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Macrophage Transcriptional Responses following In Vitro Infection with a Highly Virulent African Swine Fever Virus Isolate

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We used a porcine microarray containing 2,880 cDNAs to investigate the response of macrophages to infection by a virulent African swine fever virus (ASFV) isolate, Malawi LIL20/1. One hundred twenty-five targets were found to be significantly altered at either or both 4 h and 16 h postinfection compared with targets after mock infection. These targets were assigned into three groups according to their temporal expression profiles. Eighty-six targets showed increased expression levels at 4 h postinfection but returned to expression levels similar to those in mock-infected cells at 16 h postinfection. These encoded several proinflammatory cytokines and chemokines, surface proteins, and proteins involved in cell signaling and trafficking pathways. Thirty-four targets showed increased expression levels at 16 h postinfection compared to levels at 4 h postinfection and in mock-infected cells. One host gene showed increased expression levels at both 4 and 16 h postinfection compared to levels in mock-infected cells. The microarray results were validated for 12 selected genes by quantitative real-time PCR. Levels of protein expression and secretion were measured for two proinflammatory cytokines, interleukin 1β and tumor necrosis factor alpha, during a time course of infection with either the virulent Malawi LIL20/1 isolate or the OUR T88/3 nonpathogenic isolate. The results revealed differences between these two ASFV isolates in the amounts of these cytokines secreted from infected cells.

African swine fever virus (ASFV) causes inapparent persistent infections in its natural hosts, warthogs (*Phacochoerus aethiopicus*), bushpigs (*Potamochoerus porcus*), and soft ticks (*Ornithodoros moubata*), which inhabit warthog burrows. In contrast, the virus causes an acute hemorrhagic fever with high mortality rates in pigs, although some low-virulence isolates have been reported (6, 33, 50, 52, 53).

The virus replicates in cells of the mononuclear phagocytic system and reticulo-endothelial cells of lymphoid tissues and organs. Widespread apoptotic cell death occurs in both T and B lymphocytes of lymphoid tissues (9, 21) and in arteriolar and capillary endothelial cells (10, 15–17, 22). Disseminated intravascular coagulation develops during the late phase of acute infections, and this may lead to the characteristic hemorrhagic syndrome (20, 22, 41).

ASFV is a large, icosahedral, double-stranded DNA virus which replicates in the cytoplasm of infected cells and has been classified as the only member of a new virus family (13), the *Asfarviridae.* The genome encodes between 160 and 175 proteins, including a number that interfere with host defense systems. These include proteins such as the A238L protein, which inhibits both the activation of the host NF-κB transcription factor and calcineurin phosphatase activity. The latter inactivation results in the inhibition of calcineurin-dependent pathways, such as activation of the nuclear factor of activated T cell transcription factors (23, 24, 37, 38, 44, 47). Members of ASFV multigene families 360 and 530 have been implicated in inhibiting the transcription of the beta interferon (IFN-β) gene and interferon-activated pathways (2).

To help understand how ASFV infection manipulates the function of macrophages, we have used a porcine cDNA microarray containing 2,880 genes to investigate changes in macrophage gene transcription that occur during in vitro infection with a virulent isolate of ASFV (Malawi LIL20/1). We validated our microarray data by quantitative real-time PCR of 12 selected differentially expressed genes.

MATERIALS AND METHODS

Cells and viruses. Alveolar macrophages were collected by lung lavage from Large White/Landrace crossbred piglets and cultured in RPMI 1640 medium supplemented with 10% fetal cattle serum, penicillin-streptomycin, and fungizone. Bone marrow cells were obtained from piglet femurs. Adherent cell populations were selected after overnight culture.

The high-virulence isolate Malawi LIL20/1 (26) and the low-virulence isolate OUR T88/3 (6) were grown in primary pig bone marrow cells, and virus was purified by OptiPrep (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation and ultrafiltration. Hemadsorption or an indirect immunofluorescence assay which detects an early viral protein, p30 (1), was used to titrate virus by limiting dilution. Indirect immunofluorescence was also used to monitor the infection rate of macrophage cultures.

Infection and stimulation of cells. Macrophages were infected at a multiplicity of infection of 5 to 10. Uninfected cells were stimulated by culturing them in the complete medium supplemented with 1 μg/ml of lipopolysaccharide (LPS; Sigma). In parallel, cells were mock treated without the addition of virus or LPS.

Microarray. Details of the porcine cDNA immunomicroarray used are available at www.arkgenomics.org, and the data are available from ArrayExpress under accession number A-MEXP-494. This array contains 2,880 porcine cDNAs selected by targeted gene cloning and from subtracted libraries.

RNA isolation. Total RNA was isolated from cells using TriReagent (Sigma) followed by purification on QIAGEN RNeasy columns. RNA quality was assessed on an Agilent 2100 bioanalyzer with an RNA 600 LabChip kit.

