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Review Article

Extracellular vesicles in host-pathogen interactions and immune regulation — exosomes as emerging actors in the immunological theater of pregnancy

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ABSTRACT

This review correlates and summarizes the role of the maternal-fetal interface in the immune tolerance of the fetus and the processes that lead to infection avoidance, emphasizing the participation of exosomes and other extracellular vesicles in both situations. Exosomes are released into the extracellular medium by several cell types and are excellent carriers of biomolecules. Host-derived exosomes and the transport of pathogen-derived molecules by exosomes impact infections in different ways. The interactions of exosomes with the maternal immune system are pivotal to a favorable gestational outcome. In this review, we highlight the potential role of exosomes in the establishment of an adequate milieu that enables embryo implantation and discuss the participation of exosomes released at the maternal-fetal interface during the establishment of an immune-privileged compartment for fetal development. The placenta is a component where important strategies are used to minimize the risk of infection. To present a contrast, we also discuss possible mechanisms used by pathogens to cross the maternal-fetal interface. We review the processes, mechanisms, and potential consequences of dysregulation in all of the above-mentioned phenomena. Basic information about exosomes and their roles in viral immune evasion is also presented. The interactions between extracellular vesicles and bacteria, fungi, parasites and proteinaceous infectious agents are addressed. The discovery of the placental microbiota and the implications of this new microbiota are also discussed, and current proposals that explain fetal/placental colonization by both pathogenic and commensal microbes are addressed. The comprehension of such interactions will help us to understand the immune dynamics of human pregnancy and the mechanisms of immune evasion used by different pathogens.

1. Introduction

In humans, recognition of self and nonself antigens, tissues or even whole organisms encompasses both local and systemic immune reactions. In the context of pregnancy, the intimate contact of fetal cells and maternal immune cells and tissues represents a substantial immune challenge. The maternal immune system must be shaped to tolerate the developing fetus, which can be compared to a semiallogeneic graft (Trowsdale and Betz, 2006; Vianna et al., 2011; Svensson-Arvelund et al., 2015). The search for factors involved in such immune adaptation has led many researchers in the field of reproductive immunology to examine the new universe of extracellular vesicles.

Extracellular vesicles (EVs) are secreted by cells from all eukaryotes and by prokaryotic organisms through shedding mechanisms (Colombo et al., 2014). Various biological fluids contain EVs, which can cross physical and physiological barriers and perform essential roles in cell-to-cell communication. Thus, EVs are critical modulators of the immune response under normal and pathological conditions (Nair and Salomon, 2018). EVs are usually classified according to their size and tissue or cell of origin (Colombo et al., 2014). However, it is difficult to assume the origin of a specific EV unless it is captured at the time of shedding by adequate imaging techniques. Therefore, it is now strongly recommended that operational terms encompassing size, shape, and biochemical composition be used for identifying EV subtypes (reviewed in Théry et al., 2018).

The term "EVs" encompasses microparticles, microvesicles (MVs), nanovesicles, nanoparticles, ectosomes, exosomes, exovesicles, and exosome-like vesicles (Colombo et al., 2014). The diversity of EVs, in terms of origin and function, makes an individual classification difficult for each type, and EVs have usually been differentiated based on their size, cargo, and origin (Nair and Salomon, 2018). Although we are in agreement with the recommendation of MISEV2018 (Minimal

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information for studies of extracellular vesicles 2018) that "terms such as exosome and microvesicle that are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis" (Théry et al., 2018), the purpose of this review is to present an overview of the role of exosomes in host-pathogen interactions and immune regulation in human pregnancy; therefore, the nomenclature for the EVs will be identical to that used by the authors of the original articles.

One specific group of EVs, exosomes, has received special attention, mainly due to its reported roles in both normal pregnancies as well as in pregnancy-related disorders (Mitchell et al., 2015). Exosomes are released into the extracellular space by virtually all types of viable cells and have a prominent function in intercellular and intracellular communication and as biomolecule carriers (Théry et al., 2002a). Different cell types release distinct types of exosomes under healthy and pathological conditions (Corrado et al., 2013). Exosomes in healthy pregnancy are known for their ability to induce and maintain, at least partially, a local immunosuppressive environment at the maternal-fetal interface (Hedlund et al., 2009). This ability is fundamental to the control of the maternal immune responses that would otherwise be harmful to the "semiallogeneic" fetus (Mincheva-Nilsson and Baranov, 2010; Mitchell et al., 2015).

It seems plausible that such intimate contact between mother and fetus, allied to local immune modulation, creates a scenario that favors infection of both individuals; however, with some exceptions, viral infections during pregnancy have been considered low-risk conditions until recently (Silasi et al., 2015). Even with placental control of both the mother's immune reactions and pathogen infections, microbes have found ways to bypass the placenta (Silasi et al., 2015; Coyne and Lazear, 2016). In addition, the findings revealing a healthy, normal microbiota in the placenta (Aagaard et al., 2014; Parnell et al., 2017; Seferovic et al., 2019) led to the following question: how do these organisms or their genetic material get in contact with the fetus before birth? The mechanisms that modulate the immune system and the universe of extracellular vesicles will probably offer some hints to the answer.

It is no wonder that an intensely immunomodulated environment at the maternal-fetal interface can open a window to relatively easy development of viral or bacterial infections. Nevertheless, placental tissues have physiological characteristics that hinder the entry of pathogens (Arora et al., 2017). Such strategies, whether physical or molecular in nature, make the placenta a complex organ where nutrient and gas exchanges occur while immune responses must be carefully regulated. In this review, we address important aspects of the local immune adjustments that enable embryo implantation and acceptance, minimizing the risk of abortion/rejection, and call attention to the placental microbiota and its implications during pregnancy. In addition, some aspects of exosomes produced by cells from the male genital tract and specific facts about sexually transmitted infections during pregnancy will be discussed.

2. Main text

Immunomodulation – is there relevant systemic immune suppression in pregnancy?

That the maternal immune system is largely suppressed in pregnancy is an oversimplified concept. Several studies indicate that immune system activation is a crucial step for healthy pregnancy development. Thus, it is inappropriate to think that human pregnancy could develop better without immune responses even in the absence of pathogens (Mor et al., 2011). The first studies on the immunology of pregnancy were conducted in the 1950s when Sir Peter Medawar first questioned that the fetus was accepted by the maternal immune system. His group postulated that fetal acceptance by the maternal immune system was due to an anatomical separation of the fetus from the mother, antigenic immaturity of the fetus, and immunological indolence/inertness of the mother toward the fetus (Billingham et al., 1953). Since then, various publications have addressed immune functions and dynamics during the gestational period. Nevertheless, in the 1990s, human pregnancy was still viewed as a period of immune inertness. Since T helper type 2 (Th2) cells are classically considered to be involved in anti-inflammatory responses, it seemed plausible to classify, at that time, human pregnancy as a "Th2 phenomenon" (Wegmann et al., 1993). In this line, as T helper type 1 (Th1) cells and cytokines were predominantly linked to inflammatory situations, Th1 cell responses were classically related to pregnancy complications (Raghupathy et al., 2000; Piccinni, 2002), and inadequate physiological outcomes of pregnancy or unsuccessful pregnancies were considered the result of a "Th1 response."

A subtle inflammatory process that involves the presence of numerous immune cells is essential for successful implantation. However, inflammation at the implantation site, more than a response against the fetus, promotes tissue remodeling and enables embryo implantation (Mor et al., 2011). Additionally, it is important to emphasize that the blastocyst is highly adhesive and travels throughout the fallopian tube to the implantation site. The endometrium is extensively covered by molecules that avoid blastocyst implantation, and it has been hypothesized that cytokines and chemokines produced by macrophages and dendritic cells (DCs) promote the degradation of these molecules covering the implantation site (Mor et al., 2011).

Considering that inflammation is a process characterized by the presence of a large number of Th1 cells and molecules derived from these cells, parturition was considered to be a pro-inflammatory phenomenon (Romero et al., 2006). Supporting this idea, inflammation has been detected in the cervix, myometrium, chorioamniotic membranes, and amniotic cavity of women in labor. Prior to parturition, there is a large influx of immune cells to the myometrium, creating an inflammatory profile and culminating in uterine contractions and delivery (Romero et al., 2006). Initially, it was believed that if such an inflammatory profile happened early in the gestational period, preterm delivery, miscarriage or other pregnancy complications would follow as a logical consequence (Wegmann et al., 1993; Raghupathy et al., 2000; Piccinni, 2002), but later, the need for a more complex Th1/Th2 balance was observed (Chaouat et al., 2002). These observations led to a new paradigm suggesting that slight inflammation at the beginning of pregnancy is followed by a longer period featured mainly by anti-inflammatory characteristics. Finally, signaling created by high levels of inflammation was expected near and at delivery. Since the description of this theory, several studies have supported it, but others have also contradicted the described dichotomy (Wegmann et al., 1993; Hill et al., 1995; Piccinni, 2002; Raghupathy et al., 2000; Chaouat et al., 2002; Mor et al., 2011).

Given such a dynamic balance at all phases of pregnancy, systemic immune suppression in pregnancy was doubted. How could an immune-suppressed organism control the dynamic fluctuations of cytokines and the migration, differentiation, and proliferation of so many immune cell types? The answer to this question is based on the absence of strong systemic immune suppression in pregnancy. Instead, tight immune regulation involves numerous cell types and molecules. Currently, human pregnancy is considered a very complex and meticulously regulated immune process. The description of a new set of cytokine-producing cells that did not fit the Th1/Th2 profile was essential to changing this pre-established paradigm (Chaouat et al., 2002; Zenclussen et al., 2002). Studies revealing the role of T regulatory (Treg) and Th17 cells and the molecules they produce during the gestational period are examples of recent discoveries that challenged the dichotomous Th1/Th2 view of human gestation (Saito et al., 2010). In this context, we highlight Interleukin-17 (IL-17), a pro-inflammatory cytokine that induces the expression of several inflammatory mediators (Witowski et al., 2004) and is produced mostly by T cells (Th17 cells) (Fu et al., 2014). Importantly, IL-17 has been shown to induce the production of proangiogenic molecules and to favor neovascularization (Numasaki et al., 2003). Concerning the human maternal-fetal interface, it was already been demonstrated that decidual cells recruit peripheral Th17 cells into the decidua by secreting CCL2 (Wu et al., 2014). At this location, Th17 cells

promote the proliferation and invasion of human trophoblast cells through the secretion of IL-17, which also inhibits apoptosis during the first trimester of pregnancy (Wu et al., 2014). Moreover, it had already been observed that IL-17 levels continuously increase throughout pregnancy (Martínez-García et al., 2011; Kaminski et al., 2018). In the context of mammalian pregnancy evolution, IL-17 can be assumed to be one of the molecules responsible for the maintenance of prolonged periods of gestation (Fu et al., 2014). The absence of IL-17A in marsupials suggests that it is an essential signaling molecule for the maintenance of the prolonged pregnancy observed in eutherian mammals, which differs from that of marsupials (Chavan et al., 2017). Of note, a new "Th1/Th2/Th17 and Treg" cell paradigm of pregnancy has been suggested and is the current trend. In this context, Th17 cells favor implantation and induce a protective immune response against microbes by the induction of inflammation, and Tregs, in contrast, are important for the immunoregulation and induction of tolerance (Saito et al., 2010).

