Trends in Molecular Medicine

CellPress

Review

Immunobiology of pregnancy: from basic science to translational medicine

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Embryo implantation failure and spontaneous abortions represent the main causes of infertility in developed countries. Unfortunately, incomplete knowledge of the multiple factors involved in implantation and fetal development keeps the success rate of medically assisted procreation techniques relatively low. According to recent literature, cellular and molecular mechanisms of 'immunogenic tolerance' towards the embryo are crucial to establish an 'anti-inflammatory' state permissive of a healthy pregnancy. In this review we dissect the role played by the immune system in the endometrial–embryo crosstalk, with a particular emphasis towards the fork-head-box-p3 (Foxp3$^+$) CD4$^+$CD25$^+$ regulatory T (Treg) cells and discuss the most recent therapeutic advances in the context of early immune-mediated pregnancy loss.

Highlights

A successful pregnancy relies on finely tuned immune adaptations, which allow fetus survival and development, while protecting the mother. The interaction between maternal lymphocytes and trophoblast-derived pro-tolerogenic molecules induces an immunotolerant microenvironment at the interface between the mother and the embryo.

A systemic and local increase of maternal CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (Treg) cells is essential for induction and maintenance of tolerance towards paternal antigens during pregnancy.

Immune modulation by maternal hormones is pivotal in favoring a healthy pregnancy.

Assisted reproduction techniques should be able to induce pro-tolerogenic immune responses to embryo antigens in order to avoid pregnancy loss.

The identification of the cellular and molecular mechanisms necessary for an optimal immunological microenvironment should improve therapeutic opportunities aimed at promoting a healthy pregnancy.

Immune tolerance at the feto-maternal interface: basic biology and clinical perspectives

Fertility rates are declining in western developed countries; currently, approximately 15% of couples are infertile [1]. Increasing stressful lifestyle and age at first pregnancy are believed to be among the factors at the base of those biological alterations that prevent a successful embryonic implantation and development. Notwithstanding the recent advances of assisted reproduction techniques (ARTs), the mechanisms behind their still relatively low rate of success (~45%) remain unclear [2,3]. Factors such as low receptivity of the **endometrium** (see Glossary), embryonic chromosomal abnormalities, and hormonal disorders are relevant but the immune system (Box 1) also deserves serious consideration since it is key in fostering an immune-compatible environment and improving the outcome of infertility treatments [4,5]. It has been described that, during the first trimester of pregnancy, the feto-maternal interface is characterized by an inflammatory microenvironment, conducive to embryo implantation and pregnancy establishment, followed by an immunotolerant phase throughout the rest of pregnancy, conducive to pregnancy maintenance and fetal development [6]. Therefore, uterine immune cells (Box 2) together with the production of pregnancy hormones (Box 3), influence each stage of implantation and gestation, ensuring a tolerogenic milieu essential for fetal health and placental development.

In this review, we dissect the main adaptive immune cell subsets involved in the regulation of tolerance during pregnancy; in particular, we describe the emerging role of fork-head-box-p3 (Foxp3$^+$) CD4$^+$CD25$^+$ **regulatory T (Treg) cells**, a specialized subpopulation of CD4$^+$ T lymphocytes, believed to curb the immune response against the semi-allogeneic fetus and we also highlight the studies that have unveiled the association between Treg cell dysfunction and infertility and/or repeated spontaneous abortions [6,7]. Moreover, we describe how immunological dysregulation impacts on the maintenance of a term pregnancy, pointing at infectious, personal habits or immune disorders as possible etiologies. Finally, we summarize the major ARTs acting on the modulation of immune system with the aim to induce pro-tolerogenic responses against

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Box 1. Role of the immune system in embryo implantation

The maternal innate and adaptive immune responses play a critical role in the establishment, maintenance, and completion of a healthy pregnancy. To ensure the correct progression of these events, generation of an immunotolerant microenvironment is necessary; this is induced by the expression of polymorphic human leucocyte antigen (HLA) of both maternal and paternal origin on **extravillous trophoblasts (EVTs)**. Specifically, inherited paternal HLA antigens from the semi-allogenic fetus are able to prime maternal immune responses through the production of embryo-specific HLA antibodies [122]. It has been reported that EVTs do not express the classical antigen-presenting HLA-A and -B molecules; instead, they express HLA-G, -E, -F, and -C that directly interact with surface receptors of APCs residing in the maternal decidua, particularly with uNK cell receptors [123]. Among them, HLA-G has been studied for its specificity of expression on the membranes of EVTs; its function seems to be the modulation of cytokine secretion from decidual lymphocytes for generation of immune tolerance through several mechanisms, such as inhibition of $CD8^+$ T cell killing and accumulation of anti-inflammatory Treg cells [124]. The HLA-G locus contains several polymorphisms in the noncoding regions, such as those present at the 3′-untranslated region (UTR), that can influence its expression and pregnancy progression [125]. The most studied 3′-UTR HLA-G polymorphisms are insertion/deletion (Ins/Del) of 14 base pairs (bp) that affect HLA-G mRNA stability [126]. These polymorphisms are associated with lower levels or even absence of soluble (s)HLA-G in plasma of pregnant women, resulting in more frequent spontaneous and recurrent miscarriages [127,128]. HLA-G also contributes to the remodeling of the spiral arteries to maximize the delivery of maternal blood to the intervillous space, another important factor ensuring an adequate decidua [129]. These changes promote the increase of blood flow necessary for normal fetal development, since an inefficacious arterial transformation deprives the feto-maternal unit of oxygen and nutrients and leads to miscarriage and/or pregnancy complications such as pre-eclampsia, maternal hypertension, or preterm labor [130].

fetal antigens. The comprehension of these mechanisms may lead to identifying the cellular and molecular determinants favoring an immunological microenvironment able to promote a healthy pregnancy.

The adaptive immune response at the interface of embryo and mother

The adaptive immune response is critical to regulate tolerance during pregnancy [8,9]. In the human **decidua**, T cells are abundant and their levels are highly dynamic, contributing to successful

Box 2. Innate immune system at the feto-maternal interface

For the establishment of a pregnancy-specific immune microenvironment, innate immunity mainly involves uNK cells (65–70%) and dDCs (2%) [131]. They promote blood vessel remodeling at the feto-maternal interface, maintain the stability of the immune microenvironment, enable the healthy growth of the fetus, and protect the mother from harmful pathogens [132].