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RNA labeling and hybridizations to microarrays. Labeled cDNA probes were produced using the Stratagene Fairplay microarray labeling kit. Twenty micrograms of total RNA was labeled for each sample to be used in a hybridization. Labeled cDNAs were then purified using a DyeEx spin column. Labeling efficiency was checked by running a sample on an agarose gel and visualizing the fluorescence using an LSIV scanner (Genomic Solutions, Cambridge, United Kingdom). Labeled cDNAs were added to the hybridization solution (Ultrahyb; Ambion, Huntingdon, United Kingdom) and hybridized to microarrays for 18 h at 42°C, and then slides were washed at 42°C. Slides were scanned using a ScanArray 5000XL instrument (PerkinElmer, Cambridge, United Kingdom).

Data analysis. Raw data (spot intensity and background measurements) were extracted by using Quantarray and normalized in Genespring per spot and per chip (Lowess); then, in-slide averaging was done of the duplicate targets on each array. A one-way analysis of variance algorithm was used to identify differentially regulated targets with a *t* test P of <0.05.

Real-time PCR. RNA was converted into cDNA by using Onimus reverse transcriptase (QIAGEN). A QuantiTect SYBR green PCR kit (QIAGEN) was used for the PCRs on a GeneAmp 5700 system (PerkinElmer), and cycle threshold values were extracted by using GeneAmp 5700 SDS software. The relative expression levels of the selected genes were calculated (42), using beta-actin as the internal control.

Enzyme-linked immunosorbent assays (ELISA). Quantikine immunoassay kits (R & D Systems) were used to measure levels of porcine interleukin 1β (IL-1β).

SDS-polyacrylamide gel electrophoresis and Western blotting. Alveolar macrophages were either mock infected, treated with LPS (1 μg/ml), or infected with the ASFV Malawi LIL20/1 or OUR T88/3 isolate. At designated time points, culture supernatants were harvested and cells were washed with cold phosphate-buffered saline and lysed in 200 μl of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). Clarified supernatants were resolved on 12% SDS-polyacrylamide gels before transfer to nitrocellulose membranes. To detect IL-1β, a mouse monoclonal antibody (clone 77724; R & D Systems) was used at a concentration of 1 μg/ml, and as a loading control, an antibody against β-actin (code no. JM-3598-100; MBL International) was used at a concentration of 1 μg/ml. A goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase conjugate (Promega) was used as the secondary antibody at a dilution of 1:2,500, and bound antibodies were detected by enhanced chemiluminescence. To detect tumor necrosis factor alpha (TNF-α), a goat IgG anti-porcine TNF-α (AF690; R&D) was used, and bound antibodies were detected using protein G conjugated to horseradish peroxidase (catalog no. ab7460-1; Abcam) followed by ECL.

RESULTS

Experimental design. RNA was harvested from porcine macrophages infected with the virulent ASFV isolate Malawi LIL20/1 at one time point (4 h postinfection) early in the virus replication cycle when virus had entered cells and early protein synthesis had begun and at one time point late in infection (16 h postinfection) when the switch to late virus gene expression has occurred and the production of progeny virions was in progress but cell death was not yet evident (13). Pilot experiments showed little change in macrophage gene expression at 4 and 16 h following mock- infection, and we therefore used pooled samples from mock-infected macrophages at 4 and 16 h as the control in our experiments. A triangular-loop design was used comparing RNA from mock-infected macrophages with that from macrophages infected for either 4 h or 16 h. In addition, a direct comparison of RNA from macrophages infected for 4 h and for 16 h was carried out. This third comparison provided validation of the data from the other two comparisons. Dye swaps were included for each experimental comparison. Macrophages were infected with purified concentrated stocks of Malawi LIL20/1 virus, and infection rates of at least 70%, were obtained. We pooled the RNA from two sets of experimental infections and carried out experiments using four separate pools of RNA (i.e., from eight separate animals).

Temporally regulated genes. Approximately 140 of the targets were eliminated from the analysis because of poor quality. By using one-way analysis of variance analysis in the Genespring software package, 125 targets were identified as containing differentially regulated genes. Thus, 2,615 targets did not change in expression level. Differentially regulated genes were broken down into eight clusters by the K-Mean algorithm. Due to the lack of independent experimental conditions, this clustering is mainly for the purpose of data visualization and presentation. The eight clusters were then manually separated into three groups (I, II, and III) exhibiting different temporally regulated expression patterns (Table 1).

Group I contains 87 targets which have increased expression levels at 4 h postinfection compared to mock-infected cells but show decreased expression levels at 16 h postinfection compared to levels at 4 h postinfection. Since some of these targets contained cDNAs encoding the same genes, a total of 77 different differentially expressed genes were identified in this group, including 62 of known function and 15 of unknown function.

Group II contains 34 targets which increased in expression level at 16 h postinfection compared to levels at 4 h postinfection. Each of these encoded unique genes, including 20 of known function and 14 of unknown function.

The third group of genes, group III, contains four genes. Their expression levels were increased at both 4 and 16 h postinfection compared to levels in mock-infected cells. Three of these genes were ASFV genes included in the array as an infection control.