Exosomes

As previously described, the term EV refers to exosomes (30–150 nm in diameter), microvesicles (0.1–1 μm) and apoptotic bodies (0.5–5 μm) released during both pathologic and healthy physiological situations (De Toro et al., 2015; Lo Cicero et al., 2015; Yáñez-Mó et al., 2015). Exosomes originate from multivesicular bodies (MVBs) and are formed by a lipid bilayer derived from their cells of origin. Various biomolecules, such as proteins and nucleic acids, are found attached to the lipid bilayer and/or inside the exosomes (Théry et al., 2002a). The secretion of proteins and nucleic acids through exosomes confers interesting features and advantages to this process: (I) the three-dimensional structure and biological role of the cargo molecules are preserved; (II) the delivery of molecular signals can occur independently of direct cell-cell contact; (III) the concentration of specific proteins inside exosomes can be maintained at high levels; (IV) the accurate delivery of biomolecules to the target (due to specific surface markers) is assured and can be achieved with long distances between the cells; and (V) *de novo* secretion in the target cell is not necessary (Mincheva-Nilsson and Baranov, 2010).

Due to the diversity of the cargos and target cells, exosomes can interfere with distinct pathways and affect different body systems. Taking into account the interests specific to the present review, it is important to emphasize that exosomes can act as modulators of immune responses. In this sense, exosomes derived from antigen-presenting cells have immune-activating properties (Théry et al., 2002b; Hwang et al., 2003). Additionally, syncytiotrophoblast-derived exosomes from non-pathological human placenta seem to participate in pathogen infection resistance pathways, although they can be immune suppressive or tolerogenic, such as exosomes from the majority of tumors and epithelial cells (Karlsson et al., 2001; Andreola et al., 2002; Mincheva-Nilsson and Baranov, 2010).

There is great debate over the most appropriate methods for isolating and characterizing exosomes. Diverse EV isolation techniques can be found in the original articles cited throughout this review. These methods are mainly based on differential and/or density gradient ultracentrifugation, size-based isolation techniques, coprecipitation, and immunoaffinity enrichment.

The most widely used exosome isolation technique is ultracentrifugation, which is considered the gold standard method. Ultracentrifugation isolation is based on the weight and size of the exosomes, and its low cost presents a major advantage over the other available methods; however, the exosome recovery is low. Size-based methodologies (which also consider molecular weight) produce a high yield through rapid processing; however, they lack specificity and require specific equipment, which are disadvantages. Based on the surface proteins present in the exosomes, the fastest and easiest method to isolate them is coprecipitation, which is characterized by high cost, low recovery, and a relative lack of specificity. At high cost and with low recovery capacity, the method of immunoaffinity enrichment recovers many exosomes of high purity (Bu et al., 2019).

Such techniques vary in adequacy depending on the sample of interest and are in continuous need of improvement (Bu et al., 2019). Considering these variables, we highlight the importance of following the latest proposals from the International Society for Extracellular Vesicles that are featured in MISEV2018 (Théry et al., 2018), the gold standard reference that presents the latest scientific advances for better handling of samples, from collection to storage, and are quite suitable for use with cell culture, biological fluids, or tissues.

An overview of exosome isolation methods is shown in the studies addressing placental exosomes from maternal circulation. For example, enriched fractions of these specific nanovesicles with minimal "contamination" from other EVs can be obtained through methods based on the proposed use of buoyant density centrifugation (Salomon et al., 2014; Sarker et al., 2014) and immunoaffinity capture using antibody-conjugated agarose beads (Lai et al., 2018). Alternatively, some studies have obtained exosomes from the supernatant of placental explant cultures using sequential centrifugation and ultracentrifugation, followed by identification and characterization by Western blotting, immune electron microscopy, and immuno-flow cytometry based on the proteins expressed on the surface of the isolated placental exosomes (Hedlund et al., 2009; Stenqvist et al., 2013). It is also important to consider the following limitation: most isolation methods cannot ensure the complete purity of the obtained vesicles, and it is possible to coisolate other nontargeted EVs and viral particles with the desired exosomes (Ellwanger et al., 2017).

To date, the most studied exosome markers are ALIX, TSG101, CD9, CD63, and CD81 (Ellwanger et al., 2017). However, the list of exosome markers is continuously revised, with new markers being incorporated at the same time that previously established markers are considered not sufficiently specific for exosomes. The direct consequence of such a dynamic research field is that different studies use different markers to identify exosomes. Thus, it is always a challenge to know when the authors actually worked with exosomes or with another type of extracellular vesicle (Ellwanger et al., 2017). Taking this into consideration, although we use the term "exosomes" throughout this review, it is important to emphasize that the data discussed here can possibly extend to the other types of EVs described in the literature collectively as "exosomes." In an attempt to clarify this situation, web portals have been organized through which researchers are working together to better classify the different subsets of extracellular vesicles, including exosomes (Kim et al., 2015).

The maternal-fetal interface: a site of intense immune regulation accounting for exosomes and other EVs

Just after fertilization of the oocyte by the spermatozoon, the binding and fusion of the sperm cell and oocyte membranes promote oocyte changes that block polyspermic fertilization and drive the resumption of oocyte meiosis (Capmany et al., 1996). At 24h post fertilization, parental chromosomes have intermixed, and the first cellular division occurs. Messenger RNA (mRNA) synthesis is absent as the initial cells divide, apparently driven exclusively by the maternal cytoplasmic signals, an event designated as the 'maternal legacy' (Braude et al., 1988). Such maternal signals could originate from maternal mitochondrial DNA, which replicates during early embryonic cell division. The point at which the paternal genome is activated and undergoes transcription is called zygotic gene activation and is first detected in embryos 2–3 days after fertilization (Braude et al., 1988). From this moment, the mother's immune system addresses the emergence of nonself antigens (those of paternal origin) to enable adequate fetal development. During pregnancy, the uterine environment promotes tolerance in relation to the developing fetus, avoiding maternal rejection of the fetal allograft, and it has been suggested that exosomes may be pivotal in the establishment of such an immune-privileged environment (Hedlund et al., 2009; Stenqvist et al., 2013).

The uterine receptiveness to blastocyst implantation is modulated by cyclic secretion of estradiol, progesterone, and human chorionic gonadotropin — the first known hormonal signals of the conceptus. These hormones regulate growth factors, cytokine production and the expression of the adhesion molecules that alter the endometrial surface, openning an implantation window (Gude et al., 2004; Makrigiannakis et al., 2017). The blastocyst is composed of two cell types with an inner and an outer cell mass. The inner cell mass develops into the fetus. The outer cell mass consists of undifferentiated trophoblast stem cells that form the cytotrophoblast (CTB) and the syncytiotrophoblast (STB). Before attachment, the zona pellucida is lost, and the trophoblast cell layer rapidly proliferates and differentiates into an inner layer, the CTB, and in an outer multinucleated mass, the STB (Gude et al., 2004). Subsequently, the STB extends into the endometrial epithelium and invades the connective tissue, breaking through the endometrial surface, provoking the natural tissue damage that ultimately enables implantation. Thus, the uterine endothelium and vascular smooth muscles of the mother's blood vessels are gradually replaced by trophoblast cells, creating the optimal conditions for initiating and developing the placental-fetal blood supply (Gude et al., 2004; Mor et al., 2011).

After the development of spiral arteries due to remodeling of the mother's blood vessels, the human placenta becomes a hemochorial villous organ. Such proximity enables the maternal blood to come into direct contact with the placental trophoblast cells (Gude et al., 2004). The functions of the placenta range from the exchange of nutrients, gases and metabolic residues to the production of regulatory molecules, which reveals its role as an immunomodulatory organ. The formation of the placenta starts at the implantation of the blastocyst into the uterine mucosa within 5–6 days post fertilization (Cross et al., 1994). The placenta is composed of two types of villi, the floating villi, which comprise an inner layer of CTB covered by the STB, which is bathed in maternal blood at the intervillous space, and the anchoring villi, which attach to the decidual tissue by highly invasive CTB cells referred to as extravillous trophoblasts (EVTs) (Hamilton and Boyd, 1960). The placental villous surface is in direct contact with maternal blood through the STB in a compartment that enables nutrient and oxygen supplementation and metabolic residue and cell debris removal and that, at the same time, hinders the passage of potential pathogens from the maternal circulation to the fetus (Gude et al., 2004; Delorme-Axford et al., 2013; Arora et al., 2017). Of note, in the STB, it is possible to find physical and molecular functions that ultimately block immune activation at the maternal-fetal interface (Robbins and Bakardjiev, 2012).

