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Mammalian Genome
Genes and Phenotypes

Gene expression profiling in *Salmonella* Choleraesuis-infected porcine lung using a long oligonucleotide microarray

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Abstract

Understanding the transcriptional response to pathogenic bacterial infection within food animals is of fundamental and applied interest. To determine the transcriptional response to *Salmonella enterica* serovar Choleraesuis (SC) infection, a 13,297-oligonucleotide swine array was used to analyze RNA from control, 24-h postinoculation (hpi), and 48-hpi porcine lung tissue from pigs infected with SC. In total, 57 genes showed differential expression ($p < 0.001$; false discovery rate = 12%). Quantitative real-time PCR (qRT-PCR) of 61 genes was used to confirm the microarray results and to identify pathways responding to infection. Of the 33 genes identified by microarray analysis as differentially expressed, 23 were confirmed by qRT-PCR results. A novel finding was that two transglutaminase family genes (*TGM1* and *TGM3*) showed dramatic increases in expression postinoculation; combined with several other apoptotic genes, they indicated the induction of apoptotic pathways during SC infection. A predominant T helper 1-type immune response occurred during infection, with interferon γ (IFNG) significantly increased at 48 hpi. Genes induced by IFNs (*GBP1*, *GBP2*, *C1S*, *C1R*, *MHC2TA*, *PSMB8*, *TAP1*, *TAP2*) showed increased expression during porcine lung infection. These data represent the first thorough investigation of gene regulation pathways that control an important porcine respiratory and foodborne bacterial infection.

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Introduction

Controlling gram-negative bacterial infections in swine is important for animal health and human food safety. Yet the global transcriptional response to pathogenic infections in the pig has only recently begun to be profiled systematically using microarray technology (Afonso et al. 2003; Ledger et al. 2004; Li et al. 2004; Miller and Fox 2004; Moser et al. 2004; Dvorak et al. 2005; Niewold et al. 2005) or large-scale quantitative PCR methods (Baltes et al. 2004; Raymond and Wilke 2004; Royaee et al. 2004; Dawson et al. 2005). These studies identified immunologic genes involved in the host's response to different pathogen infections, which have contributed to a better understanding of molecular pathways relating to health and disease in pigs. Microarray technology can analyze thousands of transcripts at once, providing a more comprehensive view of the altered transcriptome compared with gene-by-gene-based methods. Such microarray data, given appropriate bioinformatic analyses, can also contribute to mapping genes with unknown function to relevant immune response pathways.

In pigs, the lung is continuously exposed to microbial challenges, and a number of cytokines have been detected in lung tissue infected with porcine reproductive and respiratory syndrome (PRRS) virus, *Actinobacillus pleuropneumoniae*, and *Mycoplasma* (Labarque et al. 2003; Baltes et al. 2004; Chung et al. 2004; Rodriguez et al. 2004; Thanawongnuwech et al. 2004). The pulmonary epithelium has been shown to be a rich source of some classes of cytokines and other immune proteins (Strieter et al. 2002). Investigations of gene expression changes in lung infection models have been conducted in mice and rabbits (Nelson et al. 2000; Geimonen et al. 2002; Jesmin et al. 2004; Jeyaseelan et al. 2004; Yanagisawa et al. 2004).

As one of the few bacteria that cause both pneumonia and diarrhea, *Salmonella enterica* serovar Choleraesuis (*S.* Choleraesuis) is the most often identified causative agent of swine salmonellosis. *S.* Choleraesuis initially causes a transient intestinal infection that can spread to the bloodstream, affecting various organs including the lungs (Gray et al.1995). Studies have reported that one of the most consistent systemic lesions in swine infected with *S.* Choleraesuis is interstitial pneumonia (Hanna et al. 1979; Reed et al. 1986; Turk et al. 1992; Anderson et al., 2000). Transmission of *Salmonella* through the upper respiratory tract may be as frequent as the classic fecal-oral route, as lung infection is suggested to be the most important cause for porcine septicemia (Fedorka-Cray et al. 1995). Although pneumonia in swine can arise from infection with *S.* Choleraesuis alone, coinfections with enzootic organisms such as *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* can intensify the severity of the respiratory disease.

In addition to serving as a source of protein in the human diet worldwide, the pig is a preferred model for biomedical research, including studies on pulmonary pathophysiology (Brown et al. 2002). Furthermore, swine are considered the most promising potential organ donor, e.g., for pulmonary xenotransplantation (Cantu et al. 2004). Before transplantation, *Salmonella* is one of the bacterial species that must be excluded from the pig organ source (Onions and Witt 2000). Thus, understanding the immune response to control *Salmonella* infection is not only important for pig production but also for human consumption and porcine organ transplantation.

The response to *S.* Choleraesuis in porcine lung has not been analyzed by gene expression profiling, which is a promising approach to understanding the molecular mechanisms of the host response in this tissue. Recently, Qiagen-Operon and the USDA NRSP8 (National Research Support Project-8) Swine Genome community collaborated to develop a novel oligonucleotide set. The set of 13,297 oligonucleotides (porcine AROS 1.0/1.0 Extension; www.qiagen.com) represents porcine cDNAs and expressed sequence tags (ESTs) from a variety of tissues. They were designed from TIGR Tentative Consensus (TC) cDNA sequences (SsGI Release 5.0, http://www.tigr.org/tigr-scripts/tgi/T_release.cgi?species=pig). In a validation study, microarrays containing each 70-mer oligonucleotide were hybridized with targets from porcine adult liver, lung, muscle, or small intestine. Transcriptome analyses showed that 11,328 (92%) of the oligonucleotides demonstrated expression in at least one tissue (Zhao et al. 2005). In this study, we used this Qiagen-NRSP8 long oligonucleotide array and quantitative real-time PCR (*qRT-PCR*) to examine gene expression changes in the porcine lung during acute *S.* Choleraesuis (SC) infection. We expect that a significant response to *Salmonella* infection will be an increase in steady-state levels of RNA and that the Qiagen-NRSP8 array will be able to detect both known and unknown gene expression changes in infected lung compared with uninfected lung.

Materials and methods

Tissue collection. Piglets were raised in isolation facilities from 10 days to 7 weeks of age at the National Animal Disease Center. Bacteriologic culture of rectal swabs was performed twice to confirm that all pigs were free of *Salmonella* spp. Pigs were randomly assigned to three groups in the experiment. After necropsy, cultures of portmortem pig tissues were performed to prove *in vivo* bacterial burden. Three control pigs were necropsied on experimental day −3. On day 0, pigs in the infected group were intranasally challenged with 1×10^9 colony-forming units (CFU) of *S.* Choleraesuis χ3246. Lung samples were aseptically collected from control, three pigs at 24 h postinoculation (hpi), and 48 hpi and immediately frozen in liquid nitrogen for future RNA isolation.