Functional classification of differentially regulated genes. Within gene group I (increased levels at 4 h postinfection), several functionally related classes of genes were identified. One of these classes encoded cytokines and chemokines. These include the CC chemokines MIP-1α, MIP-1β, MIP-2α, MIP-2β, and RANTES and the CXC chemokine AMCF1. Of these, MIP-1α and MIP-1β consistently showed the highest increase (*n*-fold) in expression at 4 h compared to levels in mock-infected cells, and this varied between three- and fivefold when we compared different cDNA array spots encoding the same genes. The only cytokines that showed increased expression levels in infected cells were those encoding the proinflammatory cytokines IL-1β, TNF-α, and IFN-β. These showed comparatively small increases in expression levels which varied from 1.5- to 2.4-fold compared to those in mock-infected cells at 4 h postinfection.

A second functional class of genes within group I are those encoding other cell surface-expressed or -secreted proteins. Genes which showed a >4-fold increase in transcript levels at 4 h postinfection compared to levels in mock-infected cells included syndecan 2 (heparan sulfate proteoglycan), galectin 3, and the mannose receptor. The other cell surface proteins in group I included CD87 urokinase plasminogen activator receptor (uPAR); IEX1, a type II membrane protein induced by radiation treatment, stress, and phorbol esters (28, 31, 43); a lectin-like, oxidized, low-density lipoprotein receptor (LOX-1); and annexin 1. An additional gene encoding a secreted protein in group I was the TNF-stimulated gene 6 product (TSG-6).

TABLE 1. Changes (*n*-fold) in expression levels of genes differentially regulated following ASFV infection

Group and accession no. for gene on each spot	Description	Fold change at:		
		4 h/mock infection	16 h/mock infection	16 h/4 h
Group I (87 targets, 15 unknown)				
AJ747345	CCL4/MIP-1β, small inducible cytokine A4	4.5	1.1	0.3
AJ747413	No hit	4.3	1.3	0.3
AJ746841	CCL4/MIP-1β, small inducible cytokine A4	3.6	1.1	0.4
AJ747490	No hit	3.1	1.0	0.5
AJ746828	CCL4/MIP-1β, small inducible cytokine A4	3.3	1.0	0.5
AJ746638	No hit	4.8	1.3	0.3
AJ746803	CCL3/MIP-1α, small inducible cytokine A3	3.5	1.2	0.4
AJ747430	No hit	3.7	1.0	0.4
BC028674	EF-1-alpha-1, elongation factor 1-alpha 1	4.5	1.1	0.3
X62001	CATS, cathepsin S	5.0	1.2	0.3
AY368183	MMR, macrophage mannose receptor	3.9	1.3	0.4
BC034992	SNXA, sorting nexin 10	4.1	1.1	0.4
AJ747009	LGALS3, galectin 3	4.1	1.2	0.3
AJ747450	No hit	2.7	0.9	0.3
J04621	SDC2, syndecan-2 precursor (fibroglycan)	4.5	1.3	0.3
AJ747122	CCL3/MIP-1α, small inducible cytokine A3	5.0	1.3	0.3
AJ747350	CCL3/MIP-1α, small inducible cytokine A3	4.6	1.4	0.3
BC009334	CTC6, protein C20 ORF126	6.4	1.1	0.2
AB006011	JAK2, Janus kinase 2	2.8	0.7	0.3
AJ279008	CATL, cathepsin L precursor	1.6	0.8	0.5
AJ747364	CXCL2 chemokine	1.5	1.0	0.5
AJ746916	ESTD, esterase D	1.6	0.9	0.5
AJ747481	No hit	1.6	0.8	0.5
AM260755	No hit	1.6	0.9	0.6
BE233980	Prostaglandin G/H synthase 2	1.8	1.1	0.6
BE232835	IL-1β	1.8	1.0	0.6
AJ747240	ANX1, annexin 1	1.5	1.0	0.6
BE031195	MT1A, metallothionein	1.7	1.0	0.7
BD132522	OBRG, leptin receptor gene-related product	1.8	1.0	0.7
AJ747349	CXCL2 chemokine	1.6	1.2	0.6
AJ747344	CXCL3 chemokine	1.7	1.0	0.6
AJ747109	OCTM4, tetraspan membrane protein	2.1	1.1	0.6
AJ747692	PAI-2, urokinase inhibitor	2.1	0.9	0.5
AJ747354	GDF8, *Sus scrofa* myostatin gene	2.3	1.1	0.6
AJ746636	NP, purine nucleoside phosphorylase	1.7	0.9	0.6
AJ746832	ARGI, arginase 1	1.5	0.8	0.5
BF442132	UPAR	1.7	0.9	0.6
AJ747334	Similar to RIKEN cDNA 2010003F10 gene	2.1	1.1	0.6
X74568	IL-1β	2.4	0.9	0.5
AJ747452	No hit	2.0	0.9	0.5
BF078747	IEX1, radiation-inducible immediate early gene	2.1	0.8	0.5
X57321	TNF-α	2.3	1.0	0.7
U95009	CREB, cyclin-AMP-responsive element binding protein	3.0	1.0	0.6
AJ747487	KIAA0266 gene	2.6	1.2	0.5
AJ279057	POL, endogenous retroviral POL polyprotein	1.3	1.0	0.9
AJ746673	UPAR	1.3	1.0	0.8
AJ747410	No hit	1.4	1.0	0.8
AJ747911	DJC7, DnaJ homolog subfamily C member 7	1.4	1.0	0.8
M29079	TNF-α	1.5	1.1	0.9
BC030634	Mortalin-2, 70-kDa shock protein 9B	1.4	1.1	0.8
AJ747102	Similar to B-cell phosphoinositide 3-kinase adaptor	1.3	1.1	0.8
AJ746786	CCL5/RANTES, small inducible cytokine A5	1.4	1.1	0.8
AJ746859	CXCL5/AMCF1, chemokine	1.5	1.2	0.8
AJ747474	APOB, apolipoprotein B	1.3	1.0	0.8
AJ747126	CIS1	1.3	1.0	0.8
AJ746642	MIP-1β, partial	1.4	1.0	0.8
AM260756	No hit	1.5	1.0	0.8
AJ747469	ERF1, eukaryotic peptide chain release factor	1.5	1.0	0.8
AJ747417	C122, putative alpha-mannosidase C1 ORF22	1.4	1.0	0.9
AJ747862	OXA1, mitochondrial inner membrane protein	1.4	1.0	0.9
Z21968	IκB-α, NF-κB inhibitor alpha	1.2	1.0	0.9
M86762	IFN-β	1.3	1.0	0.9
AJ747720	ASFV gene	1.6	1.4	1.0
BI347029	NF-κB1, NF-κB p50 precursor	1.4	1.0	0.7
AJ746951	PNPH, purine nucleoside phosphorylase	1.5	0.9	0.7
AW316433	STAT2, signal transducer and activator of transcription 2	1.7	1.1	0.8
AJ747878	S3B2, splicing factor 3b subunit 2	1.5	1.0	0.7
AJ747477	No hit	1.4	1.2	0.7
BI342110	TSG-6	1.6	1.1	0.7
BF080110	No hit	1.6	1.0	0.7
BG384374	SFRS3, splicing factor arginine/serine-rich 3	1.2	0.8	0.7
L43124	VCAM1, vascular cell adhesion molecule 1	1.3	0.9	0.8
BI345703	ND2, NADH dehydrogenase subunit 2	1.1	0.9	0.6
AM260754	PPG, platelet proteoglycan core protein, partial	0.9	0.6	0.6
BI342952	DDX5, DEAD box protein p68	1.3	0.8	0.7
BF198233	No hit	1.2	0.8	0.7