Phenotypically, uNK cells differ from peripheral NK cells ($CD56^{dim}CD16^+$) for their higher expression of CD56 and lower expression of CD16 ($CD56^{bright}CD16^-$) and for having weak cytotoxic activity characterized by the production of IL-8 and IFN-inducible protein (IP)10 that in turn regulate trophoblast migration. Similar to peripheral NK cells, uNK cells possess equivalent or higher levels of granzyme B, perforin, and granulysin [133,134].

Furthermore, uNK cells also secrete several matrix metalloproteinases (MMPs) and angiogenic factors such as VEGF-C, angiopoietin (Ang)1, Ang2, and TGF-β1 promoting angiogenesis and tissue remodeling [135]. Additionally, uNK cells express high levels of killer cell immunoglobulin like receptors (KIRs), surface inhibitory receptors specific for allelic forms of HLA-G and HLA-C class I molecules, and CD94/NKG2 heterodimer receptors that bind nonclassical HLA-E molecules expressed by invasive EVTs [136]. The interaction of maternal KIR with fetal HLA-C is crucial for pregnancy outcome (e.g., the combination of KIR2D/HLA-C contribute to greater reproductive success than other combinations), primarily due to signals received by uNK cells [137]. In addition, KIR region is defined by two groups of haplotypes, A and B, that can have different effects in pregnancy; the association between the maternal inhibitory KIR-A/HLA-C increases the risk of developing pregnancy disorders, whereas KIR-B/HLA-C has a protective effect in reproduction [137,138].

dDCs also play a function in successful implantation and placentation in **allogeneic pregnancy** [42,139]. In a normal pregnancy, at the level of feto-maternal interface, dDCs are exposed to several agents, including prostaglandin E2 (PGE2) and cytokines such as TGF-β that influence their polarization, promoting maturation of myeloid or tolerogenic DCs, respectively [140,141]. Several studies have demonstrated that the decidualization program promotes a specific subset of DC, the DC-10, which contributes to the establishment of tolerogenic and immune suppressor milieu by inducing Treg cells and favoring Tconv cell hyporesponsiveness and deletion [142]. Also, dDCs help to prevent fetal rejection by recruiting uNK cells and potentiating decidual angiogenesis via close dialogue with decidual stromal cells, through the production of IL-15, a progesterone-dependent cytokine, highly expressed in the human endometrium (Figure 1) [143].

Box 3. Hormonal control of immunological tolerance

The interaction between the immune system and the endocrine system is known to contribute to creating a favorable environment for the fetus. Hormones associated with pregnancy, such as hCG and steroid hormones (P_4 and estrogens), participate in placental angiogenesis, normal trophoblast development and invasion, and maintenance of immune homeostasis. Specifically, hCG contributes to the fetal tolerance by attracting Treg cells to feto-maternal interface, increasing both their number and suppressive capacity, besides maintaining DCs in an immature and tolerogenic state [38]. In mice hCG was also demonstrated to maintain pregnancy tolerance by inhibiting B cell proliferation and mediating their conversion into a regulatory phenotype (B10 or IL-35$^+$ Breg cells) [39]. The immunomodulatory effect of hCG is enhanced by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), two gonadotropins involved in the neuroendocrine control of menstrual cycle, ovulation, and pregnancy [40]. The combination of hCG, FSH, and LH positively influences CD4$^+$ T cell tolerance towards embryo implantation, exerting a synergistic effect that induces T cell hyporesponsiveness through the increase of cyclic adenosyl monophosphate (cAMP) synthesis and the induction of regulatory-type cytokines such as IL-10 and TGF-β [40,41].

P_4 also promiscuously binds to the GR, which promotes immune suppression by inducing enrichment of Treg cells and triggering apoptosis of Tconv cells [42–44]. High levels of P_4 show an immune-suppressive function in human cord blood, since they drive the shift of naïve cord blood T cells into suppressive Treg cells and prevent their conversion into Th17 cells [45]. P_4 also stimulates the lymphocytes to synthesize progesterone-induced binding factor (PIBF) and mediates its immunological effects, such as upregulation of Th2-related cytokines and downregulation of peripheral NK cell activity [46]. In humans, the increase of PIBF during pregnancy promotes the differentiation of CD4$^+$ T cells into Th2 cells, which in turn secrete augmented quantities of anti-inflammatory cytokines, including IL-4, IL-5, and IL-10 [47,48]. Similarly, a high concentration of E_2 favors the shift of T cells towards a Th2-phenotype and promotes Treg cell induction by decreasing the production of IL-17 from Th17 cells, both *in vivo* and *in vitro* [49,50]. In particular, this hormone is able to induce the expansion of Treg cells and enhance their suppressive functions through high expression of estrogen receptor alpha (ERα) on T cells [51]. In summary, interactions of an appropriate maternal immune system and an adequate level of circulating hormones creates a favorable environment for the developing embryo and fetus.

gestation. They represent about 10–20% of decidual immune cells in the first trimester, of which 30–45% are CD4$^+$ and 45–75% are CD8$^+$ T cells [10,11].

T helper cells controlling inflammation during implantation

During the implantation phase (Figure 1), T helper (Th)1 cells infiltrate the decidua and release several proinflammatory cytokines [interleukin (IL)-1, IL-6, IL-8, interferon (IFN)-γ and tumor necrosis factor (TNF)-α], which contribute to low grade inflammation and support embryo implantation, tissue remodeling, and recruitment of other immune cells [11,12]. The predominant role of Th1 cells is to sustain trophoblast invasion, stimulate the adequate repair of the uterine epithelium and promote the removal of cellular debris following **blastocyst** implantation. Once the implantation phase is completed, the predominant cytokine milieu is shifted to produce an anti-inflammatory Th2-type environment. In this context, decidual dendritic cells (dDCs) actively participate in naïve T cell differentiation into Th2 cells, by inhibiting Th1 cell development [13]; also the pregnancy-dependent production of progesterone (P_4), estradiol (E_2), prostaglandin (PG)-D2, and the leukemia inhibitory factor (LIF) are able to induce Th2 differentiation, promoting the release of Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 [14]. Notably, it has been reported that Th2-derived IL-10 has several roles in normal pregnancy, such as promoting successful placentation, controlling inflammation, and regulating vascular function. Impairment of IL-10 functions contributes to pregnancy loss through these mechanisms [15]. In conclusion, timely and proper quantities of Th1 and Th2 cells support a healthy pregnancy, while their imbalance can result in miscarriage.

