Genomic expression profiling of peripheral blood leukocytes of pigs infected with highly virulent classical swine fever virus strain Shimen

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Classical swine fever (CSF), caused by a virus of the same name (CSFV), is a highly contagious swine pyrexic disease featuring extensive haemorrhagic lesions and leukopenia, but little is known about the molecular mechanisms of its pathogenesis. To gain insight into the interaction between the virus and host cells, microarray analyses were performed to detect alterations in genomic expression of pig peripheral blood leukocytes (PBLs) following CSFV infection. Three healthy pigs were inoculated with a lethal dose of highly virulent CSFV strain Shimen. PBLs were isolated at the onset of typical clinical signs and total RNA was subjected to microarray analyses with Affymetrix Porcine Genome Array GeneChips. Of all 20 201 pig genes arrayed in the chip, 1745 showed altered expression (up- or downregulation) after infection. These were classified into eight functional groups, relating to cell proliferation (3.6 %), immune response (2.1 %), apoptosis (1.4 %), kinase activity (1.4 %), signal transduction (1.4 %), transcription (0.7 %), receptor activity (0.7 %) and cytokines/chemokines (0.4 %). The remaining 88.3 % of genes had unknown functions. Alterations in genomic expression were confirmed by real-time RT-PCR of selected cellular genes and Western blotting of annexin 2, a cellular protein relating to virus infection. The observed expression changes of numerous genes involved in immune and inflammatory responses and in the apoptosis process indicate that CSFV has developed sophisticated mechanisms to cause leukopenia in infected pigs. These data provide a basis for exploring the molecular pathogenesis of CSFV infection through an understanding of the interaction between viral and cellular components.

INTRODUCTION

Classical swine fever virus (CSFV) is a small, enveloped virus with a positive-stranded RNA genome that belongs in the genus Pestivirus, together with two bovine viral diarrhea virus species and border disease virus, within the family Flaviviridae (Thiel et al., 2004). The virus genome is approximately 12.5 kb in size and contains a single, large open reading frame that encodes a 3898 aa polyprotein (Meyers et al., 1999).

CSFV has a particular tropism for cells of the immune system and is known to cause severe leukopenia, in particular lymphopenia, featuring atrophy of primary lymphoid tissue (van der Molen & van Oirschot, 1981) and bone marrow (Summerfield et al., 2000), and depletion of different subsets of leukocytes (T and B lymphocytes, monocytes–macrophages and granulocytes) in infected pigs (Summerfield et al., 1998, 2000, 2001a; Carrasco et al., 2004; von Freyburg et al., 2004). All leukocytes are depleted during CSFV infection, but B lymphocytes are particularly susceptible (Susa et al., 1992). Besides the reduction in leukocyte numbers, lymphocyte activation and function during virulent CSFV infection are impaired severely, thereby significantly decreasing antibody production and host defences (Lee et al., 1999). The destruction of leukocytes following CSFV infection is largely associated with apoptosis in thymus, spleen, lymph nodes and bone marrow (Summerfield et al., 1998, 2000; Sato et al., 2000; Sánchez-Cordón et al., 2002).

Virus infections are generally associated with numerous changes in host gene expression that determine the fate of the infected cells and the eventual outcome of the infection. Molecular pathogenetic studies of virus infection involve the investigation of these specific changes within the host cell or, more completely, within a specific tissue or organ. CSFV-induced apoptosis of thymocytes in the thymus...
Pig transcriptional responses to CSFV infection

(Sánchez-Cordón et al., 2002) and of lymphocytes in the spleen (Sánchez-Cordón et al., 2005) is considered to be related to the increased expression of cytokines in monocytes–macrophages, whilst apoptosis of T lymphocytes during classical swine fever (CSF) is associated with an increase in CD49d, major histocompatibility complex II and Fas gene expression (Summerfield et al., 1998). Apart from this, however, little is known concerning the molecular markers of CSFV-induced disease, including the direct effects on host-cell gene expression. Furthermore, the molecular mechanisms and the role played by CSFV infection of leukocytes in pathogenesis and leukopenia remain unknown. A more complete, and precise, identification of cellular genes with altered expression during infection is therefore essential to understand better the host–pathogen interactions and to identify the molecules involved in the mechanisms of host resistance or susceptibility.

DNA microarray technology, in combination with bioinformatics, has proved to be a very efficient, high-throughput tool and offers great advantages in the study of genomic expression profiles of cells. Widely used for human, mouse and rat gene expression studies, high-density gene arrays have been used increasingly to evaluate the status of host gene expression following infection by viruses such as human immunodeficiency virus (HIV) (Solis et al., 2006), dengue virus (Fink et al., 2007), measles virus (Sato et al., 2008), Newcastle disease virus (Munir et al., 2005), rabies virus (Ubol et al., 2005) and simian immunodeficiency virus (George et al., 2003).