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TABLE 1—*Continued*

Group and accession no. for gene on each spot	Description	Fold change at:		
		4 h/mock infection	16 h/mock infection	16 h/4 h
BG834863	RNA-binding protein FUS	1.3	1.0	0.7
BF189184	No hit	1.2	0.9	0.8
BI338506	GLUL, glutamate-ammonia ligase	1.2	0.7	0.7
BI338939	NF-κB1, NF-κB p100/p49 subunits	1.3	0.9	0.8
AJ746964	HSPA8, 70-kDa heat shock protein 8	1.1	0.9	0.8
AW425011	Heterogeneous nuclear D-like ribonucleoprotein	1.1	1.0	0.8
BE236016	No hit	1.4	0.9	0.7
BC000178	KCMF1, potassium channel modulatory factor	1.3	0.9	0.7
Z21968	IκB-α	1.4	0.8	0.7
BI341366	Glrx1, glutaredoxin	1.3	1.0	0.7
BG383949	LOX1, lectin-like oxidized low-density lipoprotein receptor 1	1.4	0.8	0.6
Group II (34 targets, 14 unknown)				
BG384562	No hit	0.7	0.8	1.1
BG384417	NAGK, *N*-acetylglucosamine kinase	0.7	0.9	1.1
AM260789	S100a6, S100 calcium-binding protein A6 (calcyclin)	0.7	0.9	1.2
AJ747011	No hit	0.5	0.8	1.1
AW479942	TKT, transketolase	0.7	0.9	1.0
AJ747897	No hit	0.7	0.9	1.3
AJ747223	Progesterone membrane-binding protein	0.7	0.9	1.3
BG383269	PGR, 15 oxoprostaglandin 13-reductase	0.5	0.8	1.3
BG609005	No hit	0.6	0.8	1.3
BG834532	Hypothetical protein CGI-128	0.9	1.1	1.3
AJ746996	GNS, *N*-acetylglucosamine 6 sulfatase	0.8	1.0	1.2
BI338320	RPS18, 40S ribosomal protein S18	0.8	1.0	1.3
AW436618	No hit	0.8	1.1	1.3
BI467643	CARD6, caspase recruitment domain protein	1.0	1.1	1.3
BF444373	No hit	0.9	1.1	1.3
BE013887	No hit	0.8	1.1	1.3
BF190002	RPP2D, acidic ribosomal protein P2	0.8	1.1	1.3
BI346405	PRST, prostein protein	0.8	1.0	1.2
BF192896	Pparg, peroxisome proliferator-activated receptor	0.7	1.0	1.2
BF442098	No hit	0.9	1.0	1.2
BG732923	No hit	0.8	1.0	1.2
BG382031	P26452, 40S ribosomal protein P40	0.9	1.1	1.2
AW435754	CSAD, cysteine sulfinic acid decarboxylase	0.9	1.0	1.2
AW308265	PLAU, urokinase-type plasminogen activator	0.9	1.1	1.2
BE013644	Ptgis, prostaglandin I2 (prostacyclin) synthase	0.9	1.0	1.2
BI340632	No hit	1.0	1.1	1.4
BE014284	No hit	0.9	1.1	1.4
BF198933	CISY, citrate synthase, mitochondrial precursor	0.8	1.2	1.3
BI341443	No hit	1.1	1.4	1.6
BI360720	No hit	1.0	1.7	2.0
BG384920	RAD51L3, TRAD, DNA repair protein	1.3	2.6	3.0
AJ747845	No hit	1.3	2.7	2.5
AJ747820	MHC1 SLA gene	1.1	1.5	1.7
AJ747815	Lysosomal glucocerebrosidase precursor	1.1	1.9	1.8
Group III (4 targets, 1 unknown)				
AJ747727	ASFV O174L	11.9	15.2	1.4
AJ747714	No hit	4.2	3.4	1.3
AJ747717	ASFV V118	13.2	13.3	0.8
AJ747734	ASFV J319L ORF15L	8.0	10.5	1.1

