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REVIEW ARTICLE

Immunological uterine response to pig embryos before and during implantation

Inmaculada Parrilla[1,2] | Maria Antonia Gil[1,2] | Cristina Cuello[1,2] | Josep M. Cambra[1,2] | Alejandro Gonzalez-Plaza[1,2] | Xiomara Lucas[1,2] | Jose L. Vazquez[1,2] | Juan M. Vazquez[1,2] | Heriberto Rodriguez-Martinez[3] | Emilio A. Martinez[1,2]

[1]Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain

[2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), El Palmar, Murcia, Spain

[3]Department of Biomedical & Clinical Sciences (BKV/Obst&Gyn), Linköping University, Linköping, Sweden

Correspondence
Inmaculada Parrilla and Emilio A. Martinez, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain.
Emails: parrilla@um.es (I. P.); emilio@um.es (E. A. M.)

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Abstract

The establishment of a successful pregnancy can only occur through a concerted functioning of the entire female reproductive system, allowing for fertilization, subsequent embryo development and implantation of the conceptus. In this context, the uterine immunological responses responsible for rejection or tolerance of the conceptus are of critical importance. The aim of the present review is to summarize our current knowledge about those cellular and molecular immunological events occurring at the uterine level during pre-implantation and implantation stages of pregnancy in the pig. Advancing our understanding of the immune mechanisms involved in the success or failure of pregnancy will provide cues to develop novel strategies augmenting endometrial receptivity, finally increasing the efficiency of assisted reproductive technologies in pigs.

KEYWORDS
allogenic, embryo, hemiallogenic, immune tolerance, pig, pregnancy

1 | INTRODUCTION

From an immune point of view, the female reproductive tract is extremely dynamic. It is able, during the different stages of the estrous cycle, to adapt its immune response to hormonal changes and to the presence of semen and conceptus, preparing the uterine environment for pregnancy (Chase & Kaushik, 2019; Lin et al., 2015). The first preparatory events occur immediately post-mating in response to semen stimuli (seminal plasma and spermatozoa), being early and essential determinants of successful pregnancy progression (Schjenken & Robertson, 2020).

Subsequently, the secretion of estrogen by free-floating conceptuses in the uterine lumen switches on the signal to initiate maternal pregnancy recognition in pigs (around Day 12; Ka et al., 2018). Embryos secrete other molecules in addition to estrogens during the peri-implantation process, such as growth factors, microRNAs and a variety of inflammatory mediators, including prostaglandins, interferons and cytokines (Mclendon et al., 2020), leading to the

establishment of a pro-inflammatory environment during implantation (Waclawik et al., 2017).

Around Days 18–20 of pregnancy, and in response to signaling factors secreted by the conceptus after estrogen production, there is a change in the maternal immune environment, which reaches an equilibrated immune state that allows on one hand the protection of the mother from pathogen infection while, at the same time tolerating an immunologically foreign conceptus (Ka et al., 2018). This immune permissive environment will be maintained until the peripartum stages when uterine inflammatory events are again established in preparation for labor (Hansen et al., 2017).

Days 12–30 of pregnancy is a crucial period in the swine species, during which between 20% and 30% of embryos die, either following natural breeding or artificial insemination (AI; Bidarimath et al., 2021). Embryo death is dramatically increased to approximately 70% when embryo transfer (ET) is performed, (Martinez et al., 2014). Coinciding with this period, important vascular changes and angiogenic events, largely dependent on immunological cellular and molecular mechanisms, take place at the embryo–maternal interface, where the placenta is to be formed, to ensure an appropriate nutrient supply to the growing conceptuses (Linton et al., 2008; Stenhouse et al., 2019). Disturbances in immunologically promoted endometrial angiogenesis are some of the primary causes later leading to fetal loss and litter size reduction in pigs (Bidarimath & Tayade, 2017; Linton et al., 2008; Tayade et al., 2006, 2007).

A better knowledge of the immunological mechanism underlying the establishment and maintenance of pregnancy in pigs is essential for the design of strategies to improve their reproductive efficiency. This challenge is especially relevant in the context of porcine ET; a technology that can notably contribute to improve sanitary, productive and, ultimately, economic aspects of the pig commercial sector (Gonzalez-Ramiro et al., 2021). In addition, other relevant biotechnologies, such as gene editing or cloning, are highly dependent on ET (Martinez, Martinez, et al., 2019).

The application of ET at the commercial or practical level in pigs, however, is still limited compared with other livestock species, such as cattle (Martinez, Cambra, et al., 2019; Martinez, Martinez, et al., 2019). Most likely, among the causes of this situation is reduced reproductive efficiency of ET due to, as mentioned earlier, the high embryo mortality that occurs (Martinez et al., 2014). An impaired maternal immune system due to dysregulation of endometrial cytokine levels is suggested as a potential reason for this high embryo mortality after ET (Cambra et al., 2020; Martinez et al., 2020).