Porcine oligonucleotides and microarray characteristics. The set of array oligonucleotides (porcine AROS 1.0/1.0 Extension; www.qiagen.com) represents porcine cDNAs and ESTs and were designed from TIGR Tentative Consensus (TC) cDNA sequences (SsGI Release 5.0, http://www.tigr.org/tigr-scripts/tgi/T_release.cgi?species=pig). In total, there are 13,297 pig-specific ~70-mer oligonucleotides. A recent BLAST-based annotation of these

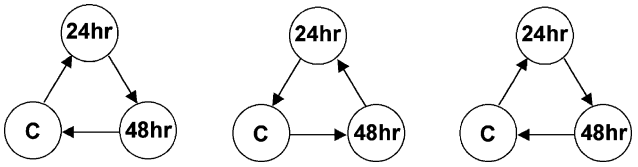

Fig. 1. Loop design of the microarray experiments. Each circle represents a single animal. Arrows represent an individual slide hybridization. Arrowheads indicate samples labeled with Cy5, and arrowends represent samples labeled with Cy3; dye balance was achieved for each biological sample type. In total, nine animals were randomly assigned to three loops so that each loop included one animal from each of the three infection status categories. C: samples from control pigs; 24hr: samples from 24-h infected pigs; 48hr: samples from 48-h infected pigs.

oligonucleotides showed that they match 8541 unique human or mouse RefSeq or pig annotated gene NCBI accession numbers (Zhao et al. 2005). Gene Ontology (GO) terms were assigned to porcine oligonucleotides by using orthologous human/mouse RefSeq accessions and ''Build Simplified Ontology'' functions in GeneSpring 6.1 or by novel PERL scripts (Orley et al., unpublished). In addition, there were 10 positive control genes and 12 negative controls including 5 *Arabidopsis* genes known to have minimal cross-hybridization with mammalian transcripts (for more information, see www.qiagen.com). For simplicity, we refer to this oligonucleotide set as the Qiagen oligonucleotide set and the microarray produced (see below) as the Qiagen-NRSP8 array.

The synthesized oligonucleotides were spotted at the University of Minnesota microarray facility. Each oligonucleotide was spotted ($\sim$ 0.5–1 nl at 20 μM) on Corning GAPS II slides with 240-μm spacing. Oligonucleotides were UV crosslinked to the slides after spotting. Each pig-specific oligonucleotide was spotted one time and each control gene was spotted 16 times. There are 48 subgrids spotted on the slide: 24 subgrids contain positive controls and the other 24 contain negative controls.

Experimental design. A loop design was used to provide direct, within-slide comparisons of the treatments of interest. Nine animals were randomly assigned to three loops so that each loop included one animal from each of the three infection status categories (control, 24 hpi, and 48 hpi). Dye balance was used throughout so that each RNA sample was measured once with each dye. A total of 9 slides (3 slides per loop) were used to obtain a total of 18 measurements from the three control and six infected pig lungs. Figure 1 shows the loop design for the above 9 samples.

RNA preparation and array hybridization. Total RNA from approximately 200 mg of frozen tissue was isolated by the RNeasy Midi kit with on-column RNase-free DNase digestion (Qiagen, Valencia, CA) according to the manufacturer's protocol for all samples. Briefly, frozen tissues were homogenized in liquid nitrogen using a mortar and pestle. RNA purity and integrity were determined by the Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies, Palo Alto, CA) analysis and by UV spectroscopy and denaturing gel electrophoresis. Target labeling, hybridization, and image processing were conducted essentially as described in Zhao et al. (2005). Briefly, amino-allyl-dUTP-labeled single-strand cDNA was prepared from 30 μg RNA and reverse-transcribed in the presence of 6 μg random and 1 μg oligo-dT primers (Invitrogen/Life Technologies, Carlsbad, CA). RNA was hydrolyzed, and first-strand cDNA was mixed with either Cy3 or Cy5 NHS-ester (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated 1 h at room temperature. Cy3- and Cy5-labeled cDNA were purified on QIAquick PCR purification columns (Qiagen), precipitated by ETOH, and hybridized to the microarray at 42°C for 12–16 h in hybridization solution containing 50% formamide, 5× SSC, 0.1% SDS, and 0.2 μg/μl sheared salmon sperm DNA. After hybridization, microarray slides were washed with 2× SSC, 0.1% SDS for 5 min at 42°C, 0.1× SSC for 1 min at room temperature four times, and 0.01× SSC for 10 sec at room temperature, and finally dried by centrifugation. Slides were scanned at 10-μm resolution using a ScanArray 5000 scanner. The intensities of spots on each image were quantified by ImaGene 5.1 software, and data were saved as .txt files for further analyses.

Differential gene expression analyses. Chip-wide LOWESS normalization was used to normalize the log Cy3 and log Cy5 signal intensities within each slide, and signal intensities from all slides were aligned to a common median signal intensity following LOWESS normalization (Dudoit et al. 2002; Yang et al. 2002). Differential gene expression analysis of the normalized data was conducted using mixed-model analyses in SAS (SAS Institute, Cary, NC). The mixed model included fixed effects for dye (Cy3 or Cy5), time point (control, 24 hpi, and 48 hpi), and random effects for slide and animal. An approximate F test for differences in expression across the three time points was conducted as part of the mixed-model analysis for each gene. Satterthwaite's method was used to determine the denominator degrees of freedom for each approximate F test. False discovery rate (FDR) (q values) was

calculated according to the Storey and Tibshirani method (Storey and Tibshirani 2003). Microarray results from this study were submitted to the NCBI GEO database (accession numbers: Platform, GPL1881; Samples, GSM 43177-43183, 43228; Series, GSE2339).

Cluster analyses. An unsupervised learning procedure in ''Find classes'' in Gene Cluster 2 (Reich et al. 2004) was used to perform the cluster analyses based on the normalized expression levels for significantly differentially expressed genes.

