IMMUNOLOGY UPDATES

Cytokines: Important for implantation?

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Abstract

Problem Cytokines are obviously very important in an established pregnancy, but what about human embryo implantation?

Methods Literature review.

Results We first discuss the necessity and limits of animal models, and then review the few cytokines which have been demonstrated by knock-out methods to be absolutely necessary for embryo implantation using in animal models. We then review what is known or discussed about the role of other cytokines as deduced from quantitative and/or qualitative dysregulation in animals and in humans.

Conclusions Cytokines are indeed involved in implantation as they are in ongoing pregnancy and delivery. Relevance to infertility and recurrent pregnancy loss is discussed.

Keywords Cytokines · Animal implantation · Human implantation

Introduction

One of the major achievements of reproductive immunology is that it has moved to solving an apparent [1, 2] paradox of maternal non-rejection of the 'fetal allograft' [3]

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to the discovery that the immune system was a Janus: Whereas some maternal immune system components do represent a threat to the fetus, others are useful and necessary. Classical views of the immune system as potentially deleterious to the fetal allograft implied a state of tolerance or immunosuppression. However, allogeneic embryo trophoblast, unlike allografts of paternal tissue, does not elicit rejection, perhaps because pregnancy was not inherently dangerous and did not elicit a pro-inflammatory T helper (Th)-1 type cytokine response. The discovery, by the late A E Beer and colleagues, that pre immunization of the mother to paternal alloantigens resulted in *enhancement* of placental weight and litter size, whereas paradoxically, animals rendered tolerant to paternal alloantigens exhibited *smaller* litter size and weight [4], was the first indication that an *active* involvement of the immune system was involved in pregnancy's well being. These experiments were repeated and extended in a murine spontaneous abortion model derived by extension on a full CBA/J background in the original observations by DA Clark's group [5] of a high abortion rate in CBA/J×DBA/2, but not in C3H×DBA/2 matings, and reduced abortion rates with anti-BALB/c male immunization [6–8] and, this time, as said Tom Wegmann, it became a matter of life for otherwise compromised fetuses, since pre immunization of the mother rescued otherwise doomed fetoplacental units and resulted in litter size and weights higher even than in controls [6, 7]. This resulted in enunciation of the "immunotrophic theory " [9], which was quickly followed by the confirmation that indeed T cell-derived cytokines , namely IL-3 and GM-CSF, were growth factors for the trophoblast [10, 11] and were effective in preventing abortions vivo [12]. In the very same time, Jeff Pollard's group discovered that CSF-1 was an important factor for implantation, was expressed at very high levels in the

uterus, and its receptor was present on early trophectoderm and subsequently placental spongiotrophoblast [13–16]. Injecting CSF-1 (M-CSF) restored macrophages but not fertility whereas mating op/op×op/+ males generated enough CSF-1 for pregnancies to succeed.

Meanwhile, we repeated [12] early experiments by Parand and Chedid [17] demonstrating that TNF-α was abortifacient, and added to the list of "bad guys" IL-2 at high doses as well as interferon-γ, and we confirmed the IL-2 data of Tezabwala et al. [20]. However, reports that (very) low doses of IL-2 plus indomethacin were abortifacient [18] were not confirmed [12, 19]. This demonstration that several inflammatory cytokines were "bad guys" was very quickly followed by enunciation by Tom Wegmann of what is now known as the "Th1/Th2 paradigm" [21, 22]. That theory was still set up in the conceptual framework of a (revised) "tolerance to the fetal allograft". Initially envisaged as "immunosupression", materno fetal relationship was still seen as a "depressed cellular immunity", with a predominance of Th2 cytokines, Th1 cytokines being seen as harmful to the feto placental unit, by activating NK cells that were seen as potentially cytotoxic/cytostatic for the fetus. It became quickly apparent that this view did not apply to pre- and peri-implantation events. As early as 1992, independently but concurrently, the groups of Mc Master and Wood published that the uterus at that period was replete with inflammatory Th1-type cytokines [23, 24]. The second blow came from studies from the group of Anne Croy in 1997 stating that "Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice" [25]: NK (at least in that strain) were required for successful pregnancy! Where are we 10 years after?

Animal models?

Of mice and women

Relatively few cytokine gene knock-out mice (KOs) have shown effects on reproduction, as will be described. It is generally assumed that this reflects the existence of redundant backup circuits, and indeed from the teleological view point, it seems odd to many that such an important function as mammalian reproduction could be compromised by the deficient expression of a single cytokine. However, there are no back up circuits for a variety of hormones, so it was possible. Also, care should be taken when extrapolating murine data to human situation. As stated repeatedly by YW Loke, call "*ruminations about the immune system during pregnancy...mostly centered on the acquisition of maternal tolerance to the allogeneic fetus*" [2] using mice as a model have distracted us from the fact