Due to limited genomic information, application of microarrays for the study of farm and domestic animals has been hindered by the lack of microarray chips designed specifically for these animals. Therefore, use of the Affymetrix Human GeneChip for cross-species hybridization with mRNA from cattle, pig and dog has been evaluated (Ji et al., 2004; Shah et al., 2004). However, large-scale porcine gene microarray analysis had to await the appearance of the first-generation porcine oligonucleotide array (Qiagen NRSP8 array), representing 13297 cDNAs and expressed sequence tags (ESTs) and designed for porcine transcriptional profiling (Zhao et al., 2005). The current Affymetrix Porcine GeneChip is a complete, pan-genomic array and has been used mainly for global transcriptional profiling of the pig genome, as it is the most sensitive and reproducible for swine genomic studies (Tsai et al., 2006). However, it has also been used to observe the transcriptional changes of 848 genes of pig mesenteric lymph nodes in response to Salmonella typhimurium infection (Wang et al., 2007), and Genini et al. (2008) reported the expression alteration of 1409 genes of primary porcine alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. The present study provides the first pan-genomic microarray analysis of pig transcriptional responses to CSFV infection, in which the genomic transcriptional levels of porcine peripheral blood leukocytes (PBLs) prepared from CSFV-inoculated pigs with severe clinical symptoms were analysed by using the Affymetrix Porcine GeneChip, showing that 1745 genes were up- or downregulated by at least 2-fold following infection.

METHODS

Animals. Three littermate pigs (breed Landrace) at age 8–9 weeks, of similar size and thermostasis, were determined to be free from CSFV infection by negative results in real-time RT-PCR detection of the virus genome and in serum antibody tests using an Idexx CSFV Antibody Test kit (lot no. 43220-P651). The pigs were acclimatized to laboratory conditions for several days prior to experimental infection.

Virus infection and PBL isolation. The three pigs were injected intramuscularly in the neck with a lethal dose of highly virulent CSFV strain Shimen (1 ml 100-fold-diluted blood stock containing 10⁸ TCID₅₀). Body temperature and clinical symptoms were recorded daily post-infection (p.i.). Prior to infection, a blood sample, with EDTA–K₂ as anticoagulant, was collected from the anterior vena cava of each animal as a health control. By the same procedure, blood samples were collected from each animal once typical CSF clinical symptoms had appeared. Immediately following collection, suspensions of PBLs were prepared by incubation of blood in NH₄Cl buffer (0.15 M NH₄Cl, 10 mM NaHCO₃, pH 7.4) for 5 min at 4 °C to lyse the erythrocytes. The PBLs were washed three times with PBS at 4 °C, each time followed by centrifugation for 10 min at 350 g. Virus infection of the PBLs was confirmed and quantified by real-time RT-PCR in an ABI PRISM 7000 cycler (Applied Biosystems), using total RNA of infected PBLs, and primers and probes prepared as described previously (Shi et al., 2007). Quantification was achieved by relating viral Cₚ value to the Cₛ value on a standard curve of a measured number of copies of a plasmid bearing a 362 bp fragment of the CSFV 5′ non-coding region, amplified by using primers 5′-GGTGGTCTAAGTC-3′ (forward) and 5′-CATGTGTAGTGTTGGGTGTACCCTC-3′ (reverse).

Leukocyte counting and T-cell subpopulation sorting. The cells in the leukocyte preparations were counted in a haemocytometer using light microscopy. Their phenotypes were determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-CD4, FITC-conjugated anti-CD8 and phycoerythrin (PE)-conjugated anti-CD3 (Becton Dickinson). T-cell subpopulations were identified by CD4/CD3 and CD8/CD3 multiple labelling with an electronic gate set on CD3 cells. The T-helper cells (Th) were identified as CD3⁺ CD4⁺ (Saalmüller et al., 1999) and cytotoxic T-cells as CD3⁺ CD8⁺ (Paul et al., 1996). Acquisition of data used a FACScan flow cytometer and the CellQuest program (Becton Dickinson).