Functional annotation of gene expression data. The Database for Annotation, Visualization and Integrated Discovery (DAVID 2.0 and 2.1 beta) provides a comprehensive set of tools to visually summarize gene annotation (Dennis et al. 2003; http://david.abcc.ncifcrf.gov/). Functional annotations were pursued for differentially expressed genes selected based on a maximum pairwise p value of 0.001 for at least one of the three possible comparisons: 24 hpi vs. control; 48 hpi vs. control; or 48 hpi vs. 24 hpi. The TC accession numbers were first updated from TIGR 5.0 to TIGR 11.0 and the Human Gene ID were noted so that the DAVID analysis software could be interrogated. The data were then mined for each of the above comparisons individually using DAVID 2.0 and 2.1 beta. Some genes are duplicated in each list but the total number of independent genes analyzed was 109. Complete data and results for each comparison are shown in Supplementary Table 3, which is an Excel workbook with each gene list documented.

Quantitative RNA analyses using real-time PCR methodology (qRT-PCR). Synthesis of cDNA was performed using Superscript reverse transcriptase (Invitrogen) and oligo-dT with 5–10 μg RNA as previously described (Royaee et al. 2004; Dawson et al. 2004, 2005). Individual RNA samples from the three animals at each time point in the microarray experiment were measured in duplicate by qRT-PCR. Using the Stratagene Brilliant kit (La Jolla, CA), qRT-PCR was performed on 100 ng RNA equivalent at 25 μl/reaction/well on an ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Amplification conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min; then 4°C. All probes and primers were designed using Primer Express (Applied Biosystems) software and nucleotide sequences were obtained from GenBank or the TIGR porcine EST database. The sequences for these probes and primers appear in our previous publications (Rodriguez et al. 2004; Dawson et al. 2005) or in the Porcine Immunology and Nutrition (PIN) database (http://www.ars.usda.gov/Services/Services.htm?docid=6065). Gene names and abbreviations are based on human gene nomenclature per International Society for Animal Genetics (ISAG) guidelines. One gene, *MYD88*, which was not on the array, was also tested in the qRT-PCR analysis as part of the panel. We also assayed *RPL32* RNA levels to use for normalization. The relative quantitative gene expression level was evaluated using the comparative C_t method (Dawson et al. 2004). The ΔC_t values were calculated by subtracting the *RPL32* C_t value for each sample from the target C_t value of that sample. The duplicates for each sample were averaged, ΔC_t values were calculated, and pairwise t tests were conducted on these averages to identify genes differing in expression between control, 24 hpi, and 48 hpi data using JMP5.1 (Computer Associates, Cary, NC). In all qRT-PCR analyses, $p < 0.05$ was considered significant.

Results

Identification of differentially expressed genes in porcine lungs during* S. *Choleraesuis infection. Seven-week-old *Salmonella* spp.-free piglets were intranasally challenged with 1×10^9 CFU *S.* Choleraesuis χ3246. Lung samples were aseptically collected from pigs at necropsy at 24 and 48 hpi and from uninfected control pigs. The two time points should be informative for the pathways controlling innate response as that is the time when the fever starts (24 h) and peaks (48 h). Only the infected pigs showed clinical symptoms such as fever and *Salmonella* shedding, demonstrating a successful infection in these animals (data not shown). Isolated mRNA from lung samples was transcribed and labeled with Cy3 or Cy5 and used for hybridization experiments (Fig. 1). Statistical analyses, using linear models based on the normalized expression levels, were performed to determine significantly differentially expressed genes. Of the 13,297 oligonucleotide targets, 57 genes showed differential expression at the $p < 0.001$ level, while 1300 genes showed differential expression at the $p < 0.05$ level (Supplementary Table 1). The estimated FDR for these threshold values was 12% and 27%, respectively. The top 25 genes (within the 57-gene list) with the largest fold change between noninfected control RNA and lung RNA at 48 hpi are shown in Table 1. One gene, protein-glutamine glutamyltransferase E3 (*TGM3*), showed the most dramatic increase after the infection (>1000-fold change be-

Table 1. Major differentially expressed genes in lung from pigs experimentally inoculated with *S.* Choleraesuis

Gene symbol	*Oligonucleotide ID*	*TC accession*	*p value*	*Fold induction*		
				24 h/control	*48 h/control*	*48 h/24 h*
TGM3	SS00003340	TC52923	0.00002	327.5	1320.5	4.03
AGR_L_2875p	SS00010184	TC62447	0.013	0.88	39.1	44.3
TGM 1	SS00002189	TC49370	0.04	19.9	31.4	1.6
FLJ3181	SS00008710	TC58693	0.007	10.0	15.1	1.5
No good matches	SS00010543	TC196868	0.0161	4.1	14.2	3.5
FLJ39383	SS00008113	TC54983	0.007	3.4	13.0	3.7
SPP1	SS00012722	TC61113	0.04	5.0	12.8	2.5
KIAA2028 protein	SS00007640	TC51899	0.03	5.4	11.8	2.1
GBP3	SS00006033	TC61663	0.007	2.0	10.7	5.3
HSPA2	SS00010881	TC180964	0.02	4.2	10.5	2.5
GBP1	SS00003495	TC53560	0.001	2.8	9.9	3.9
GBP2	SS00005079	TC182080	0.0007	1.2	8.4	6.5
mspC	SS00010712	TC54745	0.02	7.1	7.7	1.1
NADH5	SS00010667	TC181125	0.02	7.8	6.5	0.8
INDO	SS00006633	TC63855	0.04	1.8	6.3	3.4
Sus scrofa clone *TBI 3-7-3* mRNA	SS00012346	TC58724	0.04	3.6	6.2	1.7
FLJ21511	SS00005748	TC60601	0.03	30.3	5.8	0.1
Antigen *NY-CO-25*	SS00009197	TC61428	0.0001	2.4	5.7	2.3
IRF1	SS00002396	TC49895	0.0002	2.6	5.5	2.1
No good matches	SS00010282	BF079221	0.01	0.4	5.4	11.5
MHC2TA	SS00000824	TC49657	0.01	1.0	4.9	4.9
HSPA6	SS00000953	TC53194	0.002	1.6	4.6	2.9
KV1.2	SS00009663	NP275185	0.01	0.76	4.6	6.1
PA28	SS00008052	TC54486	0.0001	1.5	4.6	3.1
CD114	SS00003377	TC53068	0.01	3.1	4.6	1.5

These are the top 25 differentially expressed genes ($p < 0.05$) with the largest-fold change in lung tissue from the noninfected controls to 48 h following experimental inoculation with *S.* Choleraesuis.

tween control and 48-hpi samples). Interestingly, a second member of this family, *TGM1*, was also highly elevated (31-fold) at 48 hpi.

