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Short Communication

NEW INSIGHTS INTO IL-4 AND FOXP3 EXPRESSION IN THE PATHOGENESIS OF EQUID ALPHAHERPESVIRUS ABORTION IN THE BALB/C MURINE MODEL

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ABSTRACT: Most of the investigations using the murine model of *Equid alphaherpesvirus 1* (EHV-1) infection have aimed at elucidating the pathogenesis of abortion caused by the virus, and the mechanisms were mainly associated with the general immune response and vascular changes. We examined whether some components of the local immune response to EHV-1 during pregnancy contribute to the pathogenesis of the abortion in infected pregnant mice. Twenty-six pregnant females were used. At day 13 of pregnancy, females were intranasally infected. Samples of lungs, uteri and placentas were processed for viral isolation and DNA detection by PCR. In addition, the levels of IL-4 and Foxp3 mRNA from uteri and placentas of infected and control mice were studied 3- and 4-days post-inoculation (pi) by real time PCR. Virus isolation and DNA detection were positive only in lungs of infected groups. A significant decrease in the expression of IL-4 and Foxp3 mRNA levels was found in the uteri and placentas 3- and 4-days pi, in comparison to the control group. Here, we showed changes in the local cytokine expression that may prove critical for understanding the pathogenesis of the abortion in horses and experimental models.

Key words: Abortion, Cytokines, Equid alphaherpesvirus 1, Placenta, Uterus.

Equid alphaherpesvirus 1 (EHV-1) causes respiratory disease, neurological disorders, abortion and perinatal mortality in horses. Despite considerable research, the pathogenic mechanism by which EHV-1 induces abortions is still unknown. Normal pregnancy requires an adequate function of the cytokine network. The physiological balance between Th1 / Th2 cytokines changes towards a predominantly Th2 profile and although Th1/Th2 paradigm was useful to explain the local immune response and retention of the conceptus (Wegmann *et al.* 1993) other elements of the immune response, such as Treg cells, have been incorporated to a new paradigm. Treg cells are elevated in blood and uterus during oestrus and pregnancy (Zenclussen *et al*. 2006). These cells are essential in the early response to local viral infection by facilitating the entry of immune cells in infected tissue (Lund *et al*. 2008), and depression of these cells generates abortion in mid gestation (Zenclussen 2005, Clark 2016). Treg cells contribute to embryo or foetus tolerance and limit the inflammation during implantation. Besides, foetal specific Treg cells could generate memory cells for future pregnancy (Tong and Abrahms 2020). Transcription factor forkhead box P3 (Foxp3) expression is essential for the differentiation

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and functionality of Treg cells and it is used as Treg cell marker (Rowe *et al*. 2012). Cytokines of Th2 profile, such as IL-4, are anti-inflammatory and generate an adequate and protective microenvironment for embryonic and foetal development inhibiting the cytotoxic response (Chatterjee *et al*. 2014). Particularly IL-4 is a key regulator in adaptive and humoral response, and promotes Th0 to Th2 profile differentiation, leading to a greater production of IL-4 in a positive feedback loop, by decreasing the production of Th1 profile cells (Yang *et al*. 2019).

Our interest relies on further study the pathogenic mechanism of reproductive failures in EHV-1 experimental murine model, particularly the BALB/c strain which successfully reproduces the effects observed in horses (Zanuzzi *et al*. 2014). In previous study using this model we reported microvascular lesions, changes in cell proliferation and death, and in the cytokine expression of INF- γ, TNF-∝ and IL-10 in the placentas of mice experimentally infected with EHV-1 (Zanuzzi *et al*. 2016). Thus, here we further analysed the expression of IL-4 and Foxp3, with the aim of enhancing our understanding of the local immune response to EHV-1 during pregnancy.

