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Ovine Fetal Immune Response to Cache Valley Virus Infection

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Abstract

Cache Valley virus (CVV) induced malformations have been previously reproduced in ovine fetuses. To evaluate the development of the antiviral response by the early, infected fetus, before development of immunocompetency, ovine fetuses at 35 days of gestation were inoculated in utero with CVV and euthanized at 7, 10, 14, 21 and 28 days post infection. The antiviral immune response in immature fetuses infected with CVV was evaluated. Gene expression associated with an innate, immune response was quantified by real-time, quantitative PCR. Up-regulated genes in infected fetuses included *ISG15*, *Mx1*, *Mx2*, *IL-1*, *IL-6*, *TNF-α*, *TLR-7* and *TLR-8*. The amount of *Mx1* protein, an interferon stimulated GTPase capable of restricting growth of bunyaviruses, was elevated in the allantoic and amniotic fluid in infected fetuses. *ISG15* protein expression was significantly increased in target tissues of infected animals. B lymphocytes and immunoglobulin-positive cells were detected in lymphoid tissues and in the meninges of infected animals. These results demonstrated that the infected ovine fetus is able to initiate an innate and adaptive immune response much earlier than previously known. That presumably contributes to viral clearance in infected animals.

Introduction

Cache Valley virus (CVV) is a mosquito-borne bunyavirus of the family *Bunyaviridae*, genus *Orthobunyavirus*, Bunyamwera group (40), and endemic in North America (11). Serologic studies have shown that unlike most members of the family *Bunyaviridae*, the vectors of CVV also infect larger mammals with virus being isolated

47 from sheep, cattle and horses, and causing reproductive losses in small ruminants (7-9,
48 11-14), similar to the disease caused by Akabane virus (5, 22, 32) and the newly
49 identified Schmallenberg virus occurring in Europe (16). The virus has rarely been
50 associated with meningoencephalitis in humans (4, 41). *In utero* ovine fetal infection
51 causes abortion and fetal malformations, mainly affecting the musculoskeletal (MSS)
52 and central nervous (CNS) systems (7-9, 11, 13, 37).

53 Previous studies describing experimental CVV-induced malformations in ovine
54 fetuses showed that the development of fetal lesions depends on the fetal age when
55 infected. If the virus is inoculated between 28 and 48 days of gestation (dg), fetal death
56 and abortion or MSS and CNS malformations occur. No apparent malformations are
57 observed if the virus is inoculated after 48 dg (7, 11). Virus isolation can only be made
58 from infected fetuses in early development, and viral recovery from tissues of term
59 abortions and malformed lambs is uniformly unsuccessful. The virus is cleared from
60 infected tissues within a few weeks after infection (11, 37) and before the presumed age
61 of fetal immunocompetency, at approximately 70-75 dg (29, 33).

62 The gestation period of the ewe is approximately 147 days. Ovine fetuses develop
63 erythropoiesis, myelopoiesis and megakaryopoiesis in the yolk sac and liver at
64 approximately 17 dg (30). At approximately 20 to 25 dg, lymphocyte production begins
65 in the thymus, and lymphocytes are in the bloodstream at 48-50 dg (30). At 45-50 dg, T
66 and B lymphocytes and cells with surface immunoglobulins are present in the spleen and
67 lymph nodes (27, 28). The lymph nodes become integrated into a lymphatic system
68 around 65 dg (30). Establishing the time when fetuses are able to respond to antigens is

69 difficult. Available data on adaptive immune response of ovine fetuses have been based
70 on serum neutralization assays, which were established for use in mature animals.
71 Additionally, gestation assay points used in experiments are somewhat arbitrary. Ovine
72 fetal antibody response to viral infection at titers greater than 1:2 has been detected after
73 76-78 dg (32). Because the syndesmochorial placenta of ruminants prevents passage of
74 immunoglobulins from the ewe to the fetus (45), antibodies in fetuses and precolostral
75 newborns are those produced by the fetus. For this reason, *in utero* viral infections can
76 be diagnosed in aborted fetuses and stillborn ruminants using serum neutralization tests.
77 Such testing is necessary with CVV because the fetus clears the virus long before the
78 end of gestation (9, 11).