Decidual CD8$^+$ cell protective immunity during pregnancy

On the one hand, decidual CD8$^+$ T (CD8$^+$ dT) cells provide protective immunity against viral infections and are critical for immune tolerance and pregnancy success. On the other hand, these cells have a predominant effector-memory (EM) phenotype [16], with reduced expression of perforin and granzyme B proteins compared with peripheral blood CD8$^+$ T (CD8$^+$ pT) cells [17]. During normal pregnancy, CD8$^+$ dT cells coexpress T cell immunoglobulin mucin (Tim)-3, programmed

Glossary

Allogeneic pregnancy: a pregnancy in which the embryo derives half the genes from the mother and the other half are inherited from the father.
Blastocyst: a structure characterized by an external layer of trophoblast cells, an inner cell mass (ICM), and a fluid-filled blastocoel cavity. Its formation begins around 5–6 days after fertilization; after its implantation in the uterine wall, it becomes an embryo, then a fetus.
Decidua: a specialized membrane of the uterus characterized by embryo-receptive properties, formed from endometrium during the secretory phase of the menstrual cycle. It is composed of glandular and immune cells, blood and lymph vessels, and decidual stromal cells (DSCs). DSCs acquire specific functions related to recognition and acceptance of the embryo in the process called decidualization, which is controlled by estrogen and progesterone.
Embryo transfer (ET): the final and crucial step after a procedure of *in vitro* fertilization (IVF) for the treatment of human infertility. ET involves the placement of one or more embryos into the uterine cavity by passing a catheter through the cervical os.
Endometrium: the mucous membrane surrounding the uterus, in which a fertilized egg must embed in order to develop into an embryo.
Experimental autoimmune encephalomyelitis (EAE): mouse model of multiple sclerosis characterized by inflammatory demyelination of the central nervous system (CNS), caused by immunization with myelin antigens leading to an aberrant immune response against self-myelin.
Extravillous trophoblasts (EVTs): differentiated trophoblastic cells allowing placental villi to anchor to the maternal decidua. They reshape uterine spiral arteries for effective placentation and perfusion of the intervillous space.
Pre-eclampsia: hypertensive disorder of pregnancy that begins with abnormal placentation and release of antiangiogenic markers, resulting in endothelial dysfunction, vasoconstriction, and immune dysregulation, which in turn negatively impacts on maternal and fetal organs.
Regulatory T (Treg) cells: subset of T cells involved in the maintenance of peripheral tolerance, characterized by the expression of IL-2 receptor α (CD25), glucocorticoid-induced tumor

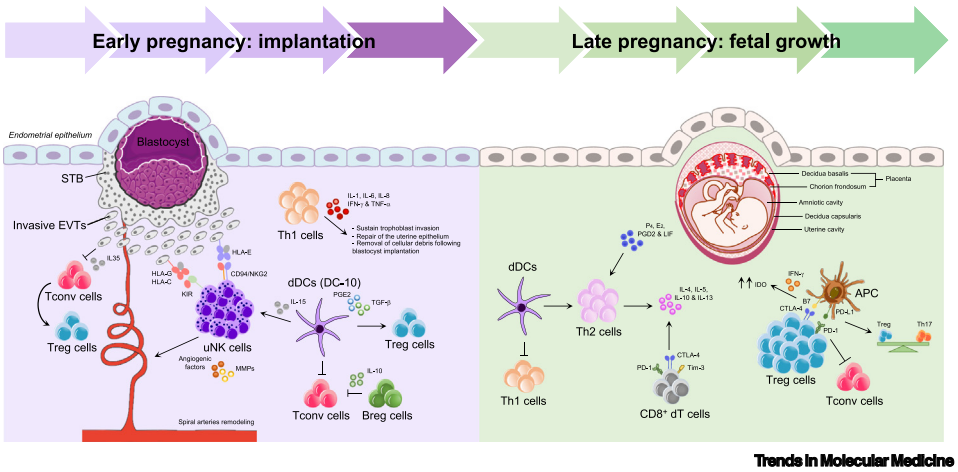

Figure 1. Innate and adaptive immune cell interactions at feto-maternal interface. Factors secreted by trophoblast cells are responsible for the recruitment of immune cells to support trophoblast invasion. Decidual DCs, uNK, and Th1 cells cooperate to the generation of a mild proinflammatory microenvironment, affecting both uterine epithelial cell receptivity and successful implantation. Embryo implantation is characterized by a complex state of immune tolerance with the recruitment and proliferation of different immunoregulatory cells and the expression of several regulatory molecules and cytokines and by the formation of the placenta and fetal membranes that also play an important immunomodulatory role. Following successful implantation, there is a stage of fetal growth and development in which the predominant milieu is that of a Treg/Th2-type or an anti-inflammatory environment. Abbreviations: APC, antigen presenting cell; Breg cells, regulatory B cells; $CD8^+$ dT cells, decidual $CD8^+$ T cells; CTLA-4, cytotoxic late antigen 4; dDCs, decidual dendritic cells; E_2, estradiol; EVTs, extravillous trophoblasts; HLA, human leucocyte antigen; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; IL, interleukin; KIR, killer cell immunoglobulin like receptor; LIF, leukemia inhibitory factor; MMPs, matrix metalloproteinases; P_4, progesterone; PD-1, programmed cell death 1; PD-L1, programmed cell death-ligand 1; PGD2, prostaglandin D2; PGE2, prostaglandin E2; STB, syncytiotrophoblast cells; Tconv cells, conventional T cells; TG-Fβ, transforming growth factor beta; Th cells, T helper cells; Tim-3, T-cell immunoglobulin mucin 3; TNF-α, tumor necrosis factor alpha; Treg cells, regulatory T cells; uNK cells, uterine-natural killer cells.

cell death (PD)-1, and cytotoxic T-lymphocyte-associated antigen (CTLA)-4, which promote an anti-inflammatory cytokine production. The blockade of these inhibitory receptors results in increased trophoblast killing and IFN-γ producing capacities of $CD8^+$ dT cells associated with miscarriage [18,19]. Further elucidation of the molecular mechanisms that regulate the expression of inhibitory markers will be key to understanding how these cells provide defense to infection yet maintain immune tolerance to fetal and placental cells.

