Molecular Pathogenesis and Immune Evasion of Vesicular Stomatitis Virus Inferred from Genes Expression Changes in Infected Porcine Macrophages

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Abstract: Molecular mechanisms associated with the pathogenesis of Vesicular stomatitis virus (VSV) in livestock remain poorly understood. Several studies have highlighted the relevant role of macrophages in controlling the systemic dissemination of VSV during infection in different animal models, including mice, cattle and pigs. To gain more insight on the molecular mechanisms used by VSV to impair the immune response in macrophages, we used microarrays to determine the transcriptomic changes produced by VSV infection in primary cultures of porcine macrophages. The results indicated that VSV infection induced the massive expression of multiple anorexic, pyrogenic, proinflammatory and immunosuppressive genes. Overall, the interferon (IFN) response appeared suppressed, leading to the absence of stimulation of interferon-stimulated genes (ISG). Interestingly, VSV infection promoted the expression of several genes known to downregulate the expression of IFNβ. This represents an alternate mechanism for VSV control of the IFN response, beyond the recognized mechanisms mediated by the matrix protein. Although there was no significant differential gene expression in macrophages infected with a highly virulent epidemic strain compared to a less virulent endemic strain, the endemic strain consistently induced higher expression of all upregulated cytokines and chemokines. Collectively, this study provides novel insights into VSV molecular pathogenesis and immune evasion that warrants further investigation.

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1. Introduction

Vesicular stomatitis virus (VSV) infection causes fever and vesicular stomatitis, one of four clinically indistinguishable viral vesicular diseases. VSV (family Rhabdoviridae, genus Vesiculovirus) is comprised of a non-segmented RNA viral genome encoding five structural proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and the large RNA-dependent RNA polymerase (L) (1, 2) along with two non-structural proteins (C and C0) of undetermined function encoded in overlapping reading frames of the P gene (3). VSV causes most of the cases of vesicular diseases reported in livestock resulting in economic losses associated to quarantines imposed by animal health authorities due to its similar clinical presentation with foot and mouth disease virus (FMDV) (4, 5).

VSV has a broad host range and cell tropism due to its glycoprotein binding to host LDLR family members that are ubiquitously expressed on host cells and conserved among mammalian species (6, 7). Besides typical vesicular lesions in specific tissues, infected animals also show systemic signs such as anorexia, lethargy and fever (https://en.wikivet.net/Vesicular_Stomatitis_Virus). Despite these clinical signs, VSV infection typically does not result in host mortality (4, 5). After infection via insect bites, animals show limited virus replication, primarily in specific tissues where the vesicular lesions occur. Infected animals usually recover completely within 2-3 weeks of infection (4, 5).

Unlike gross pathogenesis, the molecular pathogenesis of VSV is not very clear. Based on literature review, it appears that only TNF has been investigated in VSV pathogenesis, showing more rapid induction of TNF by an attenuated VSV mutant after infection, but more drastic TNF induction later in infection by wildtype VSV in mice (8). TNF knockout mice showed diminished weight loss following wildtype VSV infection, and the rapid weight loss seen in wildtype VSV infection was less pronounced in C57BL/6 mice infected by an attenuated mutant virus (8). In mice, interferons produced by VSV-infected macrophages play a key role in protection against neuropathogenesis of the virus (9). In the natural hosts such as cattle, VSV antigens were colocalized with an antibody against a marker molecule (MAC387, MRP14 or S100A9) of myeloid cells including macrophages using immunohistochemistry (10). Both wildtype VSV and matrix protein mutants productively replicate in porcine immune cells and non-immune cells (11, 12). Infection with wildtype VSV induced weaker proinflammatory cytokine responses and downregulated the expression of the costimulatory molecule complex CD80/86 and MHC class II

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compared to the matrix protein mutant virus (11). A matrix protein (M51R) VSV mutant virus replicated ~1000 times less in cultured primary porcine macrophages than its wildtype counterpart and showed significantly diminished virulence in pigs (13). The molecular pathogenesis and immune evasion in natural hosts such as pigs and cattle have yet to be investigated.

It is well-known that VSV can inhibit the host interferon response primarily via its matrix proteins (14, 15). VSV matrix protein mRNA can be translated into three proteins starting at three in-frame start codons (16). Transfection with plasmids containing the M protein gene alone can induce CPE in transfected cells (16). VSV M proteins can delay apoptosis induced by other viral components (17) and suppress transcription in infected cells by inhibiting the basal transcription factors TFIID and TFIIH and interacting with host Rae1 and Nup98 (18, 19, 20, 21, 22). VSV M proteins can also inhibit nuclear export of host mRNA and snRNAs (23) and NFκB activation (24). The suppression of IFNβ expression by the matrix protein is correlated with the inhibition of host RNA and protein synthesis (25). A systems biology approach including transcriptomic analysis has been conducted to study VSV infection in a murine macrophage cell line (26, 27); however, VSV pathogenesis and immune evasion were not explicitly explored based on transcriptional changes after virus infection. Although mice have been extensively used as an experimental model for VSV infection, they are not natural hosts for VSV infection. The transcriptomic analysis of VSV infection has not been investigated in the primary macrophages of its natural livestock hosts .

Macrophages play an important role in host defense against pathogens via positioning in all tissues where they can effectively sense danger signals with highly expressedPAMP receptors and producing a large quantity of both pro- and anti-inflammatory cytokines such as IL-1, IL-10, TGFβ and TNF via cell polarization and differentiation to regulate the immune response (28). Our previous study showed that primary porcine macrophages expressed higher levels of IFNβ and cytokines than primary fetal porcine kidney cell cultures after VSV infection (13). Given that VSV can infect and replicate in macrophages and the important role of macrophages in the immune response, *ex vivo* porcine macrophages were used as model cells to extrapolate the molecular mechanisms of VSV pathogenesis and immune evasion. The objective of this study was to formulate hypotheses for the molecular mechanisms of VSV pathogenesis and immune evasion based on gene expression changes in porcine macrophages after infection for further investigation.

2. Results

2.1 Differential gene expression

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There were no genes differentially expressed between macrophages infected with epidemic VS New Jersey (NJ0612NME6) and endemic New Jersey (NJ0806VCB) strains (minimal FDR = 0.13). There was a total of 4,346 significant differentially expressed genes at a false-discovery rate (FDR) of ≤ 0.05 with at least a 50% difference and a total of 3,345 with a difference of 2-fold or greater between epidemic VSV and mock infected macrophages. Between epidemic VSV and mock infected macrophages, there were a total of 3,345 significant differentially expressed genes (DEG) by at least 50% at a false-discovery rate (FDR) of ≤ 0.05. Among these DEG, the majority were detected as being downregulated (2,179 DEG) compared to 1,166 upregulated genes between VSV-infected and mock-infected cells, which was at approximately 2:1 ratio. Forty four percent of DEG were differentially expressed by 1.5- to 2.0-fold with 841 of these genes being downregulated, and 618 being upregulated (Figure 1). There were 54% of DEG with a fold change between 2.0 and 5.0, and the largest proportion of genes, 1288, were downregulated compared to 521 genes upregulated. Finally, the remaining 2.3% of the DEG were differentially expressed with a fold change greater than 5 (27 genes downregulated and 50 genes upregulated) (Figure 1). The most drastic differences were at a fold change of 10.2 for a downregulated gene, and 32.8 for an upregulated gene between these VSV- and mock-infected macrophages.