that both antigen expression by invading trophoblast as well as the anatomical relationship/invasiveness of the trophoblast creates a situation which *is unique* to higher primates with the possible exception of some great apes. Indeed, a disease likely linked to very early implantation defects such as preeclampsia, associated also with a "shallow invasion" of trophoblasts into maternal decidua, might indeed be observed only in humans (and some anthropologists have even expressed the concept that it might be an evolutionary difference between Neanderthals and *Homo sapiens* [26, 27]. However, for obvious technical reasons, we have no serial data on blood pressure during pregnancy in such species as gorillas, or orangutans. It is worth pointing out that in the mouse there is trophoblast invasion of the central artery feeding the placenta as described by Redline and Lu [28] but changes in the walls of maternal decidual arteries in mice are due to interferon (IFN)-γ rather than trophoblast invasion. The most obvious difference between human and animals is seen when dealing with pregnancy in ovine species! Here, the corpus luteum is maintained by a material, originally named trophoblastin by its inventor, sequencer, and cloner, J Martal, and rechristened oTP by the Americans, which is a bona fide new class of interferon (IFN-tau) and is therefore an *absolute* requirement both for successful implantation and pregnancy maintenance in the sheep, goat, and related species. It has important immunological activities, and is likely involved in establishing Th1/Th2 balance [29, 30]. However, when turning to the pig, one sees only expression of γ and δ interferons, and no oTP-like material has ever been detected in human. In the mouse, it is pituitary LH that maintains the ovary until the placenta can take over hormone production. Even though different species have developed different molecular mechanisms to achieve the same result, the objective of maintaining ovarian hormone production is the common theme.

Yet, for obvious technical and ethical reasons, we are left with no possibility to study ex vivo human uterine implantation sites. In vitro models often are "reductionist" and do not study the whole organ, so there are various possible biases introduced by cell selection during the isolation procedure, not to mention variations imposed by the culture system used. The measurements of cytokine expression in abortion tissues are always suspicious of having been modified after the induction of abortion itself, and extra-uterine pregnancies are known to differ from the physiological implantation site (e.g. by the lack of NK cells there as well as absence of spiral arteries [31, 32]). Prospective studies sampling uterine tissues post coitum and/or on the day of embryo transfer in IVF cycles are the best we can do to approach to the real situation [33, 34], and amongst several groups, the one of Sarah Robertson has excelled in that respect [35]. Thus, we are partly left

with animal models, mostly rodents, because mice and rats have a hemochorial placenta which is often (wrongly) regarded as close to the human situation as one can get. But primate models are expensive, and in the case of chimps, of very limited "availability", not to mention the rising concerns about experimentation in species closely related to humans.

The gene deficient and knockout (KO) mice

Studies in mice can be disappointing and/or misleading if not scrutinized with extreme care!

As already mentioned, CSF-1 was initially thought to be a major growth factor for trophoblasts, and CSF-1 deficient op/op mice were initially found sterile in homozygous matings, and it was then thought that implantation was deficient in the op/op mice due to lack of macrophages in the uterus [36, 37]. Then, surprisingly, op/op females mated with heterozygous males proved to be fertile, albeit at a reduced rate, and placental weights were normal, whereas systemic treatment with CSF-1 that restored macrophages failed to correct fertility. It was later on shown that the story was even more complex, since csfmop/csfmop male mice were shown to have both a reduced mating capability and too few viable sperm [38, 39]. Similarly, GM-CSF KO were first thought to have reduced implantation rate since both GM-CSF KO and GM-CSF+CSF-1 double-KO mice exhibited lower litter size than control [39, 40], but re-examination of the phenomenon showed that fetal loss was occurring by an increased resorbtion (abortion) rate post implantation [41], in agreement with the immunotrophic hypothesis and our own data on protective role of GM-CSF in a murine abortion model [12]. IL-3 KO mice also had a normal implantation rate, and IL-3/GM-CSF/IL-5βc receptor KO mice as well as IL3Rβ-deficient animals, or both, were not reported to have reproductive problems [42]. Women with pregnancy loss due to the anti-phospholipid antibody syndrome (APS) are low IL-3 producers [43] and in the CBA×DBA/2 system [12], IL-3 injection prevents abortion. In fact, a triple KO (GM-CSF+IL-3+CSF-1) is needed to test the original immunotrophic hypothesis for abortion prevention and the role of those cytokines in implantation.

As for IL-1, Simon et al. [44] published in 1994 a headline paper in *Endocrinology* showing that "Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist". However, Stewart and Cullinan [45], and then others, analyzed the reproduction of mice deficient for IL-1 and IL-1Rt1 [46] (it is an isoform of IL-1 receptor- named Il-1 receptor type 1- which is the only one which mediates transduction upon IL-1 binding). Results showed that mice lacking this receptor for IL-1 did *not* exhibit any significant alterations in their reproduction, apart from a slight reduction in litter size. Also no reproductive impairment

was seen when Horai et al. [47] examined IL-1α/β double KO mice nor with mice deficient in IL-1α, IL-1β, or the IL-1R antagonist (IL-1ra) genes. The pups were born healthy, and their growth was normal except for IL-1ra KO mice, which showed growth retardation of pups after weaning [47, 48]. Simon himself admitted in an INSERM Philippe Laudat conference which we organized that he could not repeat 100% implantation blockade. One possible explanation (which we favor) is that the IL-1R antagonist preparation, generated in *E. coli*, was contaminated by minute amounts of LPS which would have induced abortifacient levels of TNF-α. Simon, however, wrote that "it should be mentioned that, although there appears to be certain evidence for the important role of the IL-1 system in murine and human reproduction" (and indeed Simon's group has described in great detail the distribution of the IL-1 system in the human female reproductive tract) IL-1R tI$^{-/-}$ knockout mice, even while having smaller litter sizes when compared to wild type IL-1R tI$^{+/+}$ mice, were able to reproduce.