In this environment of intimate contact between the uterine region and the placenta, the exosomes secreted in the maternal blood are possibly the most abundant in the intervillous space of the chorionic villi. Moreover, the continuous release of exosomes by the STB creates an exosomal concentration gradient, accounting for stronger protection against an exacerbated maternal immune response at the maternal-fetal interface. It is said that the fetus, together with the placenta, is surrounded by a “cloud of exosomes” (Mincheva-Nilsson, 2010). Importantly, the concentration of the placenta-derived exosomes in the maternal blood increases during a healthy pregnancy (Salomon et al., 2014). In this context, it has been suggested that the concentration of placenta-derived exosomes in maternal blood could also be a potential marker of abnormal placentation (Kshirsagar et al., 2012). Trophoblast-derived exosomes could regulate the recruitment and differentiation of monocytes into tissue macrophages by inducing them to secrete the cytokines and chemokines required for trophoblast growth and survival (Atay et al., 2011). Placenta-derived exosomes lack the classical major histocompatibility complex (MHC) expression, presenting with the same characteristics as their tissue of origin. Instead, they express the nonclassical molecules MICA/B and RAE-T1/ULBP1–5, ligands of the activated Natural Killer (NK) cell receptor NKG2D (Mincheva--Nilsson et al., 2006; Hedlund et al., 2009).

The decidua is the mucosal layer of the resulting pregnant uterus. It comprises the endometrial/decidual glands, blood vessels, and the decidual stroma. Furthermore, leukocytes represent approximately 15–30% of all the cells in the early pregnant decidua of humans (Mincheva-Nilsson et al., 1994). The organization of decidual lymphoid tissue is unique and includes lymphoid cell clusters, subepithelial lymphoid cells, and individual immune cells that are randomly distributed. B cells are absent or rare; instead, there are abundant uterine NK (uNK) cells, αβ T and γδ T cells, DCs, and macrophages. Interestingly, uNK cells represent up to 70% of decidual leukocytes in the first trimester (Moffett-King, 2002), while there is no consensus regarding the distribution and number of uNK cells in later stages of gestation (Lash et al., 2010).

Immunomodulation in human pregnancy: tolerance is enhanced by exosomes and other EVs

Early studies on human preimplantation embryos reported the absence of expression of MHC class I or II genes (Roberts et al., 1992). The villous trophoblast, exposed to maternal blood, seems to lack expression of both MHC class I and class II proteins. However, the EVTs, which invade the uterus, express a particular combination of four MHC class I molecules: the classical HLA-C and the nonclassical class I molecules, HLA-E, HLA-F, and HLA-G (Moffett-King, 2002; Hackmon et al., 2017). The expression of HLA-E in EVTs enables them to evade NK cell-mediated cytotoxicity (King et al., 2000). Regarding HLA-G, it was first proposed that its expression in trophoblast cells also protected the fetus from maternal NK cell cytotoxicity, but this proposal is being debated, as HLA-G can induce secretion of cytokines and proangiogenic factors from decidual NK cells and human decidual antigen-presenting cells (APCs) *in vitro* (van der Meer et al., 2004; Li et al., 2009). Thus, it seems that HLA-G contributes to fetal tolerance by modulating decidual NK cell and APC responses (LeMaoult et al., 2004). Such nonclassical MHC molecules are of key importance not only in the establishment of the pregnancy but also in its maintenance, as revealed by studies in both healthy and pathological pregnancies (Tripathi et al., 2006; Michita et al., 2016; Persson et al., 2017; Meuleman et al., 2018). Notably, it has been shown that immunomodulatory molecules from the B7 family and the soluble HLA-G isoform HLA-G5 are secreted from the placenta during the first trimester and at term via exosomes (Kshirsagar et al., 2012).

As previously described, blastocyst implantation requires an inflammatory environment (Fest et al., 2007). The implantation process is achieved via the proper interaction of the innate uterine immune cells with the invading trophoblast (Huppertz et al., 1998; von Rango et al., 2003; Shih et al., 2006). During implantation, the uNK cells are pivotal for trophoblast invasion, and their absence is a predictor of poor vascularization of the placenta and pregnancy interruption (Hanna et al., 2006). Additionally, depletion of DCs leads to blastocyst implantation failure and prevents decidual development, likely due to failure in uterine receptivity; in healthy pregnancy, DCs orchestrate uterine receptivity through the regulation of tissue remodeling and angiogenesis (Placks et al., 2008).

In addition, equal to the importance of the role played by decidual leukocytes, cellular receptors and other molecules are important for fetal tolerance. For example, indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the degradation of tryptophan, leading to cell starvation and inhibition of T cell proliferation. Interestingly, in the human blastocyst, IDO is detected from day 6 (Kudo et al., 2004). Furthermore, immunohistochemical analysis of mice revealed the presence of IDO throughout pregnancy (Shayda et al., 2009).

Genetic variants have also been reported as important in the likelihood of different pregnancy outcomes. For example, different gene polymorphisms can impact human gestation in such ways that can enhance or attenuate inflammation-related pregnancy disorders (Michita et al., 2016, 2018; Kaminski et al., 2019).

Membrane-associated and secreted immune regulatory factors are widely produced and secreted by placental tissues and are also detected in the maternal serum during the gestation period. For example, expression of Fas and FasL can be detected in the villous CTB and in the

STB (Pongcharoen et al., 2004; Frängsmyr et al., 2005); early pregnancy factor (EPF) activity was detected in sera from pregnant women (Fan and Zheng, 1997); progesterone-induced blocking factor (PIBF), which blocks lytic NK cell activity, was observed in term placentas (Anderle et al., 2008); and a variety of cytokines are expressed and tightly regulated both locally and systemically throughout pregnancy (von Rango et al., 2003). An important role for the Leukemia Inducing Factor (LIF) in implantation was shown in *LIF*-knockout mice, in which embryos failed to implant (Stewart et al., 1992). Notably, LIF is a secreted glycoprotein belonging to the interleukin 6 (IL-6) family, with pleiotropic effects that include the induction of the proliferation, differentiation, and survival of trophoblast cells (Aghajanova, 2004).

Complement activation at the maternal-fetal interface is avoided, or at least minimized, by the expression of the complement regulatory proteins CD59, MCP, and DAF in the placenta. These proteins are expressed on the trophoblast from at least 6 weeks of gestation, and their presence at the maternal-fetal interface (Holmes et al., 1992) implies a pivotal role in the maintenance of pregnancy, probably by protecting the developing fetus against maternal complement-mediated immune responses.

As previously stated, the human placenta constitutively expresses Fas and Fas-ligand (FasL) (Abrahams et al., 2004; Stenqvist et al., 2013). These molecules participate with other factors that regulate the maternal immune system. Fas is a type I membrane protein that is a member of the tumor necrosis factor (TNF) receptor family and is expressed by a wide variety of cells. FasL is a type II transmembrane protein expressed by activated T cells, some tumor cells and epithelial and other cells at immune-privileged sites (Ferguson and Griffith, 2006). Cross-linking of Fas expressed on the cell surface with its natural ligand FasL (also called CD95L) induces apoptosis (Nagata and Golstein, 1995). The FasL protein and mRNA transcripts were detected in human term placenta, with higher expression being detected in the STB layer (Uckan et al., 1997). It has been suggested that Fas determinants are expressed in lymphocytes when maternal lymphocytes are activated by fetal antigens. In this case, the interaction of the activated Fas-expressing T lymphocytes with the Fas-expressing STB cells would lead to apoptosis of the activated maternal lymphocytes, thus mitigating or preventing inflammatory responses towards the fetus (Uckan et al., 1997). Subsequent to this proposal, more studies confirmed the role of Fas and FasL in the human placenta. Apoptotic leukocytes, mainly T lymphocytes, can be seen at the maternal-fetal interface, strongly suggesting that these cells are negatively affected by immune suppression (Mor et al., 1998; Hammer and Dohr, 2000). In this context, it was proposed that clonal deletion of the activated immune cells through the Fas/FasL apoptotic pathway is involved in the establishment of the immune-privileged maternal-fetal interface (Runic et al., 1996; Uckan et al., 1997; Ohshima et al., 2001). In summary, the activated maternal lymphocytes that express the Fas receptor will undergo apoptosis when they interact with the FasL-expressing trophoblast. Experimental data demonstrate the importance of such molecules in human pregnancy. Abrahams et al. (2004) showed that, despite the absence of membrane-associated FasL in isolated first-trimester trophoblast cells, a cytoplasmic form of FasL is expressed in association with a specialized secretory lysosomal pathway (Abrahams et al., 2004). Later, this association was further investigated, and the abovementioned FasL association was found to be related to placenta-derived exosomes (Stenqvist et al., 2013).

TNF-Related Apoptosis-Inducing Ligand (TRAIL) is a type II membrane protein detected in a variety of tissues. TRAIL is constitutively expressed by the placenta, and its receptors DcR1 and DcR2 are located predominantly in the STB, and DR4 and DR5 are preferentially found in the CTB (Bai et al., 2009). DcR1 and DcR2 act as decoy receptors, while DR4 and DR5 are death receptors that are responsible for activating the apoptotic pathway (Truneh et al., 2000). TRAIL activates intracellular apoptotic pathways in a way similar to that of FasL, indicating a potential functional redundancy between these two ligands. Thus, TRAIL has been proposed, together with FasL, to be a cooperating factor for inducing apoptosis in activated lymphocytes (Wiley et al., 1995; Bai et al., 2009). These two molecules represent the most important apoptosis pathways and can be observed in the human placenta throughout pregnancy, where they participate in important processes such as trophoblast invasion and differentiation (Huppertz et al., 1998; Mor et al., 2002) and in the development of maternal immune tolerance towards the fetus (Phillips et al., 1999; Mincheva-Nilsson et al., 2000; Clark, 2005).

Importantly, the intracellular localization of FasL and TRAIL in the human placenta is intimately connected to the biogenesis of exosomes. These molecules, in their membrane form, are associated with induction of apoptosis. The observation of a constitutive release of FasL- and TRAIL-expressing exosomes from the apical microvillous surface of the STB suggests an important role of such structures in the protection of the fetus from maternal lymphocytes (Stenqvist et al., 2013). This finding is in accordance with the first demonstrations showing that FasL is targeted to the MVB of the secretory lysosomes and is expressed on exosome-like microvesicles (Martínez-Lorenzo et al., 1999; Jodo et al., 2000; Mincheva-Nilsson et al., 2000; Monleón et al., 2001; Andreola et al., 2002; Smith et al., 2003; Frängsmyr et al., 2005).

