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CELLULAR AND MOLECULAR BIOLOGY

IFN-γ and IL-10: seric and placental profile during pig gestation Seric and placental cytokines in pig gestation

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Abstract: Concentration of interferon-gamma and interleukin-10 in maternal serum and in maternal and fetal porcine placental extracts from different gestation periods was determined. Crossbred pigs' placental samples of 17, 30, 60, 70, and 114 days gestation and non-pregnant uteri were used. Interferon-gamma concentration was increased at the placental interface at 17 days, in maternal and fetal placenta, and decreased significantly in the remaining gestation periods. Interferon-gamma showed a peak in serum at 60 days. Regarding interleukin-10, placental tissue concentrations were unaltered, there were no significant differences with non-gestating uteri samples. In serum interleukin-10 increased at 17, 60, and 114 days gestation. At 17 days there are uterus structural and molecular changes that allow the embryos implantation and placenta development. The presence of interferon-gamma found at this moment in the interface would favor that placental growth. Moreover, its significant increase in serum at 60 days, would generate a proinflammatory cytokine pattern that facility the placental remodeling characteristic of this moment of porcine gestation. On the other hand, a significant interleukin-10 increase in serum at 17, 60 and 114 days could indicate its immunoregulatory role at a systemic level during pig gestation.

Key words: Cytokines, immunology, pig placenta, pig reproduction.

INTRODUCTION

Porcine conceptus loss is more numerous than in other farm animals, as a negative consequence of poly-implantation in this species. There are abundant studies that analyze peri-implant embryonic losses that occur between 10 and 30 days gestation (dg) and that represent the loss of about 30% of the conceptus in this species (Stroband & Van der Lende 1990, Geisert & Schmitt 2002, Ross et al. 2009, Edwards et al. 2012, Bazer & Johnson 2014). However, there is another period, between 50 and 70 dg, when 10- 15% of the conceptus that gets lost and that has not been so studied (Wessels et al. 2007). Although this loss has been attributed to competition for space in the uterus and some authors consider this can be related to endometrial remodeling processes (Vallet et al. 2013). Specifically, at 60 dg, period in which the size of the fetuses is increased, the presence of numerous cells with phagocytic apoptotic bodies in the stroma has been observed (Cristofolini et al. 2013). Also at 70 dg the expression of some adhesion molecules is minor (Vélez et al. 2018). At 90 dg, an increase in vascular apoptosis related with a vascular remodelation was observed (Sanchis et al. 2017). On the other hand, angiogenesis is fundamental for fetal survival in late porcine pregnancy (Stenhouse et al. 2019) and the expression of the angiogenic factor VEGFA (Vascular Endothelial

Growth Factor) is increased from 70 dg (Fiorimanti et al. 2018).

The relationship between the early loss of pig pregnancy and the action of different cytokines has been studied in depth in the peri-implantation loss. Thus it was shown that between days 15 and 23 of gestation, the expression of proinflammatory cytokines (Th1) such as interferon gamma (IFN-γ), tumor necrosis factor alfa (TNF-α) and interleukin 1 (IL-1) is increased in biopsies from conceptus with embryonic death (Tayade et al. 2006), these changes were not found in conceptus of 50 dg (Murphy et al. 2009). In a previous report, we analyze the concentration of IL-1β (Th1 cytokine), IL-2 (Th1 cytokine) and IL-4 (anti-inflammatory Th2 cytokine) in serum, fetal placenta and maternal placenta during gestation, and we found an increase of both types of cytokines in the placental tissue at 70 dg (Vélez et al. 2019). These results seem to corroborate the importance of this point during pig gestation.

IFN-γ is a Th1 cytokine produced mainly by stimulated lymphocytes, in the case of pigs is also synthesized in trophoblast cells (Tayade et al. 2006). This cytokine has been associated with embryonic and fetal death in various species (Casazza & Lazear 2019). However, it has also been demonstrated that IFN-γ is necessary for normal implantation in humans (Murphy et al. 2009) and it has an angiogenic role in the pig placenta (Tayade et al. 2007). We did not find reports about the presence of this cytokine after 50 dg in pigs.