79 Previously, we have demonstrated that fetuses infected with CVV early in
80 gestation (around 35 dg) have low viral antigen and RNA signal in tissues around 56 dg
81 and are able to clear the infection before development of an adaptive immune system at
82 75 dg (37). Similarly, age-based findings have been described in Akabane virus-infected
83 fetuses (5, 29, 32). Because no effective serum neutralization antibody (an adaptive
84 immune response) has been detected in ovine fetuses at the time when CVV is cleared
85 from fetuses (30), it may be that the ovine fetus mounts an innate immune response for
86 early viral clearance. To test this hypothesis, the expression of selected genes associated
87 with the innate immune response was determined in tissues of CVV-infected and non-
88 infected ovine fetuses, and CVV mRNA was quantify ed in selected tissues of these
89 fetuses to correlate the measured innate response with viral clearance. In addition, fetal
90 *Mx* protein, an interferon-stimulated GTPase previously associated with antiviral activity

against bunyaviruses (15, 23, 25, 38, 44), was quantified in the fetal allantoic and amniotic fluids. Expression of the *interferon stimulated gene 15* (*ISG15*), that has been shown to be produced by fetuses in response to viral infection *in utero* (6, 42, 43), was evaluated in CVV-infected fetal tissues. Finally, distribution of B and T lymphocytes and immunoglobulin-positive cells was evaluated in infected and non-infected, ovine fetal tissues in early gestation.

Materials and methods

Virus inoculation and sample harvesting

A group of 15, seronegative, pregnant Rambouillet ewes was housed in BSL2 confinement buildings according to protocols approved by The Institution Animal Care and Use and the Institutional Biosafety Committee. At 35 dg, ewes were inoculated *in utero* with a 1ml inoculum containing 10^5 50% tissue culture infectious doses ($TCID_{50}$) of CVV (infected group) or 1 mL of minimum essential medium (MEM) (mock infected/control group), as previously described (37). The viral inoculum was derived from the second passage of an isolate from allantoic membrane from an experimentally infected fetus (7). At 7, 10, 14, 21 and 28 dpi, three ewes (one mock-infected and two virus-infected) were humanely euthanized. Selected fetal tissues and their fluids were harvested and immediately frozen at -80ºC or placed in RNA later (Ambion, Life Technologies, Carlsbard, CA) and frozen at -80ºC. The remaining fetal tissues and placenta were fixed in 10% buffered formalin diluted in deionized water or in Davidson's AFA (glacial acetic acid, 37% formaldehyde and 95% ethanol) fixative.

113

114 *Real time quantitative polymerase chain reaction (qPCR)*

115 To quantify relative numbers of CVV RNA copies, and abundance of genes of
116 interest, real time qPCR was performed on samples of brain and muscle harvested from
117 CVV-infected or mock-infected ovine fetuses according to a previously published
118 protocol with minor modifications (10). Briefly, RNA was isolated from harvested
119 samples and homogenized in Trizol reagent (Gibco BRL, Bethesda, MD) according to
120 the manufacturer's recommendation. In order to eliminate contamination with genomic
121 DNA, the extracted RNA was treated with the DNase1 amplification reagent (Invitrogen,
122 Carlsbard, CA), and the RNA concentration was quantified by spectrophotometry. RNA
123 quality was determined by denaturing samples in a 1.5% agarose gel electrophoresis
124 stained with ethidium bromide and visualized on a UV transilluminator. The cDNA was
125 synthesized from 500ng of total RNA combined with primer mix containing oligodT
126 primer (0.2 µg/ml), random hexamer primer (300 µg/ml)(Invitrogen), dNTPmix (10mM
127 each) and incubated at 65ºC for 5 min. After incubation, SuperScript II reverse
128 transcriptase (Invitrogen) was added to the reaction according to manufacturer's
129 recommendations and reverse transcribed under the following conditions:
130 25°C for 10 min; 42°C for 60 min and 70ºC for 5 min. To test for genomic DNA
131 contamination, control reactions were prepared without reverse transcriptase.

132 Specific oligonucleotide primers (Table 1) were generated in Oligo 5 program
133 (Molecular Biology Insights, Inc) for the following genes: M segment of glycoprotein 1
134 of CVV, *interferon-α* (*IFN-α*), *IFN-β*, *TNF-α*, *IL-1*, *IL-6*, *Mx1*, *Mx2*, *ISG15*, *TLR7* and

135 *TLR8*. The real-time qPCR reaction was performed using the ABI prism 7900HT system
136 (Applied Biosystems, Foster, CA) with power SYBR green PCR Master Mix (Applied
137 Biosystems). Primer specificity and efficiency (-3.6>slope>-3.1) were confirmed using a
138 test amplification run. The data were normalized using cycle threshold (Ct) values for
139 the ovine *glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*) gene in each sample.
140 Semiquantitative analysis was calculated with the ΔΔCT method, and expressed as
141 relative fold-changes compared with the lowest value for mock-infected samples. To
142 calculate relative amounts of viral mRNA, relative fold-changes were compared with the
143 infected sample with the lowest ΔΔCT.
144
145 *Protein slot blot*
146 The protein slot blot assay was used to determine and compare relative concentrations of
147 the Mx1 protein in the fetal fluids of infected versus non infected fetuses. The starting
148 concentration of protein in allantoic and amniotic fluids was determined using the
149 Bradford Assay as recommended by the manufacturer (Bio-rad Laboratories). According
150 to the protein concentration, samples were prepared and diluted to 12 µg of protein for
151 the amniotic fluid and 50 µg of protein for the allantoic fluid in a final volume of 200 µl
152 of Tris-Buffered saline (TBS). The volume was deposited onto a nitrocellulose
153 membrane (Bio-Rad Laboratories, Richmond, CA) in a Bio-dot SF micro-filtration
154 apparatus (Bio-Rad Laboratories) following a modified protocol as previously published
155 (39). Briefly, nonspecific binding sites were blocked by immersing the membrane in 5%
156 skim milk in TBS-Tween (TBST) for 1 h at room temperature on an orbital shaker. The