Cluster analyses of porcine genes responding to* S. *Choleraesuis pulmonary infection. To define sets of genes with similar response to *Salmonella* infection, we clustered the 57 genes that showed differential expression at the $p < 0.001$ level (Fig. 2A) using Gene Cluster 2. All genes were classified into one of five clusters containing 7–15 genes (Supplementary Table 2). When control lung RNA was compared with lung RNA collected at 48 hpi, 24 genes were identified with decreased RNA expression levels and 33 genes with increased RNA levels. A molecular function classification of these 57 genes showed that a majority of the genes with elevated expression levels during infection (17 of 33, 52%) had annotations related to immune response, apoptosis, and tumorigenesis (Fig. 2B).

Further annotation of differentially expressed genes. Additional annotation was performed using the online NIH-DAVID software package on an expanded list of genes that demonstrated differential expression between at least two time points (pairwise $p < 0.001$; see Materials and methods section). Within this group, there were 11 genes (5 with increased expression, 6 whose expression decreased) compared at 24 hpi vs. control, 81 genes (25 decreased, 55 increased) compared at 48 hpi vs. control, and 53 genes (27 decreased, 26 increased) compared at 48 hpi vs. 24 hpi (Supplementary Table 3); some genes are counted more than once in these comparisons. Figure 3 shows the categories of genes differentially expressed following lung infection with *S.* Choleraesuis. The majority of annotated genes were found to have increased expression at 48 hpi relative to the controls (Fig. 3A) and prominently included functions related to defense and stress responses. On the other hand, annotations by DAVID indicated that genes associated with reactive oxygen metabolism decreased in expression during infection. Expression of the JAK-STAT cascade genes also increased at 48 hpi. Expression of genes in only a few classes changed at 48 hpi relative to 24 hpi; these included genes involved in defense and stress responses and kinase activity (Fig. 3B). No genes with

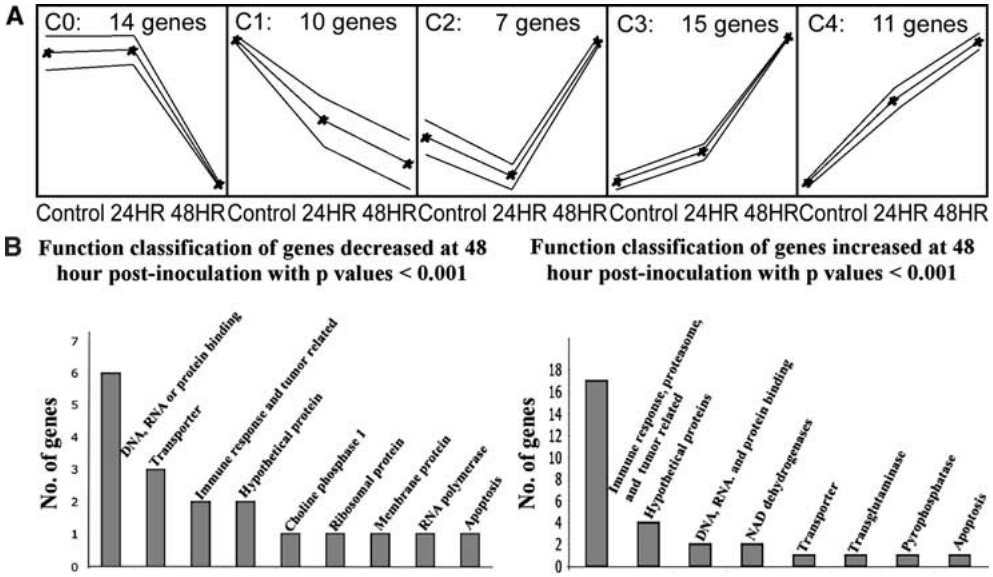

Fig. 2. Unsupervised cluster analysis (**A**) and functional classification (**B**) of 57 genes with differential expression ($p < 0.001$) during pulmonary infection with *S.* Choleraesuis. (**A**) Cluster analysis with the number of genes in each cluster (from 7 to 15) shown within each box (as determined by GeneCluster 2.0). The middle trend line in each graph is relative centroid values for each cluster. (**B**) Functional classification of genes with decreased expression (left two clusters in 1A) and increased expression (right three clusters in 1A) is indicated independently in two bar graphs.

GO terms that were recognized by DAVID had expression levels that were statistically different between 24 hpi and controls.

qRT-PCR results of selected swine genes from* S. *Choleraesuis-infected lungs. We chose 61 genes for qRT-PCR analysis, including *MYD88* that is not on the Qiagen-NRSP8 array (Table 2). These 61 genes were chosen for two purposes: (1) to validate expression patterns during infection for specific genes identified in the microarray studies, and (2) to characterize more fully the lung immune response to *S.* Choleraesuis at the molecular level. Thus, genes that did not show statistically significant differences in expression during infection by the microarray analysis were also selected for qRT-PCR analysis. These included genes involved in inflammatory response (through *NFKB* or through induction of *IFNG*), inhibition or activation of macrophages, neutrophil recruitment, B-cell responses, T helper 1 (Th1) and Th2 responses, and apoptosis.

The qRT-PCR results are shown in Table 2 and Fig. 4. Of the 61 genes assayed, 33 were chosen to confirm differential expression ($p < 0.05$ level) found in our microarray analyses. The qRT-PCR results confirmed the expression pattern for 23 of these 33 genes (Table 2, top). Furthermore, our PCR results also showed significant differential expression for an additional six genes (Table 2, middle). These genes had expression patterns in the qRT-PCR that were consistent with the microarray results, but for which statistical significance in the microarray analyses did not reach the $p < 0.05$ significance level. The single gene assayed that was not on the microarray (*MYD88*) showed no differential expression in qRT-PCR. Of the remaining 21 genes, neither the microarray nor the qRT-PCR data showed significant expression differences during infection (Table 2, bottom). These combined data were used to interpret the immune response in the lung during infection based on known pathways represented by these genes using functional annotation of these genes in pig and other species (see Figs. 3 and 4 and Discussion).