Microarray analysis. PBLs (≥1.0 × 10⁷ cells) were lysed by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and total RNA was extracted with phenol/chloroform, followed by ethanol precipitation. A quality test using a small amount of prepared RNA on a GeneChip Test 3 Array (Affymetrix) was conducted to determine whether the integrity and concentration of sample RNAs met the requirements for microarray analysis. The GeneChip Test 3 Array contains probes representing a subset of characterized genes from various organisms, including mammals, plants and eubacteria. For each eukaryotic organism represented, there are three sets of probes derived from the 5′-end, middle and 3′-end portions of the gene. By measuring signals from these probe sets, it is possible to identify sample degradation containing insufficient target that may result in poor expression array results. Following acceptable results in the
quality test, the first- and second-strand cDNAs were synthesized from 5–15 μg total RNA by using a One-cycle cDNA Synthesis kit (Affymetrix). The cRNA was synthesized and purified by using a GeneChip IVT Labelling kit (Affymetrix). After fragmentation according to the manufacturer’s instructions, about 15.0 μg cRNA was hybridized for 16 h at 45 °C to Porcine Genome Array GeneChips (Affymetrix). The arrays were washed at 25 °C with 6 × SSPE (0.9 M NaCl, 60 mM NaH2PO4, 6 mM EDTA and 0.01 % Tween 20), followed by a vigorous wash at 50 °C with 100 mM MES sodium salt (100 mM MES, 0.074 M Na+). The arrays were then stained with PE-conjugated streptavidin and fluorescence intensities were determined by using a GeneChip Scanner 3000 (Affymetrix). The scanned images were analysed by using the GeneChip Operating Suite (Affymetrix). Sample loading and staining conditions were standardized by scaling the mean of the fluorescent intensities of all genes on an array to a constant target intensity for all arrays used. The expression data were analysed as described previously (Lockhart et al., 1996). The signal intensity for each gene was calculated as the mean intensity difference, represented by [APM–MM]/(no. of probe pairs)], where PM and MM denote perfect-match and mismatch probes, respectively. Genes in which expression was increased or decreased p.i. by more than 2-fold in at least two of the three infected pigs were considered to be regulated reproducibly by the virus.

Real-time PCR for confirmation. Microarray results were validated by real-time RT-PCR of some genes showing altered expression by using SYBR green-based detection with an ABI PRISM 7000 cycler (Applied Biosystems), i.e. total leukocyte RNA (1 μg) prepared as described above was reverse-transcribed in a 20 μl reaction mixture containing 1 μl avian myeloblastosis virus (AMV) buffer, 10 mM dithiothreitol, 50 μM random hexamers, 0.5 mM dNTPs, 10 U RNase inhibitor and 20 U AMV reverse transcriptase (Promega). All primers were synthesized based on the sequences of the corresponding porcine mRNAs in GenBank. Triplicate PCRs were carried out to guarantee the reproducibility of amplification of the leukocyte cDNA sample from each animal. Changes in gene expression revealed by RT-PCR were calculated by the 2−ΔΔCt method (Livak & Schmittgen, 2001).

Western blotting. Freshly trypsinized cells of the pig kidney cell line PK-15 were added in minimal essential medium/10 % newborn calf serum to six-well microwell plates (4 × 106 cells per well) and incubated at 37 °C in 5 % CO2. When 80–90 % confluent, cells were infected with CSFV strain Shimen (106 TCID50 per well). At 60 and 72 h p.i., the culture media were aspirated, the cell monolayers were washed twice with cold PBS, then harvested by addition of lysis solution [8 M urea, 4 % CHAPS, 40 mM Tris, 65 mM dithiothreitol, 1 mM protease inhibitor (PMSF)]. As controls, uninfected PK-15 cells were treated in parallel in the same way. After determination of protein concentration using the Coomassie Plus – the Better Bradford Assay kit (Amersham Biosciences), the lysed cell samples were subjected to SDS-PAGE and protein bands were transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked overnight with 3 % bovine serum albumin in PBS and then incubated with antibodies and hors eradish peroxidase (HRP)-conjugated second antibodies. Bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Total cellular protein samples of PBLs from each pig before and after infection were prepared and analysed in the same way.

Confocal laser-scanning microscopy (LSCM). For LSCM analysis, coverslips of PK-15 cells infected with CSFV strain Shimen (105 TCID50 per 150 mm2) were fixed in 4 % paraformaldehyde overnight at 4 °C. The fixed cells were permeabilized with PBS/0.1 % Triton X-100 and incubated with 0.1 M glycine for 10 min at room temperature to quench free aldehyde groups. The fixed cell sheets were then blocked with 3 % goat serum albumin at room temperature for 1 h, and incubated with either anti-CSFV E2 monoclonal antibody (mAb) WH 303 (Lin et al., 2000) or goat polyclonal antibody against selected porcine cellular proteins. After washing three times (15 min each) at room temperature, the coverslips were incubated sequentially with FITC-conjugated anti-mouse IgGs (Sigma) and Texas red-conjugated anti-goat IgGs (Santa Cruz) for 30 min at room temperature. After several rinses with PBS, the coverslips were mounted on gelatin-covered slides and examined in a Leica SP2 Confocal system (Leica Microsystems).