Treg cell-mediated feto-maternal tolerance

Several studies demonstrate that the abundance of Treg cells is modified during pregnancy. Fluctuations in uterine or peripheral Treg cell levels make these cells more abundant during the first and second trimester, so that suppression can promote pregnancy, and they decline postpartum [20–22]. Interestingly, this increase in Treg cell proportion is not restricted to feto-maternal interface and their expansion is also observed in other peripheral tissues of pregnant women [23].

Specifically, during implantation, maternal and fetal Treg cells play an essential role in preventing immune responses against paternal antigens in fetal cells. Circulating Treg cell numbers increase during early pregnancy, with a peak during the second trimester, and decrease postpartum; these events occur in concomitance with the maximal decidua invasion by **trophoblast cells** [10,24,25]. During embryonic development, double-positive cells initiate expression of CD25, GITR, CTLA-4, and CD122 in the fetal thymus. Moreover, $CD4^+CD25^+$ fetal thymocytes already have the potential to suppress proliferation of conventional T (Tconv) cells and, upon maturation, these cells enter the periphery and circulate in elevated numbers when compared with adult peripheral blood [26].

necrosis factor receptor (GITR), and cytotoxic T-lymphocyte-associated antigen (CTLA)-4 as crucial markers for the induction of immune tolerance. The transcription factor fork-head-box-p3 (Foxp3) is the major regulator of Treg cell development and function.

Syncytialization: a process in which the trophectodermal cells of the blastocyst penetrate the maternal uterine epithelium. This occurs around the time of embryo implantation until delivery and is involved in the production of hormones and the control of feto-maternal exchanges.

Trophoblast cells: cells derived from trophectoderm and constituting the outer layer of the blastocyst; they provide nutrients to the embryo and form the placenta. These cells regulate the immune system at the implantation site.

Decidual Treg cells are composed of both thymus-derived Treg (tTreg) cells and inducible Treg (iTreg) cells (arising from $CD4^+$ Tconv cells in peripheral tissues) and exhibit phenotypic heterogeneity according to the cycle and pregnancy phase [27–30]. Moreover, decidual Treg cells express the Foxp3 gene at levels comparable with those of 'classic' Treg cells, but they are numerically more abundant in the pool of decidual $CD4^+$ T cells than those circulating in the blood. The enrichment of Treg cells in the decidua is a critical event: on the one side, those cells represent a reservoir of 'inactive' or 'naïve' $CD4^+CD25^-Foxp3^+$ Treg cells that can be rapidly converted into a pool of 'classic' $CD4^+CD25^{+-}$ $Foxp3^+$ Treg cells; on the other, they are able to attenuate the activation of Tconv cells by transient acquisition of the transcriptional factor Foxp3, thereby switching off the immune response [31].

The development and function of decidual Treg cells during pregnancy is promoted by the PD-1/PD-ligand (L)1 pathway, which inhibits the activation and proliferation of T cells by altering the production of proinflammatory cytokines and inducing apoptosis [32,33]. Indeed, lack of PD-L1 function in mice causes fetal resorption and hyperactivation of proinflammatory Th17 cells, paralleled by Treg cell reduction [32,34,35]. Therefore, the interaction between PD-1 and PD-L1 supports pregnancy by regulating Treg and Th17 cell balance [36,37]. A fundamental mechanism by which Treg cells lead to fetus tolerance is the catabolism of tryptophan to kynurenine indoleamine 2,3-dioxygenase (IDO), which is toxic for T cells neighboring the DCs, and compromise T and natural killer (NK) cell activation/proliferation [38,39]. The binding between CTLA-4 on Treg cells and the B7 complex on antigen-presenting cells (APCs) induces IDO production and favors IFN-γ secretion by APCs, which in turn enhances IDO expression on dDCs and macrophages [40]. This interaction favors the decidual M2 macrophage phenotype, which represents the predominant macrophage population sustaining feto-maternal tolerance; on the contrary, macrophages with an M1 phenotype are mainly present during preimplantation period [41].

In the crosstalk between trophoblast and T cells, a crucial role is mediated by IL-35, an inhibitory cytokine predominantly produced by Treg cells and required for their suppressive activity [42], with IL-35 levels significantly higher in normal pregnancy than in age-matched non-pregnant female donors. Similarly, IL-35 secreted by trophoblast cells suppresses T cell proliferation and induces the conversion of naïve Tconv cells into IL-35-producing induced Treg (iTR35) cells, providing immune protection for the fetus [43].

Taken together, these studies suggest that the generation and recruitment of Treg cells at feto-maternal interface are critical factors involved in the survival and protection of the allogeneic fetus. Indeed, Treg cell dysfunction has been associated with infertility, repeated spontaneous abortions, and pregnancy-related complications, including **pre-eclampsia**.

B cell emerging functions during pregnancy

While T cell functions in pregnancy are well documented, scant information is available on the role of B cells, a major component of the immune system with pleiotropic functions, including antibody and immunomodulatory cytokine production and also antigen-presenting capabilities.

In particular, a functional regulatory B (Breg) cell subset contributes to the maintenance of immune tolerance by producing IL-10, IL-35, and transforming growth factor (TGF)-β [44]. Jensen *et al*. have recently shown that $CD19^+CD5^+CD1d^+$ IL-10-producing anti-inflammatory Breg cells are diminished in abortion-prone animals when compared with those with normal rates of pregnancies; also, that a population of $CD19^+CD24^{hi}CD27^+$ Breg cells increases in the first trimester of pregnancy, suppressing unwanted immune response of maternal Tconv cells. Interestingly, the study also found that the levels of Breg cells in women suffering from spontaneous abortions remain low, similar to non-pregnant women [45]. Therefore, the anti-inflammatory role of IL-10-producing

Breg cells in suppressing Th1 responses, and preventing allogeneic responses against the fetus, may emerge as key in the establishment of maternal immunological tolerance [46]. Understanding the pathways that induce naïve B cells to become Breg cells could be crucial to ensure fetal unperturbed growth.

Immunological mechanisms involved in implantation failure and pregnancy loss

The dysregulation of those immunological mechanisms normally engaged in the maintenance of a term pregnancy is heavily implicated in recurrent implantation failure (RIF) and repeated pregnancy loss (RPL) [47,48]. Genetic, hormonal, infectious, personal habits, or immune disorders were reported as possible etiologies (Figure 2) [49–51].