This review provides an overview of the current knowledge about immunological events that take place at the uterine level during early pregnancy in pigs, particularly pre-implantation and during implantation. Particular attention has been given to the information available about differences existing in the immune response of the endometrium under hemiallogenic (AI conceived) or allogenic (embryo transfer pregnancies) conditions since they could be the basis for designing strategies to improve reproductive output after the application of assisted reproductive technologies in pigs.

2 | NATURAL MATING OR ARTIFICIAL INSEMINATION: HEMIALLOGENIC PREGNANCY

Natural conception in polytocous animals, such as the pig, assumes the presence of numerous conceptuses interacting with the female genital tract (Mathew et al., 2016). These embryos and later fetuses express 50% maternal antigens and 50% paternal antigens, the latter being genetically foreign to the mother thus creating, from an immunological point of view, a hemiallogenic situation for the mother, challenging the female immune system (Robertson et al., 2015).

2.1 | Pre-implantation period

Preparation of the uterine environment to tolerate hemiallogenic embryos starts immediately after insemination components (seminal plasma, spermatozoa and/or extender) enter the genital tract and switch on the so-called 'post-mating inflammatory cascade' (Robertson, 2014). This reaction consists of the endometrium increasing its expression of pro-inflammatory mediators (see Figure 1), along with an important increase in uterine leucocyte infiltration, mainly neutrophils, macrophages, dendritic cells and T lymphocytes (Jiwakanon et al., 2011; O'Leary et al., 2004; Taylor et al., 2009). The main functions of this inflammatory response it to eliminate bacteria and excess sperm debris and, more importantly, to prime the maternal immune system to accept the presence of paternal antigens, facilitating embryo attachment and growth (Alvarez-Rodriguez et al., 2019; Bidarimath & Tayade, 2017; Samardzijaa et al., 2020). While the infiltrated neutrophils are short-lived (24–36 hr after insemination), the macrophages and dendritic cells remain in the uterus until Day 6 of gestation, and their effects in promoting immune tolerance status can remain until Day 13 of gestation (Robertson, 2005; Ziecik et al., 2011).

When the embryos enter the uterine lumen (approximately 4–5 days after breeding), the pro-inflammatory reaction has already ended, and a permissive immune status favourable for conceptus development before implantation has been established (Mathew et al., 2016; Ziecik et al., 2011). Different studies have demonstrated that during this pre-implantation stage (Days 5 to 12), there is a reduced expression of pro-inflammatory signaling factors together with an increase in anti-inflammatory cytokine expression, such as IL-10, in pregnant females (Jalali et al., 2014; O'Leary et al., 2004). Along with these changes in cytokine expression, increased numbers of dendritic cells, activated macrophages, and T-helper (Th) and T-regulatory (Treg) cells in the endometrium of gilts were identified by O'Leary et al. (2004) and Jalali et al. (2014) between Days 5 and 9 of pregnancy (see Figure 1). Interestingly, these regulatory immune cells are related to tolerogenic functions and therefore contribute to creating the permissive environment necessary for the free-floating conceptus before its attachment to the endometrial surface (Bromfield, 2016; Robertson et al., 2015; Schjenken & Robertson, 2014).

In pigs, as in other mammalian species, these changes in molecular and cellular immunological factors seem to be closely linked