RESULTS

Experimental infection and depletion of PBLs

Experimental infection of susceptible pigs can cause acute CSF. After infection with the highly virulent strain Shimen, all three pigs developed the disease by day 2 p.i., showing elevated body temperatures (40.0–40.5 °C), varying degrees of anorexia and constipation. Slight diarrhoea, erythema of the skin and an ocular discharge associated with conjunctivitis were present from days 4–5 p.i., following which rapid deterioration occurred, with rectal temperatures of 42 °C and markedly decreased activity. From day 7 p.i., the typical staggering gait and a cyanotic discoloration of the skin of the abdomen, snout, ears and legs were observed. From 8–9 days p.i. onward, deterioration continued. Body temperatures decreased to <38.5 °C, with all inoculated pigs dying by day 10 p.i. The blood samples used in the study were collected at day 7 p.i., the most severe phase of the disease. Real-time RT-PCR showed that the numbers of gene copies of CSFV replicating in PBLs of the infected pigs were 106.03 ± 526 copies (100 ng total RNA)−1 (mean ± sd), indicating that a high proportion of the leukocytes were infected. A rapid onset of leukopenia was detected, with levels of PBLs in all three infected animals declining from 20 667 ± 1379 cells μl−1 before infection to 12 000 ± 1646 cells μl−1 at day 4 p.i. This loss of PBLs progressed further until day 10 p.i., reaching levels as low as 7100 ± 1176 cells μl−1 (Fig. 1a). This result confirms the depletion of PBLs in the infected pigs.

To investigate further the loss of lymphocytes – the main target of CSFV during infection – the T-cell subpopulations of the three pigs were analysed by FACS (fluorescence-activated cell sorting) at days 4, 7 and 10. Results showed that the severest depletion of CD3+CD4+ and CD3+CD8+ T-cell subsets (P<0.05) was observed by day 7 p.i. (Fig. 1b). This is consistent with the findings of a previous study (Summerfield et al., 2001b) showing that CD3+CD4+ cells and CD3+CD8+ cytotoxic T-cell subsets were influenced strongly by the virus infection.

To explore the factors causing leukopenia, apoptosis analysis was applied to PBLs by using an Annexin V: FITC Apoptosis Detection kit (ACTGene) and FACScan flow cytometry (Becton Dickinson) and following the manufacturers’ protocols. Results showed clearly that apoptosis was observed in PBLs from the same blood samples of the three pigs as were used for microarray, with
the proportion of apoptotic cells in a total of 10,000 leukocytes per test being increased significantly from 7.03% before infection to 25.56% \((P < 0.05)\) afterwards, whilst cell death increased correspondingly, from 1.48 to 6.46% \((P < 0.05)\) (see Table 1). This increase in the numbers of apoptotic PBLs is consistent with the observations of a previous study (Summerfield et al., 2001a).

### CSFV-induced alterations of porcine genomic expression

To analyse the genomic expression of CSFV-infected pigs, total RNA was extracted from PBLs collected before and after infection. After validation of purity, concentration and integrity by the test chip, GeneChip Test 3 Array, the RNA preparations were subjected to analysis based on the above-described protocol using Affymetrix Porcine Genome Array GeneChips housing 23,937 probe sets that can detect approximately 20,201 porcine genomic transcripts. Results showed that expression of 2919, 2859 and 2995 genes from the three pigs was altered p.i., of which altered expression of 2662 genes was identified in two or three animals and subsequently subjected to one-way ANOVA statistical analysis \((P < 0.05)\). In all, 1745 genes (8.64% of all genes present in the array) were confirmed as having \(>2\)-fold-altered expression, with 877 being upregulated and 868 downregulated. Of 877 upregulated genes, 24.3% (213/877) were found in all three animals, 61.2% (537/877) were found to be upregulated in two animals whilst not changed in another and 14.5% (127/877) were found to be upregulated in two animals whilst downregulated in another. Of 868 downregulated genes, 37.2% (323/868) were found in all three animals, 55.6% (483/868) were found to be downregulated in two animals whilst not changed in another and 7.2% (62/868) were found to be downregulated in two animals whilst upregulated in the third. The functions of the 1745 genes were analysed by the Gene Ontology tool, available from the Affymetrix website (https://www.affymetrix.com/analysis/netaffx/index.affx). Surprisingly, 88.3% (1540/1745) were not functionally annotated, with only 205 genes (11.7%) showing clear functional annotation. These clustered into eight functional groups: cell proliferation and cycle (62/1745; 3.6%), immune response (37/1745; 2.1%), protein kinase activity (25/1745; 1.4%), apoptosis (24/1745; 1.4%), signal transduction (24/1745; 1.4%), transcription (13/1745; 0.7%), receptor activity (13/1745; 0.7%) and cytokine/chemokine (7/1745; 0.4%) (Fig. 2).

The complete microarray dataset of 205 genes with functional annotations and expression level changes has been summarized in Supplementary Table S1 (available in JGV Online).

To validate the microarray analysis, 13 genes with different levels of altered expression were selected for RT-PCR confirmation, in which a PBL sample from one infected pig was used. As shown in Table 2, the altered expression of

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pre-infection</th>
<th>Post-infection</th>
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<tbody>
<tr>
<td></td>
<td>Apoptotic cells (%)</td>
<td>Dead cells (%)</td>
</tr>
<tr>
<td>Pig 1</td>
<td>5.68</td>
<td>1.51</td>
</tr>
<tr>
<td>Pig 2</td>
<td>7.67</td>
<td>1.06</td>
</tr>
<tr>
<td>Pig 3</td>
<td>7.73</td>
<td>1.87</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.03 ± 1.17</td>
<td>1.48 ± 0.41</td>
</tr>
</tbody>
</table>
those 13 genes identified by microarray was highly consistent with the results of real-time RT-PCR, although the extent of the changes as measured by the two methods did not match well. Nevertheless, the data indicate that the results from the microarray analysis are good indicators of overall changes in gene expression.

