| gi|864509599    | IL-6      | Interleukin-6                                    | 1.33                           |
| gi|14210488     | DCTN5     | Dynactin 5 (p25)                                 | 1.32                           |
| gi|511831346    | TNFAIP3   | Tumor necrosis factor, alpha-induced protein 3   | 1.32                           |
| gi|511916377    | CTS2L2    | Cathepsin L2                                     | 1.32                           |
| gi|119690561    | HSPE1     | Heat shock 10 kDa protein 1                      | 1.32                           |
| gi|511883884    | SUMF2     | Sulfatase modifying factor 2                     | 1.32                           |
| gi|545527366    | NRPB1     | Nuclear receptor binding protein 1               | 1.32                           |
| gi|511869866    | ETV6      | Ets variant gene 6 (TEL oncogene)               | 1.32                           |
| gi|511914585    | FAM83G    | Family with sequence similarity 83, member G     | 1.31                           |
| gi|355696495    | IRAK2     | Interleukin-1 receptor-associated kinase 2       | 1.31                           |
| gi|511904212    | DUS3I     | Dihydouridine synthase 3-like                    | 1.31                           |
| gi|511951618    | PPP1R12B  | Protein phosphatase 1, regulatory (inhibitor) subunit 12B | 1.30                           |
| gi|511901047    | C11orf68  | UPF0696 C11orf68 homolog                         | 1.30                           |
| gi|511906727    | CNP       | 2',3'-cyclic nucleotide 3' phosphodiesterase     | 1.30                           |
| gi|13385318     | KDELRE2   | KDEL endoplasmic reticulum protein retention receptor 2 | 1.30                           |
| gi|511834309    | BPGM      | 2,3-bisphosphoglycerate mutase                   | 1.30                           |
| gi|511992880    | GGH       | Gamma-glutamyl hydrolase                         | 1.30                           |
| gi|511825419    | PDLUM7    | PDZ and LIM domain 7                             | 1.29                           |
| gi|511886519    | WLS       | Wntless homolog (Drosophila)                     | 1.29                           |
| gi|511921959    | RAD54L2   | RAD54 like 2 (S. cerevisiae)                     | 1.29                           |
| gi|332856788    | PRMT1     | Protein arginine N-methyltransferase 1           | 1.28                           |
| gi|511910087    | LNP       | Limb and neural patterns                         | 1.28                           |
| gi|532072898    | POLR3H    | Polymerase (RNA) III (DNA directed) polypeptide H | 1.28                           |
| gi|511914328    | SAMD9I    | Sterile alpha motif domain containing 9-like     | 1.27                           |
| gi|511833014    | DHDDS     | Dehydrodolichyl diphosphate synthase             | 1.27                           |
| gi|511974382    | SERPINB2  | Serine (or cysteine) peptidase inhibitor, clade B, member 2 | 1.27                           |
| gi|355707086    | NFXB2     | Nuclear factor of kappa light polypeptide protein enhancer in B-cells 2 | 1.27                           |
| gi|511923903    | TNF-a     | Tumor necrosis factor alpha                      | 1.27                           |
| gi|511841350    | PARL      | Presenilin associated, rhomboid-like             | 1.27                           |
| gi|511862001    | FOXC3     | Forkhead box C3                                  | 1.27                           |
| gi|11345462     | SPCS3     | signal peptidase complex subunit 3               | 1.26                           |
| gi|511834349    | CEP41     | Centrosomal protein 41kDa                       | 1.26                           |
| gi|511857535    | HPSS5     | Hermansky-Pudlak syndrome 5                      | 1.26                           |
| gi|511876736    | PURG      | Purine-rich element binding protein G            | 1.26                           |
TABLE 2 | Continued