Immune system involvement in pregnancy failure

Alteration of T cell-mediated immune responses is among the main mechanisms responsible for **embryo transfer (ETs)** failures; in particular, reduced Treg cell suppressive capacity or exhausted Treg cells are associated with implantation failure [52]. Similarly, impaired number and function of PD-1^{+}Tim-3^{+}CD8^{+} T cells is associated with miscarriage. According to their

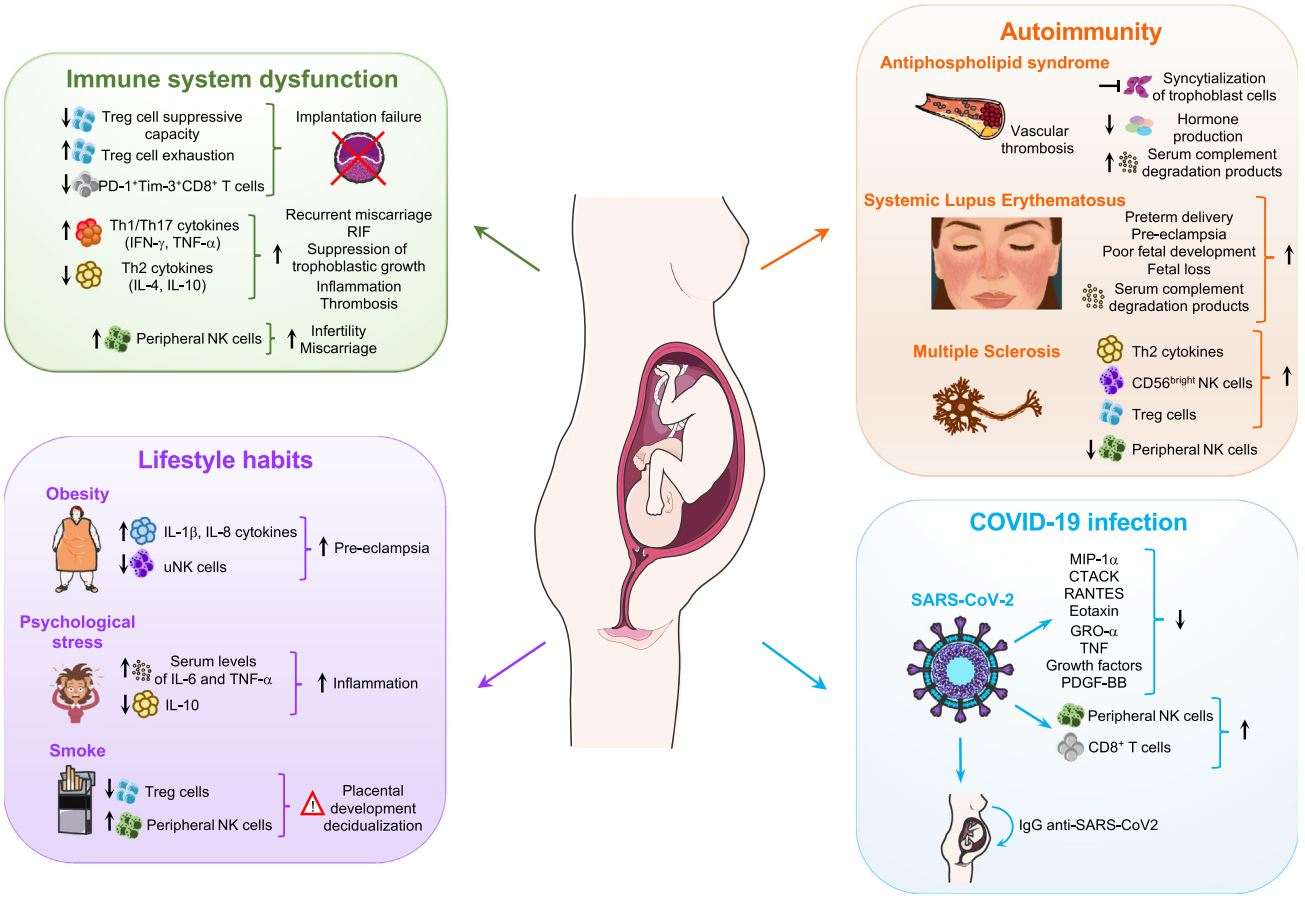

Figure 2. Immunological mechanisms involved in implantation failure and pregnancy loss. Schematic representation of immune system alterations, lifestyle habits, autoimmune diseases, and infections implicated in recurrent implantation failure (RIF) and repeated pregnancy loss (RPL). Abbreviations: COVID-19, coronavirus disease 2019; CTACK, cutaneous T cell-attracting chemokine; GRO-α, growth-regulated protein alpha; IgG, immunoglobulin G; IL, interleukin; MIP-1α, macrophage inflammatory proteins 1 alpha; NK cells, natural killer cells; PDGF-BB, platelet-derived growth factor BB; RANTES, regulated upon activation normal T cell expressed and presumably secreted; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Th cells, T helper cells; TNF-α, tumor necrosis factor alpha; Treg cells, regulatory T cells; uNK cells, uterine-natural killer cells.

study, inhibition of PD-1 and/or Tim-3 pathways results in fetal loss in pregnant CBA/J mice due to impaired $CD8^+$ T cell activity [19].

Moreover, recurrent miscarriage and RIF are associated with high levels of Th1 and Th17 cytokines (IFN-γ and TNF-α) and low levels of Th2 cytokines (IL-4 and IL-10) [53,54]. This also determines the suppression of trophoblastic growth, favoring inflammation and thrombosis in maternal uterine blood vessels [54]. Therefore, evidence in humans and mice suggest that pregnancy failure is triggered when immune responses or their regulators are perturbed.

The influence of lifestyle habits in pregnancy

During gestation, a number of factors such as obesity, stress, and smoking can influence normal fetal development. It has been reported that obesity leads to a chronic state of inflammation, which not only leads to insulin resistance and type 2 diabetes, but also limits the quality of oocytes [55,56]. Increased levels of IL-1β and IL-8 and decreased uterine (u)NK cell numbers in the placenta of obese women were found to be associated with pre-eclampsia [57]. In addition, maternal psychosocial stress is associated with risks for maternal and offspring health. A study from Coussons-Read *et al*. demonstrated that a cohort of pregnant women with increased stress showed a higher serum level of proinflammatory IL-6 and TNF-α and lower levels of the anti-inflammatory cytokine IL-10 [58]. Other data suggest that sleep disturbance and depression may interact in promoting inflammation in pregnancy [59].