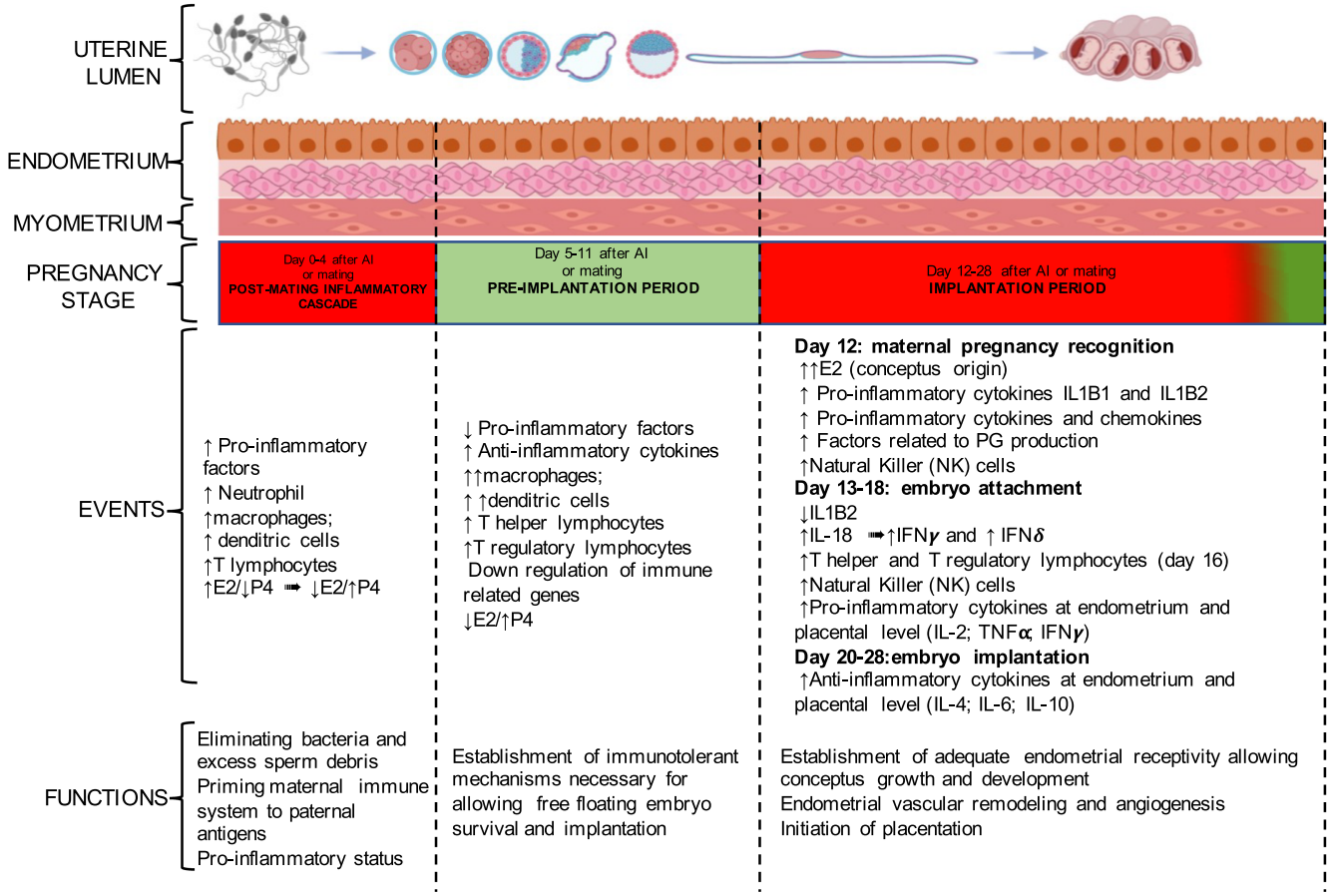

FIGURE 1 Schematic overview of some immunological events taking place at the uterine level to achieve a successful pregnancy in pigs. Post-Mating Inflammatory Cascade: During the first days after mating, increased expression of pro-inflammatory factors, such as cytokines (i.e., GM-CSF) and other factors (i.e., MCP-1 and COX-2) together with an important increase in leukocyte infiltration (neutrophils, macrophages, dendritic cells and T lymphocytes) occurs. A pro-inflammatory environment is predominant in this period with two main functions, namely, the elimination of bacteria and excess sperm debris and, more importantly, priming of the maternal immune system with paternal antigens. Pre-Implantation Period: During this stage, the neutrophils disappear, but macrophages and dendritic cells remain, acting as antigen-presenting cells and they temporarily activate the immune system toward immunotolerance, therefore tolerating free-floating conceptuses before their attachment to the endometrial surface. Implantation Period: During the early implantation period (Day 12), E2 secreted by the elongating conceptuses is the signal for maternal pregnancy recognition in pigs. Conceptus-secreted E2 maintains adequate P4 levels for optimal endometrial receptivity and modulates the expression of endometrial genes related to cell growth, adhesion mechanisms, PG synthesis and immune regulation, all of which are essential for conceptus development. During the mid-implantation stages (Day 18), a pro-inflammatory status at the cellular and molecular levels is established, and vascular changes and angiogenesis allowing embryo implantation are evident. Finally, during the late implantation period, the immune environment shifts again to an anti-inflammatory status that allows for placental development and is maintained until the peripartum stages. COX-2, cyclo-oxygenase-2; E2, estrogens; GM-CSF, granulocyte-macrophage colony stimulation factor; MCP-1, monocyte chemoattractant protein-1; P4, progesterone; PG, prostaglandin

to the delayed immunomodulatory effects of insemination components, mainly seminal plasma (Martinez, Cambra, et al., 2019; Martinez, Martinez, et al., 2019; Schjenken & Robertson, 2020). However, it should not be forgotten that most likely effective fertilization will take place after mating or AI, resulting in the presence of several embryos in the uterus. Therefore, embryos should also be considered a potential factor involved in the modulation of the immune reproductive system at this time point. Similarly, Almiñana et al. (2012) demonstrated that the uterine transcriptomic profile is influenced by embryo presence as early as Days 2–4 after insemination, before their entry to the uterus, and certainly far from the time when the maternal recognition of pregnancy in pigs is established. Moreover, this study demonstrated that the presence of blastocysts (Day 6) downregulate the expression of immune-related genes in the uterus. These results suggest that insemination components have delayed effects that, together with embryo presence, effectively regulate immune responses at the uterine level during the pre-implantation period in pigs (Almiñana et al., 2012; Jalali et al., 2014; O'Leary et al., 2004).