| Accession No. | Gene       | Description                                      | Log2 ratios (infection/control) |
|--------------|------------|--------------------------------------------------|---------------------------------|
| gi| 511846480 | GLIPR2     | GLI pathogenesis-related 2                       | 1.26                            |
| gi| 511837127 | C1orf23    | UPO44 transmembrane C1orf23 homolog             | 1.26                            |
| gi| 535707083 | NFkB1      | Nuclear factor of kappa light polypeptide protein enhancer in B-cells 1 | 1.26                            |
| gi| 511827086 | FLNB       | Filamin, beta                                   | 1.26                            |
| gi| 511899679 | LPCAT1     | Lysocephosphidylcholine acyltransferase 1        | 1.26                            |
| gi| 511876709 | MAK16      | MAK16 homolog (S. cerevisiae)                   | 1.25                            |
| gi| 511900404 | CD2BP2     | CD2 antigen (cytoplasmic tail) binding protein 2 | 1.25                            |
| gi| 511827872 | HMCES      | RIKEN cDNA 8430410A17 gene                      | 1.25                            |
| gi| 511857770 | MICAL2     | Microtubule associated monooxygenase, calponin and LIM domain containing 2 | 1.25                            |
| gi| 472384836 | HSPG2      | Heparan sulfate proteoglycan 2                  | 1.25                            |
| gi| 512007599 | CLCN7      | Chloride channel, voltage-sensitive 7           | 1.25                            |
| gi| 511900977 | YIF1A      | Yip1 interacting factor homolog A               | 1.25                            |
| gi| 472343859 | CD47       | CD47 antigen                                    | 1.25                            |
| gi| 511893624 | SCAMP4     | Secretery carrier membrane protein 4            | 1.25                            |
| gi| 511876596 | WHSC1L1    | Wolf-Hirschhorn syndrome candidate 1-like 1 (human) | 1.25                            |
| gi| 511850086 | SH3PDX2A  | SH3 and PX domains 2A                           | 1.25                            |
| gi| 511936255 | TST        | Thiosulfate sulfurtransferase (rhodanese)       | 1.25                            |
| gi| 511911865 | PHF11      | PHD finger protein 11                           | 1.25                            |
| gi| 564300780 | TRIM33     | Tripartite motif-containing 33                  | 1.25                            |
| gi| 511902036 | TAPBP      | TAP binding protein (tapasin)                   | 1.24                            |
| gi| 511882769 | MYO10      | Myosin X                                       | 1.24                            |
| gi| 511893223 | KANK1      | KN motif and ankyrin repeat domains 1           | 1.24                            |
| gi| 511907397 | MYL6B      | Myosin, light polypeptide 6B                    | 1.24                            |
| gi| 511883719 | TBL2       | Transducin (beta)-like 2                        | 1.24                            |
| gi| 511884480 | TOR3A      | Torsin family 3, member A                       | 1.23                            |
| gi| 511873534 | TRAF2      | TNF receptor-associated factor 2                | 1.23                            |
| gi| 555290040 | AK6        | Adenylate kinase isoenzyme 6                   | 1.23                            |
| gi| 511923928 | ANO9       | Anoctamin 9                                    | 1.23                            |
| gi| 511902555 | COL12A1    | Collagen, type XII, alpha 1                     | 1.23                            |
| gi| 511887963 | RAB3B      | RAB3B, member of RAS oncogene family            | 1.22                            |
| gi| 511906384 | DHX8       | DEAH (Asp-Glu-Ala-His) box polypeptide 8        | 1.22                            |
| gi| 488526784 | FCF1       | FCF1 small subunit (SSU) processome component homolog | 1.22                            |
| gi| 511844820 | PTGIS      | Prostaglandin I2 (prostacyclin) synthase        | 1.22                            |
| gi| 432094860 | TUBA3A     | Tubulin, alpha 3A                               | 1.22                            |
| gi| 511869593 | RAP2C      | RAP2C, member of RAS oncogene family            | 1.22                            |
| gi| 511926986 | GSTK1      | Glutathione S-transferase kappa 1               | 1.22                            |
| gi| 472384437 | GOLPH3     | Golgi phosphoprotein 3                         | 1.21                            |
| gi| 419017087 | RPRD1A     | Regulation of nuclear pre-mRNA domain containing 1A | 1.21                            |
| gi| 511906773 | BCL2L3     | BCL2-like 13 (apoptosis facilitator)            | 1.21                            |
| gi| 511841295 | ATP11B     | ATPase, class VI, type 11B                     | 1.21                            |
| gi| 511976077 | AKAP2      | Uncharacterized protein                         | 1.21                            |
| gi| 544446238 | PRMT5      | Protein arginine N-methyltransferase 5          | 1.21                            |
| gi| 511976770 | MNPP1      | Multiple inositol polyphosphate histidine phosphatase 1 | 1.20                            |