Discussion

In this study the Qiagen-NRSP8 oligonucleotide array was used to profile gene expression in noninfected and SC-infected porcine lung. We found 1300 genes ($p < 0.05$) that were increased or decreased in expression within the first 48 hpi. For the 57 genes with $p < 0.001$, we used software to cluster genes based on their expression pattern similarity (Fig. 2A). Many (52%) of the genes that showed an increase in gene expression have been annotated as members of an immune pathway (Fig. 2B). This is much higher that one would expect in a random selection because across the entire Qiagen-NRSP-8 array there are 526 (6%) annotations for immune function, apoptosis, and cancer of 8937 total anno-

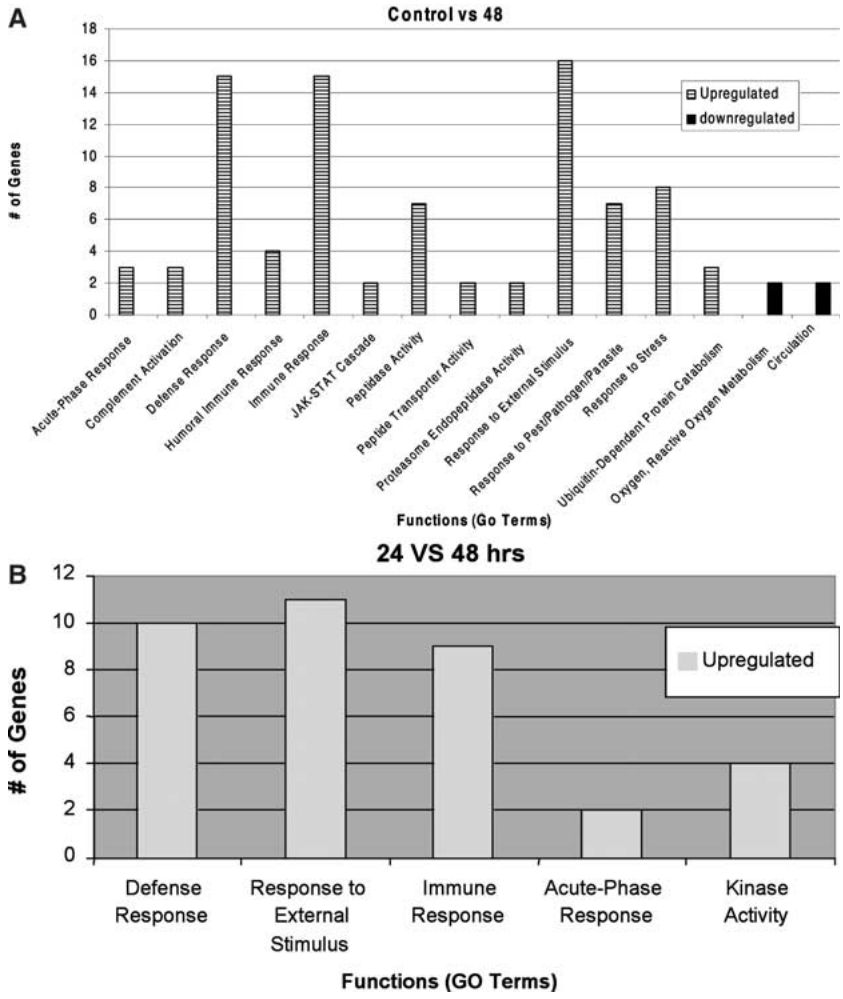

Fig. 3. Gene Ontology (GO) annotation of 109 differentially expressed genes (pairwise $p < 0.001$) revealed using DAVID analyses. The TC assignments were updated from TIGR 5.0 to TIGR 11.0 (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=pig) to verify gene assignments for the respective oligonucleotides and to select the relevant human GenBank reference sequence to use to probe the DAVID Bioinformatic Resources (http://david.abcc.ncifcrf.gov/) as detailed in Supplementary Table 3. (**A**) Genes whose RNA abundance increased or decreased significantly at 48 hpi compared with control. (**B**) Genes whose RNA abundance increased significantly at 48 hpi compared with 24 hpi.

tations (Zhao et al. 2005). However, the genes that exhibited a significant decrease in expression were much less likely to have an immune annotation. Genes whose expression was induced during infection are clearly important in immune response, but genes repressed during the early immune response are also of interest. Our data are the first report of an investigation of the transcriptional response of the porcine lung to an infection that used a long oligonucleotide microarray. While these data are most useful for describing the response to *S.* Choleraesuis in pig lung, the gene expression information from this study should also be useful for studying the immune response to other gram-negative infections in the pig. The differentially expressed genes could be candidate genes to investigate association with disease resistance traits for potential improvement of pig health through molecular breeding strategy.

We validated the expression pattern for a number of these genes using qRT-PCR. Our qRT-PCR analysis of 60 genes present on the microarray showed statistically significant differential expression for 29 genes and no change in expression for another 31 genes. Most of these 60 genes showed the same expression pattern in the qRT-PCR as that observed in the microarray results (increased, no change, or decreased expression during infection), although for some differentially expressed genes, the expression differences detected by microarray data did not reach a statistically significant level. Of the 33 genes tested that had differential expression in the microarray ($p < 0.05$), ten were not confirmed by the qRT-PCR analyses. As the FDR for these genes was estimated to be up to 27%, the observed confirmation rate of 70% was close to expectation. Furthermore, for the nine genes tested (*TGM1*, *TGM3*, *SPP1*, *GBP1*, *GBP2*, *INDO*, *NY-CO-25*, *IRF1*, *MHC2TA*) that belonged to the group of 57 genes with highest statistical confidence ($p < 0.001$, FDR = 12%), only *SPP1* was not confirmed by qRT-PCR. An additional six genes with no significant expression differences in the microarray analysis showed significant differences in qRT-PCR (Table 2, middle). It has been reported that because of the kinetic differences

Table 2. Quantitative PCR results for gene expression in control and *S. choleraesuis*-infected lungs