Attempting to increase our understanding of the molecular immune mechanisms taking place during this early pre-implantation period, we mapped the endometrial cytokine profile in blastocyst-bearing (BB; inseminated) and cyclic (uninseminated) sows at Day 6 of the cycle using an intact endometrial explant model in combination

TABLE 1 Differences in anti- and pro-inflammatory cytokine levels in endometrial explants of blastocyst-bearing (BB) and uninseminated cyclic (U-IC) sows

Cytokines	Levels in BB vs. U-IC sows	*p* Value
Anti-inflammatory		
IL-1ra	↑↑↑	$p < .001$
IL-4	↑↑	—*
IL-6	↑↑↑	$p < .001$
IL-10	↑↑↑	$p < .001$
TGFβ1	↑↑↑	$p < .001$
TGFβ2	↑↑↑	$p < .001$
TGFβ3	↑↑↑	—*
Pro-inflammatory		
IL-1α	↑↑↑	$p < .001$
IL-1β	↑	$p < .05$
IL-2	↑↑↑	$p < .001$
IL-8	↑↑	$p < .01$
IL-12	↑↑	—*
IL-18	↑↑	$p < .01$
GM-CSF	n.s.	
INFγ	↑↑↑	$p < .001$
TNFα	↑↑	—*

Note: ↑: increased cytokine levels in BB sows compared to U-IC sows; *Cytokine level below the detection limit in U-IC sows. In this case, the differences were not analyzed; n.s., no significant differences were obtained.

Abbreviations: GM-CSF, granulocyte-macrophage colony stimulation factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor. (Modified from Parrilla et al., 2020.)

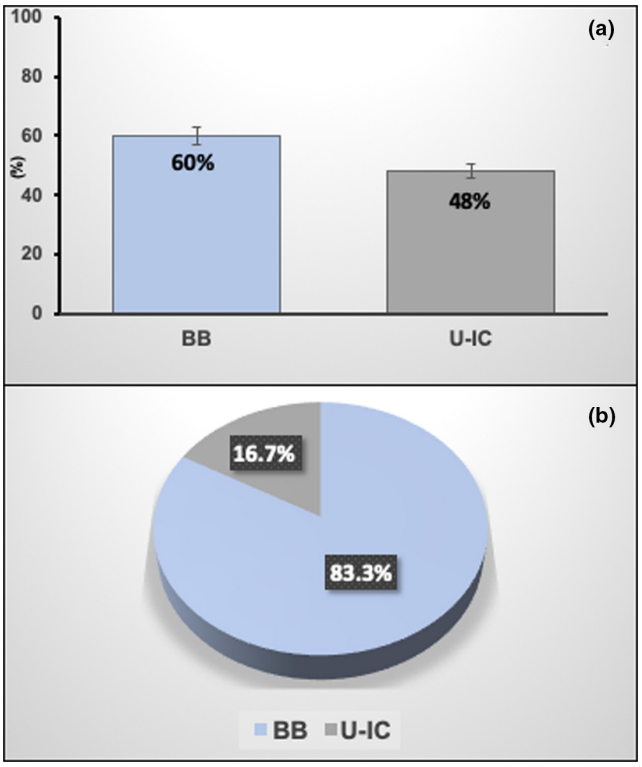

FIGURE 2 Percentage of anti- to pro-inflammatory cytokine ratios shifted to anti-inflammatory status in the endometrium of blastocyst bearing (BB) and uninseminated cyclic (U-IC) sows. (a) Among 25 ratios showing significant differences between BB and U-IC sows, 15 (60%) shifted toward anti-inflammatory status in BB sows and 10 in U-IC sows (48%). (b) Within the 25 ratios showing significant differences between BB and U-IC sows, 12 shifted toward anti-inflammatory status in both groups of sows, and among them, 10 (83.3%) were higher in endometrial samples from BB sows. (Modified from Parrilla et al., 2020)

with cytokine analysis through Luminex® xMAP® technology (Parrilla et al., 2020). A total of 16 cytokines, 7 anti-inflammatory cytokines and 9 pro-inflammatory cytokines, were analyzed (see Table 1), the results showing that whereas the uteri of BB sows produced high concentrations of pro- and anti-inflammatory cytokines, the response in uterine samples from uninseminated sows was much less evident, depicting a lower profile of cytokine production. Most likely, these differences are due to the presence of the different stimuli to which the BB were exposed, including insemination components and an embryo presence. The ratios of anti-/pro-inflammatory cytokines were also analyzed as a potential precise indicator of the optimal conditions for adequate embryo survival and progression. Our findings indicated that at this period, the uterine environment is progressing or has progressed from an initial pro-inflammatory stage towards an anti-inflammatory stage (see Figure 2), which, in accordance with previous studies, appears necessary for the establishment of immunotolerant mechanisms preventing embryo rejection (Almiñana et al., 2012; Jalali et al., 2014; O'Leary et al., 2004; Samardzijaa et al., 2020). Further research is necessary to decipher the mechanisms underlying the modulatory effects of insemination components and the embryo presence on the uterine response of pig females at pre-implantation moments.