**Western blotting analysis and LSCM of proteins with altered expression**

Based on the availability of commercial antibodies against porcine cellular proteins, annexin 2 and haem oxygenase 1 (HO-1) were selected for Western blotting (annexin 2 only) and LSCM analysis (both proteins). Microarray analysis showed that the expression of the genes for annexin 2 and HO-1 was upregulated by about 2-fold and 5-fold, respectively, in PBLs following CSFV infection (see Supplementary Table S1). To confirm this result, the levels of annexin 2 in the PBLs of one infected pig and PK-15 cells were analysed by Western blotting as described above. Results showed that CSFV infection led to a significant increase of annexin 2 protein in PK-15 cells at two time points (60 and 72 h p.i.), as well as in PBLs (Fig. 3a, b), i.e.

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**Table 2.** Validation of microarray data by real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence 5′-3′</th>
<th>Change (fold)</th>
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<tbody>
<tr>
<td>CD163</td>
<td>F: CTTGAAGATGCTCAGGTTG            16.43</td>
<td>13.76</td>
</tr>
<tr>
<td></td>
<td>R: TGAGGAATGCAGATGAGCG            8.49</td>
<td>1.75</td>
</tr>
<tr>
<td>Natural resistance-associated macrophage protein 1</td>
<td>F: CGGGAGGACATCCGAGAA            4.45</td>
<td>2.21</td>
</tr>
<tr>
<td>(Nramp1)</td>
<td>R: AAGACCCACCCCAAAAT            4.60</td>
<td>4.18</td>
</tr>
<tr>
<td>Caspase 1 (CASP1)</td>
<td>F: ACAGCCCTTGCCTCCTCAT            4.79</td>
<td>15.72</td>
</tr>
<tr>
<td></td>
<td>R: CACCTTTGGTTGTGCTCTTCA         4.60</td>
<td>4.18</td>
</tr>
<tr>
<td>Toll-like receptor 2 (TLR2)</td>
<td>F: GCCAATAGCATTCATACCG            4.45</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>R: ATTCGCAGATTTGGGAGAT            4.44</td>
<td>7.47</td>
</tr>
<tr>
<td>Caspase-3 (CASP3)</td>
<td>F: AAGGGGAGGAAGACTGGA            2.03</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>R: CGAGCCTCTCAATCTCTTTG         2.03</td>
<td>2.00</td>
</tr>
<tr>
<td>Interleukin-10 (IL-10)</td>
<td>F: GAGACCCCGTGCAAGGCA             2.03</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>R: CCAGCCTCTCAATCTCTTTG         2.03</td>
<td>2.00</td>
</tr>
<tr>
<td>Glutathione peroxidase 4 (GPX4)</td>
<td>F: TTACGAGTCAGAGCGAGGCC           2.03</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>R: ACATATTCCGATCATCGGAACG        2.03</td>
<td>2.00</td>
</tr>
<tr>
<td>IgG heavy chain</td>
<td>F: AGTTTTGCTTTTGCCTATAAGC         -62.11</td>
<td>-769.32</td>
</tr>
<tr>
<td></td>
<td>R: TGAGGCTTGCTCTTGGGT            -62.11</td>
<td>-769.32</td>
</tr>
<tr>
<td>Complement cytolysis inhibitor (CCI)</td>
<td>F: CAGAGAAGACGACGAGATGA          -6.17</td>
<td>10.81</td>
</tr>
<tr>
<td></td>
<td>R: CGGTATCCCTGTTGATGTCCTCA       -5.26</td>
<td>1.51</td>
</tr>
<tr>
<td>Glutaminase (GLS)</td>
<td>F: AAGTATCAGAGAAGATTTG            4.90</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>R: TTACTGTGGAGTGGGT              -5.26</td>
<td>1.51</td>
</tr>
<tr>
<td>Tyrosine–protein kinase (TPK)</td>
<td>F: TTACCTCAGCAAGGTCTGTT           -4.90</td>
<td>-3.69</td>
</tr>
<tr>
<td></td>
<td>R: ACATACCTCCTCGTCTCTCAAA        -4.22</td>
<td>-8.39</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 4</td>
<td>F: TCCGCAATTCCTCAGAGA            -4.22</td>
<td>-8.39</td>
</tr>
<tr>
<td>(STAT4)</td>
<td>R: TTGGGAGAACTGAAATTG             -2.78</td>
<td>-21.72</td>
</tr>
<tr>
<td>Apoptosis-related protein (PNAS-5)</td>
<td>R: ACATTGAGGGTGGAGA             -2.78</td>
<td>-21.72</td>
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</tbody>
</table>
the results are consistent with the levels of mRNA as determined by microarray. LSCM was used to locate the intracellular expression of annexin 2 and HO-1 in PK-15 cells and to explore a possible interaction of cellular genes with viral proteins. Results showed that clear perinuclear synthesis and accumulation of annexin 2, HO-1 and CSFV E2 proteins occurred in the cytoplasm of infected cells (Fig. 3c), with a colocalization of the three proteins, thereby providing more evidence for an interaction between CSFV structural proteins and cellular proteins.

