

Th1/Th2 Paradigm in Pregnancy: Paradigm Lost?¹

Cytokines in Pregnancy/Early Abortion: Reexamining the Th1/Th2 Paradigm

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Abstract

In this paper, we briefly survey the history of concepts in reproductive immunology from antibody-mediated tolerance to the 'fetal allograft' to the current concept of an embryo 'bathing in a sea of cytokines'. We then review the paradigm that 'allopregnancy is a Th2 phenomenon' and some of the evidence gained in animals and humans supporting it. We continue by discussing the light it sheds on immunologically caused recurrent abortion, and the present status of the concepts. We next show the limits of the Th1/Th2 paradigm by reviewing the role of 'inflammatory' cytokines in implantation (as first seen with leukemia inhibitory factor). We go on to discuss recent data showing that interferon- γ is not solely a 'bad guy', e.g. abortifacient as the paradigm would predict, but is needed at low doses for the vascular development and transformation of uterine spiral arteries required for implantation and successful pregnancy. We conclude by discussing the emerging role of NK and IL-12, IL-15, IL-18 tripods and other cytokines in local angiogenesis and tissue remodelling, a series of new data bringing us well beyond the Th1/Th2 paradigm in pregnancy which, in

this context, appears now obsolete and an oversimplification, although it has indeed been useful at first. Rather, step-specific events have to be considered and a key role is seen in local tissue remodelling, in which immune cytokines play an important role while not always being secreted by immune cells.

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'This review is dedicated to the living memory of Tom Wegmann and the missed presence in the field of Charlie Loke'

Introduction: Jurassic Park and Others

For a very long period of time, many reviews dealing with the fetomaternal relationship started by referring to Medawar's citation classic [1], and thus the field was dominated by the concept of 'the fetus as an allograft'. As a consequence, most of the studies were dealing with the need to understand mechanisms of an implicitly T cell-mediated 'tolerance' to the 'fetal allograft'. Thus, at first,

The opinions expressed in this review are mainly those of the corresponding author.

¹ The title of this paper was inspired by the original paper of Kelsö [110], to whom we are indebted.

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1018–2438/04/1342–0093\$21.00/0

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'masking' the target to T cell, 'immunodeviating' maternal immune responses was essential. It could rely eventually on an active recognition mechanism (see for example 'facilitation'), provided maternal antipaternal cellular immunity was depressed or blocked, but there was a target, since the confirmation that neither was the uterus an immune privileged site, nor was the placenta class I MHC-negative fuelled the search for adaptive mechanisms.

It was the era of 'facilitation' and 'enhancing antipaternal antibodies' whose elution and rebinding to the proper paternal strain only trophoblasts was the first demonstration by Voisin and Chaouat [2] of paternal MHC antigens in the murine placenta; this was followed almost immediately by the demonstration of their enhancing properties by Chaouat and colleagues [2, 3], 'blocking factors', 'nonspecific systemic immune suppression' (pregnancy-specific proteins, alpha fetoprotein, human chorionic gonadotrophin (hCG), all were thought to be involved) with as an example the rather (in)famous early pregnancy factor detected by immunosuppression as inhibition of T cell rosette formation with sheep red blood cells (SRBC) as described by Morton et al. [4].

Some of these phenomena do indeed exist, as proved by us. But in cooperation with Chaouat and Monnot [5], we had the dubious privilege of almost immediately demonstrating in a very Popperian approach that they were *not* necessary for successful allopregnancy but in fact other more prestigious individuals such as Mitchison [6] and Lanman et al. [7] had *already* done the crucial experiments.

Enhancing/facilitating antibodies were demonstrated first by us [2, 3]; then in the early 1970s, the existence of systemic T cell suppression was demonstrated by cell transfer systems first by Smith and Powell [8], and almost simultaneously by us [3] and later on confirmed in totally independent studies on the HY antigen by the group of Simpson et al. [9]. We then proposed on the basis of in vivo experiments that the placenta contained T suppressor inducer factors, making the scheme very reasonable, and indeed we [10] traced some, and some of these factors were even described in humans, for example by Hamaoka et al. [11], using a choriocarcinoma cell line. But these experiments were quickly followed by the realization that neither antifetal alloantibodies – which, in mice, Bell and Billington [12] found only in some 'producer' strains – nor systemic suppression were a requisite for a successful pregnancy as stated by Chaouat and Monnot [5]. Indeed, Sulila et al. [13] and Mattson et al. [14] described that B cell-depleted and Ts-depleted animals, or both, had nor-

mal pregnancies. (Some even went to study marsupials as a proof for the absence of the requirement for such immune components!)