The study

Virus strain culture

The Argentine AR8-EHV-1 strain was selected for the experimental study. This strain was isolated from an aborted equine foetus in 1996 and it has shown abortigenic effect in the murine model (Zanuzzi *et al*. 2014). The viral inoculum consisted of 50 µl (20.000 $CCID_{50}$) of viral stock, obtained from culture supernatant of AR8-EHV-1 infected RK13 cells until the occurrence of extensive cytopathic effect. The final infectious supernatant was fractionated and was stored at -70° C until use. Virus titre was determined using the Reed and Muench method (Reed and Muench 1938).

Animals and clinical assessment

Twenty-six specific pathogen-free, six- to eight-week-old BALB/c mice were purchased from the Laboratory of Experimental Animals and kept in conventional animal rooms of the Department of Virology. All procedures were carried out in compliance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council and were approved by the Institutional Committee for Care and Use of Laboratory Animals (School of Veterinary Sciences, National University of La Plata, Buenos Aires Argentina CICUAL-Res. T35-6-13). The weight of animals was recorded. The oestrous cycle was synchronized using the Whitten effect (Whitten 1996). Pregnancy was confirmed by the presence of vaginal plugs (day 0 of pregnancy). At day 13 of pregnancy mice were weighed to confirm the increase in body weight and then they were infected by intranasal route under light anaesthesia with isoflurane (Baxter Co., Deerfield, IL, USA).

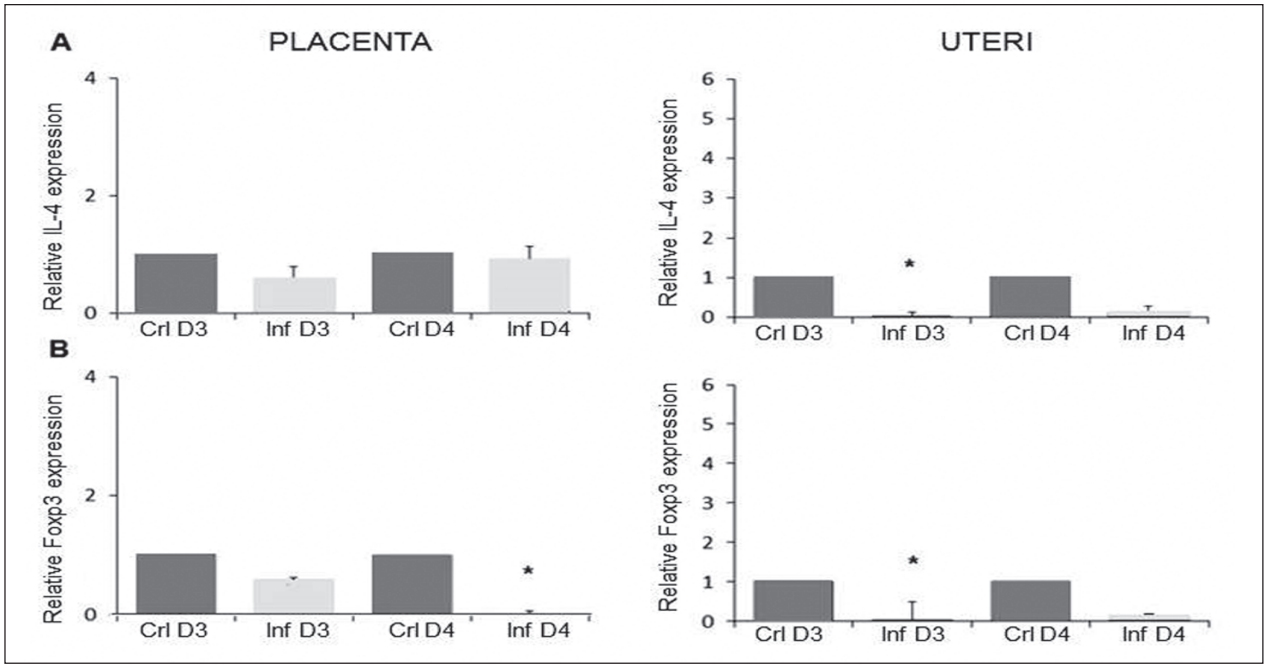

Fig. 1. mRNA IL-4 and Foxp3 transcription determined by qRT-PCR in the uteri and placentas of control and infected mice. (*) Significant differences between infected and control group at $p \leq 0.05$.