RNA extracted from ASFV Malawi LIL20/1 isolate-infected or mock-infected porcine macrophages was labeled with Cy5 or Cy3 dye and hybridized to porcine cDNA microarrays. Data were extracted and analyzed as described in Materials and Methods. "No hit" indicates that no matches with known genes were identified. Shown are the changes (*n*-fold) in expression level for the three comparisons: infected macrophages at 4 h versus mock-infected macrophages (4 h/mock infection), infected macrophages at 16 h versus mock-infected macrophages (16 h/mock infection), and infected macrophages at 16 h versus infected macrophages at 4 h (16 h/4 h). Values were calculated as described in Materials and Methods.

A third class of genes in group I encodes components of intracellular signaling pathways. These include the cyclic-AMP-responsive transcription factor CREB (delta isoform); the tyrosine kinase JAK2 and transcription factor STAT 2, which are involved in IFN-activated signaling pathways; the NF-κB p50 subunit precursor; the IκB-α inhibitor of NF-κB; and cryopirin (cold-induced autoinflammatory syndrome 1 [CIAS1] protein) (14, 40, 49).

A fourth class of genes in group I is involved in protein trafficking pathways. These include genes encoding sorting nexin (SNX) 10 and a leptin receptor gene-related product. The leptin receptor gene includes the first two untranslated exons of the leptin receptor gene but expresses a different protein. This protein contains a Vps55 (PF04133) domain and is involved in trafficking from late endosomes to vacuoles (4).

A fifth class of genes in group I encode proteins activated by stress. Of these, open reading frame 126, found on chromosome 20, is up-regulated by greater than sixfold, whereas heat shock protein HSP70 and mortalin-2 shock protein 9B show relatively small increases in expression level.

A sixth gene class in group I encodes the endosomal/lysosomal cysteine proteases cathepsin S and L. Cathepsin S is up-regulated almost 5-fold, whereas cathepsin L is up-regulated 1.5- to 2-fold.

TABLE 2. Comparison of changes (*n*-fold) determined by microarray analysis and real-time PCR[a]

Gene	Change (*n*-fold)					
	Microarray			Real-time PCR		
	4 h/mock infection	16 h/mock infection	16 h/4 h	4 h/mock infection	16 h/mock infection	16 h/4 h
TNF-α	1.8	1.1	0.8	9.8	4.1	0.4
MIP-1β	3.8	1.0	0.4	6.9	1.9	0.3
IL-1β	2.1	0.9	0.6	2.3	1.0	0.4
VCAM	1.3	0.9	0.8	2.0	0.8	0.4
JAK2	2.8	0.7	0.3	1.7	0.8	0.5
ICAM1	1.4	1.1	0.8	3.6	1.8	0.5
AMCF1	1.5	1.2	0.8	2.1	0.9	0.8
PTGS2	1.8	1.1	0.6	2.2	0.9	0.4
CD87	1.7	0.9	0.6	2.3	1.1	0.5
P50	1.4	1.0	0.7	2.6	2.1	0.8
IκB-α	1.3	0.9	0.8	0.5	0.3	0.7
HSP70	1.1	0.9	0.8	0.6	0.5	1.0

[a] Quantitative real-time PCR was carried out as described in Materials and Methods for the selection of the indicated differentially regulated genes. Shown are changes (*n*-fold) between infected cells at 4 h and mock-infected cells (4 h/mock infection), infected cells at 16 h and mock-infected cells (16 h/mock infection), and infected cells at 16 h and infected cells at 4 h (16 h/4 h). The values from the microarray analysis are geometric means based upon replicates with each gene.