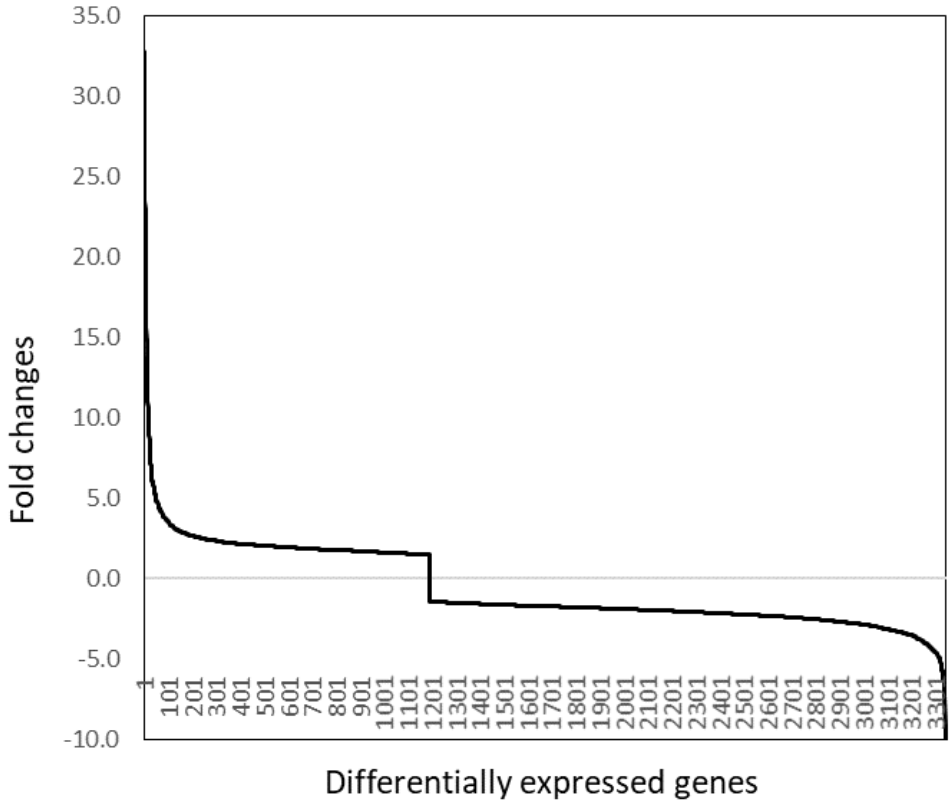

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Figure 1. The distribution of fold-changes of annotated genes with significantly differential expression equal to or greater than 2-fold between VSV- and mock-infected macrophages from most upregulated (32.8-fold) to most down-regulated (-10.2-fold) genes in VSV-infected macrophage compared to mock-infection

2.2 Pathway analyses

To identify biological pathways/processes most impacted by the differential expression, the lists of DEG with differential expression of a fold change of at least 2-fold to remove DEG with minor effects were used in the DAVID analysis. GO term analysis showed that NFκB signaling pathway was the most over-represented by the DEG with three other significant biological processes in protein ubiquitination, Toll-like receptor signaling, and mRNA transcription regulation (Table 1). KEGG pathway analysis identified eleven over-represented biological pathways with top five pathways (TNF, TLR, NFkB, RIG-I-like receptor, and NOD-like receptor signaling) that are known to play key roles in the immune response (Table 1). Only one biological pathway (TNF-induced apoptosis) was detected with REACTOME analysis.

When this list of DEG was further narrowed to those with a fold change of at least 4 (more biologically impactful DEG), GO term analysis identified thirteen over-represented biological processes; six involved in the immune response, four in apoptosis, two in signaling pathways and one in RNA transcription. The two most over-represented GO terms were in inflammatory response (GO_0006954) and apoptotic process (GO_0006915) (Table 1). The significant over-represented KEGG pathways included: KEGG_hsa04060-Cytokine-cytokine receptor interaction; KEGG_hsa04668-TNF-signaling pathway; KEGG_hsa04064-NF-kappa B-signaling pathway; KEGG_hsa04621- NOD-like receptor signaling pathway and KEGG_hsa04620- Toll-like receptor signaling pathway. Only one Reactome pathway (HSA-380108: Chemokine receptors bind chemokines) was significant.

	Pathway analysis	Count	Benjamini
DEG with at least 2-fold	GO_0007249: I-kappaB kinase/NF-kappaB signaling	23	8.20E-05
	GO_0016567: protein ubiquitination	62	0.026
	GO_0035666: TRIF-dependent toll-like receptor signaling pathway	12	0.036
	GO_0045944: positive regulation of transcription by RNA polymerase II	135	0.045
	KEGG_hsa04668: TNF signaling pathway	34	6.40E-07
	KEGG_hsa04620: Toll-like receptor signaling pathway	33	1.01E-06
	KEGG_hsa04064: NF-kappa B signaling pathway	28	5.35E-06
	KEGG_hsa04622: RIG-I-like receptor signaling pathway	21	5.73E-04

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	KEGG_hsa04621: NOD-like receptor signaling pathway	16	0.011
	KEGG_hsa04144: Endocytosis	43	0.012
	KEGG_hsa05160: Hepatitis C	27	0.022
	KEGG_hsa05169: Epstein-Barr virus infection	25	0.026
	KEGG_hsa04210: Apoptosis	16	0.029
	KEGG_hsa05220: Chronic myeloid leukemia	17	0.043
	KEGG_hsa04140: Regulation of autophagy	9	0.050
	REACTOME_HSA-5357786: TNFR1-induced proapoptotic signaling	8	0.039
DEG with at least 4-fold differential expression	GO_0006954: inflammatory response	19	1.19E-05
	GO_0006915: apoptotic process	20	5.38E-04
	GO_0045944: positive regulation of transcription by RNA polymerase II	25	0.003
	GO_0006955: immune response	15	0.009
	GO_0043065: positive regulation of apoptotic process	12	0.019
	GO_0071222: cellular response to lipopolysaccharide	8	0.020
	GO_0051897: positive regulation of protein kinase B signaling	7	0.021
	GO_0042981: regulation of apoptotic process	10	0.022
	GO_0070373: negative regulation of ERK1 and ERK2 cascade	6	0.026
	GO_2001244: positive regulation of intrinsic apoptotic signaling pathway	5	0.029
	GO_0030593: neutrophil chemotaxis	6	0.036
	GO_0051384: response to glucocorticoid	6	0.037
	GO_0070098: chemokine-mediated signaling pathway	6	0.047
	KEGG_hsa04060: Cytokine-cytokine receptor interaction	15	3.82E-05
	KEGG_hsa04668: TNF signaling pathway	11	4.69E-05
	KEGG_hsa04064: NF-kappa B signaling pathway	7	0.016
	KEGG_hsa04621: NOD-like receptor signaling pathway	6	0.020
	KEGG_hsa04620: Toll-like receptor signaling pathway	7	0.027
	Reactome-HSA-380108: Chemokine receptors bind chemokines	7	0.003