2.2 | Implantation period

This period comprises Days 12 to 26–28 of pregnancy (Ka et al., 2018; Lin et al., 2015). Two events determinant to reach a viable pregnancy take successively place during these days (see Figure 1), namely, the maternal recognition of pregnancy (Day 12) and implantation, which includes embryo attachment (Day 18) and full implantation reached around Day 25 (Lin et al., 2015). Both events are highly dependent on a correct cross-talk between the developing conceptuses and the female endometrium (see reviews by Geisert et al., 2017; Samardzijaa et al., 2020; Waclawik et al., 2017).

In species with epitheliochorial placentation, such as pigs, rapid conceptus elongation is an essential mechanism for embryo/maternal communication and pregnancy progression (Geisert et al., 2017). Secretion of estrogen by the elongating conceptuses occupying a

large surface within the uterine lumen is the main signal to initiate maternal pregnancy recognition in pigs (Ka et al., 2018). The estrogens produced by the elongating embryos inactivate the luteolytic production of endometrial $PGF_{2\alpha}$ to ensure adequate levels of the progesterone required for the establishment of endometrial receptivity for embryo implantation (Bazer & Johnson, 2014; Geisert et al., 2014). In addition, conceptus estrogen upregulates the expression of numerous endometrial genes related to among others, mechanisms of cell growth, adhesion, prostaglandin synthesis and immune regulation, all essential for optimal conceptus development (Almeida & Alvarenga Dias, 2022).

Among the immune factors with major roles in pregnancy establishment in pigs are interleukin 1 beta 1 (IL1B) and beta 2 (IL1B2; Geisert et al., 2014; Ka et al., 2018; Mathew et al., 2016; Geisert et al., 2017). While IL1B1 is expressed in macrophages and endometrial tissue, IL1B2 is a novel and specific IL1B isoform whose expression has been described uniquely in the pig conceptus, with maximal expression during the rapid elongation and estrogen synthesis periods (Geisert et al., 2014). These pro-inflammatory cytokines act synergistically to induce and modulate the pro-inflammatory uterine environment necessary for implantation and establishment of pregnancy (Mathew et al., 2016).

Studies using porcine uterine epithelial cells have demonstrated that the IL1B1 signaling pathways are related to cell proliferation and the production of pro-inflammatory factors (ERK1/2 and p38 MAPK, respectively; Mathew et al., 2016). IL1B2 triggers a pro-inflammatory reaction in the porcine endometrium, which elicits increased expression of different pro-inflammatory molecules [cytokines: TNFalpha, IL1, IL2, IL6, IL12, LIF and GMCSF; chemokines: IL8 (CXCL8), RANTES (CXCL5); and factors related to prostaglandin production (PTGS2 or COX-2)] implicated in the immune response as well as in the regulation of other important processes, such as cell survival, proliferation and differentiation (Geisert et al., 2017). Since an excessive inflammatory response could be damaging to the hemiallogenic developing conceptus, the action of IL1B2 is tightly regulated by estrogens and progesterone, creating optimal uterine conditions and highlighting the importance of maintaining a balanced uterine environment for conceptus implantation (Mathew et al., 2016).

The attachment phase occurs around Day 13 and continues until Day 20 of pregnancy. At the end of this phase, IL1B2 levels decrease to almost disappear, with interleukin 18 (IL-18) overtaking its role in modulating immune events (Geisert et al., 2014). In response to IL-18, the trophoblasts of filamentous conceptuses secrete two types of interferons (IFNs), IFN gamma (IFNγ) and IFN delta (IFNδ), which are involved in antiviral activity at the uterine level and in the regulation of the uterine immune response to enhance conceptus development (Bazer & Johnson, 2014). Interestingly, both IFNs can induce different responses in different uterine locations. IFNs secreted by porcine conceptuses induce a defensive immune response only in the uterine glandular epithelium and stromal cells, while at the luminal epithelium level, which is in direct contact with the conceptus trophoblast, this response is silenced, most likely as a protective mechanism against rejection of the conceptuses (Bazer & Johnson, 2014; Geisert et al., 2017; Ka et al., 2018).