IL-10 is a cytokine produced by the Th2 subgroup of CD4+ helper cells. It is also produced by some activated B cells and by some non-lymphocytic cells such as activated macrophages, keratinocytes and trophoblasts (Roth et al. 1996). The two main activities of IL-10 are to inhibit the production of cytokines by macrophages (TNF, IL-1 and IL-2) and to inhibit the accessory functions of macrophages in T cell activation. Besides, IL-10 has stimulatory actions on B cells (Walter 2014). In human and murine studies, during pregnancy, the cellular source of IL-10 was assumed to be T lymphocytes or trophoblast. However, in mice, a subpopulation of B cells, regulatory B10 cells, are presented as an important source of IL-10 (Bommer et al. 2016, Dilillo et al. 2010). The main function of the IL-10 produced by these B10 cells is to be able to maintain the fine immune balance required in pregnancy to generate the tolerance of the fetal allograft. It has been shown that IL-10 produced by B10 cells keeps dendritic cells in a state of immaturity, inhibiting their ability to present antigens and the consequent activation of T cells (Jensen et al. 2013). In pigs, the expression of IL10 mRNA was determinate in the initial (Choi et al. 2018) and term placenta (Zhou et al. 2019), but this cytokine was not studied along the porcine gestation.

In the last decade the classic Th1/Th2 paradigm was extended taking into account the importance of other cytokines and cells and replaced by a Th1/Th2/Th17 and regulatory T-cell paradigm (Polese et al. 2014, Saito et al. 2010.)

In pig was demonstrated that some cytokines, as IL-4, due to their functions can not be clearly included as Th1 or Th2 cytokine (Murtaugh et al. 2009). In a previous paper (Vélez et al. 2019) we found that cytokines considering as Th1 or Th2 during pig gestation did not follow the patterns described in mice and humans gestations, and Choi et al. (2018) found that the ratio Th1/Th2 cytokines is not the only factor that determines embryo survival in early pig pregnancy. Considering these antecedents and the absence of any report that study the IFN-γ levels at the period after 50 dg, in this work we analyzed the levels of IFN-γ (like Th1 cytokine) in serum and placental interface during pig gestation. IL-10 (traditional Th2) was also studied

in the same tissues, because is still unknown its behavior at any time of the pig gestation. The objective was to determine if these Th1 and Th2 cytokines present a different pattern in the classic paradigm as we observed with other cytokines.

MATERIALS AND METHODS

Animals

Twenty crossbred pigs (Landrace x Large White) between their second and third births with gestations between 17 and 114 days, and 4 uteri of non pregnant pigs, were analyzed. The pigs were healthy as certified by assessment of medical records provided by the farmer owner. The organs were obtained from slaughterhouses near General Pico, La Pampa, Argentina. Animals were sacrificed according to the animal welfare manual of National Agrifood Health and Quality Service (known in Spanish as SENASA) (SENASA 2015). All experimental procedures were revised and approved by a Scientific Committee of Faculty of Veterinary Science of the National University of La Pampa (research code: Res CD 311/2017).

The reproductive tracts were washed promptly with Hanks' saline solution (SSH), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2.5 mg/ml fungizone and stored at 4°C until processing in the laboratory. Conceptus age was stablished using the crown-rump length of the embryos/fetuses according to the Marrable's table (Marrable 1971). Specimens were divided into six groups according to stages of development: 17 dg (n=4, implantation window) (Spencer et al. 2004), 30 dg (n=4, onset of ossification and immune system development) (Butler et al. 2009), 60 dg (n=4, completed exponential placenta growth) (Wooding & Burton 2008), 70 dg (n=4, placenta development plateaued and exponentially fetus growing) (Cristofolini et al. 2013) and 114 dg (n=4, full-term placenta). In addition, uteri of 4 non pregnant pigs were obtained. The non pregnant pigs were in luteal phase of their estrous cycle, information based on their medical history.

Serum

Pigs were bled from the carotid artery following the protocol established in SENASA's animal welfare manual (SENASA 2015). Blood samples were kept at room temperature until clot retraction occurred and the serum exudate was formed. The serum was centrifuged at 500g for 20 min at room temperature, aliquoted, labeled and stored at -20 °C.

Maternal placental homogenates (MPHo), fetal placental homogenates (FPHo) and non pregnant uterus homogenates (NPUHo)

Placental extracts were obtained as previously described (Vélez et al. 2019). Briefly, the uterine horn was opened along the mesometrial side. Then, a portion of 5 g of uterus (onwards maternal placenta) and chorioallantoic membrane (onwards fetal placenta), were extracted separately from each implantation site. The samples were macerated with three parts of saline solution to obtain a homogeneous mass that was centrifuged at 500g for 20 min. The supernatant was stored in 1,5 ml aliquots at -20°C. These samples were identified as porcine MPHo and FPHo. Homogenates from NP uteri (NPUHo) were processed in the same way (Koncurat et al. 1999). One sample was taken from each uterine horn for NPUHo.