| gi| 511960500 | P2X4       | Purinergic receptor P2X, ligand-gated ion channel 4 | 1.20                            |
| gi| 511875437 | TENM3      | Teneurin transmembrane protein 3               | 1.20                            |
| gi| 511901261 | CDC45      | Cell division cycle associated 5               | 1.20                            |
| gi| 512002654 | TRABD      | TraB domain containing                          | 1.20                            |
| gi| 511855781 | TRMT6      | tRNA methyltransferase 6 homolog (S. cerevisiae) | 1.20                            |
| gi| 511839811 | E2F4       | E2F transcription factor 4, p107/p130-binding  | 1.20                            |
| gi| 511889285 | EEF1A1     | eukaryotic translation elongation factor 1 alpha 1 | 0.22                            |
| Accession No. | Gene        | Description                                      | Log2 ratios (infection/control) |
|--------------|-------------|--------------------------------------------------|---------------------------------|
| gi|511857546   | L-LDH     | L-lactate dehydrogenase                          | 0.25                            |
| gi|511861258   | RPH3A     | Rabphilin 3A                                     | 0.27                            |
| gi|511866746   | TDRD9     | Tudor domain containing 9                       | 0.33                            |
| gi|511911235   | SMURF1    | SMAD specific E3 ubiquitin protein ligase 1       | 0.37                            |
| gi|511974128   | COL14A1   | Collagen, type XIV, alpha 1                      | 0.38                            |
| gi|511869618   | MGP       | Matrix Gla protein                               | 0.39                            |
| gi|511836785   | GATA6     | GATA binding protein 6                           | 0.4                             |
| gi|511837372   | SH3BP1    | SH3-domain binding protein 1                     | 0.46                            |
| gi|511904960   | CLDN25    | Claudin 25                                       | 0.46                            |
| gi|512021328   | WWC3      | WWC family member 3                              | 0.48                            |
| gi|511863285   | ACSF3     | acyl-CoA synthetase family member 3              | 0.49                            |
| gi|11978906    | CCNT2     | Cyclin T2                                        | 0.5                             |
| gi|511842520   | CTPD1     | CTD phosphatase, subunit 1                       | 0.5                             |
| gi|511889359   | SULT1C2   | Sulforotransferase family, cytoplasmic, 1C, member 2 | 0.51                          |
| gi|511910161   | C1orf123  | Chromosome 1 open reading frame 123              | 0.51                            |
| gi|7657315     | LSM3      | LSM3-like protein, U6 small nuclear RNA associated | 0.51                            |
| gi|511864485   | DTD1      | D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae) | 0.52                            |
| gi|431906893   | KLF5      | Kruppel-like factor 5                            | 0.52                            |
| gi|511849400   | NHE-RF    | Na(+)/H(+) exchange regulatory cofactor NHE-RF    | 0.53                            |
| gi|257900516   | REEP1     | Receptor accessory protein 1                     | 0.53                            |
| gi|4723535383  | RASA2     | RAS p21 protein activator 2                      | 0.54                            |
| gi|511898541   | SKI       | Ski sarcoma viral oncogene homolog (avian)       | 0.54                            |
| gi|511890018   | CDK12     | Cyclin-dependent kinase 12                       | 0.55                            |
| gi|511910134   | AGPS      | Alkylglycerone phosphate synthase                 | 0.56                            |
| gi|51183096    | PPIC      | Peptidylprolyl isomerase C                        | 0.58                            |
| gi|511855302   | CDAN1     | Codanin 1                                        | 0.58                            |
| gi|511972404   | SMTNL2    | Smoothelin-like 2                                | 0.59                            |
| gi|51185637    | TADA2A    | Transcriptional adaptor 2A                       | 0.59                            |
| gi|511922352   | EFEMP1    | EGF containing fibulin-like extracellular matrix protein 1 | 0.59                          |
| gi|511893959   | KIAA1671  | Riken cDNA 29000026A02 gene                      | 0.6                             |
| gi|511873494   | CLIC3     | Chloride intracellular channel 3                 | 0.61                            |
| gi|511960748   | ZFP592    | Zinc finger protein 592                         | 0.61                            |
| gi|472387045   | PHPT1     | Phosphohistidine phosphatase 1                   | 0.62                            |
| gi|511876643   | RAB11FIP1 | RAB11 family interacting protein 1 (class I)     | 0.62                            |
| gi|472387045   | PHPT1     | Phosphohistidine phosphatase 1                   | 0.62                            |
| gi|511903670   | MKL1      | Megakaryoblastic leukemia (translocation) 1       | 0.63                            |
| gi|511848742   | LPRC45    | Leucine rich repeat containing 45                | 0.65                            |