Table 1. Gene ontology terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) and REACTOME biological pathways over-represented by genes differentially expressed by at least 2- and 4-fold between VSV-infected and mock-infected porcine macrophages using the NCBI DAVID program with Benjamini p-value correction

2.3 Interferon expression and signaling

VSV infection significantly induced the expression of IFNB by 6.2-fold, but not other interferons, in infected macrophages compared to mock-infected cells (Table 2). The endemic strain induced higher IFNB expression (2-fold) than the epidemic strain but not at the significant level. VSV infections significantly suppressed the expression of an IFNA homologous to human IFNA17 by approximately 2-fold and did not significantly alter expression of other interferons including types II and III. The expression of type I (IFNAR1 and IFNAR2) and II (IFNGR1) receptors were suppressed in VSV-infected cells compared to mock-infection (Table 2). The type III IFN receptor (IFNLR1) was

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expressed at a very low level in the macrophages (signal intensity = 69, SNR < 2). The expression of typical interferon stimulated genes (ISGs) was not significantly changed by VSV-infection (only 10 genes listed in Table 2). These results indicate that VSV infection suppressed type I IFN and II IFN signaling.

Six genes, AHR (30, 31), ATF3 (32), DUSP1 (33), FOS (34), HES1 (35) and PRDM1 (36) known to negatively regulate type I IFN expression were significantly induced mostly by > 10-fold in VSV-infected cells (Table 2). Among all DEG, PRDM1 was the most induced gene (~33-fold higher) after infection and FOS was the fifth most-induced gene in this study (Table 7). EGR1, a PRDM1 expression inducing gene (37), was also highly upregulated (13.4-fold) in VSV infected cells. These results indicate that VSV infection induces expression of genes suppressing IFNB expression.

Group	Gene	EXP	EP/M	FDR	EP/EN	FDR
IFN and signaling	IFNA17	77	-1.9	0.01	-1.1	0.96
	IFNB	179	6.2	0.04	-2.1	0.79
	IFNAR1	3774	-2.0	0.01	1.0	0.97
	IFNAR2	2223	-2.9	0.03	-1.2	0.90
	IFNGR1	1000	-2.7	0.02	-1.2	0.92
Ten typical interferon stimulated genes	IFI44L	955	1.1	0.88	1.2	0.82
	IFIH1	107	-1.1	0.92	-1.0	1.00
	IFIT1	231	1.7	0.52	-1.4	0.89
	IFIT2	420	-2.4	0.23	1.0	0.99
	IFIT3	3298	-1.6	0.27	1.2	0.89
	IFIT5	364	-2.0	0.10	1.4	0.80
	ISG20	580	1.2	0.91	1.3	0.96
	MX1	3882	-1.2	0.90	-1.0	0.99
	MX2	1330	-1.5	0.56	1.2	0.94
	OAS1	155	-1.1	0.95	1.1	0.98
IFN expression inhibitors	AHR	193	6.1	0.01	-1.0	0.98
	ATF3	1432	8.6	0.05	-1.6	0.91
	DUSP1	2208	13.8	0.01	-1.6	0.88
	EGR1	891	13.4	0.01	-2.1	0.79
	FOS	1993	22.9	0.01	-1.7	0.90
	HES1	218	10.6	0.02	-1.2	0.97
	PRDM1	796	32.8	0.01	-1.9	0.86

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Table 2. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of interferon signaling genes differentially expressed between infected-, and/or mock-infected macrophages

2.4 Immune signaling pathways

The expression of a transcription factor (ATF2) and five MAPK kinases (MAP2K5, MAPK14/p38, MAPK4, and MAP3K18) in the MAPK signaling pathways was significantly down-regulated in VSV infected cells compared to mock-infected cells (Table 3). Likewise, the expression of six activator genes (CARD6, IKBKB, IRAK1, NLK, TAB1, TAB2, and TAK1) in the NFκB pathway was significantly downregulated in VSV-infected cells, whereas the expression of three inhibitors of NFκB [NFKBIA, NFKBID, and TNFIP3/A20 (38) was significantly upregulated (Table 3). Three genes (IRF5, MAVS and TBK1) in the RIG-I signaling pathway were expressed at significantly higher levels in VSV-infected cells than in mock-infected cells (Table 3). The expression of four TLR receptors (TLR1, TLR2, TLR4 and TLR6) and two signal transducers (BTK and TRIF) was downregulated in VSV-infected cells compare to mock-infected cells, whereas TLR7 was upregulated (Table 3). These results indicate that VSV infection suppresses the signaling of MAPK, NFκB, RIG-I and TLR pathways.

Pathway	Gene	EXP	EP/M	FDR	EP/EN	FDR
MAPK	ATF2	300	-2.4	0.01	1.3	0.75
	MAP2K5	1804	-1.7	0.02	1.0	0.99
	MAPK14/p38	539	-3.2	0.03	1.0	1.00
	MAPK4	176	-2.8	0.02	1.4	0.79
	MAP3K18	2279	-4.3	0.00	1.1	0.98
NFκB	CARD6	404	-2.6	0.01	1.1	0.95
	IKBKB	907	-2.2	0.01	-1.0	0.99
	IRAK1	5234	-3.5	0.00	-1.0	0.98
	NLK/NEMO	170	-3.3	0.01	1.2	0.88
	TAB1	1817	-2.2	0.03	1.1	0.98
	TAB2	416	-2.7	0.00	1.1	0.91
	TAK1	440	-2.1	0.01	1.2	0.80
	NFKBIA	10873	7.9	0.01	-1.2	0.95
	NFKBID	1182	4.2	0.03	-1.6	0.81
	TNFAIP3/A20	799	8.3	0.00	-1.4	0.88
RIG-I	IRF5	3796	-2.1	0.01	-1.1	0.95
	MAVS	452	-2.1	0.03	-1.1	0.95
	TBK1	459	-2.0	0.05	-1.1	0.97
Toll-like re-	BTK	4636	-2.0	0.01	1.2	0.82
	TLR1	361	-2.8	0.02	1.2	0.93
	TLR2	11817	-3.8	0.00	-1.1	0.94