membranes were incubated overnight at 4°C in 2.5% milk-TBST containing a rabbit anti-ovine *Mx1* polyclonal antibody (gift from Dr. Troy Ott, Penn State University) (24) at a concentration of 1:10000. The membrane was washed three times and then incubated for 1 h with goat anti-rabbit IgG (1:10000 dilution in 2.5% milk-TBST). Following incubation with the secondary antibody, the membrane was washed three times and further incubated for 1 min with Bio-Rad chemiluminescence reagent. The relative concentration of the Mx1 protein in triplicate samples was determined quantitatively in relative light units using the Bio-Rad chemidoc imager and assessed via use of Quantity One Image analysis software (39).

Immunohistochemistry

Immunohistochemistry was performed on 5 µm, deparaffinized sections of paraformaldehyde-fixed tissues mounted onto positively-charged, silanized slides using an automated system for immunohistochemistry (DakoCytomation Autostainer®, Dako, Carpinteria, CA) with minor modifications to the IHC protocol previously described (37). Antigen retrieval was performed in a decloaker chamber (Biocare Medical, Concord, CA) with slides soaked in citrate buffer pH 6.0. Sections were incubated with anti-*ISG15* polyclonal antibody (diluted 1:500) for 30 min (the antibody was a gift from Dr. Thomas Hansen, Colorado State University) (3), CD3 (diluted 1:400) polyclonal antibody for 35 min (Dako), anti-CD79a polyclonal antibody (diluted 1:200) for 30 min (Dako), anti-ovine IgG (diluted 1:500) for 35 min and anti-ovine IgM (diluted 1:2000) for 30 min (KPL, Inc, Gaithesburg, MA). The primary antibody was followed by

incubation with the secondary antibody (MACH 2 anti-rabbit Biocare Medical [CD3, IgG, IgM]; or ImmPress anti-Mouse Ig, Vector Labs [*ISG15*, CD79a]) for 20 min. A chromagen complex, 3,3-diaminobenzidine tetrachloride (DAB, Dako), was used to detect the targeted antigens, and sections were counterstained with hematoxylin. Slides were coverslipped with permount mounting solution. Tissues were evaluated to determine percentage of infected cells in each examined organ and graded as follows: less than 3% of cells positive (mild); between 3-15% of cells positive (moderate); more than 15% of cells positive (marked).

Statistical analysis

The quantitative data for mRNA expression and protein levels on fetal fluids were subjected to the Wilcoxon signed rank test to determine differences between infected and non-infected groups. Differences between groups at different dpi were subject to the one pair students t-test. In order to reduce heterogeneity among samples, the data were log-transformed if necessary, and *p*-values below 0.05% were considered statistically significant. The data are presented as means and overall standard errors.

Results

Real-time qPCR

No significant difference in gene expression was observed either between infected animals at each different dpi or between control animals at different dg. Therefore, the data for each gene for each evaluated tissue were pooled into two groups: control and

infected animals (Fig. 1). qPCR analysis revealed, that *IL-1, IL-6, Mx2, ISG15*, *TLR7* and *TLR8* were up-regulated (p-value <0.05) in the brain and skeletal muscle of CVV-infected fetuses as compared to non-infected fetuses. Only in the brain was *TNF-α* significantly up-regulated. All examined genes but not *ISG15* and *Mx1*, had higher up-regulation in the brain as compared to the skeletal muscle (p-value <0.05). Similarly, the relative number of copies of CVV was significantly higher (p-value <0.05) in the brain when compared to the skeletal muscle (Fig. 2). Progressively lower means of relative number of copies of CVV were observed in both brain and skeletal muscle with the progression of the infection. A statistically significant difference (p-value <0.05) was observed in the brain of fetuses between 14-28 dpi (CVV in the brain of 10 dpi fetuses were excluded from analysis due to high variability among those samples), and in the SKM when comparing 10-14 dpi, 10-21 dpi, and 10-28 dpi.

Protein slot blot

A significant (p-value <0.01) increase in the amount of *Mx1* protein in the allantoic and amniotic fluid occurred in infected fetuses *versus* non-infected fetuses at each time point examined (Fig. 3). Similar to the quantitative data collected with the real time qPCR, no effect of day or interaction of day versus infection was detected in evaluated samples.