Cytokine determination

IFN-γ and IL-10 concentrations were determined using Eagle Biosciences and R&D Systems Swine Interleukin ELISA (enzyme linked immunoassay) kits, respectively. These kits are useful for the quantitative determination of pig cytokines

concentrations in cell culture supernatants, serum, and plasma.

IFN-γ determination

IFN-γ was measured in serum and in NPUHo, MPHo and FPHo. Briefly, 100 µl of samples and standards were added in ELISA plate that contains primary monoclonal antibody against porcine IFN-γ (IFNG51-K01, Eagle Biosciences, USA). Then, 50 µl of biotinylated IFN-γ porcine antibody was added to each well. The plate was covered from light and incubated overnight at 4°C. Samples and standards were removed and the plate was washed four times by filling the wells with 200 µl of washing solution. Next, 100 ml of streptavidin-horseradish peroxidase (streptavidin/HRP) was added at each well and incubated for 30 min at room temperature with gently shake and avoiding the direct light. The solution was removed and the plate was washed four times. After, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) solution was added to each well and incubated for 20 min at room temperature with gently shake and avoiding the direct light. Finally, 100 µl of stop solution was added to stop the reaction. Measurements were performed at 450 nm using a microplate reader (BioTeK® Instruments, Inc. USA).

IL-10 determination

IL-10 was measured in serum and in NPUHo, MPHo and FPHo. Briefly, 100 ul of assay diluent RD1W and 100 µl of samples and standards were incubated for 2 hours at room temperature in a linear shaker (DPC, USA) at 200 rpm in ELISA plate containing primary monoclonal antibody against porcine IL-10 (P-1000, R&D Systems, USA). Then, samples and standards were removed, and the plate was washed five times by filling the wells with 200 µl of washing solution. Next, 100 µl of IL-10 antibody conjugated to horseradish peroxidase (HRP) was added. Samples were incubated for 2 hours at room temperature in a linear shaker and then washed. After, 120 µl of TMB (3,3',5,5'-tetramethylbenzidine) solution was added to each well and incubated for 30 min at room temperature on the benchtop, protected from light. Finally, an equal volume of stop solution was added. Measurements were performed at 450 nm using a microplate reader (BioTeK® Instruments, Inc., USA).

Statistical analysis

Statistical data of IFN-γ and IL-10 concentrations from serum, NPUHo, MPHo and FPHo were performed using the InfoStat statistical software (Di Rienzo et al. 2010). One-way ANOVA and the Tukey's test were applied to calculate significant differences among samples ($p<0.05$). All values are indicated as mean ± EE. In cases where the assumption of homogeneity of variance and normality was not fulfilled, the test nonparametric variance, Kruskall-Wallis was used. Values for $p<0.05$ were considered statistically significant and there are indicated by asterisks.

RESULTS

IFN-γ in serum, maternal placental homogenates (MPHo) and fetal placental homogenates (FPHo).

A comparison between IFN-γ concentrations in serum samples of non-pregnant and pregnant pigs of different gestation periods was performed. As shown in Figure 1a, IFN-γ concentration was found elevated at 60 dg (84.64±12.55 pg/ml) and in serum of non- pregnant pigs (NPS, 61.04±16.93 pg/ml). When we compared seric IFN-γ concentration between different periods during gestation we found that this cytokine was significantly lower at 17 and 114 dg (11.52±4.83