	TLR4	543	-3.8	0.01	1.0	0.99
	TICAM2/TRIF	129	-2.8	0.02	1.3	0.84
	TLR6	143	-3.3	0.01	1.2	0.91
	TLR7	293	1.7	0.03	1.0	0.99

Table 3. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of interferon expression regulating genes differentially expressed between infected- and/or mock-infected macrophages

2.5 Cytokines, chemokines, and receptors

VSV infection significantly induced the expression of five immune cytokines (CSF3, IL1A, IL10, IL27, TNF and TNFSF9) and suppressed TNFSF11 expression (Table 4). Four non-typical immune cytokines (AREG, HBEGF, LIF and VEGFA) were expressed at significantly higher levels in VSV-infected than mock-infected cells (Table 4). Among those cytokines, AREG, IL1A, IL10, LIF and TNF were upregulated by > 11-fold. Overall, the endemic strain induced consistently higher expression (averaging 1.7-fold) of the upregulated cytokines than the epidemic strain though at not significant levels, whereas the receptor expression was very similar (Table 4). There were three significantly downregulated (IL17RA, LTBR and TNFRSF1A) and three upregulated (TNFRSF10, IL1R2 and IL20RB) cytokine receptors in VSV-infected cells compared to mock-infected cells (Table 4). All these DEG are proinflammatory genes with the exception of IL10, IL1R2, IL20RB, and the four non-typical immune cytokines. These results show that VSV infection induced both pro- and anti-inflammatory cytokine expression and suppressed the expression of IL-17 and TNF receptors.

VSV infection significantly induced expression of seven chemokines (CCL3, CCL4, CCL5, CCL20, CXCL1, CXCL2 and CXCL3) by ~3- to 42-fold compared to mock infection (Table 5). As for cytokines, the endemic strain also induced higher expression of the upregulated chemokines by 1.6-fold than the epidemic strain though not significant and nearly identical receptor expression (Table 5). The expression of three chemokine receptors [CCR5 (the receptor of CCL3, CCL4 and CCL5), CCLRL2 and CX3CR1] was significantly downregulated by VSV infection (Table 5), whereas CCR7 and CXCR4 expression was significantly induced after VSV infection when compared to mock-infected cells (Table 5). The results suggest that chemokines upregulated by VSV infection could cause infiltration of neutrophils, macrophages and Th17 cells in the infected tissue according to their chemotactic activities (39). On other hand, the infection could also alter the response of the infected cells to chemokines.

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Group	Gene	EXP	EP/M	FDR	EP/EN	FDR
Cytokines	CSF3	750	4.0	0.05	-1.3	0.93
	IL1A	698	13.9	0.02	-1.9	0.87
	IL1B	1793	7.7	0.13	-1.7	0.92
	IL10	1109	11.5	0.00	-1.9	0.76
	IL27	318	3.8	0.01	-1.9	0.67
	TNF	2641	23.4	0.01	-1.7	0.87
	TNFSF9/CD137L	369	5.2	0.02	-2.4	0.66
	TNFSF11	729	-4.5	0.02	1.2	0.94
	AREG	258	17.5	0.01	-1.6	0.91
	HBEGF	673	4.7	0.04	-1.3	0.94
	LIF	217	12.9	0.01	-1.9	0.79
	VEGFA	384	5.7	0.03	-1.6	0.86
Cytokine receptors	IL1R2	219	2.9	0.04	-1.3	0.88
	IL17RA	4899	-1.7	0.02	1.0	0.98
	IL20RB	92	2.7	0.03	-1.4	0.80
	LTBR	8211	-3.5	0.01	1.1	0.95
	TNFRSF1A	3943	-2.3	0.01	-1.0	1.00

Table 4. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of cytokine, chemokine and the receptor genes differentially expressed between infected- and/or mock-infected macrophages

Group	Gene	EXP	EP/M	FDR	EP/EN	FDR
CCLs	CCL3	2336	7.4	0.01	-1.8	0.80
	CCL4	3028	29.1	0.00	-2.1	0.79
	CCL5	6487	3.1	0.06	-1.4	0.88
	CCL5_v	385	23.0	0.01	-1.6	0.90
	CCL20	412	17.7	0.01	-1.4	0.94
ELR+ CXCLs	CXCL1	1791	6.4	0.03	-1.1	0.98
	CXCL2	3008	21.2	0.00	-1.9	0.81
	CXCL3	2388	6.8	0.02	-1.5	0.90
CCL/CXCL receptors	CCR5	246	-2.8	0.02	-1.0	1.00
	CCR7	206	4.5	0.00	-1.3	0.84
	CCRL2	1839	-1.8	0.02	1.1	0.94
	CX3CR1	129	-2.2	0.02	1.1	0.92
	CXCR4	1060	4.3	0.02	-1.1	0.98

Table 5. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/N, epidemic vs endemic infection: EP/EN) of chemokine and the receptor genes differentially expressed between infected- and/or mock-infected macrophages

2.6 Apoptosis, autophagy, and unfold protein response

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The expression of three pro-apoptotic genes, BCL2L13 (40), DAPK1 (41) and DIDO1 (42) and a key caspase (CASP8) in the apoptosis activating pathway were significantly downregulated in VSV-infected macrophages (Table 6). On the other hand, two apoptosis inhibitors, BIRC3/cIAP2 and SGK1 (43) and an activator of the apoptosis inhibitor expression, REL (44), were upregulated (Table 6). Two negative regulator genes of TNF-induced apoptosis, BRE (45) and IER3 (46), were also downregulated in VSV-infected cells (Table 6).

The expression of eight autophagy-associated genes including seven ATGs and FLCN and positive autophagy regulator, ULK1 (47) and RB1CC1 (48) Rab33b (49) was significantly lower in VSV-infected cells than in mock-infected cells (Table 6). Two autophagy inhibitors, BCL2L11/BIM (50) and Gadd45b (51) were expressed at significantly higher levels in VSV-infected cells than in Mock-infected cells (Table 6). GADD45B was one of the top 10 most induced genes after VSV infection (Table 10).

The expression of XBP1, a key regulator in stress-induced unfolded protein response (UPR) (52) and ERN1, the ER stress sensor of UPR (53), were significantly downregulated in VSV-infected cells compared to mock-infected cells (Table 6). PPP1R15A (GADD34) mediates dephosphorylation of eIF2alpha in a negative feedback loop and inhibits the unfolded protein response (UPR) (54), and its expression was significantly upregulated by 7-fold in VSV infected macrophages compared to mock infection (Table 6). The results of gene expression changes after infection suggested that VSV suppresses apoptosis, autophagy, and the UPR response.