| gi|194211939   | CACNB3    | Calcium channel, voltage-dependent, beta 3 subunit | 0.66                          |
| gi|511923560   | INPPS5J   | Inositol polyphosphate 5-phosphatase J           | 0.66                            |
| gi|511915011   | GABPB2    | GA binding protein transcription factor, beta subunit 2 | 0.66                          |
| gi|511875266   | DNAJC1    | DnaJ [Hisp40] homolog, subfamily C, member 1     | 0.67                            |
| gi|511836048   | LZTR1     | Leucine-zipper-like transcriptional regulator, 1 | 0.67                            |
| gi|511849384   | TMEM104   | Transmembrane protein 104                        | 0.67                            |
| gi|511838875   | WDSUB1    | WD repeat, sterile alpha motif and U-box domain containing 1 | 0.68                          |
| gi|555975747   | NSA2      | NSA2 ribosome biogenesis homolog (S. cerevisiae)  | 0.68                            |
| gi|511870441   | PRS5L     | Proline rich 5 like                             | 0.68                            |
| gi|511829786   | BCAP29    | B cell receptor associated protein 29            | 0.69                            |
| gi|511913605   | CCD97     | Coiled-coil domain containing 97                | 0.69                            |
| gi|301766733   | FAM127A   | FAM127-like                                     | 0.69                            |
| gi|511865423   | NDFIP2    | Nedd4 family interacting protein 2               | 0.69                            |
| gi|511881790   | CCD51     | Coiled-coil domain containing 51                | 0.7                             |
| Accession No. | Gene Description                      | Log2 ratios (infection/control) |
|--------------|--------------------------------------|---------------------------------|
| gi|511847011 | ALAD Aminolevulinate, delta-, dehydratase | 0.7 |
| gi|511833880 | LZIC Leucine zipper and CTNNBIP1 domain containing | 0.7 |
| gi|511903211 | Wars2 Tryptophanyl tRNA synthetase 2, mitochondrial | 0.7 |
| gi|545881843 | AGAP3 ArfGAP with GTPase domain, ankyrin repeat and PH domain 3 | 0.7 |
| gi|511841170 | PDLIM4 PDZ and LIM domain 4 | 0.71 |
| gi|511913696 | BLVRB Biliverdin reductase B [flavin reductase (NADPH)] | 0.71 |
| gi|511906284 | TMUB2 Transmembrane and ubiquitin-like domain containing 2 | 0.71 |
| gi|511876053 | ECHDC3 Enoyl CoA hydratase domain containing 3 | 0.71 |
| gi|512011090 | UPK3A Uroporphyrinogen 3A | 0.71 |
| gi|355707095 | NF-E2B Nuclear factor of kappa light polypeptide enhancer in B-cells inhibitor, beta | 0.72 |
| gi|511896151 | BLOC1S3 Biogenesis of lysosome-related organelles complex 1 subunit 3 | 0.72 |
| gi|73960021 | PTP4A2 Protein tyrosine phosphatase 4a2 | 0.72 |
| gi|51183659 | HSPB1 Heat shock protein 1 | 0.73 |
| gi|511826747 | CDH6 Cadherin 6, type 2, K-cadherin (fetal kidney) | 0.74 |
| gi|511854070 | RHBD1 Rhomboid 5 homolog 1 (Drosophila) | 0.74 |
| gi|512011195 | LPP LIM domain containing preferred translocation partner in lipoma | 0.74 |
| gi|511926830 | DPP7 Dipeptidyl-peptidase 7 | 0.74 |
| gi|511931993 | LRP8 Low density lipoprotein receptor-related protein 8 | 0.75 |
| gi|511862458 | MTRF1L Mitochondrial translational release factor 1-like | 0.75 |
| gi|511906536 | COASY bifunctional coenzyme A synthase isofrom X3 | 0.75 |
| gi|511890142 | SP2 Sp2 transcription factor | 0.75 |
| gi|511910713 | KRT7 Keratin 7 | 0.75 |
| gi|511887578 | TMEM126B Transmembrane protein 126B | 0.75 |
| gi|281349685 | ZFAND5 Zinc finger, AN1-type domain 5 | 0.75 |
| gi|511853128 | SRRM2 Serine/arginine repetitive matrix 2 | 0.75 |
| gi|511966770 | BDH2 3-hydroxybutyrate dehydrogenase, type 2 | 0.76 |
| gi|545501819 | TNRC6A Trinucleotide repeat containing 6a | 0.76 |
| gi|511880033 | TSC22D3 TSC22 domain family, member 3 | 0.76 |
| gi|2286213 | GNAQ Guanine nucleotide binding protein, alpha q polypeptide | 0.76 |
| gi|511836121 | MROPL40 Mitochondrial ribosomal protein L40 | 0.76 |
| gi|511834186 | NDUF10 Mitochondrial ribosomal protein L40 | 0.76 |
| gi|511830220 | PLA2G7 Phospholipase A2, group VII | 0.76 |
| gi|511896100 | CD3EAP CD3E antigen, epsilon polypeptide associated protein | 0.76 |
| gi|511907610 | R3HDM2 R3H domain containing 2 | 0.76 |
| gi|511843304 | COL4A1 Collagen, type IV, alpha 1 | 0.77 |
| gi|511878807 | ELF1 E74-like factor 1 | 0.77 |
| gi|511918709 | UBNL4 Ubiquilin 4 | 0.77 |
| gi|511890619 | SCFD1 sec1 family domain-containing 2 | 0.78 |
| gi|511830488 | CNPY3 Canopy 3 homolog (zebrafish) | 0.78 |
| gi|73965148 | ARF2 ADP-ribosylation factor 2 | 0.78 |
| gi|511909743 | ZBTB10 Zinc finger and BTB domain containing 10 | 0.78 |
| gi|511970268 | CD2AP CD2-associated protein | 0.78 |
| gi|511854253 | SLC12A6 Solute carrier family 12, member 6 | 0.79 |
| gi|511888766 | LUMCH1 LUM and calponin homology domains 1 | 0.79 |
| gi|511900684 | SLC16A1 Solute carrier family 16, member 1 | 0.79 |
| gi|511888312 | GPAM Glycerol-3-phosphate acyltransferase, mitochondrial | 0.79 |
| gi|511951833 | PCM1 Pericentriolar material 1 | 0.79 |
| gi|511848172 | KANK2 KN motif and ankyrin repeat domains 2 | 0.79 |
| gi|511925038 | MPRPS18B Mitochondrial ribosomal protein S18B | 0.79 |
| gi|511913714 | C19orf47 RIKEN cDNA 2310022A10 gene | 0.79 |

