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Induction of interferon-gamma and downstream pathways during establishment of fetal persistent infection with bovine viral diarrhea virus

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ABSTRACT

Development of transplacental infection depends on the ability of the virus to cross the placenta and replicate within the fetus while counteracting maternal and fetal immune responses. Unfortunately, little is known about this complex process. Non-cytopathic (ncp) strains of bovine viral diarrhea virus (BVDV), a pestivirus in the *Flaviviridae* family, cause persistent infection in early gestational fetuses (<150 days; persistently infected, PI), but are cleared by immunocompetent animals and late gestational fetuses (>150 days; transiently infected, TI). Evasion of innate immune response and development of immunotolerance to ncp BVDV have been suggested as possible mechanisms for the establishment of the persistent infection. Previously we have observed a robust temporal induction of interferon (IFN) type I (innate immune response) and upregulation of IFN stimulated genes (ISGs) in BVDV TI fetuses. Modest chronic upregulation of ISGs in PI fetuses and calves reflects a stimulated innate immune response during persistent BVDV infection. We hypothesized that establishing persistent fetal BVDV infection is also accompanied by the induction of IFN-gamma (IFN-γ). The aims of the present study were to determine IFN-γ concentration in blood and amniotic fluid from control, TI and PI fetuses during BVDV infection and analyze induction of the IFN-γ downstream pathways in fetal lymphoid tissues. Two experiments with *in vivo* BVDV infections were completed. In Experiment 1, pregnant heifers were infected with ncp BVDV type 2 on day 75 or 175 of gestation or kept naïve to generate PI, TI and control fetuses, respectively. Fetuses were collected by Cesarean section on day 190. In Experiment 2, fetuses were collected on days 82, 89, 97, 192 and 245 following infection of pregnant heifers on day 75 of gestation. The results were consistent with the hypothesis that ncp BVDV infection induces IFN-γ secretion during acute infection in both TI and PI fetuses and that lymphoid tissues such as spleen, liver and thymus, serve both as possible sources of IFN-γ and target organs for its effects. Notably, induction of IFN-γ coincides with a decrease in BVDV RNA concentrations in PI fetal blood and tissues. This is the first report indicating the possible presence of an

Abbreviations: APC, antigen presenting cells; BVDV, bovine viral diarrhea virus; cp, cytopathic; CXCL10, chemokine C–X–C motif ligand 10; CXCL16, chemokine C–X–C motif ligand 16; CXCR6, chemokine C–X–C motif receptor 6; IFN, interferon; IFN-γ, interferon gamma; IFI16, IFN-γ induced protein 16; IL, interleukin; MEM, Minimum Essential Media; NK cells, natural killer cells; ncp, noncytopathic; PAMP, pathogen associated molecular pattern; PI, persistently infected; qRT-PCR, semi-quantitative Real Time PCR; RIG-I, retinoic acid-inducible gene; SE, standard error; STAT1, signal transducer and activator of transcription 1; TAP1, transporter 1, ATP binding cassette; TI, transiently infected; TLR3, Toll like receptor 3.

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adaptive immune response in persistent BVDV infections, which may be contributing to the observed reduction of viremia in PI fetuses.

1. Introduction

Many questions regarding the mechanisms of transplacental infections in human and animal viral diseases remain unanswered despite the importance of the issue. The sum of multiple processes in both the dam and the fetus, including viral replication in the mother, transmission of the virus across the placenta to the fetus, and ability of the virus to avoid both maternal and fetal immune responses is the key to the establishment of persistent infection in the fetus throughout gestation and after birth. Studying human transplacental infections is possible only retrospectively for ethical reasons; therefore, examination of these questions about *in utero* infections requires the use of relevant animal models.

Fetal infection with bovine viral diarrhea virus (BVDV) represents one of the most interesting natural animal models allowing one to study development of fetal persistent infection *in utero*. BVDV, along with classical swine fever virus and border disease virus of sheep, belongs to the genus *Pestivirus* in the family *Flaviviridae*. BVDV are small enveloped viruses with a single-stranded positive sense RNA genome of ~12.5 kb (Ridpath et al., 1994; Vilcek et al., 2005). There are 2 biotypes – cytopathic (cp) and non-cytopathic (ncp) – defined based on ability to cause cytopathology in infected cells *in vitro* (Gillespie et al., 1961). Both biotypes cause acute viral infections in immunocompetent animals, ranging from mild subclinical to severe, sometimes fatal, systemic disease, which can lead to embryonic death, abortions, and stillbirths if pregnant cows are infected (Brownlie et al., 1989; Murray, 1990). However, the ability of ncp BVDV to cross the placenta and establish persistent viral infection in early gestational (<150 days) fetuses (Bielefeldt-Ohmann, 1995; Brownlie et al., 1989; McClurkin et al., 1984) despite complete viral clearance by the immunocompetent mother, makes ncp BVDV a unique model for studying complex mechanisms of the development of fetal persistent infection. Infection with ncp BVDV in late gestation (>150 days) results in transient infection in infected fetuses, which clear the virus and develop an antibody response to the infecting BVDV strain (Done et al., 1980).