Group	Gene	EXP	EP/MM	FDR	EP/EN	FDR
Apoptosis and Death receptor signaling	BCL2L13	1167	-2.0	0.01	1.2	0.77
	CASP8	337	-2.1	0.02	1.1	0.90
	DAPK1	246	-2.2	0.01	1.1	0.96
	DIDO1	162	-2.1	0.02	1.3	0.75
	BIRC3/cIAP2	656	3.5	0.01	-1.2	0.90
	REL	100	4.8	0.02	-2.4	0.66
	SGK1	2392	4.1	0.01	-1.4	0.86
	BRE	184	2.9	0.05	-2.0	0.68
	FADD	145	-2.2	0.04	-1.0	1.00
	IER3	988	5.6	0.02	-1.5	0.87
	RIPK1	1864	-2.1	0.01	-1.1	0.91
	TRADD	3504	-2.1	0.03	-1.0	0.98
Autophagy	ATG3	2041	-1.8	0.03	1.1	0.92
	ATG4B	1154	-2.8	0.01	1.0	0.99
	ATG5	524	-2.1	0.05	1.2	0.88
	ATG9A	977	-2.2	0.00	1.1	0.96
	ATG16L1	351	-2.3	0.01	-1.3	0.72
	ATG16L2	1146	-2.1	0.00	-1.1	0.95

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	ATG101	1026	-2.7	0.01	-1.1	0.95
	FLCN	771	-4.8	0.01	-1.1	0.97
	RAB33B	241	-3.0	0.00	1.4	0.70
	RB1CC1	147	-1.9	0.03	1.1	0.97
	ULK1	1083	-1.8	0.03	-1.1	0.96
	BCL2L11/BIM	152	4.3	0.00	-1.6	0.71
	GADD45B	649	22.6	0.00	-1.6	0.86
UPR	ERN1	146	-2.6	0.02	1.0	0.99
	PPP1R15A	850	7.0	0.01	-1.5	0.85
	XBP1	4634	-6.1	0.01	1.1	0.98

Table 6. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of apoptosis-, autophagy-, and unfold protein response (UPR)-related genes differentially expressed between infected- and/or mock-infected macrophages

2.7 Host mRNA transcription, modification, and stability

Thirteen genes involved in transcription of host RNA based on KEGG pathways were significantly downregulated in VSV-infected cells compared to mock-infected macrophages (Table 7). The expression of five genes [CMTR2 (mRNA cap methylation), DICER1 (microRNA processing), MIR132 (microRNA), TIAL1 (selective binding to several mRNAs to control expression of translation regulatory proteins) (55) and ZFP36/TTP (AT-rich mRNA degradation) (56) in post-transcriptional mRNA processing were significantly affected by VSV infection being the first two downregulated and the last two upregulated, respectively (Table 7). These results suggested that host mRNA transcription and processing could be negatively impacted by VSV infection to facilitate VSV replication.

Group	Gene	EXP	EP/M	FDR	EP/EN	FDR
mRNA transcription	CDK7	359	-3.1	0.01	1.2	0.91
	GTF2A1	534	-2.5	0.00	1.3	0.72
	GTF2B	1429	-2.2	0.05	1.2	0.92
	GTF2E1	176	-2.2	0.02	1.1	0.97
	MNAT1	217	-1.7	0.03	1.2	0.84
	RPAP3	161	-1.6	0.05	1.0	0.99
	TAF1C	481	-3.1	0.03	-1.2	0.94
	TAF7	716	-2.7	0.01	1.2	0.89
	TAF11	882	-3.2	0.03	1.1	0.97
	TBP	1191	-1.8	0.01	1.2	0.92
	TCEANC2	130	-2.6	0.01	1.3	0.75
	TCF20	348	-2.2	0.03	-1.0	0.98
	TFCP2	248	-1.9	0.01	1.1	0.91
mRNA processing	CMTR2	180	-3.5	0.01	1.3	0.83
	DICER1	1024	-2.4	0.02	-1.1	0.98
	MIR132	78	3.3	0.03	-2.7	0.54
	TIAL1	93	-3.7	0.04	-1.3	0.88
	ZFP36/TTP	5049	4.5	0.03	-2.0	0.74

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Table 7. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of transcription- and translation-related genes differentially expressed between infected- and/or mock-infected macrophages

2.8 Inflammation-related genes

Two proinflammatory mediator genes, ADM, (57), and a prostaglandin E (PGE) synthase (PTGS2) (58) were expressed at significantly higher levels (5.6 and 15.6 times, respectively) after VSV infection (Table 8). MALAT1 and DUSP2 play a role in prostaglandin E2 production in macrophages (59, 60), and their expression was also upregulated by VSV infection (Table 8). There were three C5a or purinergic receptor genes involved in macrophage M1 activation including C5AR1 (61), P2RY1 and P2RY6 (62) being downregulated and a suppressor (IL4I1) of macrophage M1 activation (63) upregulated in VSV-infected cells (Table 8).

A multitude of genes associated with pro-inflammatory responses were also differentially affected by VSV infection compared to mutant vs. mock infection (Table 8). Two genes MAP3K8 (64) and MEFV (65) critical for IL-1 and TNF production were upregulated in VSV infected cells. On the other hand, the expression of five proinflammatory genes, CEBPD (66). DUSP6 in macrophages enhances cytokine production (67), MAFB (68), MAPK8IP3/JIP3 (69) and MTRES1 (70) was downregulated. These results indicate VSV can alter host gene expression via both up- and downregulation of proinflammatory genes.

Group	Gene	EXP	EP/M	FDR	EP/EN	FDR
Inflammation mediator	ADM	331	5.9	0.01	-1.3	0.90
	DUSP2	2264	3.8	0.01	-1.0	0.99
	MALAT1	125	6.0	0.01	-1.7	0.75
	PTGS2	300	15.6	0.02	-1.3	0.95
Macrophage activation	C5AR1	10355	-3.0	0.01	1.1	0.94
	IL4I1	297	3.6	0.01	-1.2	0.89
	P2RY1	505	-7.3	0.00	1.1	0.96
	P2RY6	290	-2.6	0.05	1.1	0.97
Immune stimulators	MAP3K8	779	10.0	0.00	-1.5	0.85
	MEFV	1158	6.7	0.03	-1.7	0.85
	CEBPD	2210	-9.3	0.02	-1.1	0.99
	DUSP6	4285	-6.3	0.01	1.0	1.00
	MAFB	3002	-10.0	0.00	-1.3	0.91
	MAPK8IP3/JIP3	1932	-5.8	0.01	1.0	1.00
	MTRES1	1804	-9.7	0.00	1.2	0.93