In regard to smoke, carbon monoxide can cause a toxic depletion of oxygen and nicotine can lead to vasoconstriction and decreased nutrients to the fetus due to maternal appetite suppression [60]. Moreover, tobacco smoke contains many substances that may also affect placental development and decidualization [61]. It was observed that cigarette consumption during pregnancy affects circulating maternal lymphocytes and alters levels and functions of peripheral NK and Treg cells, both related with adverse pregnancy outcomes: percentages of Treg cells were lower, while peripheral NK cells were increased in smoke-exposed mice and smoking women compared with controls, causing an impaired vascularization [62,63]. In summary, healthy lifestyle habits (diet, psychological environment, and smoking) spanning from preconception to postpartum are considered as a major safeguard for the prevention of miscarriage.

Association between autoimmune disease and reduced pregnancy outcomes

The relationship between pregnancy and autoimmunity is bidirectional: autoimmune diseases can be affected by pregnancy and, vice versa, they can increase miscarriage risks and perinatal mortality. For instance, antiphospholipid syndrome (APS), an autoimmune disease characterized by the presence of anti-phospholipid antibodies (aPL), associates with vascular thrombosis but also pregnancy morbidity [64]. The most common autoantibodies in APS are anticardiolipin antibodies immunoglobulin (Ig)G, IgM, lupus anticoagulant, anti-β2-glycoprotein I antibodies that can directly affect trophoblast cell function by inhibiting **syncytialization** and decreasing hormone production. Systemic lupus erythematosus (SLE) also increases the risk for preterm delivery, pre-eclampsia, poor fetal development, and fetal loss [64]. In both diseases, the contribution of complement to disease pathogenesis has been extensively documented: the activation of both the classical and the alternative pathways of complement causes thrombotic events and pregnancy loss. Furthermore, pregnant women with SLE and APS display higher serum levels of complement degradation products compared with healthy pregnant mothers [65,66].

However, immunological tolerance induced by pregnancy suppresses the inflammatory activity of many cell-mediated autoimmune diseases, including rheumatoid arthritis, uveitis, psoriasis, and multiple sclerosis (MS), whereas after the delivery, the disease activity returns, often in a more

aggressive manner than before pregnancy. The factors potentially involved in this phenomenon are the decrease of estrogen levels after the delivery and the loss of pregnancy-associated immunosuppressive state [67]. In this context, MS during pregnancy is characterized by increased secretion of Th2 cytokines and increased levels of $CD56^{bright}$ NK cells associated with reduction of disease activity; conversely, the increased relapse rate after the delivery is associated with a diminished proportion of $CD56^{bright}$ NK cells [68].

In addition, it was observed that the total number of peripheral NK cells is reduced, while a higher prevalence of Treg cells has also been found during MS pregnancy, hampering disease progression, phenomena that are reversed postpartum [69,70]. Another mechanism by which pregnancy induces tolerance in MS is mediated by P_4, which increases Treg cell frequencies via its binding to the GR in T cells. *In vivo*, T cell-specific glucocorticoid receptor (GR) deletion in pregnant animals with **experimental autoimmune encephalomyelitis (EAE)** resulted in a reduced peripheral frequency of Treg cells and a selective loss of pregnancy-induced improvement of EAE [71], suggesting a specific role in these factors in pregnancy-induced immune regulation, not only towards the fetus but also against self-antigens.

In all, these data indicate that pregnancy steroid hormones and immunoregulatory factors can shift the immunological balance in favor of tolerance via differential engagement of various immune cell subsets during autoimmunity. However, autoimmune patients remain at higher risk of pregnancy failure due to dysregulated systemic and local immune responses.

Immunological adaptation of pregnancy during coronavirus disease 2019 (COVID-19) infection

The decidual and peripheral immune cells play a pivotal role in regulating the balance between immune tolerance and the defense against pathogens. Throughout pregnancy, this equilibrium is constantly subject to microbial challenge. A rapid, effective response against invasive pathogens is therefore essential in order to avoid maternal infection and consequent fetal loss. Despite extensive progress in unraveling the immunological adaptations of pregnancy, pregnant women remain more susceptible to certain viral infections, such as cytomegalovirus, herpes simplex virus type 1 and 2, varicella zoster virus, and hepatitis virus E [72–74].

Emerging data also indicate an increased risk associated with severe acute respiratory syndrome (SARS) coronavirus 2 (CoV-2) during pregnancy [75]. To date, there are conflicting data on the course of COVID-19 and the influence of the infection on pregnancy. Several studies have shown that pregnant women affected by COVID-19 developed mild symptoms in the absence of comorbidities, while those experiencing gestational hypertension, pre-eclampsia, or gestational diabetes mellitus have an increased risk of developing serious infection and exhibiting negative pregnancy outcomes [76,77]. The parallel activation of proinflammatory and anti-inflammatory immunity characterizing the immune status of pregnant women is hypothesized to protect them from the 'cytokine storm' typically present during COVID-19 infection [78,79]. Specifically, the macrophage chemokines involved in COVID-19-related cytokine storm, such as MIP-1α, CTACK, RANTES, Eotaxin, GRO-α, TNF, and growth factors [FGF, LIF, granulocyte colony stimulating factor (G-CSF), and platelet-derived growth factor (PDGF)-BB isoform] showed significantly lower expression in pregnant as compared with non-pregnant women [80,81]. In addition, numerous clinical reports have observed that pregnant women, compared with non-pregnant women, show a slightly increased number of $CD8^+$ T and NK cells, which are important also for virus-infected cell killing [82].

Finally, during infection, placenta acts as an immunological barrier, preventing the fetus being reached by the virus, but it allows the transfer of immunological components such as immunoglobulins. Data regarding the transmission of SARS-CoV-2 from the mother to the fetus in early

pregnancy are limited, but the rate of IgG in the serum of newborns indicates an active communication between the maternal immune system and fetus. Indeed, at around 10–12 weeks of gestation, maternal IgGs are transferred across the placenta to the developing fetus to protect it from infection. This process is known as passive immunization and peaks in the last trimester; in particular the majority of IgGs anti-SARS-CoV-2 are transferred during the final 4 weeks of gestation. These immunoglobulins have been detected in neonates in the absence of IgM or a PCR-positive nasopharyngeal swab, indicating that they received the IgGs passively from the exposed mother [83–85]. All the aforementioned considerations refer to the intricate regulation of immune response of pregnant COVID-19 women and they could have implications for COVID-19 immunotherapy.