**DISCUSSION**

Leukopenia with severe depletion of leukocytes (lymphocytes, monocytes–macrophages and granulocytes, etc.) in infected pigs is the most typical clinical sign of CSF, and is mainly associated with apoptosis of cells of the immune system (Susa et al., 1992; Summerfield et al., 1998, 2001a, b; von Freyburg et al., 2004). The kinetics of leukocyte depletion, apoptosis and virus infection of leukocyte subsets may vary depending on the particular CSFV strain used (Summerfield et al., 2001a; Choi et al., 2004; Sánchez-Cordón et al., 2005). The present study confirmed that severe leukopenia can be induced by infection with highly virulent CSFV strain Shimen; this leukopenia was ascribed to apoptosis, as the percentage of apoptotic PBLs increased markedly after infection (Fig. 1; Table 1). The observed high level of apoptotic PBLs was probably related to the virus strain and sampling time point used, reflecting the pathogenic nature of strain Shimen and also reinforcing previously published observations on CSFV-induced apoptosis of pig leukocytes. During CSFV infection, apoptosis of immunocompetent cells is mediated by direct virus infection of target cells or by indirect virus action, involving an inflammatory response in which cytokines and chemokines released during the virus infection induce apoptosis of uninfected leukocyte subsets (Summerfield et al., 1998, 2000; Choi et al., 2004; Sánchez-Cordón et al., 2005).

Apoptosis is a common mechanism by which many viruses cause cell death, and is usually initiated by viral gene expression and/or the altered expression of cellular genes following virus infection. To characterize the nature of the host-cell response to CSFV infection, the transcriptional profiles of cultured porcine macrophages following highly virulent CSFV infection were analysed by microarray comprising 7712 sequences of porcine macrophage; this resulted in 30 genes that were identified to have altered mRNA levels, with 11 being upregulated and 19 downregulated (Zaffuto et al., 2007). Borca et al. (2008) used quantitative RT-PCR to assess changes in expression of 58 selected host immune response-modulating genes of cultured primary porcine macrophages and 18 were identified to have altered expression following highly virulent CSFV infection, including cytokines, cytokine receptors, chemokines, interferons and Toll-like receptors. In the present study, the in vivo global genomic transcriptional responses of porcine PBLs to CSFV infection were elucidated by using a porcine pan-genomic microarray. In all, 1745 genes were found to be up- or downregulated following infection with highly virulent CSFV strain Shimen. Although 88.3 % (1540/1745) have not been functionally identified, the remaining 11.7 % (205 genes) with clearly defined functions still provide a challenge in understanding their roles in CSFV replication and pathogenesis.

From our microarray analysis, 24 apoptosis genes were found to be changed in expression, with 10 being upregulated and 14 downregulated (see Supplementary Table S1), which probably triggered the depletion of PBLs following CSFV infection. The process of apoptosis in mammalian cells involves two main signalling pathways: extrinsic and intrinsic (Shedlock et al., 2008). The extrinsic
pathway, also known as the death-receptor pathway, is activated by binding of the ligands on cell membranes with death receptors produced by external stimuli, such as various pathogens (Chattergoon et al., 2008). Receptors known to be involved in activation of the death-receptor pathway include Fas/APO-1/CD95, TNFR I, DR3, TRAIL R1/DR4, TRAIL R2/DR5 and DR6 (Locksley et al., 2001), which can bind ligands on the cell membrane and subsequently recruit procaspases through the Fas-associated protein with the death domain (FADD) (Boldin et al., 1996). In turn, procaspases, in particular caspases 8 and 10, can activate caspase 3 and other executioner caspases directly, leading to apoptosis. Here, the microarray identified an altered expression of the genes for death receptors CD40 (about 2-fold upregulation) and DR3 (about 3-fold downregulation). CD40 induces cell death and cytokine secretion in colorectal cancer (Georgopoulos et al., 2007). In this study, increased expression of CD40 could promote apoptosis of PBLs following CSFV infection, whilst the role of downregulated DR3 in CSFV infection needs further investigation. The intrinsic apoptosis pathway is activated by a complex process involving the release of cytochrome c (Wei et al., 2001). In this pathway, cytochrome c interacts with cytosolic apoptotic protease-activating factor 1 (Apaf-1), which is followed by activation of caspase 9. The activated caspase 9 activates caspase 3 (Li et al., 1998; Luo et al., 1998) and other executioner caspases, such as caspase 7 and 6, to act on key cellular substrates responsible for promoting apoptosis (Green, 2000). Usually caspases are present in an inactive zymogen form in viable cells, and overexpression of any caspase can induce apoptosis in mammalian cells (caspases 2, 8, 9 and 10 as initiator caspases, with 3, 6 and 7 as executioner caspases) (Thornberry & Lazebnik, 1998). Our results showed that the transcriptional responses of the genes for caspases 3, 7 and 9 to CSFV infection were increased by about 4-, 2- and 4-fold, respectively, whilst transcription of the gene for caspase 6 decreased by about 2-fold, indicating that the intrinsic apoptosis pathway participated in the CSFV-induced apoptotic process. Interestingly, and pertinent to our finding, the increased caspase 3 and caspase 9 activities in the mitochondrial apoptotic pathway have been observed previously in apoptotic bone-marrow haematopoietic cells of virulent CSFV-infected pigs (Summerfield et al., 2000).