Immunohistochemistry for ISG15

ISG15 was detected in several tissues of all infected fetuses, but no *ISG15* immunolabeling was observed in control fetuses (Fig. 4). In infected fetuses, a strong signal was observed in numerous cells of the cerebral parenchyma and meninges, and fewer cells were positive for *ISG15* in the spinal cord. Most fetuses at 7-21dpi had marked *ISG15* antigen signal in the brain, with moderate signal observed at 28 dpi. The *ISG15* antigen signal in the spinal cord of infected fetuses varied between mild to moderate at all test points. Multifocal clusters of cells with positive *ISG15* immunolabeling were observed in the SKM of infected fetuses through 21 dpi with only rare cells having a positive signal at 28 dpi. Other tissues with clusters of cells with positive signal included: heart, smooth muscle of intestine, tongue, fibroblasts in the subcutaneous tissue, wall of large arteries, cells around small blood vessels, spleen (one fetus at 21dpi), and rarely in the lungs, tonsils and thymus of earlier infected fetuses.

Immunohistochemistry of B and T lymphocytes, IgG and IgM

At 7 dpi/42 dg, scattered CD79a positive cells were observed in the dorsal abdominal cellular lymphoid aggregates around the abdominal aorta, which correspond to sites of development of rudimentary renal or mesenteric lymph nodes, and rarely in the hepatic sinusoids in both control and infected animals. At 10 dpi/45 dg, slightly increased numbers of CD79a, IgG and IgM-positive cells were observed in the same locations in infected and control animals (Fig. 5, A-C). Rare IgM-positive cells were observed in the meninges of one infected animal. At 14 dpi/49 dg, slightly increased numbers of CD79a, IgG and IgM-positive cells were observed in the sites described

above, and at this timepoint, CD79a and IgM-positive cells were in the spleen of one of the infected animals (Fig. 5, D-F). Surprisingly, another infected animal also had a marked infiltration of CD79a, IgG and IgM-positive cells in the meninges of the brain and spinal cord and in rare cells within the parenchyma (Fig. 5, G-I). Increased numbers of CD79a, IgG and IgM-positive cells were arranged around splenic arterioles in both infected and control animals at 21 dpi/56 dg and 28 dpi/63 dg, besides also being in the previously described sites. Numerous IgG and IgM-positive cells were observed in the meninges of the spinal cord of two infected fetuses at 21 dpi, and rare immunoglobulin-positive cells were observed in one fetus at 28 dpi. Moderate to marked numbers of CD3 positive cells, evaluated only in sections of brain and transverse sections of the abdomen including the lumbar spinal cord of control and infected fetuses at 21 and 28 dpi, were detected in the spleen, renal and mesenteric lymph nodes in all fetuses. Scattered CD3 positive cells were in the meninges of the BRA and SPC of one infected fetus at 14dpi and in the meninges of the SPC in one fetus at 21 dpi.

Discussion

Ovine fetuses are able to mount an immune response associated with CVV infection earlier in gestation than previously described (22, 29, 32, 33). Although the fetal immune response was not able to completely clear virus by the end of the study at 63 dg (37), infected ovine fetuses were able to reduce the viral load within the CNS and MSS. Viral signal was also markedly reduced or absent in the ganglion, retina, kidney and heart.

CVV targets mainly cells within the CNS and MSS, and viral signal was markedly reduced in the brain and almost completely cleared in the skeletal muscle after 21-28 dpi in fetuses infected at 35 dg (37). Similarly, a progressive reduction in viral load, as shown by mean relative amounts of CVV RNA copies was observed in the brain and SKM in this study, with significant differences observed between most days of infection in the SKM, and in the brain between 14 and 28 dpi. In addition, in all groups, the relative number of CVV RNA copies in the brain was significantly higher than in the skeletal muscle.

The *Mx* protein, an interferon stimulated GTPase involved in intracellular trafficking, membrane remodeling and fusion processes, is capable of restricting growth of viruses, including influenza virus, measles virus and bunyaviruses (including orthobunyavirus, phlebovirus and hantavirus genuses) (15, 17-20, 23, 25, 26, 44). In viral infections, the *Mx* protein acts by interfering with transport of viral components in infected cells. In a model proposed by Haller and Kochs (2002) (18), *MxA* forms large, membrane-associated self-assemblies that store monomers of this protein. With infection, monomers bind to viral target structures, forming new assemblies involving viral intermediates that lead to mislocalization of viral components and consequent inhibition of viral replication (18). Human *MxA* has been shown to inhibit replication of La Crosse virus by binding to the viral nucleocapsid protein, and forming large copolymers that accumulate in the perinuclear region (25). Previous studies have demonstrated antiviral activity of bovine *Mx2* in cattle infected with BVDV (21, 42). Because *Mx* has a demonstrated antiviral effect against bunyaviruses, this study

quantified expression of the *Mx* molecule in infected tissues. A significant up-regulation of ovine *Mx1* and *Mx2* was observed in evaluated tissues from infected fetuses compared to controls. Increased secretion of *Mx1* was identified in fetal fluids in infected ovine fetuses. Thus, this protein likely contributes to the clearance of CVV infection.