Transgenic models are excellent tools to examine functions driven by single genes. This, however, is not the case for most reproductive functions, which are based in redundancy from the processes of implantation to parturition. Implantation is one example wherein redundant mechanisms are critical. Transgenic models therefore cannot be considered the ultimate validation of physiologic processes of reproduction that depend on redundancy for the survival of the species". This alternative explanation is not necessarily only a "*pro domo*" argument: It is indeed conceivable that absence of a cytokine during early embryonic life results in the expansion of a redundant alternative pathway, and that such a development cannot take place in adult life. Nevertheless, data from Simon suggest that IL-1 is important, though not critical [49–53].

However, there DO exist cytokines whose KO, or KO of their receptor, results in total implantation failure. The best known one is LIF, which was *the first* cytokine to be shown to be absolutely necessary for implantation in mice: LIF KO results in total implantation failure, and even LIF$^{+/+}$ embryos do not implant in a LIF$^{-/-}$ mother. This can be corrected by continuous injection of recombinant LIF [54]. In humans, a certain number of sterile women have LIF deficiency as assessed by measurement of LIF production in the supernatants of endometrial explants and/or in uterine flushings [55–57]. CSF-1 and IL-1 are LIF-inducers in a progesterone-prepared environment [55], and it is generally agreed (except by Hambartsoumian [58, 59] that LIF production in humans is boosted by progesterone as in animals. In the mink, where delayed implantation exists, a progesterone boost also induces LIF and subsequent implantation of "dormant" blastocysts [60]; it has been shown in a murine delayed implantation system that

progesterone induces production of IL-1 and IL-6 by the previously dormant blastocysts [61]. There are LIF over producers among sterile women [62–64] so not all sterility is due to LIF deficiency! Also, in our hands excess LIF and an abnormal localization are associated with failure of human implantation, not unlike a 'too high' CSF-1 environment which is abortifacient [65, 66], high levels of LIF might reflect (or trigger) an ongoing "chronic Th1 response" [67]. It must be mentioned that many people are aware that a well known multinational pharmaceutical company has performed in sterile women a rather large scale trial of recombinant LIF. Unfortunately, LIF was given to women without, surprisingly enough, selection of only those women displaying LIF deficiency (a small fractions of female infertilities), and thus the effect of LIF in those most likely to benefit from treatment was diluted by the result in the non LIF-deficient infertile population. It is therefore not a surprise that no significant improvement was observed in the treated group. Unfortunately this rather badly designed study impacts on the prospect of future trials being launched by other firms. Recently, in mice, LIF has been linked to the wnT beta catenin pathways and the importance of this pathway will be discussed below.

LIF is considered to be a pro-inflammatory Th1-type cytokine, and another pro-inflammatory cytokine whose defects render mice sterile is IL-11. Further, mice KO for the IL-11R receptor have impaired decidualisation leading to implantation defects [68–70]. In humans, IL-11 has also been shown to play a role in decidualisation [71], and it was once thought it was acting via IL-1β. However, the IL-1β released in response to IL-11 in vitro is not the bioactive [72] isoform, so the mechanism by which IL-1β acts is still obscure, though it seems to involve the STAT3 and SOCS3 pathway [73]—and indeed, in an elegant study conducted in mice, Stat3 peptide inhibitor reduced embryo implantation) specifically by 70%[73]. Interestingly, there is a report linking IL-11 with differentiation of NK cells [74] Trophoblast cells differentiated and expressed placental lactogen-1 in IL-11Rα⁻ mice, but they did not seem to proliferate. There were marked anomalies in the decidual vasculature, and differentiated perforin-expressing uterine natural killer (NK) cells were virtually absent from implantation sites of IL-11Rα mutant mice. Direct evidence for a role of IL-11 deficiency in women with defective implantation is still lacking, albeit a decreased synthesis of IL-11 in the endometrial epithelium was noted in studies of recurrent aborters [71, 74], and reduced levels of endometrial IL-11 (and LIF) was noted in women suffering implantation defects as well as when women who were compared to fertile women were selected as infertile with endometriosis [75–77]. These data are consistent with data suggesting that natural cycles may be better for implantation, and reduced expression of IL-11 and IL-6 occurs in the peri-implantation endometrium of excessive ovarian responders [78, 79].