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Table 8. Expression levels (EXP), false discovery rates (FDR) and fold differences (epidemic vs mock infection: EP/M, epidemic vs endemic infection: EP/EN) of genes that are associated with macrophage immunity and were differentially expressed between infected- and/or mock-infected macrophages

3. Discussion

The molecular mechanisms of VSV pathogenesis remain unclear. Multiple studies suggest that interaction with immune cells and regulation of the immune response play a role in determining the outcome of infection (10, 11, 12, 13, 14, 15). Because of the important roles of immune cytokines in the pathogenesis of virus infection (29) and macrophages in production of proinflammatory cytokines and their wide tissue distribution (28), VSV-infected porcine macrophages are an excellent model to extrapolate the molecular mechanisms of VSV pathogenesis. Our transcriptomic analysis shows that VSV infection induces massive (>10-fold) expression of proinflammatory cytokines including IL1A and TNF especially TNF (23-fold), chemokines e.g., CCL4, CCL5 and CXCL2 (>20 times) and a PGE synthetase gene (PGTS2). CCL3, CCL4 and CCL5 share the same receptor (CCR5). Likewise, CXCL1 and CXCL3 also share the same receptor (CXCR1) with CXCL2 (39). These chemokines were also massively induced after VSV infection. High production of these cytokines and chemokines and PGE is known to induce fever (72, 73, 74). Interestingly, LIF expression was highly upregulated after VSV infection, and LIF injection can induce fever in animals (75, 76). Based on these results, it is hypothesized that high fever is mainly caused by VSV-induced high production of CCR5 and CXCR1 ligands, IL1A, LIF, PGE and TNF. These cytokines, chemokines, and PGE are potent mediators of inflammation. IL1A and TNF are well-known potent proinflammatory cytokines. PGE induces vasodilatation and local recruitment of neutrophils, macrophages, and mast cells at early stages of inflammation (77, 78). Chemokines induced by VSV infection including CCL3, CCL4, CCL5, CCL20, CXCL1, CXCL2 and CXCL3 recruit macrophages, NK cells, neutrophils and/or Th17 cells (39). CSF3 stimulates neutrophil generation in the bone marrow (79). The expression level of CSF3 was significantly increased after VSV infection. Neutrophils are known to play a key role in clearance of viruses via phagocytosis and neutrophil extracellular traps (80). Therefore, the high production of PGE, cytokines and chemokines by VSV-infected cells could play a role in the pathogenesis within infected tissues by recruiting proinflammatory immune cells and inhibiting virus infection and spread.

TNF is one of the most potent proinflammatory cytokines, and it also induces cell death via apoptosis, necroptosis and pyroptosis pathways (81). Two genes, MAP3K8 (64) and MEFV (65), critical for TNF production were upregulated after VSV infection. Additionally, TIAL1 binds to 3’

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end noncoding sequences of several mRNA such as eIF4A, eIF4E, eEF1B and c-Myc to control the expression of translation regulatory proteins, which repress protein biosynthesis in cells responding to stress (55). TIAL1-deficient mice develop arthritis and elevated TNF expression (82). Upregulated MAP3K8 and MEFV and downregulated TIAL1 in VSV infected cells strongly support a very important role of TNF both in VSV local and systemic pathogenesis.

Other cytokines highly induced by >10-fold include AREG, IL-10 and LIF, which are known to have immune-suppressive effects. High levels of IL-10 suppress the innate and adaptive immune responses (83). TNF leads to IL-10 production by monocytes and together with IL-10, inhibit of CD4 T-cell expansion and function (84). LIF can suppress IFNγ and LPS signaling (85, 86). LIF appears to be an immune-tolerogenic cytokine based on promoting Treg differentiation and inhibiting pro-inflammatory Th17 cell differentiation (87). Several growth factor such as VEGF and EGFs can inhibit IFNB expression (35) or suppress the anti-VSV activity of IFNα and IFNβ (88). The expression of VEGF and two EGFs (AREG and HBEGF) were induced after VSV infection in this study. PGE_2 selectively suppresses effector functions of macrophages and neutrophils and the Th1-, CTL-, and NK cell-mediated type 1 immunity, but it promotes Th2, Th17, and regulatory T cell responses (77, 78). Therefore, we hypothesize that high levels of EGFs, IL-10, LIF, PGE and VEGF could play a key role in suppressing the immune response of infected and non-infected cells to facilitate VSV infection and cause disease.

Our results also indicate that VSV can evade the immune response of infected cells by various mechanisms. It is known that VSV can activate IFN response via RIG-I-MAVS and TLR4/CD14 signaling pathways to induce an antiviral response (14, 89). The expression of two key signaling transducers, TBK1 and MAVS (90, 91) in the RIG-I-MAVS signaling pathway was downregulated in VSV-infected cells compared to mock-infected cells. It has been previously reported that VSV glycoprotein binds to the TLR4/CD14 dimer leading to the induction of interferon expression, mainly mediated by IFNB via a TICAM1/TICAM2-dependent but MyD88- and NFκB-independent signaling pathway (92). In our study, the expression of TLR4 and TICAM2 was significantly suppressed by VSV infection. Infection of monocytes by VSV has been reported to suppresses type I IFN and cytokine (IL-27 and TNF) responses in a viral RNA-specific and TLR7-dependent pathway (93), and TLR7−/− mice show significantly reduced VSV titers in the draining lymph nodes and diminished viral replication in subcapsular sinus macrophages (94). Our results showed that TLR4, TLR7 expression was significantly upregulated in both VSV- and mutant-infected cells (Table 7). Therefore, VSV-altered expression of genes in the virus-sensing pathways could be another immune evading mechanism in infected cells.

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Previous studies of IFNB promoters showed that ATF2-JUN, IRF, and NFκB transcription factors regulate IFNB expression (95). The expression of ATF2 and IRF5 transcription factors was downregulated by VSV-infection. There were several downregulated signaling transducer or up-regulated signaling inhibitor genes that could inhibit MAPK, NFkB, signaling pathways (Table 2). Interestingly, there were six suppressor genes of IFNB expression (AHR, ATF3, DUSP1, FOS, HES1 and PRDM1) upregulated by up to 32.8-fold in VSV-infected cells. This result supports published results that VSV suppresses the interferon response.

Our results show that VSV infection did not induce expression of other type I interferons except IFNB in pig macrophages. IFNB induction is known to induce IRF7 expression, which is needed for induction of IFNA (96). Although IFNB expression was induced in infected macrophages, the expression of IRF7 and IFNA was not increased by VSV infection. This could be explained by the suppression of interferon signaling mediated by downregulated expression of type I and II interferon receptors as shown in Table 6. This seems to a novel immune evasion mechanism of VSV in addition to the inhibition of mRNA nuclear export mediated by VSV matrix protein (14, 15). It has been previously reported that VSV infection inhibits the expression of interferon-stimulated genes via miR-132 to facilitate viral replication (97, 98). We found that miR-132 is highly upregulated by VSV infection (Table 11) which could also explain the lack of induction of interferon stimulated genes by IFNB.