It must be stated that systemic phenomena do exist and often coexist during pregnancy: transient and for example systemic suppressor T cell-mediated suppression, recoverable T cell anergy and clonal deletion were, respectively, demonstrated by us [3], Smith and Powell [8], Simpson et al. [9] for the former effects, and Tafuri et al. [15], Jiang and Vacchio [16] and Bonney and Matzinger [17] for the latter, while isotypic regulation was demonstrated by using anti-SRBC immunization and enumeration of plaque-forming cells by Bell and Billington [12]. They have all been demonstrated to (co)exist during a successful pregnancy.

But the aforementioned preimmunization experiments by Mitchison et al. [6], Lanman et al. [7], and ourselves [5] have shown that it is possible to obtain a state where one would detect circulating cytotoxic antipaternal alloantibodies and activated cytotoxic T lymphocytes (CTLs) without any harm to the fetus [see 5]; in fact, one can observe a second set rejection of paternal strain major histocompatibility complex (MHC) antigen tumor or skin allograft, while the fetus remains unharmed [5–7]. In contrast, Beer et al. [18] observed that when female animals were made (T cell) tolerant of (future paternal) alloantigens, fetal weight was shown to be reduced.

Nevertheless, boosted by the idea that maternal antipaternal alloantibodies were required for successful pregnancy, some, amongst them the group of Faulk and McIntyre [19], began to describe what would ultimately become termed by Faulk et al. [20] as the 'TLX (trophoblast leukocyte cross-reactive – hence the X) antigen system' and as stated by Labarrere et al. [21] the 'search' began. According to this concept, trophoblast antigens especially the 'system' named TLX were eliciting antibodies, which would in turn bind to cross-reactive antigens on lymphocytes and block their development function.

A whole TLX 'system' was described, supposedly in linkage disequilibrium with HLA, which the mother had to recognize as 'foreign'. TLX homologies between mother and father would thus spell doom for a pregnancy. It took a long time, till it was shown that the TLX system did *not* exist when Johnson et al. [22] showed that the so-called TLX antigens were not polymorphic and related to a complement-regulating factor but prior to that, this search led to the first attempt to treat recurrent spontaneous abortions (RSA) with lymphocyte alloimmunization, in this case directed against third party lymphocytes [23], to immunize against the disparate antigens missing

in case of mother/father TLX homologies. These open study results were excellent [23].

While the search for TLX was extended, and ultimately collapsed, others, and especially Beer et al. [18, 24], had deduced from their own experiments as well as from some of the aforementioned ones on systemic events that there was in any case ground for the 'belief that some kind(s) of active response(s) on the part of the female, following early cognitive events, plays an important, if not essential, role, in affording it protection from the possible hazards of a cellular immunity'. Evidence was indeed widely perceived as 'emerging' that maternal 'immune response aid in the establishment and maintenance of a harmonious state of immunological coexistence with her foetus'. Thus, this group engaged in the treatment of human abortion with lymphocyte alloimmunization against paternal alloantigens. Again, open study results were excellent, both from Beer et al. [25, 26] or Reznikoff-Etievant et al. [27], and in an open study review by Beer et al. [28], almost all the available theories were mixed in a package supporting lymphocyte immunization therapy (LIT).

We will at the end of this review come back to the present status of LIT, for, except for the first original one by Mowbray et al. [29], double-blind studies and meta-analysis data soon proved to be (by far) less impressive [30, 31].

In marked variance with Hill [32] and Chaouat and Mowbray [33] I believe the first double-blind study was very honest and was conducted with all experimental rigour available at its onset. Very quickly, however (and even during the course of such studies), it was realized that designing a real placebo was much more difficult than expected, for in alloimmunization the women might be aware that 'a placebo' was not a placebo, if the alloimmunization induced a local reaction (seen as 'positive') which the placebo did not elicit. Very similar problems were encountered during a double-blind assay of intravenous immunoglobulins (IVIG) in Belgium [pers. commun.].

This is important since we now also know that there is a molecular basis for stress-induced abortion (see below). Thus, conversely the 'love and tender care-only approach', which yielded to the surprise of many and the confusion of some results as good as LIT [34, 35], has a neuroendocrine immune basis.

Placenta as a Barrier?