		Expression level ($\Delta C_t^{\dagger} \pm$ SD)[‡]			Confirm microarray results?
Oligonucleotide ID	*Gene symbol*	*Control*	*24 h*	*48 h*	
SS00000615	*IFNG*	12.3 ± 0.4[a]	11.1 ± 0.4[ab]	9.9 ± 1.6[b]	These genes had the same expression direction and reached statistically significant levels in both microarray and qRT-PCR results.
SS00000872	*CASP1*	4.8 ± 0.4[a]	4.3 ± 0.7[ab]	3.5 ± 0.3[b]	
SS00000824	*MHC2TA*	7.2 ± 0.7[a]	7.4 ± 1.0[a]	4.9 ± 0.7[b]	
SS00000483	*ITGB2 (CD18)*	6.0 ± 0.1[a]	6.0 ± 0.1[a]	5.2 ± 0.5[b]	
SS00002396	*IRF1*	6.1 ± 0.8[a]	4.9 ± 0.8[a]	3.1 ± 0.5[b]	
SS00006608	*SOCS1*	15.3 ± 0.8[a]	13.1 ± 1.3[b]	12.7 ± 0.3[b]	
SS00003171	*TLR4*	5.6 ± 0.3[a]	4.8 ± 0.6[ab]	4.7 ± 0.2[b]	
SS00000838	*TPS1*	11.2 ± 1.0[a]	10.1 ± 0.4[a]	14.4 ± 0.4[b]	
SS00001171	*TNF*	12.4 ± 0.4[a]	11.3 ± 1.3[ab]	10.4 ± 0.4[b]	
SS00006633	*INDO*	6.7 ± 1.4[a]	5.4 ± 1.1[a]	3.1 ± 0.5[b]	
SS00005475	*JUNB*	13.2 ± 0.7[a]	10.9 ± 1.0[b]	12.7 ± 0.7[a]	
SS00000591	*IL15*	7.0 ± 0.6[a]	6.3 ± 0.7[ab]	5.5 ± 0.2[b]	
SS00003340	*TGM3*	12.7 ± 0.5[a]	8.3 ± 1.0[b]	5.8 ± 2.0[c]	
SS00003495	*GBP1*	8.3 ± 1.3[a]	6.6 ± 0.2[b]	3.7 ± 0.2[c]	
SS00005079	*GBP2*	9.4 ± 0.6[a]	8.7 ± 0.5[a]	5.8 ± 0.6[b]	
SS00010027	*TAP1*	6.2 ± 0.75[a]	5.1 ± 0.8[b]	4.1 ± 0.3[b]	
SS00004493	*WARS*	11.8 ± 0.2[a]	11.2 ± 1.1[a]	8.99 ± 0.5[b]	
SS00002173	*TAP2*	4.7 ± 0.8[a]	4.5 ± 0.2[a]	3.0 ± 0.3[b]	
SS00004610	*PTDSR*	7.1 ± 0.1[a]	6.3 ± 0.2[b]	5.5 ± 0.4[c]	
SS00000703	*PSMB8*	5.3 ± 0.3[a]	4.6 ± 0.4[ab]	3.8 ± 0.5[b]	
SS00002453	*C1R*	9.2 ± 0.3[a]	9.1 ± 0.6[a]	7.7 ± 0.2[b]	
SS00000703	*C1S*	3.9 ± 0.5[a]	3.6 ± 0.3[a]	2.6 ± 0.2[b]	
SS00013121	*FCGR3B*	2.4 ± 0.5[a]	2.2 ± 0.5[a]	1.3 ± 0.1[b]	
SS00000478	*TNFRSF5*	5.4 ± 0.3[a]	5.1 ± 0.4[ab]	4.7 ± 0.2[b]	These genes had the same expression direction in both microarray and qRT-PCR experiments; however, statistically significant differences were found in qRT-PCR but not microarray.
SS00000135	*CRABP1*	5.7 ± 0.6[ab]	5.3 ± 0.8[b]	6.8 ± 0.5[a]	
SS00001079	*TNFSF6*	11.3 ± 0.4[a]	9.6 ± 0.1[b]	9.8 ± 0.5[b]	
SS00010173	*IL1B*	11.0 ± 0.3[a]	8.2 ± 1.8[b]	9.5 ± 0.8[ab]	
SS00000951	*IL4*	15.6 ± 0.5[ab]	14.1 ± 1.5[b]	16.2 ± 0.3[a]	
SS00002529	*NOS2A*	18.7 ± 0.6[ab]	20.0 ± 0.6[a]	18.3 ± 1.0[b]	
SS00000546	*BAK1*	17.1 ± 0.5	16.8 ± 0.8	16.8 ± 0.3	These genes showed significant differential expression in microarray analysis but not in qRT-PCR.
SS00002273	*IRF2*	7.6 ± 0.7	7.3 ± 0.1	7.6 ± 0.3	
SS00006643	*NFKB1*	8.8 ± 1.4	8.9 ± 0.4	8.8 ± 0.8	
SS00009806	*RARG*	9.4 ± 0.5	8.9 ± 0.4	9.1 ± 0.5	
SS00003927	*RBP1*	8.0 ± 2.5	9.1 ± 0.7	9.6 ± 1.0	
SS00000922	*IL13*	15.5 ± 2.1	12.1 ± 2.3	16.3 ± 2.5	
SS00010185	*CD59*	6.4 ± 1.1	6.0 ± 0.7	5.2 ± 0.3	
SS00012722	*SPP1*	9.8 ± 0.3	9.4 ± 1.1	9.6 ± 1.0	
SS00008286	*STAT3*	6.7 ± 1.2	6.5 ± 0.6	5.4 ± 0.6	
SS00008143	*PSMB10*	14.7 ± 0.8	13.1 ± 3.4	13.7 ± 0.3	
SS00000469	*TNFRSF1A*	4.3 ± 0.6	4.1 ± 0.7	4.1 ± 0.1	These genes failed to show significant differential expression in either microarray or qRT-PCR.
SS00007514	*MAPK14*	5.0 ± 0.2	4.8 ± 0.2	4.5 ± 0.1	
SS00009842	*IL10*	12.1 ± 1.2	11.1 ± 0.8	10.4 ± 0.2	
SS00000530	*ARG1*	9.8 ± 1.1	10.3 ± 2.3	11.0 ± 0.8	
SS00000904	*IFNA*	14.7 ± 1.8	12.2 ± 4.0	15.9 ± 0.8	
SS00000832	*TGFB1*	10.2 ± 1.0	10.3 ± 0.5	10.6 ± 0.2	
SS00000662	*TGFB2*	4.7 ± 0.6	4.4 ± 0.7	5.4 ± 0.5	
SS00000996	*CD80*	10.9 ± 0.6	10.0 ± 0.6	10.4 ± 0.2	
SS00001060	*IL8*	7.6 ± 1.0	7.8 ± 0.9	7.4 ± 0.4	
SS00001087	*IL6*	9.2 ± 0.9	8.8 ± 0.5	10.1 ± .8	
SS00001121	*CD3E*	8.0 ± 0.6	7.6 ± 0.4	8.4 ± 0.2	
SS00001132	*NRAMP1*	11.6 ± 1.0	12.3 ± 1.0	10.6 ± 0.6	
SS00001141	*IL18*	7.7 ± 0.4	7.1 ± 0.3	7.4 ± 0.6	
SS00001168	*LEPR*	10.4 ± 1.0	10.3 ± 1.3	10.0 ± 0.3	
SS00004427	*STAT6*	11.6 ± 0.1	11.0 ± 1.0	11.4 ± 0.1	
SS00009677	*LBP*	10.0 ± 1.0	10.3 ± 1.1	12.2 ± 1.7	
SS00009841	*IL12B*	14.5 ± 0.3	14.3 ± 0.5	14.7 ± 0.4	
SS00009986	*CD69*	9.3 ± 0.8	8.4 ± 0.1	8.9 ± 0.7	