(Continued)
Table 2 continued

| Accession No. | Gene Description                                      | Log2 ratios (infection/control) |
|---------------|--------------------------------------------------------|---------------------------------|
| gi|511943046       | HS1BP3 HCLS1 binding protein 3                          | 0.8                             |
| gi|511919532       | NSL1 NSL1, MIND kinetochore complex component, homolog (S. cerevisiae) | 0.8                             |
| gi|511906743       | RABL3 Rab, member of RAS oncogene family-like 3         | 0.8                             |
| gi|511851382       | REPS1 RaBP1 associated Eps domain containing protein    | 0.8                             |
| gi|511914923       | RFX5 Regulatory factor X, 5 (influences HLA class II expression) | 0.8                             |
| gi|511857364       | GGA1 Goji-associated, gamma adaptin ear containing, ARF binding protein 1 | 0.8                             |
| gi|511845503       | RFFL Ring finger and FYVE like domain containing protein | 0.8                             |
| gi|511839695       | CDH11 Cadherin 11, type 2, OB-cadherin                  | 0.81                            |
| gi|511849128       | EVPL1 Envolakin                                        | 0.81                            |
| gi|511840215       | WWP2 WW domain containing E3 ubiquitin protein ligase 2 | 0.81                            |
| gi|511866085       | CKB Creatine kinase, brain                             | 0.81                            |
| gi|532066199       | RPL10A Ribosomal protein L10a                          | 0.81                            |
| gi|511853705       | CHTF18 CTF18, chromosome transmission fidelity factor 18 | 0.81                            |
| gi|511829476       | FIGNL1 Fidgetin-like 1                                 | 0.81                            |
| gi|511975646       | TFCP2 Transcription factor CP2                          | 0.81                            |
| gi|5118566781      | CACU1 CDK2 associated, cullin domain 1                  | 0.82                            |
| gi|511868239       | JUB Ajuba                                              | 0.82                            |
| gi|32880141        | DNAJA1 DnaJ homolog subfamily A member 1               | 0.82                            |
| gi|511885805       | STIM2 Stromal interaction molecule 2                    | 0.82                            |
| gi|511916124       | TCF12 Transcription factor 12                          | 0.82                            |
| gi|345786001       | NAA35 N(alpha)-acetyltransferase 35, NatC auxiliary subunit | 0.82                            |
| gi|511897376       | ARHGEF17 Rho guanine nucleotide exchange factor (GEF) 17 | 0.82                            |
| gi|511866734       | AHNAK AHNAK nucleoprotein isofrom 1                    | 0.83                            |
| gi|511856200       | KANK4 KN motif and ankyrin repeat domains 4            | 0.83                            |
| gi|301762790       | ZFP148 Zinc finger protein 148                         | 0.83                            |
| gi|511849083       | RHBPF2 Rhomboid 5 homolog 2 (Drosophila)               | 0.83                            |

FIGURE 2 | Subcellular localization of the DEPs in Mv.1.Lu cells infected with PS.

Interaction network provides clues for further illumination of the pathogenic mechanism and immunomodulation between CDV and the mink host.

Confirmation of the iTRAQ-MS Data by Western Blotting or Real-Time RT-PCR

To confirm the iTRAQ-MS data, we selected significantly changed proteins, including NFκB1, RelA, MHC-I, RPS29, and NFκBIB, which reliably cross-reacted with polyclonal antibodies to the corresponding human proteins for western blotting analysis. As shown in Figure 5A, the five representative proteins showed up-regulated or down-regulated expression in PS-infected Mv.1.Lu cells at 12 and 24 hpi (the original blots are shown in Figure S1), in accordance with the results of the iTRAQ analysis (Figure 5B). However, due to the limitation of the availability of antibodies to Neovison vison proteins, the confirmation of DEPs by immunoblotting was restricted. Thus, eight other proteins involved in the immune response process were selected and tested using real-time RT-PCR. As illustrated in Figure 5C, compared to the mock group, mRNA expression of TRAF6, TRAF2, IRAK4, IRAK2, NFκB2, CCL2, TNF-α, and IL-6 in PS-infected cells was significantly up-regulated in a time-dependent manner, which further confirmed the iTRAQ-MS data.