It is also important to consider the regulation of NK cell activity during pregnancy. In this context, both activating and inhibitory receptors should be taken into account. For example, the receptor Natural Killer Group 2 Member D (NKG2D) is a type II transmembrane protein belonging to the C-type lectin-like family and is expressed on the surface of NK, NKT, αβ T, and CD8+ γδ T cells. In these cells, NKG2D acts as an activating receptor (Bauer et al., 1999). NKG2D ligands are divided into two families: the MHC chain-related proteins A and B (MICA and MICB, respectively) and the UL16-binding protein (ULBP) 1–6, which is also known as retinoic acid early transcript 1 (RAET1). These ligands are distantly related to MHC class I molecules and are themselves signals of cellular stress instead of antigen-presenting molecules (Stern-Ginossar and Mandelboim, 2009). NKG2D stands out as a major activating NK cell receptor, and its ligand/receptor system is a potent inducer of cytotoxicity through a mechanism directed to the elimination of stressed, foreign, transformed or infected cells.

NKG2D ligands are expressed at low levels in normal cells. However, NKG2D ligands are upregulated or expressed *de novo* in response to a great variety of biological stress signals, such as those triggered by DNA damage, irradiation, oxidative stress, and inflammation, as a strategy to display stress, danger or pathological conditions in the cell (Raulet, 2003). Soluble NKG2D ligands downregulate the cognate receptor, suppress cytotoxicity and, upon release from tumors, protect tumor cells from host immune attack through an evasion strategy (Groh et al., 2002; Song et al., 2006). Interestingly, the release of soluble NKG2D ligands has been associated with exosomes in the context of cancer (Clayton et al., 2008) and pregnancy (Mincheva-Nilsson et al., 2006). MIC proteins A and B, the human ligands of the receptor NKG2D, are expressed by the placenta, delivered to the MVB of the STB and released via MIC-bearing exosomes into the circulating blood. In sera from pregnant women, a constitutive MIC is produced and released in its soluble form by the STB. It was suggested that this MIC release is associated with placenta-derived exosomes. Notably, the soluble MIC is able to downregulate the NKG2D receptor on peripheral blood NK cells and T cells, impairing NKG2D-mediated cytotoxicity (Mincheva-Nilsson et al., 2006). The second family of human NKG2D ligands, ULBP, is also expressed by the placenta (Hedlund et al., 2009). Immunoelectron microscopy revealed that ULBP1–5 are produced and retained in the MVB of the STB on microvesicles/exosomes. In addition, it has been confirmed that exosomes bearing NKG2D ligands are released by the human placenta. The isolation of placental exosomes indicated their ability to carry ULBP1–5 and MIC on their surface and to induce the downregulation of NKG2D on NK, CD8+ and γδ T cells, which culminated in the reduction of their cytotoxic effects without affecting the perforin-mediated lytic apoptosis pathway *in vitro* (Hedlund et al., 2009). Placental delivery of NKG2D ligands via exosomes suggests a bioactive role for the soluble forms of these ligands (Hedlund et al., 2009). Such discoveries emphasize a role

for NKG2D ligand-bearing placental exosomes in the evasion of the fetus from the maternal immune responses and reinforce the view of the placenta as an important temporary immune organ.

It is noteworthy that placental EVs can also be pro-inflammatory (Holder et al., 2016; Tannetta et al., 2017a, 2017b). In agreement with the slight inflammation required in early pregnancy, the syncytiotrophoblast-derived EVs from the initial gestational periods have more inflammation-inducing characteristics than has been observed for EVs secreted by the term placenta (Tannetta et al., 2017a). During normal healthy pregnancy, the exosome concentration in plasma can be as much as 50-fold greater in pregnant women than in nonpregnant women, with levels increasing significantly with gestational age. Such an increase is observed for both placenta- and nonplacenta-derived exosomes (Salomon et al., 2014). Since the characteristics of EVs resemble the cell type from which they were derived, Tannetta et al. (2017b) reviewed and called attention to the potential use of STB-derived EVs from the maternal circulation in pregnancy monitoring. According to this suggestion, alterations in cellular responses would likely alter the EV content, thus enabling the identification of potential imbalances in tissues located in regions of the body where an optimal biopsy cannot be performed. Moreover, EV levels could be measured throughout gestation and in a personalized manner.

With regard to other important features worth noting, EVs are very transitory in the maternal circulation and do not accumulate such that the analysis of a sample would represent an up-to-date picture of "placental well-being" in terms of EV levels and molecular fingerprints. One great example of the applicability of this proposed monitoring tool involves cases of preeclampsia, which is an important heterogeneous pregnancy disorder with symptoms, which include systemic inflammation, that are triggered by the placenta because of its impaired functioning. Notably, it has been shown that release of microvesicles and nanovesicles from the placenta is greatly augmented in preeclampsia, and all fractions of such EVs from preeclamptic placenta can induce activation of endothelial cells, likely via sequestration of Vascular Endothelial Growth Factor (VEGF) by fms-kinase 1, a vasoactive factor (Tong et al., 2017); VEGF is a component of the EVs isolated from normal gestation (Tong et al., 2016) and is also found in high levels in the circulation of women with preeclampsia (Tannetta et al., 2017b).

Avoiding the vertical transmission of pathogens at the maternal-fetal interface

The defense mechanisms by which the placenta limits microbial access to the fetus are still unknown. Notably, the intervillous space could contain as much as 500 mL of maternal blood, exposing the villous surfaces to microbes present in the mother (Arora et al., 2017). In addition to the place where the placenta implants, the decidua basalis is also the location where the semiallogeneic fetal trophoblast is in direct contact with these maternal cells and acts on immune tolerance. It is believed that the decidua keeps its immune privileged condition because of its immune cell components. This composition limits lymphocyte access, and precise regulation of chemokine expression is responsible for controlling cell traffic (Red-Horse et al., 2004; Nancy et al., 2012). This regulated immune environment is maintained due to constant maternal-fetal cross-talk between the invading fetal trophoblast cells and various maternal immune cell subsets (Mor and Cardenas, 2010; Arck and Hecher, 2013; Erlebacher, 2013; Zenclussen, 2013).

The syncytial surface of the human placenta acts as a first line of protection with unique physical properties, such as the presence of dense, branched microvilli at the apical surface and a complex cortical actin network that might limit microbial invasion (Cantle et al., 1987; Fisher et al., 2000; Koi et al., 2001; McDonagh et al., 2004; Maidji et al., 2010; Robbins et al., 2010; Zeldovich et al., 2011, 2013). In this context, it was demonstrated that disruption of the actin cytoskeleton subtly facilitates the invasion of *Listeria monocytogenes* (Zeldovich et al., 2013), indicating the existence of direct physical barriers that restrict pathogen infections (Arora et al., 2017). In a healthy pregnancy, the STB layer is greatly resistant to infection by viruses such as human cytomegalovirus (HCMV), herpes simplex virus-1 (HSV1), and Zika virus (ZIKV), and other pathogens such as *L. monocytogenes* and *Toxoplasma gondii* (Fisher et al., 2000; Koi et al., 2001; Maidji et al., 2006, 2010; Robbins et al., 2010; Delorme-Axford et al., 2013; Bayer et al., 2015, 2016). In addition to relying on physical barriers, resistance could be acquired by transfer from the STB of a full-term placenta to the nonplacental cells in a paracrine manner. Experiments have shown that this transfer involves placenta-specific microRNAs (miRNAs) and type III interferons that are both packaged within exosomes (Delorme-Axford et al., 2013; Bayer et al., 2015, 2016; Ouyang et al., 2016).

In an interesting experiment, primary human trophoblast cells were infected with different RNA and DNA viruses — coxsackievirus B3 (CVB), poliovirus (PV), vesicular stomatitis virus (VSV), vaccinia virus (VV), HSV-1, and HCMV. The cells showed high resistance to these infections. Additionally, when nonplacental cells, normally permissive to these viruses, were cultured with a medium containing material isolated from naïve primary human trophoblast cells, the nonplacental cells also presented some degree of resistance to the infection. In fact, the authors showed that this antiviral profile was due to exosomes released by primary human trophoblast cells (Delorme-Axford et al., 2013). These exosomes contain miRNA members of the chromosome 19 miRNA cluster (C19MC) that are almost exclusively expressed in the human placenta (Noguer-Dance et al., 2010; Donker et al., 2012). The trophoblast-derived exosomes packing these miRNAs from C19MC are capable of attenuating viral replication in target cells by inducing autophagy, thus representing a striking evolutionary adaptation that enhances protection of the fetus against viral infections (Delorme-Axford et al., 2013). Another study demonstrated this attenuated infection by the human immunodeficiency virus (HIV)-1, varicella zoster, rubella and other togaviruses in nonplacental cells previously exposed to the same abovementioned trophoblast-conditioned medium, emphasizing that human trophoblast cells can confer resistance to viruses implicated in perinatal infection (Bayer et al., 2015).

Other molecules important to the immune response to pathogens should also be cited here. Interferons (IFNs) are pro-inflammatory cytokines that enhance adaptive immunity and antiviral responses (Schneider et al., 2014). According to Bayer et al. (2016), primary human trophoblast cells isolated from full-term placenta are resistant to infection caused by two strains of ZIKV. Exposure to the conditioned medium isolated from these cells conferred resistance against these same ZIKV strains to nontrophoblast cells, likely due to the release of IFNλ1; as a result, the ZIKV must evade this strong antiviral response or bypass these cells and use another mechanism to access the fetal compartment *in vivo* (Bayer et al., 2016).

Defensins are part of a large family of antimicrobial peptides (Ganz, 2003) directed against specific gram-negative and gram-positive bacteria, yeasts, filamentous-phase fungi, and enveloped viruses (Svinarich et al., 1997). At the transcriptional level, defensins are also present in the human placenta, amnion, and chorion, suggesting their participation in the protection of the fetus against pathogen infection (Svinarich et al., 1997).