During this pregnancy period, changes in the immune cell populations infiltrating the maternal–conceptus interface coincide with the dramatic vascular remodeling of the endometrium, key for implantation and pregnancy progression (Tayade et al., 2007). T lymphocytes, including Th, Treg and cytotoxic T cells (Tc cells) and dendritic cells, macrophages and natural killer (NK) lymphocytes are among the recruited cells that can be found in uterine tissues during this attachment period (Bidarimath et al., 2021; Robertson et al., 2015). Regarding T lymphocytes, Dimova et al. (2006) demonstrated that both the total T-cell population and the different T-cell subsets are more abundant in the pregnant than in the non-pregnant endometrium at Day 15 of pregnancy. Recently, McLendon et al. (2020) demonstrated that on Day 16 of pregnancy, most T cells present within the endometrium are Th cells. These findings confirm previous results and indicate activation of the maternal immune system in response to INFγ secreted by the conceptus at peak concentrations around these days of pregnancy in pigs (Lefevre et al., 1990).

Interestingly, coinciding with the increased presence of Th cells, a considerable number of Treg cells (potent anti-inflammatory and proimmune tolerance agents) were also found in the endometrium by McLendon et al. (2020). The coexistence of these two T-cell subsets most likely determines an equilibrated situation during implantation. Thus, to avoid any potential damaging consequences during the establishment of pregnancy, the Th cells support endometrial inflammation, while the Treg cells limit this reaction (Bidarimath et al., 2021; Robertson et al., 2015).

Within the innate immune system, dendritic cells and macrophages play a pivotal role in regulating the local immune response to the conceptus presence, with both cell types seemly participating in processes such as angiogenesis regulation and tissue remodeling at the maternal–conceptus interface during this gestational period (Bidarimath & Tayade, 2017; Robertson et al., 2015). The NK cells ought to be specially mentioned. The NK cell is a lymphocyte defined as a major effector of the innate immune response in allograft rejection situations and, therefore, with a potentially relevant role in the recognition and allowance of conceptus trophoblasts during the implantation phase (Dimova et al., 2008).

In humans, NK cells found at the uterine level (uNK cells; CD56+CD16-) have a different phenotype than blood NK cells (CD56+CD16+), showing poor cytolytic activity and with their main function related to the production of cytokines, proangiogenic factors, enzymes involved in vascularization changes and conceptus tolerance (Stas et al., 2020). In pigs, endometrial recruitment of lymphocyte NK cells is largely dependent on the presence of the conceptus (Bidarimath et al., 2021). These cells are detectable around gestation Days 12 and 15 and are localized below the luminal epithelium and around the uterine glands and blood vessels (Bidarimath & Tayade, 2017). According to the literature, between Days 12 and 28 of pig pregnancy, an enrichment of NK occurs at conceptus attachment sites (Bidarimath & Tayade, 2017; Dimova et al., 2008), coinciding with the onset of the important angiogenic and vascular

changes that occur in the endometrium architecture, allowing embryo implantation (Tayade et al., 2007).

Currently, a direct association between uterine NK cells and vascular changes in the pig endometrium remains unclear. However, studies performed in pregnant gilts (Dimova et al., 2008; Tayade et al., 2006) reported the presence of lymphocytes with a transcriptional profile (expressing angiogenic factors such as VEGF and transcribing IFNγ) and a phenotype (CD56+CD16−) similar to those described for human uNKs at the attachment conceptus sites. The authors suggest that these cells could be porcine uNK cells and play a determinant role in the vascular events occurring at the maternal–conceptus interface, as it occurs in species with hemochorial placentation (i.e., humans and rodents). Interestingly, with the advancement of implantation, endometrial NK cells are relocated to deeper endometrial layers, avoiding direct contact with trophoblasts, and they are most likely involved in the mechanisms underlying conceptus immune tolerance (Croy et al., 2009; Dimova et al., 2008).

In summary, in pigs, superficial, noninvasive and nondeciduate placentation means that the conceptuses are mainly responsible for the induction and regulation of a proper endometrial reaction that implies a well-coordinated secretion of numerous immune and nonimmune factors, together with the recruitment of immune cells. During a normal pregnancy, all these events give rise to endometrial vascular modifications and collaborate in the establishment of an immune tolerant environment for the hemiallogenic conceptus (Bidarimath et al., 2021; Zang et al., 2021).

Immunologically, an equilibrated state that allows for protection of the mother from infections and, at the same time, tolerates the embryo and later the fetus is set up during this period to achieve successful implantation. Alterations in the bidirectional communication between the conceptus and the endometrium at the molecular or cellular levels can result in defective vascular changes and inadequate cellular recruitment, leading to developmental delay and spontaneous embryo loss (Tayade et al., 2006; Zang et al., 2021). The importance of the immune response for the establishment of a successful pregnancy calls for further exploration of the mechanisms to discover new pathways to reduce embryonic loss in pigs after the use of artificial insemination or any other reproductive biotechnology.