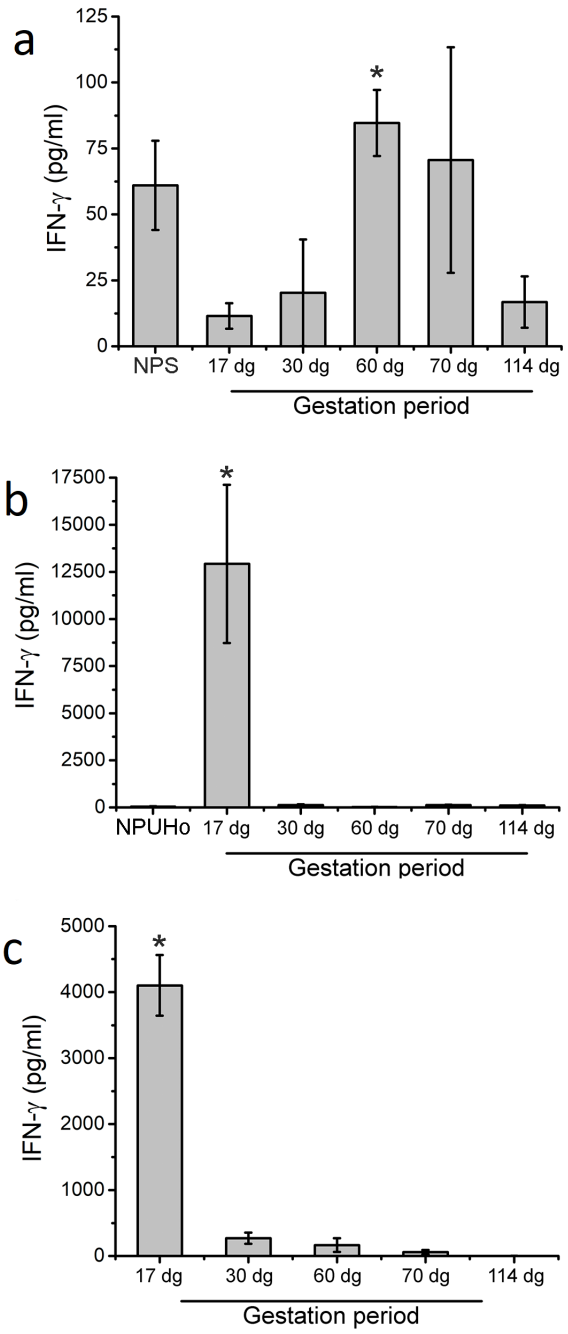

Figure 1. IFN-γ concentration (pg/ml) in serum, maternal placental homogenates (MPHo), fetal placental homogenates (FPHo) and non pregnant uterus homogenates (NPUHo), from 17, 30, 60, 70, and 114 days of gestation (dg). a) IFN-γ concentration (pg/ml) in serum from non pregnant pigs (NPS) and gestating pigs from different gestational periods. * indicates p<0.05 compared with 17 and 114 dg periods. b) IFN-γ concentration (pg/ml) in NPUHo and in MPHo from different gestational periods. c) IFN-γ levels (pg/ml) in FPHo samples from different gestational periods. b-c) * indicates p<0.05 compared with other periods.

and 16.79±9.71 pg/ml, respectively) than 60 dg (p=0.005) (Figure 1a).

On the other hand, at placental level, we observed a significant increase in IFN-γ concentration at 17 dg in MPHo (12924.78±4196.96 pg/ml; p=0.02) (Figure 1b), respect the NPUHo IFN-γ concentration (46.40±18.83 pg/ml). Otherwise, the IFN-γ concentration in FPHo showed a peak at 17 dg (4103.07±458.04 pg/ml) that significantly decreased in the others periods studied (30 dg: 270.21±85.93 pg/ml; 60 dg: 165.92±102.93; 70 dg: 59.10±32.72 and 114 dg: 0.74±0.72; p<0.0001, Figure 1c).

IL-10 in serum, maternal placental homogenates (MPHo) and fetal placental homogenates (FPHo)

A comparison between IL-10 concentrations in serum samples of non-pregnant and pregnant pigs in the different gestation periods was performed. As shown in Figure 2a, IL-10 concentration was significantly high in serum at 17 (11.59±0.58 pg/ml), 60 (15.53±0.01 pg/ml) and 114 dg (19.44±0.69 pg/ml), compared with serum from non-pregnant pigs (0.3±0.17 pg/ml) and serum from pigs that were in another gestation periods (30 dg: 0.47±0.21 pg/ml and 70 dg: 0.38±0.16 pg/ml; p<0.0001). On the other hand, local endometrial concentration of IL-10, either in NPUHo and in MPHo, was similar and even very low between non-pregnant pigs (0.22±0,12 pg/ml) and pigs in the gestation periods studied (p= 0.9) (Figure 2b, c). Otherwise, as shown in Figure 2c there were no significant differences in IL-10 concentration in FPHo between the different gestational periods studied (17 dg: 0.23±0.23 pg/ml; 30 dg: 0.5±0.10 pg/ml; 60 dg: 0.33±0.23 pg/ml; 70 dg: 0.34±0.17 pg/ml and 114 dg: 0.41±0.12 pg/ml; p= 0.68).