Il-15

IL-15 is expressed in the reproductive tract [80], and is a growth factor for uterine NK cells in mice and human [81, 82], and it also activates the cells to display high levels of granzyme and perforin [83]. In a series of elegant experiments, using recombination-activating gene (Rag) 2/common cytokine receptor γ chain deficient mice, (Rag2$^{-/-}$γc$^{-/-}$) Barber and Pollard demonstrated that uNK cells likely originate from the bone marrow and require IL-15 to develop in the uterus [84]. Surprisingly, those mice proved rather resistant to Listeria monocytogenes infection. The most important finding in this series of experiments was that IL-15 KO mice lacked uterine NK cells but did *not* show implantation or peri-implantation defects nor fetal resorptions, in contrast to the results of Guimond et al. [131] using the TgE26 mouse. The embryonic problems in the TgE26 mouse may be due to loss of the T cells, or perhaps an altered intestinal flora (as LPS is known to cause both implantation failure and resorptions). However, in agreement with the Croy group studies, the loss of uNK cells in IL-15 KO mice did result in failure to remodel (thin) the maternal arterial walls and there was a hypocellular decidua basalis. These defects in uNK-deficient Rag2$^{-/-}$ γc$^{-/-}$ mice were correctable by bone marrow transplantation that restored the uNK cell population [84]. In humans, *elevated* IL-15 [34, 85] (and, surprisingly, in one study, the "Th2 like" IL-13 [85]) have been associated implantation failure and recurrent spontaneous abortions (see Table 1). IL-15 levels, as other cytokines, seem to be controlled by sHLA-G [86].

IL-6

IL-6-deficient mice have yielded conflicting results: For Poli, homozygous males and females are fertile [87] but for Robertson, these mice have a decreased litter size associated with fewer implantation sites compared to controls [88]. Zenclussen et al. [89] found elevated serum IL-6 levels in a murine abortion-prone mating combination, but Robertson's group found a reduced expression of IL-6 and IL-1α mRNAs in secretory phase endometrium of women with recurrent miscarriage [35].

IL-5

Robertson reported that implantation rates and subsequent fetal development were comparable in IL-5$^{-/-}$ and IL-5$^{+/+}$ C57BL/6 mice, irrespective of whether pregnancies were sired by syngeneic (C57Bl/6) or allogeneic (CBA or

Table 1 Selected cytokines in implantation versus post-implantation events

Cytokine	Implantation		Post-implantation (abortion/resorption)	
	Success	Failure	Success	Failure
IL-1		Ab neut (?)	Helps	
IL-2		High doses cause		High doses cause
IL-3	High doses help			High doses prevent
IL-4	High doses help			
IL-5		No effect		No effect
IL-6		High doses cause (?)	High doses (?)	
IL-8		ND		ND
IL-10	No effect		Helps in resorb prone strains	
IL-11		KO causes		
IL-12		High doses cause		High doses cause
IL-13				Causes (?)
IL-15	KO no effect except arteries			KO no effect except arteries
IL-18		High doses cause		High doses cause
IL-23		High doses cause		ND
IL-27		High doses cause		High doses cause
LIF		KO causes		
CSF-1		KO causes		
GM-CSF		KO no effect	Prevents	KO increases
TNF-α		High doses cause		High doses cause
IFN-γ	KO no effect			High doses cause
TGF-α	Required for adhesion			ND
TGF-βs			Prevents	KO may cause (?)

BALB/c) males. There was a 10% increase in placental size and a 6.5% decrease in placental/fetal ratio seen on day 17.5 in pregnancies sired by CBA males [90].

Transforming growth factors (TGFs)

TGF-βs are well known to be immunosuppressive and also to act on the LIF/IL-6 pathway [91, 92], enhancing the former and decreasing the later for in vitro cultured endometrial cells, including in the human. In mice, besides immunosuppressive properties, TGF-α promotes mouse blastocyst outgrowth and secretion of matrix metalloproteinases. TGF-β1—and mildly TGF-β2 or TGF-β3—also stimulates mouse blastocyst outgrowth [93–95]. Other results in rats also suggest that under hormonal regulation TGF-βs repress sialomucin and thus the sialomucin complex (SMC/Muc4), SMC being an anti-adhesive glycoprotein, and thus permitting implantation [96]. Therefore, TGFs are likely very important in implantation. However, KOs studies are seriously hampered by the fact that 50% of TGF-$\beta 1^{-/-}$ embryos die in the uterus and the other 50% early after birth, before reproductive age [97], and thus it is often concluded that the studies did not yield conclusive results. However, using a human TGF-α transgenic mouse, it has been shown that its inappropriate expression down-regulates uterine expression of TGF-β receptor subtypes and delays the attachment reaction with deferred uterine expression of amphiregulin [98]. I will not detail here the well-known effects of the TGF-β2 isoform in pregnancy and invite the readers to refer to [99–101].

WnT

Recently, very elegant studies by the group of Dufort et al. [102] have that activation of the Wnt/β-catenin signaling pathway is required for implantation, and this depends on soluble embryonic factors; The Wnt/β-catenin involvement occurs at two different stages. Implantation first requires a transient activation in circular smooth muscle on early day 4. Subsequently, activation is restricted to the luminal epithelium at the prospective site of implantation. Subsequent experiments have shown that the pathway is involved in LIF regulation.