(Continued)

Table 2. Continued

SS00010183	*ICAM1*	12.7 ± 0.5	12.0 ±0.2	12.2 ± 0.7	
SS00010194	*IL2*	15.3 ± 0.5	14.2 ± 0.1	14.7 ± 1.1	
SS00010452	*IGM*	6.8 ± 0.4	6.1 ± 0.4	6.6 ± 0.7	
	MYD88	5.9 ± 0.8	5.5 ± 0.7	5.1 ± 0.1	This gene is not on the microarray.

[†]C_t = cycle threshold, i.e., the cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve; lower C_t means higher expression level. ΔC_t = target transcript C_t − RPL32 C_t, normalization of C_t for target gene relative to RPL32 RNA C_t.
[‡]ΔC_t levels not connected by same letter are significantly different at $p \leq 0.05$ level across different time points.

between PCR and hybridization reactions, qRT-PCR reveals a larger dynamic range and increased sensitivity compared with microarray analyses, e.g., there is underestimation of expression differences in microarrays (Carter et al. 2003). This could explain our observation that the qRT-PCR had greater sensitivity to detect significant differences in gene expression for those six genes; however, qRT-PCR did identify ten microarray false positives as well.

The 61 genes tested by qRT-PCR corresponded to ten general categories of immune function (Fig. 4). *IFNG* was shown to have significantly increased expression at 48 hpi. Genes known to be induced by IFNG such as acute phase response genes (*C1S*, *C1R*) and antigen processing genes (*MHC2TA*, *PSMB8*, *TAP1*, *TAP2*) also showed increased expression during infection. *C1s* and *C1r* are members of the complement system, a major component of innate immunity that recognizes and kills microorganisms (Sim and Tsiftsoglou 2004). Increased expression of the acute-phase reactants *C1s* and *C1r* suggests that the classical complement activation pathway was activated during *Salmonella* infection. Another important cellular immune response to infection in vertebrates is antigen processing. The RNA abundance of four genes involved in antigen presentation (*MHC2TA*, *PSMB8*, *TAP1*, and *TAP2*) was increased at 48 hpi. *MHC2TA* is a very important regulator of MHC class II genes (LeibundGut-Landmann et al. 2004). Other studies have also shown that expression of *PSMB8*, *TAP1*, and *TAP2* is increased in various cell types by different pathogens (Jenner and Young 2005).

Our microarray results showed that expression of three type I IFN-induced genes, *GBP1*, *GBP2*, and *GBP3*, was increased. IFNA expression increased at 24 hpi, and *GBP1* and *GBP2* followed; our qRT-PCR analysis showed their maximal expression at 48 hpi when *IFNA* was already decreasing. Studies from mouse and human macrophages demonstrated that IFN or lipopolysaccharide (LPS) can induce expression of *GBPs 1–5* (MacMicking 2004). A secondary response gene in this pathway, *IRF1*, was also shown to have significant higher expression at 24 and 48 hpi. These results indicate the early activation of type I IFN-induced immune response pathways in porcine lung infected with *S.* Choleraesuis.

Seven of the 11 Th1-associated immune genes tested showed significant increases in RNA levels at 24 or 48 hpi in both microarray and qRT-PCR analyses, while three of the seven Th2 genes tested showed significant RNA decreases (*JUNB* increased at 24 hpi but decreased at 48 hpi). These results indicate that there is a predominant Th1 immune response at 24 and 48 hpi in porcine lung infected with *S.* Choleraesuis. Th1 immunity would stimulate *IFNG* production with associated macrophage activation, factors that would aid in clearing bacterial infections (Van de Vosse et al. 2004). Genes corresponding to innate/inflammatory response such as *TLR4* (the LPS receptor), *FCGR3B*, and *IL1B* also showed increased expression at 24 or 48 hpi. *TLR4* is a key signaling element in respiratory epithelial cells in response to LPS (Guillot et al. 2004; Iwasaki and Medzhitov 2004). Increased expression of cytokines such as tumor necrosis factor (*TNF*) and interleukin 1β (*IL1B*) in porcine lung has been demonstrated after PRRS virus or *Mycoplasma hyopneumoniae* infection (Rodriguez et al. 2004; Royaee et al. 2004; Thanawongnuwech et al. 2004). Characterization of the porcine Th1/Th2 response to parasitic infections and their *in vitro* gene expression correlates has recently been published (Feezor et al. 2003; Raymond and Wilkie 2004; Dawson et al. 2005). Our results add new data to describe the cytokine response to gram-negative bacteria infection in the porcine lung. No transcriptional profiling work in porcine lung infected with gram-negative bacteria has been previously published; however, studies using LPS stimulation of gene expression in mouse or human lung have shown similar expression patterns to our results. *IFNG*, *IRF1*, *IRF7*, and *IL1B*, which increased after *Salmonella* infection in our study, all increased expression in LPS-treated mouse lung (Jeyaseelan et al. 2004; Okamoto et al. 2004). *TNF* is prominently expressed in bronchial epithelial cells, and elevated