ISG15 was markedly up-regulated in the brain and in the skeletal muscle and highly expressed in the tissues from infected animals. Similar to the *Mx* protein, *ISG15* is induced by type I interferon, and has been associated with antiviral activity (43). In recent studies, *ISG15* has been shown to conjugate to proteins in a manner similar to ubiquitin (1, 46). Modifications and interference of antiviral signaling pathways involving *ISG15* are mechanisms used by Crimean-Congo hemorrhagic fever virus, a bunyavirus, to evade the innate immune response (1). Up-regulation of numerous ISG, including *ISG15*, and continuous stimulation of the innate antiviral response have been demonstrated in blood and tissues of bovine fetuses and cattle infected with BVDV (21, 42).

Although *ISG15*, *Mx1* and *Mx2* were abundantly expressed in all infected fetuses, the expression of other selected IFN-stimulated genes was not altered. Previous studies have demonstrated that ovine fetuses within the second and third trimester of gestation are able to produce levels of interferon similar or greater to those produced by adult animals in response to a viral infection (36). Since type I interferon-induced genes were up-regulated, it is presumed that expression of type I interferon genes were also up-regulated at the same or similar times, and that due to the short half-life of type I interferons the timing of up-regulation of the type I interferon(s) was not detected at the

time points evaluated. Even though less likely, one must consider the possibility of up-regulation of both ISG15 and ovine Mx by an interferon-independent pathway.

CD79a-positive cells (B lymphocytes and plasma cells) were observed in the spleen of CVV-infected fetuses at 49 dg, which is in agreement with previous studies, that identified B lymphocytes in ovine fetal lymph nodes and spleen as early as 47-48 dg (27, 28, 34). Both infected and non-infected fetuses had scattered CD79a-positive, IgM and IgG-positive cells in lymphoid aggregates in the dorsal aspect of the abdominal cavity, sites of development of rudimentary renal and/or mesenteric lymph nodes, as early as 42 and 45 dg, respectively. The identification of CD79a-positive cells in regional lymph nodes earlier than observed in the fetal spleen, supports the previously proposed idea that extrasplenic sites have the potential of producing B lymphocytes (2, 34), and potentially explains the fact that splenectomized fetuses are also capable of colonizing Peyer's patches with B lymphocytes (34, 35).

A marked infiltration of CD79a, IgM and IgG-positive cells was observed within the meninges with fewer cells within the parenchyma of the brain and spinal cord of infected fetuses at 49 dg. It appears that the ovine fetus not only mounts an innate immune response to a viral infection, but is also able to stimulate an adaptive immune response. Unfortunately, blood samples were not available from the fetuses during these experiments; therefore, fetal neutralizing antibodies could not be assayed. In ovine fetuses infected with Akabane virus, the earliest day of gestation where IgM and IgG-positive cells were observed in tissues was 59-60 days gestation. Titers >1:4 were detected only after 100 dg (22, 31). Two other studies detected Akabane virus

neutralizing antibodies after 76-78 dg, with titers ranging from 8-64 in one of these studies (29, 32).

CVV is a viral pathogen with a tropism for the ovine fetal CNS, skeletal muscle and fetal membranes. The study of early infection demonstrated that a tropism correlated well with the CNS and musculoskeletal malformations observed in spontaneous CVV disease. With CVV, the development of arthrogryposis probably has a multifactorial pathogenesis involving effects on developing neurons, myocytes, and fetal membranes. CVV is the only viral infection shown to cause oligohydramnios. The virus is partially cleared from most fetal tissues by approximately 65 dg (28dpi), before the presently accepted onset of ovine immunocompetence.

The ovine fetus is able to mount an immune response associated with CVV infection earlier in gestation by up-regulation of genes that participate in the innate immune response. The infected fetus up-regulates *Mx* genes, which have been shown to restrict growth of bunyaviruses. In addition, cells of the adaptive immune response, CD79a-positive, IgM and IgG-positive cells, are found in large numbers in infected tissues. Whether the immunoglobulin in these cells is actually CVV specific is still unclear. Further studies should be conducted for better understanding of the immune system development in the ovine fetuses exposed to CVV and other viral infections.