The transcription factor NF-κB can modulate the expression of genes associated with innate immunity, inflammatory response and apoptosis. Activation of NF-κB expression is an important mechanism for suppressing apoptosis induced by diverse stimuli (Beg & Baltimore, 1996; Liu et al., 1996), particularly that induced by virus infection (Yurochko et al., 1995; Goodkin et al., 2003). NF-κB can inhibit NF-κB activity through binding to its Rel domain and plays an important role in responses involving growth regulation and apoptosis (Spink et al., 2007). During CSFV infection, the transcription of NFKBIA was upregulated by about 3-fold, probably resulting in blockage of NF-κB activity and subsequently furthering apoptosis of PBLs. Calpain small 1 (CAPNS1) is an important regulatory subunit of calpain, and a deficiency of calpain can strengthen the proapoptotic effect induced by ceramide (Demarchi et al., 2005). In PBLs, CAPNS1 was downregulated by CSFV infection, indicating its possible role in inducing the apoptosis pathway.

A previous study showed that ferritin plays a key role in stimulating upregulation of p53 and mediates apoptosis involving Fas (CD95) signalling, as well as addressing the intrinsic mitochondrial proapoptotic pathway in cultured hepatocytes in vitro (Bresgen et al., 2007). Apoptosis causing peripheral T-lymphocyte depletion of pigs during lethal CSFV infection has been shown to be mediated through Fas–Fas ligand interactions (Summerfield et al., 1998). In the present study, CSFV infection increased the transcription of ferritin by >2-fold in PBLs, indicating that ferritin might be a positive factor inducing apoptosis. In addition, expression of more apoptotic genes was activated by CSFV infection, such as the genes for cathepsins D and B, mitogen-activated protein kinase-activated protein kinase 3, p75 apoptosis-inducing death domain, translationally controlled tumour protein, caspase-1, PNAS-5, RPL6 and Hsp90, although their functions in apoptosis following CSFV infection require further exploration.

Although apoptosis of PBLs was an outcome of CSFV infection, the microarray analyses have also provided evidence that anti-apoptotic processes probably co-exist during the infection. CSFV has been reported to suppress interferon production and double-stranded RNA-induced pathways via its Npro protease (Bensaude et al., 2004; Ruggli et al., 2005), although the molecular mechanism by which Npro induces the anti-apoptosis antagonistic effect has not been elucidated. Additional findings from our pan-genomic microarray have shown that some anti-apoptosis genes were also upregulated following CSFV infection. Overexpression of HO-1 via chemical induction or gene therapy was used to reduce the deleterious effects of oxidative stress and apoptosis in various cell types and animal models (Tobiasch et al., 2001; Tsuburai et al., 2002). The significant upregulation of its gene following CSFV infection indicated that HO-1 might play a role in the anti-apoptosis process. In addition, the gene for another anti-apoptosis protein, heat-shock protein 27 (Hsp27), was upregulated during CSFV infection, indicating that a concomitant anti-apoptosis pathway probably exists. Hsp27 is responsible for the regulation of apoptosis through its interaction with protein kinase B (Akt), and activation of Akt has been demonstrated to inhibit apoptosis in a variety of systems (Kauffmann-Zeh et al., 1997). Hsp27 has also been found to inhibit apoptosis through a direct inhibition of caspase activation (Samali et al., 2001). In the intrinsic apoptosis pathway, it has been shown that Hsp27 can inhibit caspase 3 activity by interacting with procaspase 3 (Concannon et al., 2001). Furthermore, Hsp27 downregulates the activation of procaspase 9 by interaction with cytochrome c, thereby
preventing the correct formation/function of the apoptosome complex (Bruyé et al., 2000). Taken together, a complicated network is likely to be activated in host cells following CSFV infection, containing up- and down-regulated apoptotic genes belonging to different apoptotic pathways.