Pathogen-associated molecular patterns (PAMPs) are microbe-derived molecules which act as critical regulators of the innate immune response (Medzhitov and Janeway, 1997) and can be a threat to the development of a healthy pregnancy. In this context, Koh et al. (2014) addressed the release of pro-inflammatory cytokines and the expression of *NF-kβ* gene by JEG-3 and BeWo human choriocarcinoma cell lines under the influence of lipopolysaccharide (LPS), a common PAMP recognized by the immune system. Interestingly, an elevated inflammatory response was observed in JEG-3 cells in comparison to the BeWo cell line, indicating that LPS influence trophoblast cells in different ways. Moreover, despite the lack of NF-kβ response in BeWo cells, this study corroborates that bacterial products such as LPS can trigger an inflammatory response in trophoblast cells, thus representing a risk factor for

pregnancy disorders like preterm labor. Toll-like receptors (TLRs) are, in humans, a family of ten molecules that recognize and respond to PAMPs. Both TLR-2 and TLR-4 are expressed by amniotic epithelial cells (Kim et al., 2004; Adams et al., 2007), and TLR-2 expression is limited to the basolateral side of these cells (Kim et al., 2004). In situations where inflammation occurs, this expression pattern is lost, and both TLR-2 and TLR-4 are upregulated. Decidual cells, decidual macrophages, and neutrophils also express TLR-2 and TLR-4 (Kim et al., 2004). Decidual cells from the first and second trimester express TLR-2 and TLR-4 (Krikun et al., 2007), and at term, these cells express TLR-1 and TLR-6 (Canavan and Simhan, 2007). Regarding mRNA expression, all ten TLRs have been identified in term placentas (Zarember and Godowski, 2002; Abrahams, 2008). In the first trimester, EVTs and villous CTB cells highly express TLR-2 and TLR-4. The STB lacks expression of TLRs; however, in the third trimester, expression of TLR-2 and TLR-4 can be found in the outer STB layer and in intermediate and EVT cells (Holmlund et al., 2002; Kumazaki et al., 2004; Ma et al., 2007; Rindsjö et al., 2007). This change in the TLR expression pattern shows the ability of the placental villi to promptly respond to an infection at the placental surface. Additionally, this shift in TLR expression could reflect changes in placental function throughout the gestational period and might suggest how infection can impact pregnancy at each trimester (Abrahams, 2008).

The trophoblast also expresses cytoplasmic-based Nod-like receptors (NLRs) (Costello et al., 2007). Nucleotide-binding Oligomerization Domain (NOD) proteins recognize peptides derived from the degradation of bacterial peptidoglycans during normal bacterial growth or destruction (Girardin et al., 2003). NOD proteins are thought to be a second line of defense in cases where TLR signaling is defective, reduced, absent or has been evaded (Abrahams, 2008). In the first trimester of pregnancy, NOD1 and NOD2 proteins are detected in the CTB and STB (Costello et al., 2007); in term placentas, only NOD1 expression has been observed (Abrahams, 2011). In the decidual stroma and glandular epithelium, NOD1 and NOD2 are also expressed (King et al., 2000).

Interestingly, transplacental trafficking of EVs from the mother to the fetus was also demonstrated. Holder et al. (2016) showed that macrophage-derived exosomes are internalized by the human placenta. This process likely occurs in a time- and dose-dependent manner via clathrin-dependent endocytosis. Such internalized exosomes have the ability to prompt the secretion of proinflammatory cytokines, thus potentially enhancing the responses to maternal inflammation and infection and thereby thwarting harm to the developing fetus. This is an important finding that indicates the existence of a highly controlled and bidirectional extracellular vesicle-mediated transfer of protein and nucleic acids that accounts for the balance of immune responses at the maternal-fetal interface (Holder et al., 2016).

The different pathways discussed here highlight the diverse immune mechanisms that protect the developing fetus from pathogen infections without invoking harmful immune responses. This process results in an immune uterine environment that must undertake controlled responsiveness such that a slight inflammatory state is created for embryo implantation. In a second, concomitant task of the uterine immune environment, immune responses are developed towards potential infections in such a careful manner that the first task is not disrupted.

Pathogens bypassing maternal-fetal immune defenses: an arms race in the biological world

The STB has been shown to be refractory to several infections. However, this feature seems to be almost exclusive to this cell type, since neither the amniotic epithelium and CTB cells of the chorionic villi isolated from mid- and late-gestation placentas nor explants from the first trimester showed such resistance (Tabata et al., 2016). Interestingly, experiments with ZIKV showed that early trophoblasts are quite susceptible to infection, but this susceptibility is lost as the STB is formed, with the trophoblast cells becoming increasingly resistant to ZIKV infection (Sheridan et al., 2017).

The classic ways of vertical infection of a developing fetus by pathogens are (I) infection of endothelial cells in the maternal microvasculature that spread to invasive extravillous trophoblasts (EVTs); (II) trafficking of infected maternal immune cells across the placental barrier; (III) paracellular or transcellular transport from maternal blood across the villous trees and into the fetal capillaries; (IV) damage to the villous tree and breaks in the STB layer; and (V) transvaginal ascending infection (Coyne and Lazear, 2016). However, except for the infections caused by some specific pathogens included in the TORCH group (toxoplasmosis, "other," Rubella, CMV, and HSV), viral infections during pregnancy are often considered of little concern from a clinical point of view. Women are frequently infected by viruses during pregnancy without severe consequences to their developing progeny (Silasi et al., 2015). Nonetheless, the recent ZIKV epidemic and the developmental problems related to ZIKV infection in newborns reveal the possible consequences of neglecting pathogens in pregnant women, including undesirable gestational outcomes and a concomitant high cost in terms of health services (Schuler-Faccini et al., 2016; Ellwanger and Chies, 2018).

When pathogens breach the STB and reach the underlying villous core, inflammation of the placental villous is the result. Such inflammation eventually induces monocyte binding to the syncytial surface through ICAM-1 (Juliano et al., 2006). This reaction can cause an immune-mediated breakdown of the STB, which creates damage that could predispose the individual to infections mediated by other pathogens (Mor and Cardenas, 2010).

As already discussed, there are two anatomical interfaces between maternal cells and fetal cells — the trophoblasts, which constitute the villous region where maternal blood bathes the STB for nutrient exchange, and the maternal decidua, where the EVT anchors the villous region to the uterus. Using first-trimester human placental explants, it was shown that the interface composed of the EVT is significantly more vulnerable to infections, despite having a much smaller surface area (Koi et al., 2001; McDonagh et al., 2004; Robbins et al., 2012). Furthermore, it has been shown that EVT cells are not as resistant to infections as STB cells, and distinct studies suggested EVTs as favorite targets of some pathogens (Robbins et al., 2010, 2012; Tabata et al., 2016). In this context, an experiment demonstrated the preference of *L. monocytogenes* for infecting EVTs by penetrating the intrauterine space (Robbins et al., 2010). This is probably due to the lack of E-cadherin expression by the STB, which is the receptor for internalin, a surface protein required for the entry of *L. monocytogenes* into epithelial cells (Mengaud et al., 1996). Additionally, a study used cultures from first trimester placentas to define where and how *L. monocytogenes* breaches the maternal-fetal barrier and demonstrated that the EVT is the preferred site for the initial placental infection (Robbins et al., 2010). A cell culture model system of primary human EVT was used to study the intracellular life cycle of *L. monocytogenes* inside EVTs. Isolated EVTs were able to restrict intracellular bacterial growth and spread, preventing vacuolar escape, and were also capable of guiding vacuolated bacteria towards lysosomes for degradation. This finding suggested that the EVT has effective defense mechanisms against intracellular pathogens and is a significant bottleneck to transplacental infections (Zeldovich et al., 2011).

Among the different strategies of immune system evasion, an interesting example comes from *T. gondii*. It was suggested that a fetal infection from *T. gondii* starts with maternal immune cells of the decidua acting as "Trojan horses" (Oz, 2017). Subsequently, the pathogen is transferred from the infected leukocytes to susceptible EVT cells or even to other cell types. Once successful in those two steps, *T. gondii* bypasses the villous core and infects fetal vascular tissues such that it reaches the central nervous system (Arora et al., 2017). The use of immune cells as Trojan horses by *T. gondii* facilitates parasite entry into immune-privileged sites. Additionally, *T. gondii*-derived exosomes have the ability to change the cytokine profile of the macrophages to modulate their activation *in vitro*. A positive aspect of this mode of infection is that *T. gondii*-derived exosomes are excellent therapeutic candidates since they have been shown to trigger humoral and cellular immune responses

and induce partial protection against acute parasitic infection in mice (Li et al., 2018).

The HCMV replicates in the underlying CTB of the floating and anchored villi, which is a place where STB is sparse. Therefore, this virus must first breach the STB and its defenses. As suggested, HCMV may bypass the STB through transcytosis of the virions in an antibody-mediated manner throughout the neonatal Fc receptor that serves as an IgG transporter instead of through direct infection (Arora et al., 2017). In addition, studies using decidual tissue cultures with clinically derived and laboratory-derived viral strains *ex vivo* showed that the HCMV could also target the EVT as well as the microvasculature and leukocytes to reach the CTB (Weisblum et al., 2011).