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360 **References**

361 1. **Akutsu, M., Y. Ye, S. Virdee, J. W. Chin, and D. Komander.** 2011. Molecular
362 basis for ubiquitin and ISG15 cross-reactivity in viral ovarian tumor domains.
363 Proc Natl Acad Sci U S A **108:**2228-2233.
364 2. **Alitheen, N., S. McClure, and P. McCullagh.** 2007. Detection and
365 quantification of IgM(+) lymphocytes in fetal lamb spleen, liver and lymph
366 nodes by flow cytometry. Immunol Cell Biol **85:**391-393.
367 3. **Austin, K. J., A. L. Carr, J. K. Pru, C. E. Hearne, E. L. George, E. L.**
368 **Belden, and T. R. Hansen.** 2004. Localization of ISG15 and conjugated proteins
369 in bovine endometrium using immunohistochemistry and electron microscopy.
370 Endocrinology **145:**967-975.
371 4. **Campbell, G. L., J. D. Mataczynski, E. S. Reisdorf, J. W. Powell, D. A.**
372 **Martin, A. J. Lambert, T. E. Haupt, J. P. Davis, and R. S. Lanciotti.** 2006.
373 Second human case of Cache Valley virus disease. Emerg Infect Dis **12:**854-856.
374 5. **Charles, J. A.** 1994. Akabane virus. Vet Clin North Am Food Anim Pract
375 **10:**525-546.
376 6. **Chen, S., J. A. Short, D. F. Young, M. J. Killip, M. Schneider, S.**
377 **Goodbourn, and R. E. Randall.** 2010. Heterocellular induction of interferon by
378 negative-sense RNA viruses. Virology **407:**247-255.
379 7. **Chung, S. I., C. W. Livingston, Jr., J. F. Edwards, B. B. Gauer, and E. W.**
380 **Collisson.** 1990. Congenital malformations in sheep resulting from in utero
381 inoculation of Cache Valley virus. Am J Vet Res **51:**1645-1648.
382 8. **Chung, S. I., C. W. Livingston, Jr., C. W. Jones, and E. W. Collisson.** 1991.
383 Cache Valley virus infection in Texas sheep flocks. J Am Vet Med Assoc
384 **199:**337-340.
385 9. **Concha-Bermejillo, A. d. l.** 2003. Cache Valley Virus is a cause of fetal
386 malformation and pregnancy loss in sheep. Small ruminant research **49:**1-9.
387 10. **Dorniak, P., F. W. Bazer, and T. E. Spencer.** 2011. Prostaglandins regulate
388 conceptus elongation and mediate effects of interferon tau on the ovine uterine
389 endometrium. Biol Reprod **84:**1119-1127.

11. **Edwards, J. F.** 1994. Cache Valley Virus. Vet Clin North Am Food Anim Pract **10:**515-524.
12. **Edwards, J. F., A. Angulo, and E. Pannell.** 2003. Theriogenology question of the month. Journal of the American Medical Association **222:**1361-1362.
13. **Edwards, J. F., C. W. Livingston, S. I. Chung, and E. C. Collisson.** 1989. Ovine arthrogryposis and central nervous system malformations associated with in utero Cache Valley virus infection: spontaneous disease. Vet Pathol **26:**33-39.
14. **Erwin, P.** 2010. First case of Cache Valley Fever found in Kansas.
15. **Frese, M., G. Kochs, H. Feldmann, C. Hertkorn, and O. Haller.** 1996. Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein. J Virol **70:**915-923.
16. **Gibbens, N.** 2012. Schmallenberg virus: a novel viral disease in northern Europe. The Veterinary record **170:**58.
17. **Haller, O., M. Frese, and G. Kochs.** 1998. Mx proteins: mediators of innate resistance to RNA viruses. Rev Sci Tech **17:**220-230.
18. **Haller, O., and G. Kochs.** 2002. Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic **3:**710-717.
19. **Haller, O., P. Staeheli, and G. Kochs.** 2007. Interferon-induced Mx proteins in antiviral host defense. Biochimie **89:**812-818.
20. **Haller, O., S. Stertz, and G. Kochs.** 2007. The Mx GTPase family of interferon-induced antiviral proteins. Microbes Infect **9:**1636-1643.
21. **Hansen, T. R., N. P. Smirnova, H. Van Campen, M. L. Shoemaker, A. A. Ptitsyn, and H. Bielefeldt-Ohmann.** 2010. Maternal and fetal response to fetal persistent infection with bovine viral diarrhea virus. Am J Reprod Immunol **64:**295-306.
22. **Hashiguchi, Y., K. Nanba, and T. Kumagai.** 1979. Congenital abnormalities in newborn lambs following Akabane virus infection in pregnant ewes. Natl Inst Anim Health Q (Tokyo) **19:**1-11.
23. **Hefti, H. P., M. Frese, H. Landis, C. Di Paolo, A. Aguzzi, O. Haller, and J. Pavlovic.** 1999. Human MxA protein protects mice lacking a functional alpha/beta interferon system against La crosse virus and other lethal viral infections. J Virol **73:**6984-6991.
24. **Johnson, G. A., M. M. Joyce, S. J. Yankey, T. R. Hansen, and T. L. Ott.** 2002. The Interferon Stimulated Genes (ISG) 17 and Mx have different temporal and spatial expression in the ovine uterus suggesting more complex regulation of the Mx gene. J Endocrinol **174:**R7-R11.
25. **Kochs, G., C. Janzen, H. Hohenberg, and O. Haller.** 2002. Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. Proc Natl Acad Sci U S A **99:**3153-3158.
26. **Kochs, G., M. Reichelt, D. Danino, J. E. Hinshaw, and O. Haller.** 2005. Assay and functional analysis of dynamin-like Mx proteins. Methods Enzymol **404:**632-643.