The Nrampl gene encodes a divalent cation transporter that is well-known to be located in the phagolysosomal membrane of macrophages and plays an important role in innate immunity by providing resistance to intracellular bacteria and protozoan pathogens, including Mycobacterium sp., Salmonella sp., Brucella sp. and Leishmania sp. (Vidal et al., 1995; Gruenheid et al., 1997; Zwilling et al., 1999; Jabado et al., 2000; Capparelli et al., 2007). The function of Nrampl in virus infection has not yet been elucidated, but its about 8-fold upregulation in PBLs following CSFV infection indicates that it is possibly involved in the anti-CSFV cellular response. Leukocyte membrane protein CD69 is an early activation marker in response to inflammatory stimuli and acts as a costimulatory molecule for T- and natural killer-cell activation (Mihaylova et al., 2007). In addition, CD1 functions as a microbial recognition factor for activation of T-cell responses and controls T-cell reactions that are highly specific for lipid antigens that are expressed particularly abundantly by pathogenic mycobacteria (Behar & Porcelli, 2007). In the present study, both CD69 and CD1 were downregulated markedly (>10-fold), suggesting that the proteins encoded by these two genes may be associated with immune-system depression in CSFV-infected pigs.

Annexin 2 is one of the Ca\(^2+\)-dependent cytosolic proteins that can bind to cellular membranes, and its diverse functions include membrane trafficking, endosome formation and aggregation of vesicles (Drust & Creutz, 1988). It also interacts with some viruses and mediates virus entry, replication, assembly, budding and release. Previous studies have shown that annexin 2 is an HIV Gag-binding partner in macrophages and can promote HIV entry into monocyte-derived macrophages (MDMs) through an interaction with phosphatidylserine, present in infectious virus particles. It has also been found to be essential for the proper assembly of HIV in MDMs and its downregulation results in suppression of virus replication (Ma et al., 2004; Ryzhova et al., 2006). Annexin 2 is also a potential respiratory syncytial virus (RSV) receptor on Hep2 (human epithelial) cells, and its expression is increased after RSV infection (Malhotra et al., 2003). In the present study, expression of the annexin 2 gene was upregulated by >2-fold in the PBLs of CSFV-infected pigs, which is consistent with our previous proteomic analysis showing that the expression of annexin 2 protein in CSFV-infected PK-15 cells was increased by about 5-fold (Sun et al., 2009). LSCM in the present study has shown that annexin 2 and CSFV glycoprotein E2 were colocalized in the cytoplasm of infected PK-15 cells (Fig. 3c), implying that annexin 2 may interact with certain viral proteins during CSFV replication and thereby promote the virus cycle.

An important receptor involved in the entry of flaviviruses into target cells is scavenger receptor class I (SR-BI); its gene gives rise to at least two mRNA splice variants, SR-BI and SR-BII, which differ in their C termini (Webb et al., 1998). SR-BI and SR-BII mediate the internalization of hepatitis C virus (HCV) into cells through their interaction with HCV soluble E2 envelope glycoprotein. Increased expression of SR-BI and SR-BII in the Huh-7.5 hepatoma cell line enhanced HCV infectivity, which was inhibited by anti-SR-BI antibodies, indicating a critical role for these proteins in HCV infection (Grove et al., 2007). Both HCV and CSFV belong to the family Flaviviridae and their E2 proteins are type I transmembrane glycoproteins that mediate virus entry into cells (Bartosch et al., 2003; Wang et al., 2004). The present microarray analysis detected upregulation of the SR-BII gene, suggesting that CSFV infection can modulate the expression of its receptor and probably use a mechanism similar to that of HCV for virus internalization by interaction of its E2 with SR-BI/II.

It is well-established that increased mucosal production of thromboxane plays a significant role in the pathogenesis of human inflammatory bowel disease (ulcerative colitis and Crohn’s disease) and experimentally induced colitis of animal models (Clapp et al., 1993; Rampton & Collins, 1993). Here, we detected a 2-fold upregulation of thromboxane synthase expression during the severe clinical stage of CSF in CSFV-infected pigs. This finding provides a strong clue in the pathogenesis of colitis with button ulcers, a very specific intestinal lesion of CSFV-infected pigs (van Oirschot, 1999).

In conclusion, the present study has described a complete transcriptional response of pigs to CSFV infection. Microarray analysis has shown that expression of 1745 genes in PBLs was altered following CSFV infection, an observation confirmed by real-time RT-PCR and Western blotting of some selected genes and their products. This work has established a most comprehensive differential transcriptional profile of CSFV-infected pigs, although the application of these data to elucidation of virus pathogenesis is still limited. Further functional investigation of the altered genes may facilitate understanding of the pathogenic mechanisms and molecular responses of host cells to CSFV infection.

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**REFERENCES**