Additional functional classes in group I encode translation factors and nucleotide binding and metabolism factors, such as translation elongation factor 1, termination factor ETF1 (eRF1), splicing factors 3b subunit 2, and arginine/serine-rich factor 3.

Other group I genes encode proteins which may alter the function of macrophages. One of these, arginase 1, is involved in the degradation of arginine to provide polyamine biosynthesis precursors. Arginine is also used as a precursor for NO synthesis, and arginase activation can negatively influence NO production. The activation of arginase is an indicator of the alternative activation pathway of macrophages induced by type II rather than type I cytokines (7, 8). A cDNA encoding prostaglandin G/H synthase 2 was also in group I (27, 48, 54).

Temporally regulated group II contained 34 genes whose expression levels were either slightly down-regulated or slightly up-regulated at 4 h postinfection compared to levels in mock-infected controls but increased during the infection period. For example, the transcriptional levels of DNA repair protein RAD51 homolog 4, cytochrome P450, and major histocompatibility complex class I swine leucocyte antigen in cluster 7 showed just a small increase at 4 h, and by 16 h, their expression levels had increased two- to threefold compared to levels in mock-infected cells.

The one host gene included in group III is of unknown function.

Real-time PCR analysis of differentially expressed genes. Transcript levels of 12 genes were measured by real-time PCR using β-actin as the internal control. The comparisons carried out were the same as in the microarray experiments. For most genes, the relative expression levels obtained by real-time PCR were consistent with those obtained in the microarray experiments (Table 2). In the comparison between RNAs from macrophages infected for 16 h and 4 h, the two techniques gave very similar expression levels for all 12 genes. There were some discrepancies in the other two comparisons. For example, real-time PCR results showed much higher increases (*n*-fold) than the results of the microarray analysis for TNF-α in the comparisons of cells infected at both 4 and 16 h with mock-infected cells. In addition, the expression levels for IκB-α and HSP70 were slightly up-regulated at 4 h and almost the same as in mock-infected cells at 16 h postinfection according to the microarray data but about two- to threefold down-regulated compared to levels in mock-infected cells according to the real-time PCR data. These inconsistencies were probably due to the intrinsic difference between these two techniques, notably, in the normalization methods. For microarray experiments, the normalization was based on a large number of genes, whereas in real-time PCR experiments, a single housekeeping gene was used as an internal control against which results were normalized.

Accumulation and secretion of IL-1β and TNF-α during ASFV infection. To investigate changes at the protein levels of two of the differentially regulated genes, we measured the expression and secretion levels of two proinflammatory cytokines, IL-1β and TNF-α. Macrophages were mock treated, continually stimulated with 1 μg/ml LPS, or infected with the virulent ASFV Malawi LIL20/1 or nonpathogenic OUR T88/3 isolate. As shown in Fig. 1A, only the 31-kDa precursor of IL-1β was detected in the cell lysates by Western blotting. At early time points, 2 and 4 h posttreatment, both LPS treatment and ASFV infection induced a greater accumulation of pro-IL-1β synthesis than mock treatment in macrophages. This correlates with the microarray result that macrophages infected with the ASFV Malawi LIL20/1 isolate showed higher transcription levels of IL-1β than uninfected cells at 4 h postinfection. By 6 h posttreatment, similar intracellular pools of pro-IL-1β were present in all four groups of cells, and these were maintained until the end of the time course, at 24 h posttreatment.

The amounts of IL-1β estimated by ELISA that were secreted from macrophages infected with the Malawi LIL20/1 isolate were about sixfold greater by 23 h postinfection than with mock treatment, LPS stimulation, or OUR T88/3 infection (Fig. 1B). As shown above, a large pool of pro-IL-1β is present in both uninfected and infected cells. Although intracellular levels increased slightly during ASFV infection, the major mechanism regulating the differential release of IL-1β into the medium must involve the control of processing and secretion pathways.

The precursor and mature forms of TNF-α were detected by Western blotting in cell lysates. The amounts of intracellular TNF-α were greatly increased following LPS stimulation (Fig. 1C). Slightly increased levels of intracellular TNF-α were detected in cells infected with both viruses compared to levels in mock-infected cells at 4 h postinfection. By 22 h postinfection, levels of intracellular TNF-α had declined in cells infected with the Malawi LIL20/1 isolate and in mock-infected cells but remained slightly elevated in cells infected with the OUR T88/3 isolate.