Immune evasion of pathogens mediated by exosomes and other EVs: a double-edged sword

The constant clash described as "pathogens *versus* immune system" involves a multifaced and very complex process. Several immune evasion mechanisms were selected during viral evolution, and many viruses usurp both exosomal trafficking and budding pathways with distinct consequences in terms of infectivity and viral spread (Gould et al., 2003; Anderson et al., 2016; Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017). For instance, exosomes packing viral particles can promote viral persistence, increasing the potential for viral infection, since the viral material is "masked." Viruses, namely those that enter cells by endocytosis, can usurp endosomal/exosomal pathways to advance their infectivity and spread (Nour and Modis, 2014; Anderson et al., 2016). Dengue virus (DENV), West Nile virus, hepatitis C virus (HCV), and ZIKV are examples of pathogens that enter cells with mechanisms related to endosome formation, and exosomes also have an endosomal origin such that their proximity may facilitate the accumulation of viral antigens in exosomes, thus increasing their spread and infection capacities (Smit et al., 2011; Anderson et al., 2016). When secreted as exosomes, intraluminal vesicles (ILVs) containing viral genomes can target uninfected cells and then penetrate them via endocytic pathways. Evidence of this process has already been observed in cases of HCV, the genome of which can be secreted in ILVs as infectious particles (Liu et al., 2014). Thus, viruses such as HCV can hijack components of the vesicular trafficking machinery and thereby integrate viral components into exosomes (Liu et al., 2014; Raab-Traub and Dittmer, 2017). Therefore, it is likely that other viruses with genomes that can be found in endosomal ILVs are also trafficked between cells via exosomes (Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017). Recently, many studies have addressed the interaction of exosomes with different viruses. Some viruses reportedly interact with exosomes, such as bunyavirus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), hepatitis A virus (HAV),

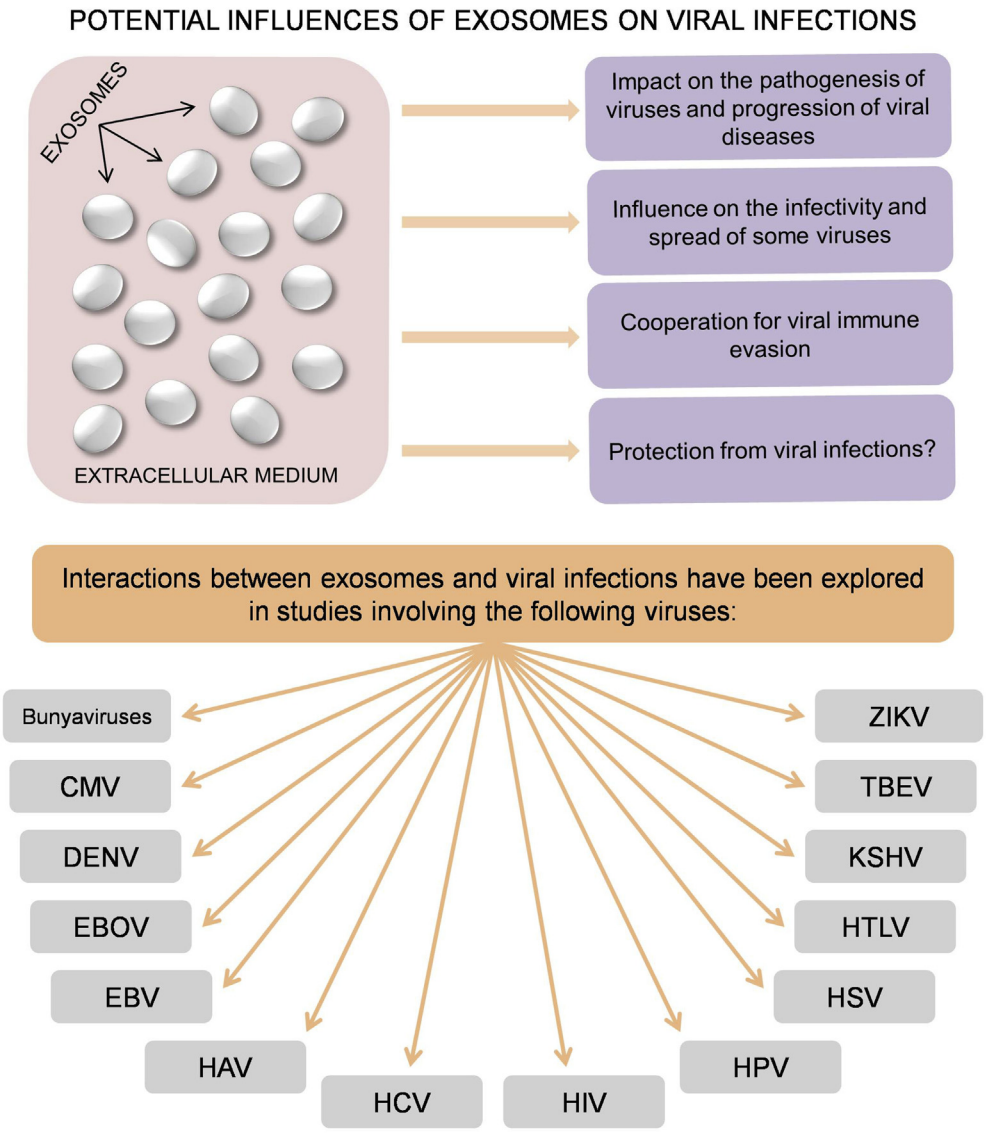

Fig. 1. Potential influences of exosomes on viral infections and examples of viruses for which their interaction with exosomes have been investigated using different methodological approaches. CMV: Cytomegalovirus; DENV: Dengue virus; EBOV: Ebola virus; EBV: Epstein-Barr virus; HAV: Hepatitis A virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HPV: Human papillomavirus; HSV: Herpes simplex virus; HTLV: Human T cell lymphotropic virus; KSHV: Kaposi's sarcoma-associated herpesvirus; TBEV: Tick-borne encephalitis virus; ZIKV: Zika virus. References are cited throughout the text.

herpes simplex virus 1 (HSV-1), HIV, human papillomavirus (HPV), human T cell lymphotropic virus (HTLV), DENV, ZIKV, tick-borne encephalitis virus (TBEV), and Kaposi's sarcoma-associated herpesvirus (KSHV) (Anderson et al., 2016; Ellwanger et al., 2017; Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017; Martins et al., 2018; Zhou et al., 2019; Ellwanger and Chies, 2019; Reyes-Ruiz et al., 2019). In addition to the viruses covered in these studies, Ebola virus (EBOV) also appears to be associated with exosomes, in particular, those that package the viral protein VP40, causing immune cell dysfunction (Pleet et al., 2016). Furthermore, a prominent research field to be explored stems from the antiviral action of exosomes (Li et al., 2013; Madison et al., 2014). Fig. 1 presents a summary of some interactions between exosomes and viral infections that have been suggested to date.

As stated in previous sections, EVs can influence other pathogens besides viruses. The interaction between exosomes and pathogens can modify the outcomes of the infections. Such modifications caused by the action of EVs can enhance the infectivity of a particular microorganism via the delivery of infective particles and pathogen-derived molecules to distant sites from the primary focus of infection. Alternatively, EVs can favor the immune evasion of the pathogens from the host (Zhang et al., 2018). For example, *Staphylococcus aureus*-derived exosomes can transfer virulence factors, such as α-toxin, to cells found in distant physiological sites from the original location of the bacteria (Husmann et al., 2009). Also, cells infected by *Bacillus anthracis* secrete exosomes containing pathogen-derived toxins, thus enabling the action of virulence factors at long-distances (Abrami et al., 2013). In the context of *Helicobacter pylori* infection, exosomes have been associated to the secretion of cytotoxins by the host gastric epithelial cells, promoting extragastric complications (Shimoda et al., 2016).

Intrauterine infections by bacteria also represent a major threat to pregnancy when they gain access to gestational tissues. Bacterial infections can take place via the maternal circulation, in the peritoneal cavity, or ascend into the uterus from the lower tract (Espinoza et al., 2006). Regarding *L. monocytogenes,* a successful infection in the EVT could rely on the capacity of this bacterium to produce extracellular vesicles, called bacterial membrane vesicles (MVs). Studies have shown that MVs help bacteria survive inside mouse embryonic fibroblasts *in vitro* (Vdovikova et al., 2017). Considering opportunistic infections of the genitourinary tract, Group B *Streptococcus*-derived MVs loaded with virulence factors led to up-regulation of pro-inflammatory cytokines and inflammation related-symptoms of chorio-amnionitis in mice. These observations suggested that these bacterial-derived MVs are capable of triggering events at the maternal-fetal interface associated with preterm birth or even fetal death (Surve et al., 2016).

Intracellular pathogens such as *Mycobacterium tuberculosis*, which primarily infect macrophages in the lungs, also interact with microvesicles. It is known that infection of macrophages with mycobacteria leads to the release of EVs by the infected host cells. These released EVs contain numerous mycobacterial lipoglycans, lipoproteins, and antigens that modulate the immune response of the host (Bhatnagar et al., 2007). Moreover, electron microscopy images of *M. bovis* BCG-infected macrophages suggested that these EVs are indeed exosomes (Giri and Schorey, 2008). However, a study brought important evidence regarding the origin of EVs in the context of *M. tuberculosis* infection. It was demonstrated that some immune modulations related to TLR2 signaling provoked by this pathogen in the host are derived from the bacterial membrane vesicles rather than exosomes derived from the infected macrophages. In summary, the study suggested that the impairments on immune effector functions are primarily driven by the exportation of *M. tuberculosis* lipoglycans and lipoproteins released from the pathogen cell membrane (Athman et al., 2015).

Other infectious agents besides viruses and bacteria present interesting links with EVs. Prions are proteinaceous infectious particles that spread in the host cells due to the ability of these proteins to interact and shape the quaternary structure of nascent proteins. The resulting quaternary structure is an exact copy of the proteinaceous infectious proteins, which can also modify other proteins, thus further spreading the infection. Prions are responsible for transmissible spongiform encephalopathies in humans and other mammals. These infections are fatal and commonly known as prion diseases (Prusiner, 1982; Watts et al., 2006). Interestingly, exosomes can contain and transport prion proteins, contributing to the spread of these proteins in the infected organism (Robertson et al., 2006; Fevrier et al., 2004; Hartmann et al., 2017). Moreover, a study demonstrated that mouse neuroblastoma cells transmit cytosolic prions in association with membrane-bound vesicles besides the classical transmission by direct cell contact. The presence of flotillin, Alix-1, and Tsg101 and cup-shaped appearance of the EVs indicated that these vesicles indeed represented exosomes (Liu et al., 2016).

Regarding parasites, interesting features involving the pathogenesis of malaria and EVs secretion by host cells were addressed. Red blood cells infected by *Plasmodium falciparum* can secrete EVs containing molecules involved in the silencing of gene expression in endothelial cells. Also, these EVs were efficiently internalized by the endothelial cells and can disrupt the mechanisms responsible for hindering the entry of pathogens, thus enhancing the infection of the parasite in the host target cells (Mantel et al., 2016).

Besides the previously mentioned aspects regarding EVs and *T. gondii* infection in pregnancy, the pathogenesis of this parasite can be further affected by exosomes through the transference of molecules that alter the host cell cycle. Such alterations eventually decrease host cell proliferation, favoring the parasite because it invades cells in the S stage more easily than in other phases of the host cell cycle (Kim et al., 2016).