CDV Infection Induces the Phosphorylation and Nuclear Translocation of NF-κB P65 and the Degradation of IκB-α Proteins

The activation of the NFκB signaling pathway requires a series of cascade reactions, followed by the recruitment and phosphorylation of NFκB protein and subsequent translocation from the cytoplasm to the nucleus, as well as the proteasome degradation of IκB proteins, which ultimately induces the...
production of inflammatory cytokines and type I IFN. Therefore, the degradation of IκB proteins (typically represented by IκB-α) and phosphorylation and nuclear accumulation of the NF-κB proteins (typically represented by NF-κB P65) are distinct features of NF-κB signaling pathway activation. The network analysis of the DEPs involved in the immune response has preliminarily indicated the induction of the NF-κB pathway by PS infection. To further validate this speculation, Mv.1.Lu cells were infected with PS at 2 MOI, after incubation for 12 or 24 h, total proteins were collected to measure the expression of IκB-α and phosphorylated NF-κB P65 proteins. As shown in Figure 6A, compared to that in mock-infected cells, phosphorylated NF-κB P65 (P-P65) and IκB-α proteins were obviously increased and decreased in PS-infected cells, respectively (the original blots are shown in Figure S2). To assess whether PS infection facilitates NF-κB P65 nuclear translocation, Mv.1.Lu cells were infected with PS at an MOI of 2 or mock infected for 24 h. As shown in Figure 6B, NF-κB P65 showed evident nuclear translocation in PS-infected cells but remained in the cytoplasm of mock-infected cells. Further, to determine whether other CDV strains could activate NF-κB P65, the expression of phosphorylated p65 and IκB-α was also detected in CDV3-infected cells, which was increased and decreased, respectively (Figure 6A). Additionally, the nuclear translocation of NF-κB P65 was also observed in CDV3-infected cells (Figure 6B).

**DISCUSSION**

CDV infection commonly causes a severe lethal disease in carnivores, including minks. However, the molecular mechanisms involved in viral pathogenesis and host immune responses have not been fully elucidated. To date, no research has focused on differential proteome analysis of host cells in response to CDV infection. Therefore, we utilized an iTRAQ approach to identify the DEPs to further explore the pathogenic mechanism and immunomodulation of CDV infection through an analysis of the effects on host cell proteins in the mink. The present study is the first to use Mv.1.Lu cells for iTRAQ analysis due to their ability to efficiently support CDV replication in vitro, and this cell line is homologous to the natural host system of minks.

As a starting point, we determined an optimal time to perform proteomic analysis by monitoring the CPEs and analyzing the one-step viral growth curve in PS-infected Mv.1.Lu cells.
results revealed that PS infection induced serials CPE changes from 12 to 60 hpi, with the virus load exhibiting a plateau between 24 and 60 hpi. Considering the high virus load was maintained at 24 hpi and most cells showed little CPE, we conducted the following proteomic analysis based on 24 hpi.

In total, we identified 151 up-regulated and 369 downregulated proteins. Notably, an interesting observation in the present study was that CDV infection induces NF-κB activation in Mv.1.Lu cells. The NF-κB pathway regulates the expression of numerous immune system components to efficiently modulate the innate immune, inflammatory, and antiviral responses (Bose et al., 2003; Bours, 2005) and comprises a hub of cellular signal transduction pathways involved in host immune responses to viral challenge (Moynagh, 2005). So far, NF-κB has been reported as activated following various viral infections of porcine parvovirus (Cao et al., 2017), type 2 porcine circovirus (Wei et al., 2008), and herpes simplex type 1 (Patel et al., 1998). Additionally, NF-κB activation has previously been shown in MV infection (Helin et al., 2001) and was postulated as one of the mechanisms by which CDV might induce osteoclastogenesis (Mee and Sharpe, 1993). Moreover, NF-κB was subsequently demonstrated as induced by CDV (Onderstepoort strain) infection in human osteoclast precursors (Selby et al., 2006); however, these observations are all cases in humans or found in case of one single CDV strain. No reports of different CDV strains affecting NF-κB signaling in mink cells have been previously demonstrated. In the present study, nine NF-κB signaling regulators and downstream cytokines, including TNF-α, IRAK4, TRAF6, TRAF2, NFκB1, NFκB2, RelA, TNFαIP3, and VCAM1, were significantly up-regulated, and the NF-κB complex inhibitory protein IκB-β was obviously down-regulated. Further, KEGG pathway and network analyses of the DEPs involved in the immune response process also indicated the induction of the NF-κB signaling pathway. These results preliminarily indicated the activation of the NF-κB pathway by PS infection in Mv.1.Lu cells. More profound confirmation was observed by the detection of the phosphorylation and nuclear translocation of the NF-κB p65 subunit and the proteasome degradation of IκB-α protein in PS-infected Mv.1.Lu cells. Moreover, the activation of NF-κB p65 in CDV3-infected Mv.1.Lu cells also confirmed these findings. Together with the previous finding that NF-κB activation was found in human cells after CDV (Onderstepoort strain) challenge, these findings enriched the current knowledge of NF-κB activation by CDV infection, suggesting that NF-κB activation was not specific for a certain CDV strain or a certain species cells, but was suitable at least in part for several CDV strains and different species cells. Further validation is needed to compare the ability of various CDV strains to activate NF-κB signaling in other cell lines. In addition, some DEPs involved in the NF-κB pathway, containing IRAK4, RelA, TRAF6, NFκB1, and TNF-α together with IRAK2 and IL-6, were also identified as associated with measles and respiratory diseases, such as tuberculosis and pertussis, which are similar to the respiratory symptoms of CDV infection. The causative agent of measles is MV. In dogs and ferrets, CDV causes a disease that is highly similar to measles in humans (Hutchins et al., 2004; Perry and Halsey, 2004). Several theories have proposed that IL-6 is a critical inducer in the development of pagetic osteoclasts and bone lesions in Paget's disease induced by MV (Roodman et al., 1992; Ehrlich and Roodman, 2005). Mice expressing IL-6 and TNF-α in astrocytes suffer ataxia, inflammation and neurodegeneration after MV infection (Akassoglou et al., 1997; Raber et al., 1997). Therefore, the expression of these cytokines could contribute, in part, to mink pathological symptoms during CDV infection.