Tumour necrosis factors (TNF)

TNF-α is markedly over expressed in the pre- and peri-implantation uterus, in marked contrast with its limited expression during established pregnancy [23, 24]. It is generally assumed from a lot of studies as early as the ones of Parand and Chedid [17] and ours [12] that excess local TNF-α also reduces litter size or prevents implantation in mice, rats, and humans [103]. For cattle, refer to [104–106].

One should note that of course in contrast to animal studies, human effects are deduced from correlative studies on serum and local TNF-α levels in sterilities/RSA women and no direct injection of TNF-α, nor LPS, nor a TNF-α inducer, has been ever attempted! But there have been studies in primates with neem extract, a Th1 cytokine inducer [107] However, TNF-α (as well as LIF, TGF-β, IL-1, IL-6 and insulin-like growth factor binding protein-1 (IGFBP-1) markedly influence the secretion and/or activation of MMP-2 and MMP-9 according to Bischof [108, 109], thus promoting early and late trophoblast invasion. One possible explanation would be that several effects of TNF-α are prevented in early implantation by *tweak* [110] as in other systems [111]. However, TNF-α KO mice have perfectly normal implantation rates so TNF-α is not required for normal implantation [112].

Th2 cytokines

It is noteworthy that all the KOs described above, which are amongst the (few) where implantation defects have been detected, concern pro-inflammatory Th-1 cytokines. In the CBAxDBA/2 murine abortion model where there is a pro-inflammatory cytokine response, and an IL-10, IL-3 and IL-4 defect, and anti-IL-10 enhanced resorbtion rates. However, there was *no* effect of anti-IL-10 in other, non abortion-prone murine mating combinations [113]. The first IL-10 KO mice had *no* fertility problem, and that was confirmed with single and multiple Th2 cytokine KOs, including a quadruple Th2 cytokine KO (but with normal IL-3 production) [114–117]. In humans, there is evidence from a variety of studies that Th2 cytokines defects in production/ and/or expression are associated with early pregnancy loss see [103]—albeit the very existence of a Th2 bias has been debated [118]. But there is no convincing data as far *as implantation* itself is related to Th2 cytokine deficiencies. The Th2 like cytokine, IL-13, is present as a "Th2" shield all around the peri-implantation embryo [81] but its KO does not affect implantation [115]. Excess IL-13, is associated with RSA [85], but there is *no* evidence for implantation problems.

Altogether, the KO mouse data did not *seem* to support a major role of Th2 cytokines in implantation, but rather point out that several pro-inflammatory Th1 and Th1-like cytokines are important, the presence of some of them being an *absolute* requirement . However, an important point to add is that for most of these KO, or antibody mediated cytokine neutralization, the deduction that a cytokine is not required for successful pregnancy stems from studies conducted in the KO strain itself, e.g. with congenic matings, which means syngeneic pregnancies where there are no paternal allloantigens. Further comments on this issue are provided at the end of this essay.

Cytokines in the semen

The uterus seems to be prepared for implantation by cellular and cytokine constituents in seminal fluid, and indeed a human endometrial cellular response comparable to what is observed after mating in mice have been reported [110]. The cellular influx, and most important, surge in local production of proinflammatory cytokines observed after mating in mice is not seen when females are mated with vasectomised mice which deliver no seminal fluid. Elegant studies by Robertson's group imply seminal TGF-β (which induces production of GM-CSF) as pivotal to such preparation of the uterus, and possibly promoting maternal immunological tolerance to paternal antigens, a phenomenon which may be important in preventing preeclampsia as described by Robillard et al. [27, 28]. The reader is referred to their excellent reviews on that topic [119, 120].

The role of the immune system on local vascularization

An embryo can implant but cannot develop without an increased blood supply form the mother. An "angiogenic" role of NK cells was predicted by Loke in 1991 [122] when he wrote that uterine NK cells "may have a role in the control of implantation and the transformation of the uterine vasculature by trophoblast on which the blood supply to the fetoplacental unit depends". In fact, there was already more and more data showing that the implantation uterus stroma was full of NK cells (up to 60 to 80% of the " stroma" in mice and human, which is more lymphocytes than are present in some lymph nodes) [123–129]. For years, NK cells were seen as "bad guys"in the context of the classical Th1/Th2 paradigm, and their "activation" towards a cytostatic/cytotoxic pathway by Th1 cytokines was believed to lead to pregnancy failure, albeit there were relatively few indications in animal models (except for activation by poly I: C) that activated NK cells could affect implantation itself (rather than causing early pregnancy loss as in the CBA×DBA/2 model [121]). NK-lineage cells infiltrating the uterus were activated as early as day 6.5 to *massively* secrete IL-18 (a cytokine that promotes secretion of IFN-γ), and there was more IL-18, in the non abortion prone mating with BALB/c than in the abortion prone one with DBA/2 [129]. Yet, IL-18, alone, or with IL-12, is abortifacient in established pregnancy, and has been implied to play a role in a murine preeclampsia model, as first reported by a Japanese group [130]. The role of uterine NK (uNK) cells was further elucidated by Guimond et al. [25, 128, 131]: NK deficient TgE26 mice (which also have a T cell defect) had profound reproductive defect with reduced placental size and lack of transformation of the uterine arteries which retained too thick arterial walls, a