We have previously described the significant impact of BVDV infection on fetal development and immune cell gene expression (Shoemaker et al., 2009; Smirnova et al., 2008; Smirnova et al., 2009), as well as the adverse effects on fetal growth and development of multiple organs, including the brain (Bielefeldt-Ohmann et al., 2012; Bielefeldt-Ohmann et al., 2008) and bone (Webb et al., 2012), and altered fetal antiviral immune responses (Shoemaker et al., 2009; Smirnova et al., 2012). The presence of persistently infected (PI) animals, continuously shedding virus, is the mechanism by which the virus persists in the population.

Two major factors are thought to be important for the development of the persistent infection with BVDV. First, an ability of ncp BVDV to avoid innate immune response, specifically, interference of the virus replication with the production of interferon (IFN) type I, was considered a major cause for the establishment of persistent infection (Charleston et al., 2001; Peterhans and Schweizer, 2013). However, we have previously demonstrated robust production of the type I IFN and upregulation of IFN stimulated genes (ISGs) in fetuses and heifers transiently infected (TI) with ncp BVDV, while chronic modest, but significant, ISG upregulation was detected in PI fetuses (Shoemaker et al., 2009; Smirnova et al., 2008; Smirnova et al., 2012). These data and recent work of Palomares et al. (2013) indicate that ncp BVDV is able to upregulate type I IFN and associated innate immune responses in infected animals.

A second proposed component promoting the establishment of persistent BVDV infection is an evasion of the adaptive immune response due to the presence of the virus during selection of T lymphocytes, which incorporate the viral antigen into the host antigen repertoire (Peterhans et al., 2003; Potgieter, 1995). This lack of recognition of the virus by the adaptive immune system results in a failure to clear the infection and a persistent, life-long viremia.

Production of IFN gamma (IFN-γ) is a pivotal mechanism in building an effective immune response to bacterial and viral pathogens. IFN-γ secretion by natural killer (NK) cells and activated T cells early in response to infection is an important step, as it both promotes innate immune response through activation of macrophages and leads to the activation of professional antigen presenting cells (APCs). Activated APCs in turn provide a positive feedback loop by producing IL-12 to further induce IFN-γ production by NK cells and Th1 cells (reviewed in Frucht et al., 2001; Hu and Ivashkiv, 2009).

IFN-γ and associated adaptive immune responses of the bovine fetus to BVDV infection have not been previously examined. We hypothesized that development of the persistent fetal infection with ncp BVDV in early gestation is accompanied by induction of IFN-γ, which leads to stimulation of downstream pathways and thus contributes to the partial curtailing of viral replication and a decrease in viremia in PI fetuses. The aims of the study were: (i) to determine IFN-γ concentration in fetal blood and amniotic fluid from control, TI and PI fetuses collected at multiple time points during *in utero* ncp BVDV infection in two previously described studies (Smirnova et al., 2008; Smirnova et al., 2012); (ii) to determine sources of IFN-γ; and (iii) to analyze possible induction of IFN-γ downstream genes in fetal lymphoid tissues.

The results presented herein demonstrate that ncp BVDV infection induces temporal IFN-γ secretion into fetal blood during acute infection in both TI and PI fetuses, which coincides with the decrease in BVDV RNA levels in fetal blood and tissues during the establishment of persistent infection. To the best of the authors' knowledge, this is the first report demonstrating that components of an adaptive immune response might be present and can contribute to the partial clearance of viremia in PI fetuses.

2. Materials and methods

2.1. Animals

Animal experiments were performed following two experimental designs, which were previously described (Smirnova et al., 2008; Smirnova et al., 2012). For both experiments, weaned Hereford heifers, serologically negative for BVDV, were purchased from a source that did not vaccinate for BVDV. All animal experiments were approved by the Institutional Animal Care and Use Committee at Colorado State University (Experiments 1 and 2) and University of Wyoming (Experiment 1). Prior to arrival all heifers were confirmed to be (i) seronegative for BVDV in standard serum neutralization assay and (ii) free of BVDV viral antigen using a commercial antigen capture ELISA on ear notch extracts (IDEXX) as previously described (Smirnova et al., 2008; Smirnova et al., 2012) and were re-tested after arrival but prior to the *in vivo* BVDV inoculation (Smirnova et al., 2012). At ~1 year of age, estrous cycles were synchronized and heifers were artificially inseminated with