3 | EMBRYO TRANSFER (ET): ALLOGENIC PREGNANCY

The ET is a procedure by which one or more embryos removed from an, usually, AI-bred female (donor) are deposited in the genital tract of one or more females (recipient) that gestate and give birth to these embryos. In this case, the embryo is considered allogenic because neither the paternal nor the maternal alloantigens are coincident with those of the gestation carrier, which suggests a major challenge for the female immune system (Cambra et al., 2020; Martinez et al., 2020).

Currently, in pigs, the application of ET is not as widespread as might be expected despite the numerous advantages that it offers from the productive, health and animal welfare viewpoints (Martinez, Cambra, et al., 2019; Martinez, Martinez, et al., 2019). In recent years, a nonsurgical ET procedure with optimal reproductive parameters of the recipients using fresh and vitrified/warmed embryos at the farm level has been developed [farrowing rates: 80%–90%; litter size: 9.0–9.5 piglets born and farrowing rates: 50%–75%; litter size: 9.0–10 piglets born, respectively] (Martinez et al., 2014, 2015).

Despite these improvements, high rates of embryo mortality (above 70%) during the implantation period (Days 12–30) have been observed during ET as compared with natural breeding or AI (Martinez, Cambra, et al., 2019; Martinez et al., 2014; Martinez, Martinez, et al., 2019). In pigs, embryos on Days 5–6 (morulae or blastocysts) are collected from donor females and transferred to synchronized recipient females (ideally on the same day of the estrous cycle or 24 hr before; Martinez et al., 2014). At this point, the uterine tract of the recipient has not undergone the post-mating inflammatory cascade, and therefore, the subsequent favorable uterine environment created by this reaction is not present or could be considered less efficient (Martinez et al., 2020, 2022). Most likely, this fact along with the presence of allogeneic embryos totally different from the mother from an immunological point of view could result in a lower degree of maternal tolerance towards the embryo, affecting its development (Cambra et al., 2020; Parrilla et al., 2020). An altered immune response at the maternal–conceptus interface has been identified as a major component of spontaneous embryo loss during the early stages of pregnancy in pigs (Zang et al., 2021). In humans, different immunoregulation mechanisms in the response to allogenic conceptus from egg donation pregnancies compared to those occurring in natural conception pregnancies (hemiallogenic conceptus) have been reported (Martinez-Varea et al., 2014). This altered immune response in allogenic pregnancies could be most likely related to a higher incidence of pregnancy pathologies (Martinez-Varea et al., 2014).

Unlike humans and rodents, information on the immune aspects of allogenic pregnancies is currently scarce in pigs and to a lesser extent even in cattle. Being aware of the potential of ET technology for the improvement of not only reproductive parameters derived from ARTs but also the outputs of novel biotechnologies, such as cloning, gene editing and blastocyst complementation interspecies (Martinez, Cambra, et al., 2019; Martinez, Martinez, et al., 2019), different experiments have been recently performed by our group and others to enhance the knowledge of the immune response in an ET context.

As previously mentioned, studies from our group demonstrated that, at Day 6 after estrus, endometrial cytokine profile production differs among blastocyst-bearing sows (inseminated) and uninseminated sows (Parrilla et al., 2020). Within an ET program, the latter cyclic sows at Day 6 of the estrous cycle would be considered recipients, and it could be that the uterine immune conditions were not adequate to properly host the transferred embryos. Thus, creating a uterine environment in recipients similar to that in naturally pregnant sows could be a useful strategy to improve ET outcomes.

With this idea in mind, we performed a study to analyze the expression of leukemia inhibitory factor (LIF) cytokine in endometrial samples from ET (allogenic) and AI (hemiallogenic) pregnant sows during the implantation period (Days 18 and 24; Cambra et al., 2020). Among the cytokines involved in the pregnancy immune networks, we chose LIF because it is implicated in numerous relevant events and has an immunotolerant role during pregnancy by inducing an anti-inflammatory response and immunotolerance toward the embryo, which leads to successful embryo implantation (Blitek et al., 2012; Stewart et al., 1992).

Hence, quantification was performed at the gene (mRNA-RT–qPCR) and protein (WB) levels, and the results showed reduced expression of LIF in ET pregnancies at Day 24 as compared with AI pregnancies on the same day. Previous studies in mice demonstrated that animals lacking LIF were infertile due to embryo attachment failure (Fukui et al., 2021; Stewart et al., 1992), suggesting that reduced levels of LIF at implantation sites in allogenic pregnancies could induce an immunoreactive state leading to embryo rejection and contribute to the high embryo mortality rates associated with ET in pigs. These findings seem to point out the relevance of LIF as a modulating factor of embryo implantation in pigs.