feature ultimately leading to a high percentage of fetal deaths. As previously mentioned, thick vascular walls, absence of transformation also occurred in IL-15 KO mice, and fetal weight was reduced, but there was no fetal lethality. However, implantation was *not* affected in any of these NK deficient mice. Subsequent experiments proved that IFN-γ was a key activator of NK cells and as a product of NK cells, caused vascular wall remodeling directly altering arterial wall thickness in NK-deficient pregnant mice [132–136]. IFN-γR KO mice also have vascular wall anomalies. Uterine NK cells secrete several angiogenic factors, including angiopoietin 2 and VEGF [134–140]. It should be recalled here that high doses of IFN-γ can prevent implantation in mice and an "immunodystrophic hypothesis" stems from correlative studies in human [142–144]. Such *high* doses of IFN-γ are also abortifacient during "established pregnancy", synergising with TNF-α and having a procoagulant induction effect [reviewed in 103]. But, at *lower* doses, IFN-γ is involved in promoting a beneficial placental phagocytic activity [145]. IFN-γ and IL-15 activate pre-/peri-implantation murine uNK cells, and at the relatively low doses which arise post-coïtum when T cells and macrophage move in to dispose of those spermatozoa and lymphocytes which die in situ.

As far as VEGF is concerned, it is expressed in the pre- and peri-implantation uterine stroma proper, in addition to uNK cells, in human and animals [141, 146–151], including in species with delayed implantation such as the mink [152]. In some species, VEGF is expressed in placentomes [153]. VEGF is partly regulated by hormones during the cycle as well as chorionic gonadotropin at implantation and [146, 147, 153–157]. Indeed mefipristone down-regulates local VEGF production [158], whereas, conversely, HLA-G (a non polymorphic Class I HLA antigen present on extravillous human trophoblasts), while inhibiting NK cell mediated cytotoxicity [2, 122, 159–161], up-regulates VEGF production by uNK cells [162], a property fitting with a role of HLA-G in angiogenesis [163]. These observations have led to studies showing prevention of implantation in rhesus monkeys by injecting neutralizing antibody to VEGF-A "apparently through direct antagonism of the action of VEGF-A in the endometrium" [164]. In addition to VEGF, there are also soluble angiopoietins now described.

Angiopoietin-2 (Ang-2) is secreted mostly by uNK cells, in mice and human, rather than uterine stroma [136–140, 146, 164–166]. In fact, in mice Ang-2 mRNA and protein expression is seen in uterus in both the estrogen-dominated cycling phase and the progesterone-dominated mated phase, whereas Ang-1 expression was restricted to the mated phase .However, Ang-1 is also expressed by pre-implantation mouse embryos and may act as a possible complement to expression of mouse uterine: Angiopoietin-1 mRNA was found to be expressed throughout development in 78% of zygotes, 66% of 2-cell-embryos, 71% of 4-cell-embryos, 70% of 8-cell-embryos, 60% of morula stage embryos, 48% of early blastocysts, and 78% of late blastocysts. The number of Ang-1-expressing embryos in the early-blastocyst group was significantly different in comparison with zygotes, 4-cell-embryos, 8-cell-embryos and late blastocysts. However, Ang-2 mRNA and protein expression could not be detected in pre-implantation embryos [165].

Robertson et al. have shown that GM-CSF induced by TGF-β promotes implantation and can stimulate metabolism and development of pre-implantation embryos. GM-CSF is also a product of uNK cells [159]

Quantitative local dysregulations

The total (systemic+local) absence of a cytokine is in fact unlikely to be observed in human for the first time at reproductive age. For example, the aforementioned murine LIF$^{-/-}$ embryos give rise to mice which have subnormal levels of ACTH, absent nerve repair after injury, defective neural stem cell renewal in the adult brain, postnatal maintenance of distal axons and motor endplates. LIF plays a protective role in endotoxic shock and host defense, and with IGF-1 facilitates lung maturation, etc. It is thus very unlikely that women with global LIF deregulation would reach reproductive age without being affected by several other LIF deficiency-related pathologies. In infertile women LIF deficiency was diagnosed by examination of local LIF production, be it by flushing, immunohistochemistry or quantification in explant culture supernatants or by RTPCR for mRNA [55–57]. So, local dysregulation which affect only either the uterine part of the feto maternal interface or are affecting the embryo itself in an autocrine fashion are likely most important. A further example is the local production of IL-12, and IL-18 [33, 35, 83]. The local absence of those cytokines in a pre implantation uterus in our hands is associated with a very low number of NK cells, and as a correlate, low production of angiogenic cytokines, as is also the case in low IL-15 producers. It is interesting to note that in several of those women a more global lack of production of implantation related cytokines by the cycling uterus is demonstrable. A possible explanation would be a defect in an important upstream signaling pathway, such as Wnt, or such genes as homeobox HOX-7.1T/Msx 1. It is hoped that Microarray studies will eventually identify such upstream regulators. Alternatively, there may be an abnormal response to the IVF stimulation protocol. Ledée-Bataille et al. [78] have suggested this by comparing IVF in natural cycles to cycles where oocyte production was induced, coming to the conclusion that

"Controlled natural in vitro fertilization may be an alternative for patients with repeated unexplained implantation failure and a high uterine natural killer cell count". This is further supported by the aforementioned studies showing that a reduced expression of IL-11 and IL-6 in peri-implantation endometrium is observed in excessive responders to ovulation induction [79], and may account for lower implantation rates.