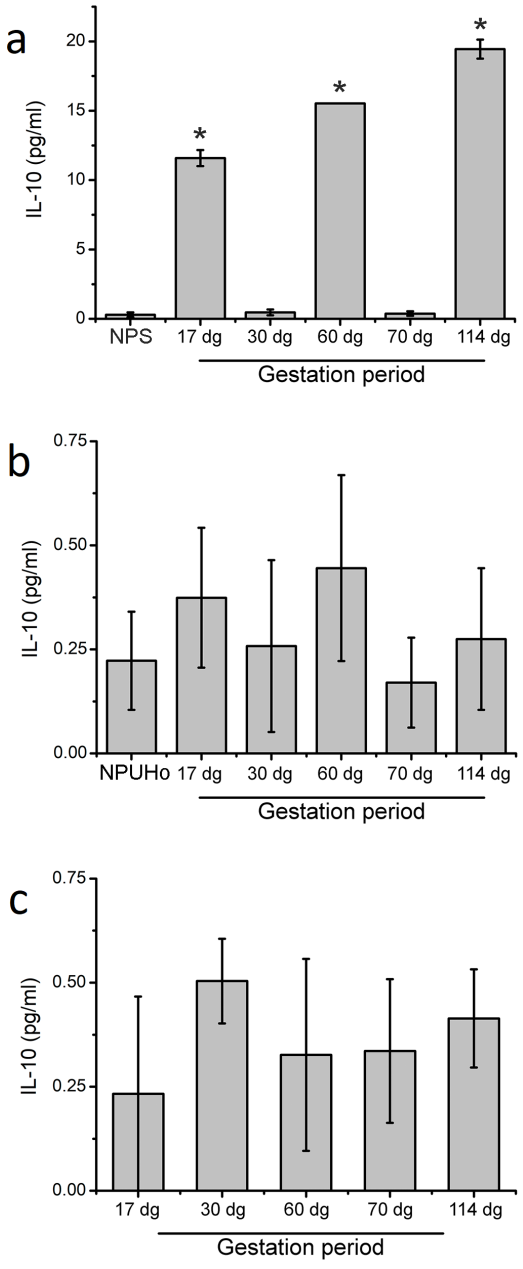

Figure 2. IL-10 concentration (pg/ml) in serum, maternal placental homogenates (MPHo), fetal placental homogenates (FPHo) and non pregnant uterus homogenates (NPUHo) from 17, 30, 60, 70, and 114 dg. a) IL-10 concentration (pg/ml) in serum from NP pig (NPS) and gestating pigs from different gestational periods. * indicates p<0.05 compared with NPS, 30 and 70 dg periods. b) IL-10 concentration (pg/ml) in NPUHo and in MPHo from different gestational periods. c) IL-10 levels (pg/ml) in FPHo samples from different gestational periods. b-c) no significant differences were found.

DISCUSSION

Early embryo loss is a very important problem in pig production, numerous studies have been conducted on the importance of cytokine expression during peri-implantation period, lapse with highest percentage of embryonic death occurs (Kridli et al. 2016). However, a minor percentage of embryo loss also occurs in the second half of pregnancy, especially when placental remodeling processes occur. For example, between 60 and 90 dg, changes in death cell processes (Cristofolini et al. 2013), in angiogenesis (Fiorimanti et al. 2018) and in adhesion molecules expression (Vélez et al. 2018) have been demonstrated. Moreover, at this gestation point, an increase of IL-1β, IL-2 e IL-4 expression (Vélez et al. 2019) was reported.

In previous studies other authors have found high levels of uterine IFN-γ at preimplantation period (Cencič et al. 2002, La Bonnardière et al. 1994, Lefèvre et al. 1990). This increment was associated with the typical conceptus loss that occurs around day 20 of gestation but not in the loss that happens on day 50 (Bidarimath & Tayade 2017). In the present work we found that the concentration of this cytokine is low after 17 dg, both in maternal and fetal placenta, and those low values are maintained through pregnancy. However, we found an increase of IFN-γ in serum at 60 dg, the same period that we previously observed high seric levels of IL-1β (Vélez et al. 2019). Its significant increase in serum at 60 dg, would generate a proinflammatory cytokine pattern that facility the placental remodeling characteristic of this moment of porcine gestation.