Meanwhile, the discovery that it was possible to bypass systemic events made Tom Wegmann and others adopt the concept that the placenta was a 'shield and a sink' or a barrier against maternal antipaternal immune responses

[36]. They referred to it as a 'sink', because it did express MHC class I alloantigens as shown first by Wegmann et al. [37] and Singh et al. [38], to which antipaternal alloantibodies did bind as originally shown in our own experiments [2, 3]; however, Wegman et al. [37] and Singh et al. [38] demonstrated that the turnover of MHC class I molecules at its surface was so high that antibodies were quickly degraded – and amino acid components assimilated – before they could harm the very trophoblast cells to which they bound. (There have even been estimates of the extra nutrients brought to a semiallogeneic fetus vs. a syngeneic fetus by this process, which was before immunotrophism and for a short period of time considered to be an explanation of hybrid vigor.)

It was referred to as a 'shield', because the placenta exhibited anticomplement activity. This phenomenon was quickly attributed to the expression by the placenta of complement regulatory proteins, membrane cofactor proteins in humans as shown by Johnson et al. [22] (whose discovery, incidentally, meant the end of the TLX 'system'). To this, the human placenta adds the expression of decay-accelerating factor as shown by Holmes et al. [39].

The importance of such components cannot be ignored, since mice knockout (KO) for the CRRY [complement receptor 1 (CR1)-related gene y] regulatory protein cannot have a successful pregnancy as shown by Xu et al. [40]. It was also a shield, because it seemed to prevent the emergence of antipaternal cellular immunity. Mice producing antipaternal alloantibodies in the first pregnancy were named by Bell and Billington [12] as 'producer strains', and in the human around 25% of primiparous and up to 75% multiparous women do produce antipaternal Ig, be they IgM or IgG. In the advent of transplantation immunology, sera from multiparous women were thus indeed often used for tissue typing. But even in antipaternal alloantibody 'producer' strains, Bell and Billington [12] and others observed that there was no sign of pregnancy-induced antipaternal CTLs, hence by then the concept of 'split tolerance', e.g. a tolerance which would affect only cellular events leading to graft rejection (with or without a possible 'enhancing' role of antibodies).

This finding of a spared maternal immune response will be one of the key phenomena at the origin of the application of the Th1/Th2 concept to pregnancy. In humans also no CTLs were ever traced in 'immune abortions', except perhaps a transient surge during the last hours of the abortion process in recurrent spontaneous aborters, observed in 3 patients. Anyhow, we [5] had shown that the placenta could resist activated CTLs when

these were induced by voluntary alloimmunization. This turned the tide to the examination of local suppression.

Indeed, as early as the 1980s, Clark et al. [41] had observed a defect of a soluble suppressor factor in the lymph nodes of CBAJ (H-2^k) females mated with DBA/2 (H-2^d) males, whereas control C3H (H-2^k) mated with DBA/2 did not exhibit this defect. In a (long patient) series of elegant experiments, they finally showed that this was in fact a reflection of a differential production of a decidua isoform of TGFβ2 [41–44].

The placental suppression was also thoroughly investigated.

Evidence was given for suppressor factors acting at the afferent phase of maternal immunity by Chaouat and Kolb [45], Menu et al. [46], and Saji et al. [47], which De Smedt et al. [48] and Voluménie et al. [49], and subsequently others, have shown to induce T cell anergy by acting on the TCR ζ chain. It appears from experiments of the group of Delves and Roitt [50] that the active moiety might well be prostaglandin E₂, but then the problem of the heat resistance of the factor and thus of a protective carrier molecule as seen by our group and confirmed by that of Delves [48–50] remains in fact unsolved.

Evidence was also found showing that placental cells were resistant to T cell- and NK-mediated lysis, as shown by us [51], Croy and Rossant [52] and Zuckermann and Head [53]. We attributed this to the placental secretion of efferent stage suppressor soluble factors [51], shown by Chaouat and Kolb [54], which were disputed by others [52].

It has now been shown that in humans such factors do exist, HLA-G, and especially sHLA-G1 and G2 being able to decrease, if not block, NK cell-mediated lysis, whereas the soluble isoforms can also induce apoptosis of activated T cells [55, 58–60]. (The role of HLA-G is beyond the scope of this review, but HLA-G is thus involved in tissue protection against NK and CTL killing, plays a role in the presentation of viral peptides to maternal T cells, and seems to regulate angiogenesis [58, 60]. Pregnancy, however, is possible in women harboring the HLA-G null mutation – but they still secrete sHLA-G2 – and in several monkey species where HLA-G homologs are missing or nonfunctional.)

In mice (and humans) another system, Fas/Fas ligand interaction, offers an additional backup as suggested by Hunt et al. [61], Runic et al. [62] and Uckan et al. [63]. Indeed, any activated T cell which will bind the placenta will thus undergo apoptosis. We have shown together with Clark [64] that the system, however, is not mandatory for successful allopregnancy and this is confirmed by Rogers

et al. [65]. However, there are limits to such a protective phenomenon. We [66] have shown that high doses of IL-2 were shown to be abortifacient in mice. This has generally been confirmed.