It is interesting also to note in that context that reduced endometrial IL-11 and/or LIF may also contribute to infertility in some women with endometriosis and/or recurrent miscarriages. Defective production of LIF, CSF-1 (M-CSF) and Th2-type cytokines by T cells at fetomaternal interface is also associated with pregnancy loss [167–169]. In line with the initial assessment of infertility in op/op mice, the number of uNK cells as well as the number of uterine leucocytes expressing CSF-1 and c-fms mRNAs was substantially lower in the uteroplacental unit of mice with pregnancy loss than in control animals [170].

In addition, in mice and humans, IL-18s regulated by EBI3 or IL-18 BP (IL-18 binding protein). In humans, abnormal regulation correlates with a pathologic sub-endometrial vascular flow index (VFI), and IL-15 levels correlate with high IL-18 levels in sterile patients, but not completely with excess NK counts so that the main effects are likely NK cytotoxic activation rather than replication which seems more strongly correlated to the ratio IL-18/IL-18 BP [33, 34]. Experimentally, as stated above injection of high doses of IL-12 or IL-18, or both, produce abortion and /or a pre eclampsia like syndrome in mice [130, 171, 172].

Many other examples of implantation defects linked with too high a production of cytokines or abnormal localization exist! Too high levels of LIF in the luminal fluid are predictive of a poor IVF-ET outcome, and this might reflect as stated above a "chronic Th1-like hyper-activation" [67], as are poor prognosis patients with detectable levels of IL-18 in luminal fluid [173]. Similarly, too early too high levels of CSF1 are abortifacient: Pre-immunizing B6 mice with a syngeneic tumors that regressed due to an effective host anti-tumor response also prevented normal gestations when the tumor-immunized mice were mated to C57BL/6J×DBA/2 F-1 (B6D2F1) males or DBA/2 males but sustain normal pregnancies when impregnated by CBA/J or C57BL/6 males. Thus, as in other murine abortion systems, susceptibility to embryo rejection was highly strain dependent. An investigation into the cause of these male-specific pregnancy failures led Tartakowsky to propose that CSF-1 was responsible for both pregnancy-block and resorption of embryos. Indeed, injection of very small amounts of CSF-1 into mated (plugged) females, during the first 5 days of pregnancy, was sufficient to block B6xB6D2F1 gestations but had no effect on B6xCBA/J matings [174, 175]. Too high levels of

TNF-α? as a consequence of infection, or direct injection, or with very high doses of Poly IC, also prevent implantation and/or induce abortions [103]. The effect observed on abortion rates is not mediated primarily by cytostatic/cytotoxic effects on embryo, but rather by action on the coagulation pathway, and this can be prevented by anti-FGL2 prothrombinase [176]. These studies paved the way for those implying CD200/CD200R in prevention of abortion pregnancy loss [101]. Studies conducted at low doses of Poly IC which do not affect the number of implantation sites show that it also induces early post implantation impairment of uterine vascular remodeling in CBAxDBA/2 mice [103, 121]. Cytokines influencing Th1 and Th2 cell differentiation, including IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-12p40, as well as dendritic cell-regulating cytokines IL-1α, IL-1β, IL-6, LIF, GM-CSF and TNF-α were also expressed similarly regardless of fertility status. This is, incidentally, in marked contrast with data on such cytokines claimed in some murine studies, where some labs claiming the surprising "complete prevention of abortion" in a murine model of spontaneous abortion (e.g. well below the well known irreducible "genetic background" linked to chromosome anomalies).

TNF-α is also implicated in the pathways mediating stress related implantation failure or early pregnancy loss: for review see [177, 178]. However, it should be noted that 'occult' loss observed, for example, in C57BL/6 mice, which is in fact an implantation failure, did not require TNF-αR1 [179, 180].

In lines with those data and other linking excess Th1 cytokines and early pregnancy loss abortion, excess expression of IL-23 [110, 181] or IL-27 is/seem abortifacient [110] but, as stated above and paradoxically, excess Th2 like cytokine IL-13 seems to be involved in some abortions [85], though this is not corroborated by two more recent studies, which, however, do not check endometrium but serum levels [182, 183].

IL-6 data are contradictory. IL-6 was found to be elevated in abortion prone mice by Zenclussen et al. [89], found elevated in human MPLR by the Raghupathy group [184], but most other studies have not found such a correlation with either abortion or early implantation failure—see for brief review [39].