Manipulation of the immune system and potential implications for treatment

Nowadays, infertility represents a worldwide health problem and it is attributed to many factors, such as ovarian insufficiency, diminished ovarian reserve, endocrine disturbances, and genetic and immunological defects [86–88]. The success of medically ARTs associates with several factors, such as oocyte/embryo quality, maternal age, timing of ovulation, ET, the vaginal and endometrial microbiome, and sperm factors (see Clinician's corner) [89,90]. Among reproduction techniques, *in vitro* fertilization (IVF) with autologous/heterologous oocytes and spermatozoa has become an increasingly utilized option for couples unable to conceive [89–92]. Several diagnostic procedures have been approved to treat potential causes of RIF, such as those concerning uterine cavity (ultrasound, hysteroscopy, endometrial biopsy) and embryo development (time lapse and preimplantation genetic testing for aneuploidy) [93]. More recently, it was demonstrated that immune dysregulation contributes to defective implantation and placentation, resulting in pregnancy complications such as pre-eclampsia, intrauterine growth restriction (IUGR), and preterm labor [94]. In this context, we described the immunotherapies proposed as potential treatments to improve embryo implantation and pregnancy outcome and whether they are recommended or not by the European Society of Human Reproduction and Embryology (ESHRE) (Table 1) [5,93,95–98].

Specifically, the intricate interface between 'immune tolerant' Treg cells and 'immune rejectant' proinflammatory Th cells is a crucial aspect of immunotherapies in the context of ART [94,99,100]. Indeed, elevated circulating Treg cells on the day of vaginal oocyte collection are associated with increased pregnancy rates [101]. Consistently, Wang *et al*. have shown that decreased levels of Treg cells at the feto-maternal interface presented impaired implantation rates, while transfer of paternal antigen-specific Treg cells before mating increased implantation rates and reduced the incidence of spontaneous abortion in mice [102]. Further, a study of Liu and colleagues showed that the percentage of peripheral Treg cells was increased compared with controls when intrauterine human chorionic gonadotropin (hCG) was administered [103]. In RIF subjects, hCG infusion increased endometrial Treg cells and chemokine (C-C motif) ligand 2 (CCL)2 expression; however, CCL2 small interfering RNA (siRNA) or CC receptor (R)2 antagonist treatment blocked Treg cell migration to the endometrium [7].

Another immunotherapeutic approach aimed at improving implantation and pregnancy rates is intrauterine peripheral blood mononuclear cell (PBMC) therapy; this technique intends to fuel the initial 'controlled' inflammation required for implantation. Autologous PBMCs co-cultured with hCG induce the secretion of several cytokines, such as IL-1α, IL-1β, and TNF-α, which positively promote embryonic invasion and improve endometrial receptivity by inducing the expression of LIF and vascular endothelial growth factor (VEGF) [104,105].

Moreover, another immunoregulatory approach is the subcutaneous administration of G-CSF, which induces trophoblast proliferation, invasion, and maintenance during pregnancy and

Table 1. Immunotherapies currently available for treatment of recurrent implantation failure (RIF) and repeated pregnancy loss (RPL)

Immunotherapy[a]	Immune cell subset involved	Mechanism of action	Limitations	Study	ESHRE RIF position	ESHRE RPL position	Refs
IU administration of hCG	Treg cells	Increased CCL2 expression and increased migration of Treg cells into the endometrium	The mechanisms involved in improving outcomes remain poorly understood	Liu *et al.*, 2019; Huang *et al.*, 2020	Can be considered	Insufficient evidence	[7,103]
IU administration of autologous PBMCs preactivated with hCG	PBMCs	Induction of IL1a, IL1b, TNFα, LIF, and VEGF secretion	Excessive immune cell infiltration and severe inflammatory responses may result in embryo rejection	Yu *et al.*, 2014	Not recommended	Insufficient evidence	[104]
Subcutaneous administration of G-CSF	T cells	Induction of conversion of T cells into Th2 cells, promotion of IL10-producing Treg cells and tolerogenic DCs	The effects remain still poorly understood	Miyama *et al.*, 1998; Lédée *et al.*, 2008; Rutella *et al.*, 2005	Not recommended	Insufficient evidence	[107–109]
Glucocorticoids	uNK cells	Suppression of the synthesis of proinflammatory IL1b, TNFα, IL6, IL8, reduced number of uNK cells, and increased trophoblast proliferation and invasion	Increased fetal exposure to glucocorticoids can cause adverse outcomes, including IUGR, adverse effect on brain development of the fetus, and postnatal cardiovascular disease	Benediktsson *et al.*, 1993; Uno *et al.*, 1994; Barker, 1997	Not recommended	Not recommended	[111–113]
Intralipid	Peripheral NK cells	Inhibition of NK cell cytotoxicity and impairment of antigen presentation function by macrophages	No clinical evidence supports this therapy	Genest *et al.*, 2023; Kumar *et al.*, 2021; Meng *et al.*, 2016; Plaçais *et al.*, 2020	Not recommended	Not recommended	[4,114–116]
Tacrolimus	T cells	Inhibition of T cell proliferation, IL2 transcription, and cytotoxic T cell generation	More studies are needed	Nakagawa *et al.*, 2015	Not recommended	Not used	[118]
Prednisolone	Th1 cells	Inhibition of Th1 cytokine secretion and increased number of Treg and uNK cells	More studies are needed	Fawzy *et al.*, 2014	Not recommended	Not recommended	[119]
IVIG treatment	T cells	Decreased balance of Th1/Th2 cytokines, decreased peripheral NK and Th17 cell number, and increased Treg cell function	The effects remain still poorly understood	Kondo *et al.*, 1991	Not recommended	Not recommended	[120]
HCQ therapy	Th17 and Treg cells	Downregulation of Th17 cytokines and upregulation of Treg cell function	No clinical adverse effects have been observed	Sadeghpour *et al.*, 2020	Not mentioned	Recommended in research settings	[121]

[a]Abbreviations: CCL2, chemokine (C-C motif) ligand 2; ESHRE, European Society of Human Reproduction and Embryology; HCQ, hydroxychloroquine; IL, interleukin; IU, intrauterine; IVIG, intravenous immunoglobulin G.

switches the T cell-cytokine secretion profile towards the Th2 type, thus promoting immunoregulatory IL10-producing Treg cells and tolerogenic DCs [106–109]. Currently, immunosuppressive therapies that minimize excessive immune cell infiltration and severe inflammatory responses have been proposed for infertile women with immune overactivation. Among anti-inflammatory molecules, glucocorticoids positively regulate embryo implantation, as well as the subsequent development of the fetus and placenta, by suppressing synthesis of the proinflammatory IL-1β, TNF-α, IL-6, and IL-8, promoting trophoblast cell proliferation and invasion [110]. Therefore, a single dose of antenatal corticoids should be administered in women with premature rupture of membranes before the 32nd week of gestation to reduce the risk of respiratory distress syndrome, perinatal mortality, and other morbidities. Increased fetal exposure to glucocorticoids can cause adverse outcomes, including IUGR, effects on the fetal brain development, and postnatal cardiovascular disease [111–113].