Recently, Martinez et al. (2020) analyzed the endometrial and placental cytokine production profile in response to the presence of semi or allogenic embryos (AI and ET sows, respectively) during the implantation period (Days 18 and 24). Levels of 16 pro- and anti-inflammatory cytokines were determined by using multiplex assay technology. Additionally, data about embryo recovery at Day 24 were also recorded for AI and ET sows. The results of cytokine analysis showed that at Day 18, a pro-inflammatory environment was present in the endometrium and placenta (increased levels of IL2, IFNG and TNF_{α}) of AI sows but not in the ET group. At Day 24, cytokine expression in the AI endometrial samples changed, displaying a physiological anti-inflammatory profile by increasing IL-4, IL-6 and IL-10 anti-inflammatory cytokines, while only IL-6 was increased in ET samples. Moreover, in ET samples, the levels of IL-10, a cytokine with important anti-inflammatory functions in pregnancy (Mobini et al., 2016), were under-expressed at the endometrial and placental levels. These results indicated a potential failure of transferred embryos to generate a physiological-like immune response in the uterus of recipient females (a pro-inflammatory environment during initial implantation and immunotolerant status around Day 24), which most likely causes a hostile environment for the embryo and finally its rejection. These findings perfectly match the results obtained regarding embryo recovery and embryo development in the same study (almost 40% less recovered embryos in ET compared with AI sows at Day 24 of pregnancy and percentages of developmentally delayed conceptuses higher than 20% in the ET group; see Table 2).

Similarly, a very recent study (Martinez et al., 2022) reported an upregulation of several important genes involved in the pro-inflammatory response in endometrial samples from ET sows compared with AI sows (Day 24), with the results being more evident in ET sows with a reduced number of implanted embryos (less than 12). These interesting and novel results confirm the relevant role of the immune mechanism for pregnancy establishment in an ET context and could be the basis for future research that supports the design of effective protocols and strategies to improve endometrial receptivity and pregnancy rates in swine as well as in other species.

TABLE 2 Events occurring differently between hemiallogenic (AI sows) and allogenic (ET sows) pregnancies in pigs

Event	AI sows	ET sows
Increase in pro-inflammatory cytokines at endometrial level Day 18	+	−
Increase of pro-inflammatory cytokines at placental level Day 18	+	−
Increase of anti-inflammatory cytokines at endometrial level Day 24	+	−
Decrease of anti-inflammatory cytokines at endometrial and placental level Day 24	−	+
Decreased levels of endometrial LIF at Day 24	−	+
Upregulation of genes involved in the pro-inflammatory response	−	+
Normal recovered embryos at Day 24 (%)[a]	77	46
Delayed recovered embryos at Day 24 (%)[b]	5	22

Note: +/− occurring or not occurring events, respectively (modified from Martinez et al., 2020 and Cambra et al., 2020).

[a]Percentage of recovered embryos in AI and ET sows was obtained in relation to the number of corpora lutea counted on each ovary (AI sows) or in relation to the total number of transferred embryos (ET sows).

[b]Percentage of delayed embryos was calculated in basis to the number of total embryos recovered (Martinez et al., 2020).

4 | FINAL COMMENTS

It is obvious from the above that the establishment of pregnancy in pigs requires, among other mechanisms, the establishment and proper functioning of complex and tightly regulated interactions between cellular and molecular immunological factors. These interactions will be activated immediately after natural mating or AI, and their effects will condition the uterine environment to allow pregnancy success. Under an ET context, these initial events do not take place in the uterus of recipient females compromising the embryo development. Therefore, creating an adequate uterine environment in recipient females to house the embryos could be a promising strategy for the improvement of ET outputs.

At more advanced pregnancy stages, the presence of the embryo acquires a fundamental role in the regulation and adequate functioning of maternal–embryonic communication that is maintained until the end of placentation. As a result of these well-orchestrated mechanisms, a balanced immune status tolerating the developing conceptus, while remaining competent to respond against pathogens, is established in the uterus of pregnant females during early pregnancy.

A better understanding of the signaling factors modulating the immune equilibrium during these early pregnancy stages in pigs, from pre-implantation to implantation, and how they are regulated in

healthy and failed gestations will provide the knowledge necessary to develop useful strategies for improving uterine receptivity and pregnancy rates. Taking into account the complexity and the multifactorial nature of immune response networks taking place during these pregnancy stages, further research, integrating in vivo and in vitro studies from cells, tissues and embryos/conceptuses with the use of powerful 'omics' technologies (i.e., genomics, transcriptomics, proteomics and metabolomics) is necessary. This is of particular importance in the context of assisted reproductive technologies, such as ET, where reduced reproductive success is of major concern.

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CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the paper and approved the final version of the manuscript.

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Inmaculada Parrilla https://orcid.org/0000-0002-5121-758X
Maria Antonia Gil https://orcid.org/0000-0002-6955-7750
Cristina Cuello https://orcid.org/0000-0002-6202-5946
Emilio A. Martinez https://orcid.org/0000-0003-1260-9721

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