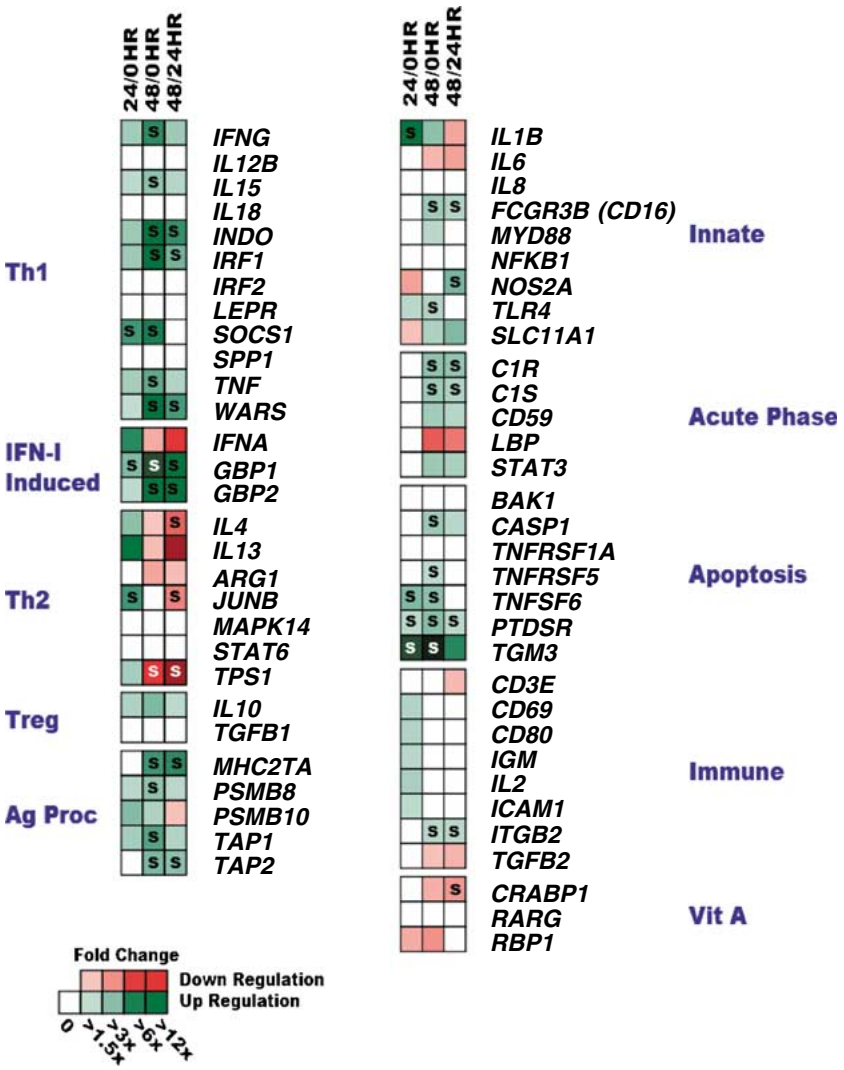

Fig. 4. Relative expression of 61 genes in the noninfected and *S.* Choleraesuis-infected lungs. qRT-PCR was used to measure gene expression; ΔC_t values were calculated relative to the housekeeping gene *RPL32*, and expression then was calculated relative to time of infection. The 61 genes were grouped according to immune functions. 24HR/0HR: expression changes at 24 hpi compared with the uninfected control; 48 HR/0HR: expression changes at 48 hpi compared with the uninfected control samples; 48/24HR: expression changes at 48 hpi compared with 24 hpi samples; S: statistical significance at $p < 0.05$.

levels have been demonstrated in mouse and human lung after LPS treatment (Khair et al. 1996; Dudoit et al. 2002; Ermert et al. 2003; Jeyaseelan et al. 2004).

The ubiquitin-proteasome pathway is a nonlysosomal protein-destroying pathway that degrades IκBα and allows NF-κB protein entry into the nucleus (Hatakeyama et al. 1999; Hayashi et al., 2000). In addition to the antigen-processing genes described above, the expression of additional proteasome genes, including proteasome activator 28 beta (*PA28 beta*) and proteasome subunit *PSMB10*, was elevated in pig lungs during SC infection. Previous studies have shown that *IFNG* could induce the expression of *PA28 beta*, *LMP2*, *PSMB8*, and *PSMB10* in human cell lines, rat, or mouse tissues (Foss et al. 1998; Tanaka and Kasahara1998; Nelson et al. 2000). Furthermore, the F-box proteins are responsible for substrate recruitment in this pathway (Liu 2004). In addition to *IFNG*, *PA28*, *PSMB8*, and *PSMB10*, the F-box-only protein 6 (*FBXO6*) and *FBXO7* genes were increased during infection, further indicating that the ubiquitin-proteasome pathway is activated in porcine lung during SC infection. However, the expression of *LMP2* showed no difference between control and infected animal lungs. Thus, further study is needed to clarify if *LMP2* is involved in this process in the pig and if there are mechanistic differences between the pig and other animals for this gene during infection.

Our data show for the first time the dramatic changes in lung expression for *TGM3*, which had a greater than 1000-fold increase from noninfected to 48 hpi with *S.* Choleraesuis. *TGM1*, found in the same gene family as *TGM3*, also showed a dramatic increase (31-fold) in gene expression. These results suggest a role of the *TGM* gene family in the porcine immune response to gram-negative bacteria infection, as has been found for PRRS (Thanawongnuwech et al. 2004). Transglutaminases are

enzymes known to be active in apoptosis (Cantu et al. 2004), and we did find ample evidence of activation of apoptotic pathways, including the proteasome, and increased expression of apoptosis-related genes such as *CASP1*, *TNFRSF5*, and *TNFSF6*, apoptotic cell clearance receptor PtdSerR, and DAP kinase-related apoptosis-inducing protein kinase. No data showing the function of *TGM1* and *TGM3* in lung infection have been reported up to now in any species, although another member in the *TGM* family, *TGM2*, was found to be induced in retinoic acid-treated bronchial epithelial cells in a microarray study aimed to understand the role of retinoids in cancer treatment and prevention (Ma et al. 2003). Unfortunately, no oligonucleotide representing *TGM2* was included in the Qiagen set. Interestingly, transglutaminase was also found to have an important role in stabilizing apoptotic cells before clearance; transglutaminase helps form the highly crosslinked apoptotic envelope during programmed cell death (Fesus et al. 1987; Knight et al.1991; Johnson et al.1998; Nanda et al. 2001; Caraglin et al. 2005). In mice, apoptosis was induced in lung endothelial cells after intravenous administration of LPS (Fujita et al. 1998). Whether *TGM3* or *TGM1* is directly involved in apoptosis in porcine lung after infection with *S.* Choleraesuis remains unknown, but clearly apoptotic signals are induced in this infection.

Overall, our results have shown that *Salmonella* Choleraesuis infection induces major changes in gene expression in porcine lung as early as 24 hpi. Using a long oligonucleotide microarray, our data revealed that numerous immune genes are activated by 48 hpi, particularly those associated with Th1- and IFN-stimulated responses, as well as apoptosis induced changes. We also uncovered the likely involvement of the *TGM* gene family in the apoptotic response to *Salmonella* infection in the pig. These results will inform new studies for control of infection by *Salmonella* pathogens and for other respiratory pathogens of pigs.

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