This is partly due to activated NK cells (see below) but lymphokine-activated killer cell (LAKC) involvement has also very quickly been suggested by the group of Head [67, 68]. LAKCs can indeed kill trophoblasts, which normal NK cannot do, and this killing is seen despite an expression of membrane or sHLA-G. Indeed, resistance of placental or choriocarcinoma cells to CTL- and NK-mediated lysis, which despite claims to the contrary has been convincingly shown to be an HLA-G-independent phenomenon by Avril et al. [69] and Sivori et al. [70], does not extend to the LAKC-mediated attack. Furthermore, there are limits to the effects of all those suppressors and protected mechanisms. For example, Munn et al. [71] have shown in very elegant experiments that if one blocks indoleamine 2,3-dioxygenase in pregnant mice, CD4 are then activated and a CD4+-mediated abortion is seen in allogeneic pregnancies, but *not* in syngeneic matings, testifying for an immune recognition-dependent mechanism.

Of interest is the fact that *no* CD8+-mediated abortion has been demonstrated so far, and in fact there is not even convincing proof that there is homing of CD8+ T cells towards the placenta: amongst others, unpublished experiments which we conducted with Tom Wegmann using ¹¹¹In-labeled T cells were barely conclusive. This and the lack of activated macrophages during pregnancy have led Fauve et al. [72] to propose the ‘immunorepulsion’ hypothesis, and it is indeed striking to observe the cellular changes immediately following the preimplantation period (see below).

It is also important to mention that for the group of Baines and colleagues [73–76] the main abortifacient cells are activated macrophages purported to be mediated by nitric oxide (and indeed an excess of oxidative stress is known to be a very effective anti-implantation and early abortifacient pathway).

In keeping with this a residual abortion rate can still be observed in a murine model of spontaneous abortion, the CBA × DBA/2 model (see below) after NK depletion, suggestive of a minor, but real, macrophage involvement as discussed by joint experiments of the group of Clark and my group [77]. Anyway, this set of data led Tom Wegmann to dedicate even more of his attention to the placental interface itself. It was the beginning of a series of experiments which would lead to immunotrophism, and then the Th1/Th2 paradigm.

Murine Abortion Models, Immunotrophism, Th1/Th2

Murine Abortion Models

In 1982, Tom Wegmann visited Paris. He was focusing on the placenta as a barrier, after his demonstration of the presence of the MHC class on trophoblasts. After a visit to Nice, where he met Page Faulk, he was intrigued by his concept of allorecognition, though not by the TLX theory. I reminded him of Clark's C3H $\times$ DBA/2, CBA $\times$ DBA/2 observations. He pointed out that it was important to use a common *maternal*, not paternal background, and thus chose CBA mothers. He then wanted to 'vaccinate' CBA mice against abortion.

Because it was icy and freezing, and dangerous outside (the antepenultimate real winter in Paris and Villejuif before global warming), instead of using mice on the B10 background, as originally planned for ease of immunogenetic studies, we decided to use the few mice still available in the building which would save us a trip to the animal compound, and we agreed on BALB/c (H-2^d).²

The results of immunizing CBA mothers with BALB/c splenocytes were an impressive reduction in abortion/resorption rates, which Nicole Kiger reproduced in the subsequent weeks in Nice. But Tom insisted on having the fetuses and placentas weighed, a process that J.P. Kolb did on the spot. The results were also impressive: not only were the immunized mice protected against abortion, but the weight of their placentas and fetuses was higher than that of the controls as seen in our first papers [78, 79] and those of Kiger et al. [80] and Bobe et al. [81].

Immunotrophism

Tom Wegmann [82] very quickly became aware of the significance of the latter finding, which was followed by an editorial: 'Is It Immunosuppression or Immunostimulation?' in parallel with the Prehn and Lappe immunostimulation of the tumor growth concept. It was the beginning of immunotrophism [82]. It was postulated that allorecognition would result in the production of trophic cytokines by T cells, which would act as growth factors for trophoblasts.

The interest of the paradigm was 3-fold:

(1) It turned the attention to a hitherto unsuspected aspect of the maternofetal relationship.

² This process underscores the role of serendipity in science: had we used B10.D2 mice for immunization as planned in the morning, it would never have worked as subsequent experiments revealed.

(2) It definitely focused on an interaction rather than a complete separation between local and systemic components.