Experimental design

Control group was composed of pregnant mice that received 50 µl of supernatant of non-infected RK13 cells. After inoculation of mice, the evolution of their general condition was daily monitored and the appearance of clinical signs was recorded. At days 3 and 4 post-inoculation (pi), the infected groups (Inf D3, n = 8 and Inf D4, n = 8, respectively) and their corresponding control groups (Crl D3, n = 5 and Crl D4, n = 5) were lightly anesthetized with isoflurane and killed by exsanguination (Eöry *et al.* 2013). Pregnant mice were killed by the end of pregnancy because abortion in horses generally occurs in the last third of pregnancy. Selection of pi days was based on previous works (Zanuzzi *et al.* 2014).

Collection, processing of samples and cytokine determination

Necropsy was performed and samples of lungs and half of uteri and placentas were collected and processed for viral isolation and DNA detection by polymerase chain reaction (PCR). The DNA of all samples and control groups was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and subsequent PCR was performed to confirm viral infection (Galosi *et al*. 2001). The other half of the uteri and placentas removed were snap-frozen with liquid nitrogen for 30 min and further stored at -80°C until RNA isolation for real time PCR (qPCR). Frozen uterine and placentas were homogenized and amplified according to Zanuzzi *et al.* (2016) to determine cytokine mRNA profile for qPCR using specific primers for IL-4 and Foxp3 (Table 1). The qPCR amplification efficiency was calculated using the equation: E = 10 [-1 / slope] (Pfaffl *et al.* 2002).

Statistical analysis

Data were statistically analysed using a student t-test. Gene expression was calculated by a relative quantification method $(2^{-\triangleleft\triangleleft CT})$ (Livak and Schmittgen 2001). All samples were normalized regarding their β-Actin mRNA content.

Results and discussion

Clinical signs

Infected mice showed slight respiratory signs and ruffled fur from 12hs PI up to 2days PI, without weight lost when compared to control.

Virus isolation and PCR.

Infection of pregnant mice with EHV-1 was confirmed by viral isolation and PCR. The presence of virus was positive in lung samples of mice infected 3- and 4-days pi, whereas virus isolation and DNA detection were negative in the uteri and placentas.

Cytokine determination

In this study, all infected mice showed a significant decrease in the expression of IL-4 and Foxp3 mRNA in the uteri and the placentas 3- and 4-days pi, compared with gestational age-matched control mice (Fig. 1).

During pregnancy, the maternal immune response is redirected to a Th2 cytokine profile. Damages that lead to embryonic or foetal death are usually generated by the cells of the maternal immune system. In different intracellular infections Th1 cytokine profile predominates, but during pregnancy this protective response against the infection may also induce abortion (Ge *et al.* 2008). EHV-1 infection activates the immune system and the consequent expression of cytokines. In this work we investigated for the first time whether the expression of IL-4 and Foxp3 mRNA is altered in the uteri and placentas of infected BALB / c mice, changes that may also contribute to the pathogenesis of reproductive failures during pregnancy.