Another intervention aimed at improving pregnancy outcomes is intralipid infusion [4,114]. Observations in women demonstrate that this can modulate immune function by inhibiting peripheral NK cell cytotoxicity and by impairment of macrophage antigen presentation function [115,116]. A significant reduction in peripheral NK activity and lymphokine-activated killer activity was found after intralipid administration, since RIF/RPL are often associated with elevated peripheral NK cell numbers and activity [117]. However, more studies are required to assess the safety of intralipid treatment prior to conception and during pregnancy.

In addition, several immunosuppressive drugs are used to prevent RIF: for example, tacrolimus restrained excessive immune cell responses against the fetus by inhibiting T cell proliferation, IL-2 transcription, and cytotoxic T cell generation in a cohort study of 25 women treated 2 days before ET [118]. Prednisolone, which inhibits Th1 cytokine secretion and increases Treg and uterine natural killer (uNK) cell number, is also used in women with RIF and recurrent abortions [119]. Finally, another immunomodulatory mechanism to improve the implantation process is intravenous immunoglobulin G (IVIG) treatment, which inhibits autoantibody production, decreases the balance of Th1/Th2 cytokines, peripheral NK, and Th17 cell number, and increases Treg cell function [120]. A recent clinical trial has demonstrated that modulation of Th17/Treg cell balance in RIF subjects, by hydroxychloroquine (HCQ) therapy, is associated with successful pregnancy outcomes. HCQ administration, before ET, downregulates Th17 cell-related cytokines and upregulates Treg cell function, contributing to the creation of a tolerant microenvironment for implantation [121]. In conclusion, further studies are required to develop personalized immunotherapies depending on the unique immunological characteristics of each subject.

Concluding remarks

Pregnancy is a dynamic process, during which diversified mechanisms intermingle to enable its successful establishment and maintenance over time. The immune cell crosstalk at the feto-maternal interface, the secretion of pregnancy hormones, and the development of placenta are essential factors leading to proper progression of pregnancy and to protect the fetus from both internal and external insults. Animal models and observational studies in humans and mice suggest that the highly tuned balance between pro-and anti-inflammatory mechanisms are pivotal, with immunoregulatory Treg cells enforcing and maintaining the immunotolerance against the semi-allogenic fetus. The progressive increase of Treg cells during pregnancy is not only associated with augmented rates of live birth upon IVF but also ameliorates disease severity in pregnant women with autoimmunity. Indeed, the majority of ARTs mainly act on the modulation of the immune system with the aim to induce pro-tolerogenic responses against fetal antigens. Further studies are needed to determine the impact of these interventions on maternal immunity, aimed at improving embryo implantation and pregnancy outcomes (see Outstanding

Clinician's corner

The elective single embryo transfer (e-SET) strategy has been introduced to optimize efficiency of ART. The combination of extended embryo culture until the stage of blastocyst with preimplantation genetic testing for aneuploidy (PGT-A) has permitted selection of euploid embryos to maintain the best rate of success (live birth rate) by reducing abortion rates and multiple gestations.

The proportion of euploid embryos failing to implant is relatively high, ranging approximately from 40% to 55%; this evidence suggests that factors other than aneuploidy, such as immune-mediated ones, contribute to early pregnancy failure and senescence.

Several studies focused on uterine mucosa and endometrium failed to identify reliable markers of endometrial receptivity and valid strategies for improving clinical pregnancy rates. For these reasons, there is increasing awareness that the interplay between the immunobiology of the embryo and of the endometrium are crucial for studying immunological factors able to predict the risk of embryonal miscarriage.

These observations suggest that a better knowledge of the role of the immune system may lead to developing clinical tests for improving e-SET and novel strategies for favoring embryo implantation and pregnancy progression over time.

questions). In conclusion, translational research in reproductive immunology is facing the challenge of identifying and characterizing the cellular and molecular determinants that favor an optimal immunological microenvironment, able to promote a healthy pregnancy.

Outstanding questions

What is the mechanism that allows expansion of antigen-specific Treg cells in the decidua?

Is it possible to identify a Treg cell marker able to predict pregnancy outcome in women with recurrent implantation failure and repeated pregnancy loss?

Would it be possible to enhance Treg cell function in order to increase the tolerance towards the donor eggs in women undergoing heterologous *in vitro* fertilization?

Acknowledgments

The authors acknowledge Maria Eugenia Lopez Naranjo (Universidad Pablo de Olavide) and Raffaella Di Girolamo (Dipartimento di Sanità Pubblica, Università degli Studi di Napoli "Federico II") for the help in table preparation. This work was supported by EU funding from Fondazione Italiana Sclerosi Multipla (FISM no. 2018/S/5 to G.M. and FISM no. 2018/R/4 to P.d.C.); Ministry of Education University and Research (MIUR) (Bando PRIN 2017 Prot. 2017K55HLC) to G.M.; the MUR PNRR Extended Partnership (INF-ACT no. PE00000007 and MNESYS no. PE00000006) to G.M.; Ministry of Health (Bando Ricerca Finalizzata 2019 RF-2019-12371111) to G.M. and the Juvenile Diabetes Research Foundation (JDRF no. 2-SRA-2022-1192-S-B) to P.d.C. Part of the images used in the figure preparation were from the Motifolio drawing toolkits (www.motifolio.com) and from Servier Medical Art (http://www.servier.fr/servier-medical-art).

Declaration of interests

T.D.H. and S.L. declare that they are Merck Healthcare KGaA employees. C.A. declares receipt of unrestricted research grants from Merck and lecture fees from Merck. G.M. reports receiving research grant support from Merck, Biogen, and Novartis and advisory board fees from Merck, Biogen, Novartis, and Roche.

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