Reports on local production of TGF-β and implantation are contradictory: Most of them find an association between TGF-β deficiency and implantation failure / recurrent pregnancy loss, but there are nonetheless reports that do not find such correlation [39]. Most have studied TGF-β1, but there are other isoforms that can have specific effects. TGF-β2-like factors are implicated in abortions in mice and in humans, whereas inhibition of trophoblast growth by TGF-β3 has been implicated in pre-eclampsia [185, 186].

Immunoregulatory T cells

Foxp3$^+$ regulatory T cells Treg cells) have been implicated in prevention of early pregnancy loss and abortion in allogeneic matings in mice [187, 188]. A similar role is purported in human [189–191]. For example, in a human study, foxp3 mRNA was reduced 2 fold in the uteri of infertile women, but although Treg cell differentiation is controlled by TGF beta, the relative abundance in endometrial tissue of TGF-β1, TGF-β2, TGF-β3 mRNAs was not changed in infertile women. Treg cells can act directly to suppress or by inducing cytokine-releasing Tr1 cells (which produce IL-10) or Th3 cells (with produce TGF-β).

Some additional mechanisms of action

Besides embryo growth promotion, from the uterus to the embryo, cytokines are involved in the induction/regulation of MMP/TIMPs which are important for implantation [108, 109, 192–195], as well as integrins integrin receptors [192, 196–200]. Cytokines are also produced by embryo itself, and for example as quoted Il-1 and IL-6 are produced upon maternal hormone activation of otherwise dormant embryos [61]. Recent afore quoted studies on Wnt further support the concept of embryo signals acting on the uterus [102]. However, results on cytokine detection in embryo culture supernatants and IVF success have so far failed to yield conclusive reproducible results, and thus, we will not detail them here Finally, cytokines might act before Implantation and act on the embryo itself. Of considerable interest in this context seem to us earlier data of Tartakovsky's group who obtained almost complete prevention of abortion in a variation (embryo transfer) of the classical CBA × DBA/2 murine model of spontaneous abortion by pre culturing embryos of resorbing mating combination in CSF-1 before embryo transfer. Similar epigenetic determinants (and let us recall that there is imprinting in the CBA × DBA/2 system [201]—though implantation and abortion were not recorded) were reported when in another embryo transfer system embryos were cultured in GM-CSF [201, 202].

As we discuss elsewhere [110] this is in keeping with the data of Girardi et al. [204] showing that the embryo resorption process is heralded very prior to implantation by complement activation. However, resorptions can be stopped by treatment much later in pregnancy, including by administering anti-asialoGM1, CD200Fc, by hirudin (a direct anti-thrombin), by anti-neutrophil antibody, or by anti-FGL2 prothrombinase. The immune system can activate the complement system, and C5a activates neutrophils that are recruited by cytokines released by thrombin-activated endothelial cells. Therefore the coagulation system and complement system may collaborate in the abortion process.

Conclusion

The data reviewed here establish that cytokines are important for implantation. In keeping with other data, pro-inflammatory Th1-type cytokines appear from KO mouse studies to be key determinants of attachment and adhesion stages of implantation but later on, also regulate integrins, TIMP/MMP balance, etc. In contrast, KO mice have NOT demonstrated a mandatory requirement for Th2 cytokines in the process. Proper NK cell "activation", not "dampening", is required for optimal local vascular bed transformation. In keeping with the Th1/Th2 paradigm, excess peri-implantation Th1 cytokines may induce early pregnancy loss/abortion. However, a growing body of evidence suggests the importance of NK controlled angiogenic network, and for example VEGF/sVEGFR/NK cells might be important in implantation related/initiated diseases such as pre eclampsia. However, careful examination of the networks suggests several distinct pathway of cytokine/cytokine dysregulation related implantation failure. These data point to tailored therapies, some as simple as natural vs. stimulated cycle, some involving tailored drug administration. As presented recently by Ledée, we believe the time has come, but also that such an era is just beginning!

An important point to add is that for most of these KO, or antibody mediated cytokine neutralization, the deduction that a cytokine is not required for successful pregnancy stems from studies conducted in the KO strain itself, e.g. with congenic matings, which means syngeneic pregnancies. Tregs are required for successful *allopregnancy* to proceed, but Treg depletion has *no* effect in syngeneic pregnancy [187]. Similarly, IDO neutralization has (quantitatively) strain dependent abortifacient effects *only* in allogeneic matings [180, 205]. Except for the effects of anti IL-10, which one of the authors has studied in a series of allogeneic pregnancies [113], it is not sure that allogeneic pregnancies would *not* be compromised by the KO, since the proper studies have not been conducted. It is easy to understand why (a) most of the workers likely did not even think of it (b) would have they done so, the fact that this would require two allogeneically disparate murine strains was certainly a powerful deterrent. As a typical example, as stated above and [206], T regs are implied as important for allo pregnancy, even though Wasp$^{-/-}$ mice have impaired T reg function [207–209], but these syngeneically bred mice have no noticeable reproductive defects. Notably, they have not a high resorption rate nor do they have smaller implantation rates and/or litter size (Snapper; Dupre; Roncarolo; Rawlings; personal communications).

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