It is well-known that IFN-γ and TNF-producing Th1 profile is incompatible with a viable pregnancy (Chaouat *et al*. 2002). In a previous study we reported changes in IFN-γ and TNF expression in the uteri and placentas of infected mice, but also an increase in IL-10 expression (Zanuzzi *et al.* 2016). Although IL-10 has a well-known protective role during pregnancy it may also be increased due to the establishment and perpetuation of viral persistence (Wilson and Brooks 2011). In addition, many viruses have developed different evasive mechanisms to interfere with the function of IFN-γ at different levels of the signalling pathway of this cytokine (Goodbourn *et al*. 2000, Haller *et al*. 2006, Sarkar *et al*. 2015). Paladino and Mossman (2009) reported that several alphaherpesviruses are able to control the immune response of the host by blocking or modulating the expression of cytokines. In this sense, Sarkar *et al*. (2015) and Wagner and Freer (2009) reported this effect using neuropathogenic strains of EHV-1 *in vitro* models. It is unknown whether other EHV-1 strains, such as those abortigenic, retain the ability to control the immune response in one way or another *in vivo* models. In addition, trophoblast cells have developed mechanisms to block IFN-γ signalling in order to contribute to a successful pregnancy avoiding foetal rejection (Murphy *et al.* 2009).

Thus, interferons and other antiviral cytokines synthesized by trophoblast cells in response to viral infections may prevent the arrival of the virus to the placenta and the foetus (Mor and Cardenas 2010). Further

Table 1. Genes analysed by RT-qPCR and primers used.

Gene	Primer	Sequence
β-Actin	F	GCTTCTTTGCAGCTCCTTCGTT
β-Actin	R	GTTGTCGACGACCAGCGC
IL-4	F	GTGATGTGGACTTGGACTCATTCA
IL-4	R	CTCATGGAGCTGCAGAGACTCTTT
Foxp3	F	TTCTCACAACCAGGCCACTTG
Foxp3	R	CCCAGGAAAGACAGCAACCTT

studies are needed to determine whether the immunological changes here described may be the result of some evasive mechanism of the EHV-1 AR8 strain or due to a protective effect exerted by the trophoblast to prevent infection.

Treg cells are effective to prevent foetal immune rejection and to create a privileged microenvironment of tolerance (Zenclussen *et al.* 2006). As a result, Treg cell deficiency in pregnancy induces abortion, as shown in a well-established murine model (Zenclussen 2005). On the other hand, Rowe *et al.* (2012) have described the protective role of Treg cells in host defence against viral pathogens. In some diseases, there is a redirection of the uterine immune response from one dominated by a Th2 cytokine profile, protective for pregnancy, towards another predominantly Th1, which facilitates the elimination of the microorganism but can also generate abortion (Quinn *et al.* 2002, Innes *et al.* 2005, Ge *et al.* 2008). During viral infections Th1 cytokine profile also predominates (Coombs *et al*. 2006), and from our results we suggest that this profile may participate in the abortion pathogenesis during EHV-1 infection.

In the present study, Foxp3 mRNA expression levels, both in infected placentas and uteri, were reduced as occur in other intracellular infections (Rowe *et al.* 2012). In a murine model of toxoplasmosis, the low expression of Foxp3 associated with high levels of TNF-∝ and IL6 generated adverse pregnancy outcomes (Sousa *et al*. 2021). In human the minor expression of Foxp3 is frequent in cases of recurrent spontaneous abortus (Keller *et al*. 2020). The importance of Foxp3 in pregnancy is extended to equine because this molecule is expressed in the endometrial cup during early pregnancy (Kammerer *et al*. 2020). IL-4 promotes an adequate and protective microenvironment for embryonic and foetal development (Bryant *et al.* 2017). However, in the present work we found low expression of IL-4. Similar changes were reported in women with recurrent spontaneous abortus (Li *et al.* 2014). Thus, based on the reduction in Foxp3 and IL 4 mRNA levels at the uteroplacental barrier along with the previously reported increased of Th1 cytokines (Zanuzzi *et al*. 2016), we presume that the local immune response may be involved in the abortion of EHV-1 infection during pregnancy without the existence of a direct effect of the virus.

It can be concluded that the effects of EHV-1 during pregnancy depend on different pathogenic mechanisms. Our study demonstrates that EHV-1 infection generates changes in the local expression of cytokines, which may represent a previously unrecognized mechanism for the pathogenesis of abortion.

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