Viral infections trigger three inter-connected biological processes including apoptosis, autophagy, and stress-induced unfolded protein response (UPR), which can inhibit virus replication (52). However, viruses can subvert or even manipulate these responses to promote infection, for example, VSV can delay the onset of apoptosis (17,99). Our results show that multiple genes associated with these three processes were differentially expressed during VSV infection as listed in Table 10. Among these genes, GADD45B which is one of top 10 most induced genes (22.3-fold) after VSV infection, suppresses apoptosis and autophagy (51). The expression of two key regulatory genes, XBP and PPP1R15A, in the UPR pathway was reduced after VSV infection. Additionally, genes in death receptor signaling including TNFRSF1A and several signaling transducers were downregulated in VSV-infected cells, which could delay the necroptosis/apoptosis induced by high expression of TNF. These results suggest that VSV can suppress these three important innate immune mechanisms during infection.

The chemokines associated with the CCR5 receptor activate macrophages to induce pro-inflammatory cytokine expression (100, 101). Expression of CCR5 was significantly downregulated in VSV-infected macrophages, potentially reducing the immunostimulatory effect of CCR5.

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Chemokines. Additionally, high IL10 and LIF expression and downregulation of IL17RA in the infected cells could mitigate the effects of Th17 cells recruited by increased CCL20 expression. Therefore, VSV appears to be able to evade the immune response associated with chemokines induced by its infection in infected cells. Published results showed that M2 macrophages were more susceptible to infection and killing by both wildtype and a M51R-M VSV mutant than M1 macrophages (102). The M1 and M2 activation of macrophages plays important roles in the innate and adaptive immune responses. Our results suggest that VSV infection could suppress M1 activation by downregulating the expression of several purinergic receptors such as C5AR1, CCR5, IL17RA, P2RY1, P2RY6 and by upregulating IL4I1 expression.

It has been reported that VSV interferes with host gene transcription (19). Our results showed that VSV downregulated several genes of the host transcription machinery as shown in Table 11. Additionally, VSV may control host protein translation by altering expression of genes involved in maintaining mRNA stability and cap-modification, microRNA processing and protein translation. VSV encodes proteins with cap methylation activity and mutants lacking this activity show attenuated virulence (103). Our results showed downregulation of CMTR2 which could negatively impact host translation. ZFP36/TTP is known for its central role in destabilizing mRNA molecules containing class II AU-rich elements in 3' untranslated regions (56), which are frequently found in cytokine mRNA 3′-end non-coding sequences. Increased expression of this gene could destabilize cytokine mRNA and reduce its translation. Dicer1 was downregulated by VSV infection in this study, and previous works have demonstrated that Dicer1-deficient mice are hyper-susceptible to VSV infection (104). VSV infection induced a 7-fold increase of PPP1R15A (GADD34) in our study, this protein mediates dephosphorylation of eIF2α, which inhibits viral replication (105, 106) and suppresses UPR (54).

In summary, VSNJV infection significantly induced the massive expression (>10-fold) of pro-inflammatory cytokines (IL1A and TNF), chemokines (CCL4, CCL20 and CXCL2) and prostaglandin E, upregulated PTGS2 and immune suppressive cytokines (IL10 and LIF), which are known to induce fever, immune suppression, and/or recruitment of immune cells. It is hypothesized that these cytokines, chemokines, and possibly PGE play important roles in local and systemic VSV pathogenesis and immune evasion. Although not significant DEG were seen between epidemic and endemic VSV strains, the endemic strain consistently induced higher expression of all upregulated cytokines and chemokines. This might help explain differences in virulence previously observed in pigs (LAURO's PAPER REF HERE) Based on our results, the mechanisms of VSV immune evasion could be achieved via suppressing (a) IFNβ expression, (b) type I and II

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interferon, IL-1 and death receptor signaling, and TLR and RIG-I signaling pathways, (c) biological processes involved in apoptosis, autophagy and unfolded protein response, (d) M1 macrophage activation, (e) host mRNA transcription, cap-methylation and stability and (f) eIF2α dephosphorylation-mediated inhibition of viral protein translation. This study provides novel insights (summarized in Table 9) that warrant further investigation of VSV virulence factors and pathogenesis.

Pathogenesis	**Mechanism inferred from differentially expressed genes**
Systemic	Fever: ↑ CCLs [3, 4, 5], CXCLs [1, 2, 3], IL1A, PGTS2, TNF Anorexia: ↑ LIF, PGTS2 Systemic infection restriction: ↑ IFNB, VSV sensitive to IFN inhibition
Local tissue	Immune cell infiltration: ↑ CCLs [3, 4, 5, 20], CXCLs [1, 2, 3], PTGS2 Inflammation: ↑ ADM, PGTS2 Vasodilatation: ↑ PGTS2 Nacroptosis/apoptosis: ↑ TNF, ↓ TIAL1
Infected/non-infected cells	↓ General immune response: ↑ IL10 ↓ Interferon response: ↑ AREG, HBEGF, VEGF, IL1A ↓ MΦ, neutrophils, Th1, CTL, and NK cell activities: ↑ PGE/PGTS2 ↓ Th17 response: ↑ LIF; ↓ IL17RA
Infected cells	↓ IFNB production: ↑ AHR, ATF3, DUSP1, FOS, HES1 and PRDM1; ↓ ATF2, ↓ MAPK signaling: ↓ MAPK4, MAPK14/p38, MAP3K18 ↓ RIG-I signaling: ↓ IRF5, MAVS, TBK1 ↓ NFκB signaling: ↑ NFKBIA, NFKBID, A20; ↓ TNFSF11, 7 DEG ↓ TLR4 signaling: ↓ BTK, TICAM2, TLR4 ↓ Interferon signaling: ↓ IFNAR1, IFNAR2, IFNGR1, ↑ ATF3
	↓ Apoptosis and/or autophagy: ↑ GADD45B, 18 DEG (Table 10) ↓ TNF signaling: ↓ FADD, RIPK1, TNFRSF1A, TRADD; ↑ BRE, IER3 ↓ Unfolded protein response: ↓ ERN1, XBP1, ↑ GADD34
	↓ AT-rich (cytokine) mRNA stability: ↑ ZFP36