The bloodstream form of *Trypanosoma brucei* secrete EVs that interact with the cell membrane of host erythrocytes. Thus, it was postulated that fusogenic EVs derived from the trypanosome may act as vehicles for pathogen-to-host cell transfer of membrane proteins. Of note, this fusion between EVs from the pathogen and host cells results in the transfer of lipids and antigens derived from the parasite to the host cells. Such traffic of molecules has the potential to cause host anemia, a clinical outcome due to modifications in the structure of the host erythrocytes probably related to the incorporation of lipids from the parasite via EV fusion (Szempruch et al., 2016).

The first discovery regarding the role of EVs in fungal infection was made addressing *Cryptococcus neoformans* (Rodrigues et al., 2007). It was demonstrated that the *Cryptococcus*-derived virulence factor glucuronoxylomannan was produced inside the cell and then released in the extracellular environment inside EVs. Of note, EVs released by *C. neoformans* facilitate the pathogen passage through the blood-brain barrier and modulate the host immune responses, enhancing *C. neoformans* pathogenesis (Huang et al., 2012; Bielska and May, 2019).

Subsequently, various studies addressing associations between fungi and EVs have emerged (Rodrigues et al., 2014; Coakley et al., 2015; Peres da Silva et al., 2015; Joffe et al., 2016; Bielska and May, 2019). Like EVs derived from other species, fungal EVs transport proteins, lipids, pigments, polysaccharides, and genetic cargoes (Joffe et al., 2016). Thus, fungal EVs can induce strong and different impacts on host immunity, including stimulation of pro- and anti-inflammatory cytokine production (Bielska and May, 2019). Therefore, it can be speculated that fungi-derived EVs may have some impact on the host's inflammatory status, triggering other health problems not directly related to the fungal infection, but due to unbalanced inflammation. Importantly, fungi EVs can also interact with other pathogenic microorganisms in co-infected hosts, generating an even more complex immune landscape. Also, considering that approximately three hundred species of fungi are pathogenic for humans and only eleven species have their EVs addressed, more studies in this field are necessary (Bielska and May, 2019).

Parasitic helminths are metazoan organisms that also produce and secrete exosomes. In this context, studies addressing trematodes demonstrated intact exosomes in the parasites' teguments, indicating that these vesicles could also reach the host environment. Initially, it was speculated that exosomes derived from these parasites participated in the

down-regulation of the host immune responses, a common feature of helminth infections. The immune manipulation of the host immune responses by these parasites eventually ensures the survival of the parasites, mainly by exporting a range of immuno-modulatory mediators that interact with host cells and tissues. Evidence for the role of exosomes in the host immune modulation by helminths was demonstrated by the internalization of helminth-derived exosomes by host intestinal epithelial cells (Coakley et al., 2016).

Open questions and emerging topics

Seminal exosomes — friends or foes in sexually transmitted infections?

The role of semen in sexually transmitted viral infections is another interesting topic that connects exosomes, infectious diseases, and reproduction. Semen is a complex fluid composed of cells and seminal plasma. Human semen contains immunosuppressive components with the ability to drive tolerance towards paternal antigens, consequently maximizing the chances of successful fertilization. This immune suppression is likely derived from the low incidence of antibodies against sperm and the soluble components of semen in the woman body (Johansson et al., 2004). However, the immunosuppressive properties of semen could also contribute to the evolutionary success of sexually transmitted viruses; that is, they may take advantage of the immune suppressed environment that follows from exposure to semen (Sabatté et al., 2011). Seminal plasma has a high concentration of subcellular lipid-bound microparticles that are morphologically and molecularly consistent with exosomes that originate from multiple cellular sources of the male genital tract (Renneberg et al., 1997). These microparticles are, in general terms, called "seminal exosomes" (Vojtech et al., 2014). In summary, the immunosuppressive properties of seminal plasma seem to be related to its exosome fraction, and therefore, exposure to seminal exosomes could facilitate the establishment of viral infections (Vojtech et al., 2014).

The wide variety of cells that secrete exosomes also dictates the spectrum of biological fluids from which they can be isolated: amniotic fluid, breast milk, bronchoalveolar lavage fluid, cerebrospinal fluid, malignant ascites, plasma, saliva, synovial fluid, urine, vaginal fluid, and semen, among others (Ellwanger et al., 2017). Considering semen, each ejaculate contains trillions of exosomes with an average of 2.2×10^{13} particles (Vojtech et al., 2014, 2016). Seminal exosomes are considered immunosuppressive particles due to their inhibitory action during lymphoproliferative responses (Kelly et al., 1991), phagocytic cells (Skibinski et al., 1992), and NK cell function (Tarazona et al., 2011). Seminal exosomes are efficiently and rapidly captured by peripheral and vaginal DCs, whereas seminal exosomes are captured by T cells in the vaginal environment at a lower rate and efficacy (Vojtech et al., 2016). The immunosuppressive properties of semen are predominantly restricted to the seminal plasma since isolated sperm cells can induce immune responses and alterations in the uterine environment, and seminal plasma alone induces tolerance to paternal antigens and confers benefits to the offspring mainly in early pregnancy (Robertson et al., 2009; Bromfield, 2014).

Although there is some evidence suggesting that semen-derived exosomes have anti-HIV activity, exosomes apparently do not have an effect on the replication of other viruses (Madison et al., 2014). Recently, it was shown that Herpesviruses hijack host exosomes, which contributes to their viral pathogenesis (Sadeghipour and Mathias, 2017). Thus, once viruses take advantage of the local altered/suppressed immune responses induced by exposure to semen, semen immunosuppressive properties can contribute to the prevalence of sexually transmitted viral infections. In addition, as described above, the tremendous number of exosomes present in semen could facilitate viral spread.

Do exosomes facilitate transplacental and sexually transmitted viral infections?

The role of exosomes as facilitators of (I) transplacental and (II) sexually transmitted viral infections (Fig. 2) should be considered based on the following four premises:

1st) Trojan exosomes: Gould et al. (2003) hypothesized that retroviruses such as HIV and HTLV could usurp the machinery that causes the budding and trafficking of exosomes to infect new cells without being recognized by the immune system. Although the Trojan exosome hypothesis is still debated, some experimental evidence supporting the cellular mechanisms consistent with this theory has been published (Nguyen et al., 2003; Booth et al., 2006; Gan and Gould, 2012; Kadiu et al., 2012). Interestingly, the presence of HCV particles in exosomes has already been demonstrated (Liu et al., 2014). Moreover, the detailed cellular mechanisms of the budding/trafficking of exosomes that could be used by HCV, HAV, HIV, EBV, and KSHV to spread from cell to cell were recently revised (Raab-Traub and Dittmer, 2017). Thus, the mechanism that leads to the budding/trafficking of exosomes may also be employed by viruses to cross the maternal-fetal barrier.

2nd) Immunomodulation in pregnancy: Pregnancy is considered a challenge to the woman's immune system. In fact, fifty percent of the fetal genome, and consequently the antigens and other immune molecules present, are of paternal origin. Thus, the immune system of a pregnant woman must be readjusted during pregnancy to avoid perturbing the developing fetus (Mincheva-Nilsson, 2010; Mor et al., 2011; Stenqvist et al., 2013). When immune adaptation fails, abortion is a likely consequence (Trowsdale and Betz, 2006). The local downregulation of the maternal immune system, especially in the first trimester of pregnancy, could favor transplacental viral infection.

3rd) The cloud of exosomes at the maternal-fetal interface: Taking into consideration the immune system adjustments during pregnancy that promote a tolerogenic environment for the fetus, the general suppression of the immune system during the entire gestational period would be expected. However, as previously discussed, systemic immunosuppression would not be desirable because the blastocyst would not implant, and the pregnant woman would be highly susceptible to a variety of infections. Here, we present a series of studies showing that placenta-derived exosomes play important roles in this tolerogenic process by carrying immunomodulatory molecules through the maternal-fetal interface (Hedlund et al., 2009; Mincheva-Nilsson, 2010; Stenqvist et al., 2013). It is believed that exosomes contribute to this process by forming a "cloud of exosomes", which would protect the fetus from exacerbated maternal immune responses (Mincheva-Nilsson, 2010). Of note, this process would not compromise the woman's immune defenses as a whole.

4th) Seminal exosomes: Semen has trillions of exosomes (Karlsson et al., 2001; Vojtech et al., 2014). Furthermore, although semen is an immune privileged biological fluid, some viruses have been detected in this fluid months after host infection (Madison et al., 2014; Abbate et al., 2016; Anderson et al., 2016; D'Ortenzio et al., 2016; Uyeki et al., 2016). Exosomes present in semen could facilitate sexual transmission of viruses through semen-derived immunosuppression and hide, in some cases, viral components from the host immunological system.

The placental/fetal microbiota

Aagaard et al. (2014) reported that placental and amniotic fluid from healthy human placenta are not sterile. Historically, the uterus was considered a sterile environment, but it is currently viewed by some researchers as a compartment where the microbiota starts to be established (Stinson et al., 2017). Lactic acid bacteria and other commensal bacteria were isolated from meconium obtained from healthy neonates born either by labor or cesarean section, indicating that mother-to-child efflux of commensal bacteria may exist through the placenta (Martín et al., 2004; Jiménez et al., 2008). In 2005, Jiménez et al. (2005) also found commensal bacteria in the umbilical cord blood of healthy neonates born by cesarean section. In addition, a study using placental tissues from low-gestational-age neonates showed that almost one-half of second-trimester placentas harbor organisms within the chorionic plate