Only the mature form of TNF-α was detected in cell supernatants, as expected. LPS treatment stimulated the greatest secretion of TNF-α, and this was detected from early times onward. At 22 h postinfection, small amounts of TNF-α were

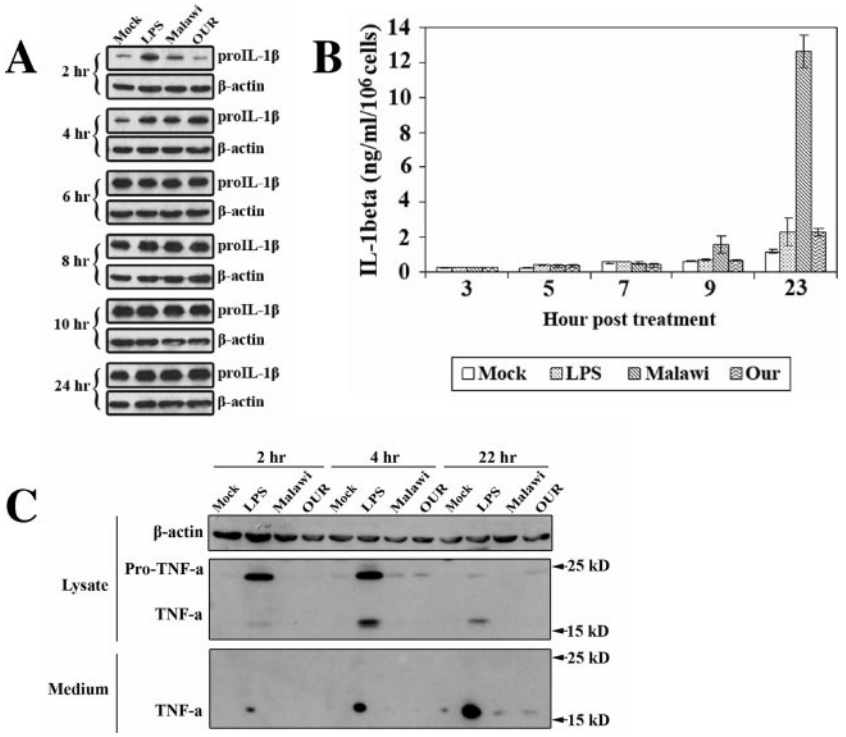

FIG. 1. Intracellular accumulation and secretion of IL-1β and TNF-α. Western blotting and ELISA assays were employed to investigate the intracellular accumulation and secretion of IL-1β and TNF-α as described in Materials and Methods. Alveolar macrophages were prepared from piglets and cultured in six-well plates. They were mock treated, continually stimulated with 1 μg/ml of LPS, or infected with the ASFV Malawi LIL20/1 or OUR T88/3 isolate. Cell lysates were prepared at 2, 4, 6, 8, 10, and 24 h posttreatment. Cell culture supernatants were also collected at 3, 5, 7, 9, and 23 h posttreatment to measure the secreted proteins. Western blotting was used to detect intracellular IL-1β (A). The positions of the 31-kDa pro-IL-1β and of beta-actin (44 kDa) are indicated. Quantitative ELISA were used to measure secreted IL-1β (B). ELISA results were expressed as geometric means ± standard deviations of three measurements. TNF-α was detected by Western blotting in extracts from cell lysates and in medium from infected or mock-infected macrophages. The positions of pro-TNF-α and mature TNF-α are marked (C).

detected in supernatants from cells infected with each virus as well as from mock-infected cells. Some variation was observed between macrophage cultures from different pigs in the levels of TNF-α and IL-1β present in cell lysates and secreted from cells. In particular, variation was observed in the background levels observed in mock-infected macrophages probably due to differences in the activation state induced by culture conditions. However, in experiments that we carried out using macrophages from six different pigs, we always observed an increased expression level at early times postinfection compared to the level in mock-infected cells.

DISCUSSION

The data presented are the first analysis of changes in porcine macrophage gene transcription that occur following in vitro infection with a highly virulent ASFV isolate. This analysis had the advantage of studying the effects of virus replication using the target species and main target cells for the virus. This showed that the majority of the genes analyzed (2,615 from 2,740) did not alter in transcription level during infection compared to levels in mock-infected cells. Thus, there is no general "switch-off" of host transcription during virus infection. This agrees with previous results which showed, by reverse transcription-PCR, that transcript levels of some constitutively expressed genes do not change during ASFV infection (19, 20, 44).

The majority of the differentially regulated genes showed a pattern of temporal expression characterized by increased expression levels at 4 h postinfection compared to levels in mock-infected cells, with a return in expression level at 16 h postinfection to levels equivalent to those in mock-infected cells. Genes in this group fell into several functional classes and included a number of genes whose transcription is predicted to be activated as part of the host response to clear virus infection. For example, we observed an early transcriptional activation of proinflammatory cytokine genes encoding IL-6, TNF-α, IFN-β, and chemokines of the CC and CXC groups. Increased expression of these cytokines and chemokines resulting in recruitment and activation of inflammatory cells might also favor virus replication by providing an additional pool of macrophages that are susceptible to infection. Transcript levels of several genes involved in intracellular signaling pathways activated by proinflammatory cytokines were also increased at early times. These included Jak 2, STAT 1, CREB, and a phosphoinositide 3-kinase adaptor.