27. **Maddox, J. F., C. R. Mackay, and M. R. Brandon.** 1987. Ontogeny of ovine lymphocytes. II. An immunohistological study on the development of T lymphocytes in the sheep fetal spleen. Immunology **62:**107-112.
28. **Maddox, J. F., C. R. Mackay, and M. R. Brandon.** 1987. Ontogeny of ovine lymphocytes. III. An immunohistological study on the development of T lymphocytes in sheep fetal lymph nodes. Immunology **62:**113-118.
29. **McClure, S., P. McCullagh, I. M. Parsonson, D. A. McPhee, A. J. Della-Porta, and A. Orsini.** 1988. Maturation of immunological reactivity in the fetal lamb infected with Akabane virus. J Comp Pathol **99:**133-143.
30. **Miyasaka, M., and B. Morris.** 1988. The ontogeny of the lymphoid system and immune responsiveness in sheep. Prog Vet Microbiol Immunol **4:**21-55.
31. **Narita, M., and K. Kawashima.** 1993. Detection of Akabane viral antigen and immunoglobulin-containing cells in ovine fetuses by use of immunoperoxidase staining. Am J Vet Res **54:**420-424.
32. **Parsonson, I. M., A. J. Della-Porta, O. H. M.L., S. W.A., F. K.J., and S. H.A.** 1981. Akabane virus infection in the pregnant ewe. 1. Growth of virus in the foetus and the development of the foetal immune response. Veterinary Microbiology **6:**197-207.
33. **Popp, S. K., D. A. Mann, P. J. Milburn, A. J. Gibbs, P. J. McCullagh, J. D. Wilson, R. R. Tonjes, and C. J. Simeonovic.** 2007. Transient transmission of porcine endogenous retrovirus to fetal lambs after pig islet tissue xenotransplantation. Immunol Cell Biol **85:**238-248.
34. **Press, C. M., W. R. Hein, and T. Landsverk.** 1993. Ontogeny of leucocyte populations in the spleen of fetal lambs with emphasis on the early prominence of B cells. Immunology **80:**598-604.
35. **Press, C. M., P. McCullagh, and T. Landsverk.** 2001. Effect of early fetal splenectomy on prenatal B-cell development in sheep. Immunology **102:**131-136.
36. **Rinaldo, C. R., Jr., J. C. Overall, Jr., and L. A. Glasgow.** 1975. Viral replication and interferon production in fetal and adult ovine leukocytes and spleen cells. Infect Immun **12:**1070-1077.
37. **Rodrigues Hoffmann, A., C. J. Welsh, P. Wilcox Varner, A. de la Concha-Bermejillo, J. Marchand Ball, A. Ambrus, and J. F. Edwards.** 2012. Identification of the target cells and sequence of infection during experimental infection of ovine fetuses with Cache Valley virus. J Virol **86:**4793-4800.
38. **Sandrock, M., M. Frese, O. Haller, and G. Kochs.** 2001. Interferon-induced rat Mx proteins confer resistance to Rift Valley fever virus and other arthropod-borne viruses. J Interferon Cytokine Res **21:**663-668.
39. **Satterfield, M. C., F. W. Bazer, and T. E. Spencer.** 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. Biol Reprod **75:**289-296.
40. **Schmaljohn, C. S., and S. T. Nichol.** 2007. Bunyaviridae. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Field's Virology. Lippincott Williams & Wilkins, Philadephia, PA.