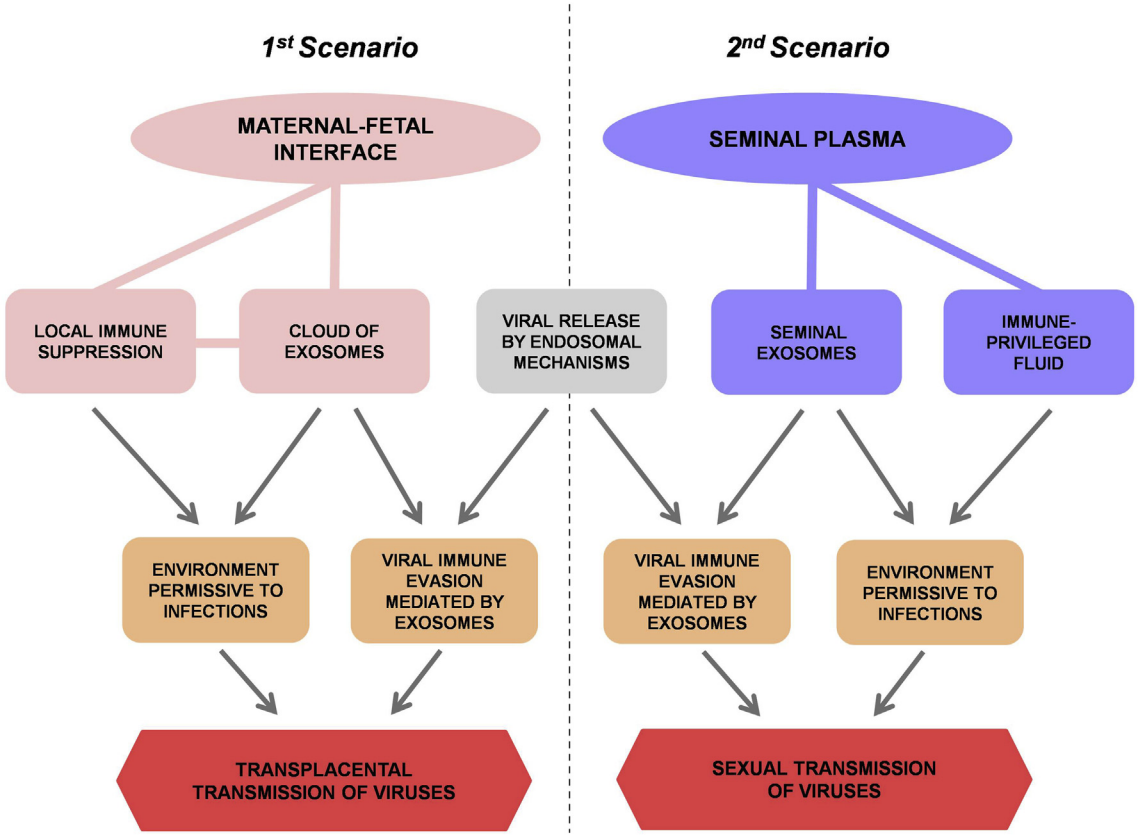

Fig. 2. Potential roles of exosomes in transplacental (1st scenario) and sexually transmitted (2nd scenario) viral infections. References are cited throughout the text.

(Onderdonk et al., 2008a). Another study showed that the chorion of placentas from preterm labor pregnancies (not related to preeclampsia) had a much higher rate of microorganism recovery than that of placentas from increasingly severe preeclampsia pregnancies (Onderdonk et al., 2008b). The presence of microorganisms in the placental parenchyma was associated with the presence of neutrophils in the fetal stem vessels of the chorion and umbilical cord, strongly indicating that the presence of microorganisms within the placental parenchyma is biologically important (Onderdonk et al., 2008b). The presence of microorganisms may correlate with the high number of neutrophils recruited to the maternal-fetal interface, as they may create an inflammatory environment similar to that necessary for the labor process but at the wrong time.

The following main question arises from these observations: how do microorganisms, or their genetic material, make contact with the fetus before birth? To date, three different origins for the fetal/placental microbiota have been proposed: maternal gut microbiota, vaginal microbiota, and oral microbiota (Stinson et al., 2017). Regarding the transference of microorganisms from the maternal gut, it is possible that the microorganisms are translocated into the maternal bloodstream from the gut epithelium, and the DCs could be quite important for this process. The intestinal epithelial barrier prevents bacteria from entering into the bloodstream. However, DCs can take up bacteria from the intestinal lumen by penetrating the gut epithelium. DCs packing bacteria could traffic to mesenteric lymph nodes via intestinal lymphatics and thus spread the bacteria to other body compartments (Stinson et al., 2017). Of note, maternal intestinal bacteria can also be found in breast milk, reportedly through the same DC-based dissemination pathway hypothesized for the delivery to the fetal tissues (Fernández et al., 2013).

The vaginal pathway by which microbes would reach the placenta is not well known, but it has been well established that microbes may ascend from the vagina and reach the amniotic cavity. One suggested mechanism involves the microbial colonization of the decidua, through mechanisms previously discussed, from which the microorganisms spread to fetal membranes and invade the amniotic fluid. Another suggested pathway involves direct microbial invasion of the amniotic fluid by penetration of a discontinuous section of fetal membranes (Stinson et al., 2017). Whatever the pathways of infection, DNA from vaginal microbes in the amniotic fluid (DiGiulio, 2012), fetal membranes (Steel et al., 2005) and the placenta (Aagaard et al., 2014) have already been found in both normal and complicated pregnancies. For example, pathogenic oral species of bacteria have been found in the placenta and amniotic fluid of pregnant women with periodontal disease (Barak et al., 2007; Katz et al., 2009). This finding indicates an opportunistic migration of bacteria from the oral cavity to the uterine environment and has been extensively correlated with preterm birth. Comparing the placental microbiome with the microbiome derived from different body compartments, the oral cavity showed the greatest similarity in terms of bacterial composition (Aagaard et al., 2014). Despite this finding, these studies were performed with the microbiota of healthy nonpregnant individuals, which makes it difficult to infer routes of transmission (Stinson et al., 2017). The effect of sexual practices in the transfer of oral and gut bacteria to the intrauterine cavity also needs further investigation. In this context, oral or anal sex preceding vaginal sex may present a mechanism of microbial transfer. The resolution of these tangled issues could not only create the possibility for studying, measuring, and mapping healthy placental/fetal microbiota from its precise beginning but could also provide an additional basis for the establishment of new public health strategies and improvement of clinical practices for complicated pregnancies with the aim of reducing the cases of newborns with severe sequelae.

However, a recent study addressing hundreds of placental samples stated that healthy placentas do not display a microbiome. This study represents the largest sample number in this field of research and was composed of 537 placental samples. de Goffau et al. (2019) performed such elegant experiments that allowed the identification of even possible contaminants from DNA extraction kits, and the results showed the

presence of only one type of microorganism in 5% of placentas: *Streptoccocus agalactiae*. Of note, this microorganism is one of the main concerns regarding the risk of neonatal sepsis, and is probably transmitted from the mother's genital tract. Besides revealing possible routes of contamination during the experimental procedure in previous related studies, these findings revealed a possible way of early detection of potential harmful agents during pregnancy. In summary, this study presented convincing evidence to support that healthy placentas lack a microbiome. The study reinforces that, despite the lack of a placental microbiome, pathogens may eventually be found in the placenta, although bacterial infection of this transient organ is not a common cause of gestational complications (de Goffau et al., 2019).

Taking together, we believe that future discussions and experimentation should consider the role of exosomes and other EVs as potential vehicles used by microorganisms in the establishment of the newborn microbiome. Finally, it is possible that the observed first microbiome in meconium samples is a result of EV-mediated traffic of bacteria from the vaginal tract, placenta or uterine cavity towards the fetus during the first signals of labor or even during delivery, thus representing the early seeds of the neonatal microbiome. Considering the emergence of studies addressing the establishment of the neonate microbiome, these hypotheses should be investigated.

3. Conclusion

The immune system of a pregnant woman, far from being in a resting state, undergoes several changes throughout the entire gestation period, encompassing distinct organs, tissues, cellular, and molecular profiles. Over the years, studies in the reproductive biology field have elegantly approached the multiple interactions at the maternal-fetal interface, which can result in normal or pathological pregnancies. Firstly considered a threat to a successful pregnancy, inflammation is currently recognized as an essential step to pregnancy establishment and maintenance, although such an immune response should be regulated. Exacerbated inflammation can cause abortion and other pregnancy complications, but the absence of inflammation precludes effective implantation due to inadequate tissue remodeling. A shift to a less inflammatory environment occurs during pregnancy, enabling fetal development. Finally, by the end of the third trimester, near parturition, a range of physiological alterations occurs, and a pro-inflammatory milieu is again predominant.

Additionally, when implantation takes place, the paternal antigens are expressed, and the maternal immune system meets two challenges: avoiding immune activation and rejection of the developing fetus while simultaneously inducing immune activation to avoid pathogen infection. Fetal tolerization is a complex process that transpires during the entire gestation period and involves modulation of local immune responses towards an anti-inflammatory profile. Interestingly, the placenta is a vigorous producer of exosomes, extracellular vesicles that have been described as key players in the regulation of maternal immune responses. The syncytiotrophoblast has important physical and molecular mechanisms that prevent microbes from bypassing the placenta and reaching the fetus, and these features range from dense, branched microvilli at the apical surface to soluble receptors carried by exosomes. Recent studies have revealed the importance of exosomes to a successful pregnancy, namely, as partners of the immune system at the maternal-fetal interface.

The trafficking of molecules, cells and even pathogens between mother and fetus during pregnancy is currently seen as a natural phenomenon. In this context, exosomes can be important mediators of transplacental infections. Additionally, the immunosuppression induced by seminal exosomes can help explain the persistence of the many viruses found in semen. In addition, this review revisited the discussion about the processes that enable viruses (and possibly other pathogens) to overcome the maternal-fetal barrier through sexual transmission. Taking into consideration the particularities of each cell type and virus *per se,* we call urgent attention to the role of exosomes and other microvesicles in viral infectivity and spread. Finally, the traditional view that establishes serious potential complications to the fetus should a microorganism succeed in crossing the placenta has been revised. In this regard, bacteria found in the normal gut, oral cavity, and vagina were detected in the amniotic cavity and in the placenta of normal pregnancies. Despite the recent emergence of controversial findings regarding this aspect, such discoveries raised important discussions about potential routes for the establishment of the newborn microbiota. Current studies are now trying to elucidate the distinct pathways used by microbes to colonize the developing fetus. Thus, we expect this review to provide insights for future investigations and new studies on all the topics addressed, since the universe of extracellular vesicles is similar to an iceberg from which, at the present moment, we are able to see only the portion that lies above the water. In this sense, a submerged “EV world” awaits our discovery, and with new methods, approaches and the establishment of connections among the several scientific fields involved, we will be able to uncover the immersed portions that will help us comprehend the immunology of gestation, fetal microbiome, and even the transplacental and sexual transmission of pathogens.

Declarations

Author contribution statement

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The authors declare no conflict of interest.

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