↓ Host mRNA cap-methylation/translation: ↓ CMTR2
↓ Host transcription: ↓ 13 DEG involved in RNA transcription (Table 11)
↓ MΦ M1 activation: ↓C5AR1, CCR5, IL17RA, Y2RY1, Y2RY6; ↑IL4I1, TNFSF9
↑ VSV replication (↓ ISG expression): ↑MIR132
↑ VSV replication (unknown mechanisms): ↓ DICER1, ↑ TLR7
↑ VSV protein synthesis via eIF2α dephosphorylation: ↑ GADD34

Table 9. Differentially expressed genes used to infer candidate mechanisms of VSV systemic and tissue pathogenesis and immune evasion in infected and non-infected cells

4. Materials and Methods

4.1 Cell culture of macrophages and viruses

Primary swine macrophage cell cultures were derived from pig peripheral blood as previously described (107). Macrophages were seeded in 6-well plates (Primaria Falcon, Becton Dickinson, Franklin Lakes, NY). VSV strains used in this study include (a) NJ0612NME6, an epidemic VS New Jersey virus (VSNJV) strain causing outbreaks in the US from 2012-2014 and isolated from a naturally infected equine in New Mexico in 2012 and (b) NJ0806VCB a VSNJV strain circulating in 2006 in an endemic area of Mexico and obtained from a naturally infected bovine in Veracruz (108). Differences between these two viruses have been reported in previous studies suggesting that NJ0612NME6 has higher virulence than NJ0806VCB in inoculated pigs (12). VSV infection experiments were conducted with three biological replicates using *ex vivo* cultured primary macrophages isolated from three different commercial domestic pigs. Macrophages were infected with a MOI of 10 $TCID_{50}$ of each virus, respectively. Mock infection was also performed in the cultured macrophages from these same pigs as non-infected controls.

4.2 RNA isolation

Total RNA was extracted from primary swine macrophage cell cultures infected with the indicated viruses, or mock infected at 5 hours post-infection. Cells were harvested and lysed with a cell lysis buffer (Qiagen, Valencia, CA) and RNA was isolated using a RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA quality was then determined using an Agilent 2100 bioanalyzer (Santa Clara, CA) using an RNA nanochip according to the procedures outlined by Agilent Technologies (Santa Clara, CA). RNA was quantified using a Nanodrop 1000 (Thermo Scientific, Waltham, MA).

4.3 DNA microarray analysis

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A 44,000 (44K) porcine whole genome expression microarray was designed based on pig expressed sequences (cDNA and EST) and porcine genome sequence homologous to non-porcine sequences as reported by Zhu et al. (109). All porcine EST and RNA sequences were downloaded from the NCBI database and assembled into unique sequences using the CAP3 software program (Huang and Madan, 1999). The assembled sequences were aligned to pig genome sequences using the UCSC genome browser to select 3' end RNA sequences or the genome sequences aligned with other expressed sequences of other species if no porcine expressed sequences were available. These selected sequences were used to design 60-mer oligonucleotide microarray probes with a low probability of cross-reacting with other genes and a bias to the 3'-end of RNA sequences using Array Designer 4.0 (Applied Biosystems, Foster City, CA). Approximately 43K porcine probes were selected to synthesize a 44K Agilent microarray for this study. The annotation of the porcine sequences was based on the results of a BLAST search against human reference proteins and RNA sequences downloaded from NCBI databases and manual curation based on all expressed sequences aligned in the porcine genome sequences using the UCSC genome browser. One hundred and eighty-six duplicated probes designed from all ASFV open reading frames were also included in this custom microarray.

The custom designed porcine microarrays were manufactured by Agilent Technologies and used for this study. Both ASFV-infected and mock-infected RNA samples were labeled with Cy3 and Cy5 individually using an Agilent low-input RNA labeling kit (Agilent Technologies). A Cy5-labeled ASFV-infected or mock-infected sample was co-hybridized with a Cy3-labeled mock-infected or ASFV-infected in one array, respectively, for each time point using a dye-swap design. The entire procedure of microarray analysis was conducted according to protocols, reagents and equipment provided or recommended by Agilent Technologies. Array slides were scanned using a GenePix 4000B scanner (Molecular Devices) with the GenePix Pro 6.0 software at 5 μM resolution.

4.4 Statistical and bioinformatic analyses of microarray data

Background signal correction and data normalization of the microarray signals and statistical analysis were performed using the LIMMA package (110). Log_2 fold changes in signal intensity were used in the statistical analysis to identify deferentially expressed genes. To account for multiple testing, the p-values were adjusted using the Benjamini and Hochberg method and expressed as a false-discovery rate (FDR). The probe sequences were aligned to the porcine genome sequence displayed in the UCSC genome browser to validate the annotation by computational methods, such as BLAST. Gene expression differences with an FDR value of 0.05 or smaller and an expression difference ≥ 50% were considered statistically significant and were considered

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differentially expressed genes (DEG). Genes down- or upregulated in the infected macrophages compared to the non-infected macrophages were expressed as negative and positive values (fold), respectively.

4.5 Pathway analyses

The identified DEG were mapped to human reference genes. Two lists of upregulated and downregulated gene associated with human Entrez gene ID were analyzed with a NCBI online bioinformatics program (DAVID Bioinformatics Resources 6.8) to identify the biological pathways (GOTERM_BP_DIRECT, KEGG_PATHWAY and REACTOME_PATHWAY) significantly over-represented by DEG ($P \leq 0.05$ with Benjamini correction). The DEG with differential expression of 2-fold or greater and 4-fold greater were used in these analyses to take the magnitudes of differentiation expression into consideration.

4.6 Biological inference

The biological functions of DEG in the identified over-represented pathways associated with the immune response were based on scientific publications obtained from PubMed. Biological inferences were based on (i) the immunological functions of the DEG, (ii) gene expression levels based on microarray averaged signal intensity and (iii) magnitudes (fold) of the differential expression, assuming higher mean signal intensity and larger differentially expressed genes play a bigger biological role in the gene groups. Genes with no significantly differential expression but are known to play important roles in the biological pathways associated with the significant DEG were also used as supporting evidence. Genes down- or upregulated in the VSV-infected samples compared to the mock-infected samples were expressed as negative and positive values (fold), respectively. In this study, genes differentially expressed between infected and mock infected macrophages were used to infer the molecular mechanisms of VSV pathogenesis and immune evasion.

Author Contributions: LV-S, JJZ and LR conceived and designed the experiments. LV-S performed the microarray experiments. JJZ designed the microarray analysis and performed acquisition and analysis of microarray data. JJZ, LV-S, JC, LR wrote the manuscript.

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Institutional Review Board Statement: The animal protocol used in this study was previously reviewed and approved by the PIADC Institutional Animal Care and Use Committee of the US Departments of Agriculture and Homeland Security (protocol number #245-200 05-14R; approved 07/12/2021).

Data Availability Statement: The microarray raw data are in the process of submitting to NCBI database. The data sets will be available to the public if this manuscript is accepted for publication.

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

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