We also observed increased transcript levels at early times for several genes which can have an anti-inflammatory role. These included the gene encoding cryopyrin/CIAS1. Cryopyrin is an inflammation mediator that can act upstream of IκB to

inhibit activation of NF-κB (40). The TNF-stimulated gene 6 protein, TSG-6, is a secreted protein with an anti-inflammatory role (3, 12, 34, 36). Increased transcription of these anti-inflammatory genes may play a role in suppressing the proinflammatory response.

Genes encoding several other cell surface or secreted proteins were up-regulated at early times postinfection. One of these encodes syndecan 2 (heparan sulfate proteoglycan), which has been shown to bind to IL-8 and, by binding to collagen, to reduce cell invasiveness (11, 25, 30, 32, 51). A second gene encodes galectin 3, a member of a family of galactose binding lectins with important roles in regulating homeostasis within the immune system. Galectin 3 can be expressed on the cell surface, nucleus, and cytoplasm or be secreted from cells. In the cytoplasm, galectin 3 binds to bcl-2 and can protect cells from apoptosis, and in the nucleus, it has growth-promoting activities. However, when present on the cell surface or secreted from cells, galectin 3 can induce apoptosis in bystander cells (18, 45, 46). If changes in the amount of active galectin 3 protein also occurs, this may provide an additional mechanism by which infected cells are protected from apoptosis until after virus replication. Conversely, galectin 3 expressed on the surfaces of or secreted from infected cells may play a role in the induction of apoptosis in bystander lymphocytes (29, 35, 45, 55).

Increased transcript levels of several genes encoding proteins which are involved in the presentation of peptides in conjunction with major histocompatibility complex class II were observed early postinfection. These genes included those encoding the mannose receptor and the proteases cathepsin S and L, the latter of which is involved in the processing of exogenous peptides. Cathepsin L is also known to cleave the precursor of IL-8.

The uPAR gene (CD87) was also up-regulated at early times. uPA and uPAR exert pleiotropic functions over both physiological and pathological processes involving inflammation, fibrinolysis, and tissue repair. By anchoring uPA at the cell surface, uPAR regulates cell migration, adhesion, and proliferation, influencing inflammatory and immune responses (5, 39).

The return to base-level transcription of genes in group I at late times postinfection may be due to the actions of virus-encoded proteins or to other host feedback mechanisms acting on infected cells. Expression of the A238L protein has already been shown to cause the down-regulation of transcription from the TNF-α and COX2 promoters (23, 24). Deletion of several members of multigene families 360 and 530 caused an increase in transcription of IFN-α (2). Although virus genes are expressed immediately following virus entry, the accumulation of proteins expressed to sufficient levels to be effective may require several hours. Hence, if these virus proteins are involved, the delay in observing an effect on "dampening" host transcription profiles is expected. The two other groups of differentially regulated genes, groups II and III, showed increased transcript levels at 16 h postinfection compared to levels in mock-infected cells. Possibly, these groups of genes may include some that encode proteins which are advantageous for virus replication.

Our results showed that infection of cells with the Malawi LIL20/1 isolate induced the secretion of much higher levels of IL-1β than the nonpathogenic OUR T88/3 isolate and that this increased secretion is likely to be regulated at the level of processing or release from cells rather than from changes in the amount of the transcript.

The amount of intracellular pro-TNF-α was increased slightly in cells infected with both the Malawi LIL20/1 and the OUR T88./3 isolate compared to the amount in mock-infected cells at 4 h postinfection. At 22 h postinfection, levels of pro-TNF-α remained elevated in lysates from cells infected with the OUR T88/3 isolate, whereas levels decreased in cells infected with the Malawi LIL20/1 isolate. We observed only slight differences in TNF-α protein levels in infected macrophages from those in mock-infected macrophages, whereas real-time PCR and microarray analysis demonstrated a greater increase in transcript levels in macrophages infected with the Malawi LIL20/1 isolate than in mock-infected macrophages. This suggests that posttranscriptional events such as protein stability are important in determining the levels of protein accumulated. One previous study (19) compared the levels of transcription of cytokines in macrophages infected with low- and high-virulence ASFV isolates. These results showed significantly increased levels of transcripts for TNF-α, IL-6, IL-12, and IL-15 in macrophages infected with a low-virulence isolate, NHP68, compared to levels in macrophages infected with the high-virulence isolate Lis 60. The differential secretion of proinflammatory cytokines from macrophages infected with the low- and high-virulence isolates may be of significance in virus pathology and will be further investigated.

This study is a useful first step in screening a wide range of host genes for potential virus-induced changes in RNA levels and provides a preliminary identification of some of the host genes that may be differentially regulated. Data from the microarray experiments provide a foundation for further studies, at the functional level, into the molecular basis of ASFV pathogenesis and virus mechanisms of immune evasion.

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