Furthermore, in the present study, NLR signaling pathway was closely associated with PS infection. This innate immunity signaling pathway may play essential roles in the production of type I interferon and in promoting inflammasome assembly upon virus activation (Kobayashi et al., 2002; Sabbah et al., 2009). Recent studies have suggested that the inflammasome NLRP3, known as the NOD-like-receptor-family, pyrin domain-containing 3, recognizes several RNA viruses, such as influenza virus (Allen et al., 2009; Ichinohe et al., 2010), VSV (Rajan et al., 2011), and EMCV (Poock et al., 2010). MV also activates the NLRP3 inflammasome, resulting in the caspase-1-mediated maturation of IL-1β (Zilliox et al., 2007; Komune et al., 2011). The NF-κB-induced activation of NLRP3 and pro-IL-1β gene expression is requisite for activating caspase-1 by the NLRP3 inflammasome to further regulate the secretion of the inflammatory cytokines IL-1β and IL-18 (Motta et al., 2015). However, whether there is signaling crosstalk between NF-κB activation and the NLR signaling pathway during CDV infection is an open question. Collectively, the findings suggested that activation of the innate immune NF-κB signaling pathway and the NLR signaling pathway was involved in mink immune responses against CDV infection, and the NF-κB signaling was associated with the pathological respiratory or other symptoms.
CDV infection could cause gastrointestinal symptoms or severe diarrhea after secondary infection. The NHERF, Na⁺/H⁺ exchanger regulatory factor, commonly locates or becomes enclosed in the intestinal brush border, thereby binding to the renal proximal tubule brush border Na⁺/H⁺ exchanger NHE3 protein, which is mainly responsible for the absorption of electroneutral salt in the intestine and is the most essential sodium absorptive transporter (Donowitz et al., 2005). Therefore, NHERF plays a crucial part in establishing and maintaining the functional integrity of the intestinal barrier. Previous reports have demonstrated that NHERF down-regulation leads to reduced Na⁺ absorption though affecting NHE3 activity, ultimately increasing intestinal epithelial permeability and the risk of inflammatory bowel disease (IBD) (Sartor, 2006; Strober et al., 2007). Butler et al. discovered that the dysregulation of sodium transit contributed to piglet diarrhea and the pathogenicity of TGEV after infection (Butler et al., 1974). In the present study, NHERF is significantly down-regulated, consistent with a previous observation of the significant down-regulation of NHERF1 (a member of NHERF family) protein in mink after CDV infection. Further research may answer these questions.

FIGURE 5 | Confirmation of the iTRAQ-MS data by western blotting or real-time RT-PCR. (A) Western blot analysis of NF-κB1, RelA, MHC-I, RPS29, and NFκBIB in PS-infected and control samples at 12 and 24 hpi. GAPDH was served as internal reference. (B) The intensity ratio of the corresponding bands (infection/mock) was quantified using ImageJ software and normalized against GAPDH. (C) Eight selected differently expression proteins related to NF-κB pathway were testified using real-time RT-PCR method. Each gene was performed in three independent experiments. The relative gene expression was calculated using 2-ΔΔCT model, representative of n-fold changes in comparison with mock-infected samples. Error bars represent the standard error for triplicate samples. *P < 0.05; **P < 0.01; ***P < 0.001. The data was analyzed by two-way ANOVA followed by Duncan’s test.
TGEV-infected PK-15 cells using quantitative proteomic analysis (An et al., 2014). Accordingly, the observation suggested that the down-regulation of NHERF by PS infection induced disordered salt and water transit through NHE3 dysfunction and further leaded to in the malfunction of the sodium pump in the intestinal barrier, ultimately resulting in gastrointestinal symptoms or severe diarrhea in infected minks. The present study provides a new view of the pathogenesis of diarrhea in CDV-infected minks.