These results suggest that the IFN-γ source would not be placental, but another organ, since we have observed that its concentration was high in serum, but not in placenta. On the other hand, an increase of IFN-γ is evident in

preterm hemochorial placenta and is related with the changes necessary to the delivery (Saito et al. 2010, Sykes et al. 2012). This IFN-γ increase does not occur in the pig, a species with epitheliochorial placenta. In a previous report, we found that IL-1β and IL-2 increased in serum but not in placental interface at term gestation (Vélez et al. 2019). Regarding these results, probably other proinflammatory cytokines replace the functions of IFN-γ in pigs' delivery.

In studies conducted in human placenta, it was found that elevated levels of IL-10 are produced in the first and second trimester, but its production decreased at term. In peripheral blood of pregnant women, IL-10 levels were higher in the first trimester, but then gradually decreased to be practically the same as in non-pregnant women at the end of pregnancy (Hanna et al. 2000). Roth et al. (1996), postulate that the production of IL-10 by the placenta serves to protect fetus from the Th1-mediated cellular response. We found that IL-10 levels in maternal and fetal placenta tissues were very low and similar along all the pregnancy. However, we found that the serum concentration of IL-10 was increased at 17, 60 and 114 dg. Studies in Rhesus monkeys (Sadowsky et al. 2003) demonstrated the inhibitory effects of IL-10 on uterine motility in late pregnancy, to prevent premature delivery. Otherwise, IL-10 concentration in cervical tissue of cattle has been determined moments before the birth, during and after the birth (Van Engelen et al. 2009). These authors postulated that its high concentration in these stages indicates that it would be fulfilling an immunoregulatory role during the great inflammation generated by the cervical maturation, thus preventing excessive tissue damage generated by this process. The high IL-10 concentration in serum at the period of the beginning of uterine remodeling (60 dg) and in partum (114 dg), could demonstrate that in pig this cytokine is associated with inhibitory effect to prevent an excessive remodeling process as was postulated in bovine cervix by Van Engelen et al. (2009). The high IL-10 levels found in serum, but not at the placental interface, could indicate its immunoregulatory role at a systemic level during the pig gestation.

IL-10 mRNA expression in pigs was previously found only in term placenta (Zhou et al. 2019) and in 30 dg placenta (Choi et al. 2018). However, in our study not relation between serum and placental levels was observed, and we must postulate an extraplacental origin of this cytokine. The production of IL-10 in pigs was determinate in different organs as tonsils (Müllebner et al. 2018) and lymph nodes (Arenas-Padilla et al. 2018). Specifically, this cytokine is produced by monocytes (Arenas-Padilla et al. 2018, Choi et al. 2018). Makris et al. (2006), demonstrated that there is not relationship between placental levels and serum levels of IL-10 in humans. This seems to show that human placenta would not be the main source of circulating IL-10, coinciding to our finding in pigs. Future experiments must be realized to determine the additional source of serum IL-10 during pig gestation.

In the classic paradigm Th1/Th2, and their actualization Th1/Th2/Th17 Treg paradigm (Murtaugh et al. 2009, Polese et al. 2014, Saito et al. 2010), mainly studied in mice and human, the expression of all the cytokines of the same type (Th1 or Th2) is similar along the gestation. This means that in those species, in early gestation there is a predominance of Th1 cytokines that are involved in the implantation window (Granot et al. 2012, Teles & Zenclussen 2014). Then, through the gestation, the environment is fully of Th2/T17 cytokines, so that allows the gestation success (Saito et al. 2010, Zenclussen 2013). Finally, at delivery, there are a predominance of Th1 cytokines again to promote the expulsion of fetuses and placentas (Bai et al.

2019, Challis et al. 2009). In pigs, are scarce the studies that analyze the cytokines expression during pregnancy. In a previous report (Vélez et al. 2019) we found that two Th1 (IL-1β and IL-2) cytokines and one Th2 (IL-4) cytokine show a similar variation during gestation. These were found elevated in placental remodeling stages (30, 60, 70 gd), different to the cytokines (IFN-γ and IL-10) determined in this work. These results strengthen the hypothesis that Th1 and Th2 cytokines during pig gestation could behave differently from what happens in other species. A better understanding of the mechanisms that mediate the interactions between the conceptus and the uterus of the sow will imply important advances to improve the productivity of this species. This will have a positive impact on the economy of the pig farms, since the embryonic / fetal losses are high.

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