41. **Sexton, D. J., P. E. Rollin, E. B. Breitschwerdt, G. R. Corey, S. A. Myers, M. R. Dumais, M. D. Bowen, C. S. Goldsmith, S. R. Zaki, S. T. Nichol, C. J. Peters, and T. G. Ksiazek.** 1997. Life-threatening Cache Valley virus infection. N Engl J Med **336:**547-549.
42. **Shoemaker, M. L., N. P. Smirnova, H. Bielefeldt-Ohmann, K. J. Austin, A. van Olphen, J. A. Clapper, and T. R. Hansen.** 2009. Differential expression of the type I interferon pathway during persistent and transient bovine viral diarrhea virus infection. J Interferon Cytokine Res **29:**23-35.
43. **Skaug, B., and Z. J. Chen.** 2010. Emerging role of ISG15 in antiviral immunity. Cell **143:**187-190.
44. **Stertz, S., J. Dittmann, J. C. Blanco, L. M. Pletneva, O. Haller, and G. Kochs.** 2007. The antiviral potential of interferon-induced cotton rat Mx proteins against orthomyxovirus (influenza), rhabdovirus, and bunyavirus. J Interferon Cytokine Res **27:**847-855.
45. **Tizard, I. R.** 1996. Immunity in the fetus and newborn., p. 237-250, Veterinary Immunology: an introduction, 5th ed. W.B. Saunders, Philadelphia, PA.
46. **Zhang, D., and D. E. Zhang.** 2011. Interferon-stimulated gene 15 and the protein ISGylation system. J Interferon Cytokine Res **31:**119-130.

496 Table 1. Primer sequences used for RT-qPCR.

Gene Symbol	Accession	Primer Sequences	
		Forward	Reverse
CVV	AF082576.1	CAC CAG CGA AAT CCC AAT CAC CA	CTC CAG ACA TAG CAC CCA CCA
TNF-α	X55152.1	CGG CGT GGA GCT GAA AGA CAA	CTG CGA GTA GAT GAG GTA AA
IL-1	NM_001009465.2	AGT GAT GGC TTG CTA CAG T	CCG AGG TCC AGG TGT T
IL-6	X68723.1	GAG GGA AAT CAG GAA ACT GT	CTC GTT TGA GGA CTG CAT CT
TLR7	HQ529279.1	ACT CCT TGG GGC TAG ATG GT	GCT GGA GAG ATG CCT GCT AT
TLR8	FJ905847.1	TCC ACA TCC CAG ACT TTC TA	GTT CTT GTC CTC ACT CTC TT
Mx1	JN377734.1	GTA CGA GCC GAG TTC TCC AA	ATG TCC ACA GCA GGC TCT TC
Mx2	NM_001078652.1	CAT CCA TAA ATC GCT CCC CTT GT	GCT CCT CTG TCG CCC TCT GGT
ISG15	FJ844480.1	TGA CGG TGA AGA TGC TAG GG	ACT GCT TCA GCT CGG ATA CC
IFN-α	X55152.1	ACC CAG CAC ACC TTC CAG CTC TT	CCT CGC AGC CCC TCC TC
IFN-β	EU276065.1	TGG TTC TCC TGC TGT GTT TCT C	CGT TGT TGG AAT CGA AGC AA

497

498 Figure legends:

499 Figure. 1. Relative levels of genes expression in brain and skeletal muscle of control and Cache
500 Valley virus infected animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

501

502 Figure 2. Relative number of Cache Valley virus RNA copies in main target tissues, brain (BRA)
503 and skeletal muscle (SKM) after 10, 14, 21 and 28 dpi. *$p < 0.05$, **$p < 0.01$.

504

505 Figure 3. Relative amounts of ovine *Mx1* protein in allantoic and amniotic fluid in control and
506 infected animals. The presented values (y axis) are the relative light units using the Bio-Rad
507 chemidoc imager and assessed via use of Quantity One Image analysis software. The starting
508 protein concentration for the samples from the allantoic fluid was 50 µg and for the amniotic
509 fluid was 12 µg. The data was log transformed for normalization. The infected fetuses had
510 relatively higher amounts of Mx1 protein in the allantoic and amniotic fluid when compared to
511 the control fetuses. $p < 0.005$

512

513 Figure 4. Immunohistochemistry for *ISG15* in brain and skeletal muscle. A rabbit polyclonal
514 antibody against ovine *ISG15* protein was stained with DAB chromagen (brown) on the brain
515 (A,B,C) and skeletal muscle (D,E,F) from 45 dg control fetuses (A,D) and from infected fetuses
516 at 14 dpi (45 dg) (B,E) and 28 dpi (63 dg) (C,F). Numerous cells in the brain and skeletal muscle
517 of infected fetuses are positive for *ISG15*. No positive cells were observed in tissues of control
518 fetuses.

519

520 Figure 5. Immunohistochemistry for B cells, plasma cells and ovine IgG and IgM in lymph node,
521 spleen and brain. A polyclonal antibody against B cells and plasma cells (CD79a), ovine IgG and
522 ovine IgM was stained with DAB chromagen (brown). CD79a (A), IgG (B) and IgM (C) positive
523 cells were observed in rudimentary lymph nodes at 42-45dg in control fetuses. CD79a (D), IgG
524 (E) and IgM (F) positive cells were observed in the spleen of infected fetuses. A remarkable
525 number of CD79a (G), IgG (H) and IgM (I) positive cells were seen in the leptomeninges of an
526 infected fetus at 14 dpi.