Ubiquitination, the covalent conjunction of ubiquitin to the target protein substrate, is the first of two successive steps associated with ubiquitin–proteasome pathway, which is responsible for a wide variety of cellular functions, including the activation of NF-κB signaling and type I IFN pathways (Giechaskiel and Kessler, 1994; Giechaskiel and Chien, 2002). Accumulated evidence has suggested that various viruses have evolved complicated mechanisms to exploit or manipulate the ubiquitin–proteasome pathway (Gao and Luo, 2006). For example, the activation of the ubiquitin–proteasome pathway is required for influenza virus replication (Widjaja et al., 2010) and is also required other viruses, such as rotavirus (Lopez et al., 2011), human cytomegalovirus (Tran et al., 2010), and porcine reproductive and respiratory syndrome virus (Zhou et al., 2014). The present study identified TRAF2, TRAF6, UBE2L6 (E2 ubiquitin ISG15-conjugating enzyme), USP48 (an ISG15 specific isopeptidase enzyme) and TRIM33 (E3 ubiquitin- ligase) as up-regulated proteins involved in protein ubiquitination. TRAF2 and TRAF6 are well-recognized as signal transducers in the NF-κB signaling pathway that function together with a dimeric ubiquitin-conjugating enzyme complex to catalyze the synthesis of K63-linked polyubiquitin chains and ultimately activate IκB kinase (IKK) and the downstream NF-κB pathway (Deng et al., 2000; Yang et al., 2016). As an IFN-induced ubiquitin-like protein, ISG15 plays a role in immunomodulation and imparting a direct antiviral activity against a wide spectrum of virus (Pincetic et al., 2010; Dai et al., 2011; Sooryanarain et al., 2017).

Although the present study failed to detect the ISG15 protein, we identified the significantly up-regulated proteins UBE2L6 and USP48, which are strongly related to the ISGylation of ISG15. Similar to the mechanism of ubiquitination, ISGylation involves the sequential co-operation of E1, E2, E3 and an ISG15-specific isopeptidase enzyme (here identified as USP48) to facilitate ISG15 combination with target proteins for the execution of antiviral responses (Kroeker et al., 2013; Falvey et al., 2017). The tripartite-motif family (TRIM) of proteins plays essential roles in the innate immune responses to antimicrobial infections. TRIM33, a member of the TRIM family and previously known as transcriptional intermediary factor 1 gamma (TIF1-γ), functions in monocyte/macrophage mediated inflammation (Gallouet et al., 2017) and inflammation activation (Weng et al., 2014). Our results provided the first evidence of multiple differentially up-regulated immune-related proteins associated with protein ubiquitination in response to PS infection in Mv.1.Lu cells, indicating that ubiquitination appeared to be a pivotal regulatory mechanism in the immune responses to CDV infection in mink.

Apoptosis plays a role in regulating the pathogenesis of various infectious diseases, which oppositely affect viral pathogenesis by either restraining viral transmission or accelerating viral propagation by the release of the virus particles (Pastorino et al., 2009). In the present study, seven up-regulated proteins, including TNF-a, RelA, NFκB1, TRAF2, a-tubulin, CTSK (Cathespin K), and CTSV (Cathespin V), were identified as apoptosis-related, suggesting the induction of apoptosis in PS infection in Mv.1.Lu cells. CTSK and CTSV are associated with a mitochondria-dependent intrinsic pathway to trigger the apoptosis of host cells, while TNF-a participates in an extrinsic receptor-mediated pathway (Benedict et al., 2002). This finding was consistent with previous reports showing that CDV induces apoptosis in the cerebellum and lymphoid...
tissues of the natural infection of dogs and in Vero cells in vitro (Moro et al., 2003; Del Puerto et al., 2010, 2011). The mechanisms of apoptosis in the pathogenesis of CDV have not yet been clearly illuminated, and the extensive study of these proteins should enhance the current understanding of the mechanisms underlying apoptosis regulation during CDV infection.

In summary, the present study provides the first overview of the protein alterations in CDV-infected Mv.1.Lu cells using iTRAQ analysis. The identification of differently expressed proteins reflects a comprehensive interaction network of Mv.1.Lu cells and CDV during infection. Although some significantly regulated proteins were suggested to be related to the pathological symptoms and the immune responses to CDV infection, further functional elucidations are needed to clarify the pathogenic mechanisms and the immune responses to additionally identify new therapeutic targets for preventing CDV infection.

AUTHOR CONTRIBUTIONS
MT and SC designed the study; MT, LY, NS, and YC performed the experiments; ZC and JW analyzed the data; SL, PL, and YS prepared the figures and tables; MT wrote the manuscript.

ACKNOWLEDGMENTS
This study was supported by Agricultural Science and Technology Innovation Project (No. 20150201006NY) and Jilin Provincial Science and Technology Development Project (No. 20150520128JH).

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02564/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.