(3) And amongst those components, it suggested the role of T cell-derived growth factors in placental growth. It also explained why the outer layers of the placenta in direct contact with maternal blood cells (spongiotrophoblasts in mice) were class I-positive, while the inner ones were negative. It postulated that the placenta would provoke/ elicit a maternal immune response to 'use it' for optimal growth.

At the cellular level, the use of the autoimmune murine strains MRL/lpr and control MRL/L (H-2^k) in a combination of cellular depletion and cell transfer experiments quickly validated the concept in joint experiments I carried out with him and Elisabeth Menu [83, 84]. It was shown that excess placental size and weight in the lpr autoimmune strain were corrected by T cell depletion. Transfer of T and B cells from the MRL/lpr autoimmune strain into a control lpr-negative (MRL), or indeed into an H-2^k-compatible CBA/J in both cases increased placental weight, whereas treatment of the transferred cells with anti-Thy 1 + C' abrogated this effect. In the meantime, in cooperation with Paetkau, the Wegmann group and especially Athanassakis et al. [85] individualized the role of IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) as the trophoblast growth factors which were involved in the phenomenon, a finding which Armstrong and Chaouat [86] confirmed on placental cells from various developmental stages.³

The ultimate confirmation came from our use always in cooperation with Tom Wegmann [87] of recombinant cytokines in vivo which showed a positive effect, the 'immunotrophic' ones correcting abortion, while the negative ones [tumor necrosis factor (TNF), interferon- γ and IL-2] induced it in a variety of strains.

We and the Mac Master University group [88] incidentally were not able to reproduce the effects, claimed by some, of very low doses of IL-2 plus indomethacin in keeping with the well-known fact that patients with rheumatoid arthritis very often had continued with the Indo-

³ The recent experiments by Galli et al. [86a] leading to the birth of Prometea, a clone of her own mother, have been taken as an argument against immunotrophism. However, except in murine abortion-prone strains (CBA $\times$ DBA/2) T cell function has never been an unequivocal matter of life and death. In mice, T cell-depleted animals or nude H-2-compatible ones have a normal litter size but (only) reduced placental and fetal weight. In equids, immunization has been tested in an abortion system, the 'donkey in horse' one, where indeed fetal life is otherwise at stake [86b, c].

cid® treatment during pregnancy without any report of induced abortion. But effects of high doses of IL-2 were reproduced by many, and some such as Shiraishi et al. [89] gave proof of CTL involvement.

In parallel, this turned the attention to the colony-stimulating factor (CSF) family, and totally independently, the group of Pollard [90] emphasized the regular increase in the CSF-1 content of the pregnant uterus, demonstrated CSF-1 expression on the outer layers of the murine placenta, the spongiotrophoblast, and not the labyrinth. Interestingly, *cfms*, the oncogene for CSF-1 receptor, was expressed on the MHC class I-positive spongiotrophoblasts only, not in the MHC class I-negative labyrinth, further in keeping with an immune confrontation/immunotrophic concept. These latter discoveries suggested indeed that the expression of allo-MHC by outer layers of the placenta was deliberate, since the placenta was able to neutralize emergence and function of lytic cellular effectors while sparing and using their cytokine secretion.

Later on, Szekeres-Bartho et al. [91–93] would complete the picture by showing that MHC class I recognition elicited progesterone receptor expression on otherwise progesterone receptor lymphocytes, permitting the production of progesterone-induced blocking factor (PIBF).

Indeed, CSF-1 proved to be essential, though not mandatory, for a successful pregnancy, and we now know that GM-CSF might in addition play a role not only in immunostimulation, but also in immunosuppression, being one of the key components of the preparation of the uterus by sperm fluid components for the future immune tolerance of the fetus as shown by very elegant experiments of Robertson et al. [94]; these experiments may have yielded very important data depending on the factors which control the onset of preeclampsia.

Th1/Th2

We [95] and Tangri and Raghupathy [96] showed that there were more TNF and interferon- γ in the uteri of aborting CBA $\times$ DBA/2 mice than in the aborting CBA $\times$ BALB/c combination, and Tangri and Raghupathy [96] also demonstrated that in ‘mixed lymphocyte placental reactions’, the former placental cells induced more of these cytokines than the latter.

Tom Wegmann was quick to realize that TNF and interferon- γ were two of the Th1 cytokines that were defined after successful Th1 and Th2 separation in mice. He then screened the literature for patterns of allopregnancy and noted the following:

(1) Humoral immune responses – and indeed humoral response-associated immune disease – had for a long time

been known to flare during pregnancy, whereas classical cell-mediated associated autoimmune diseases often remitted during allogestation, and indeed while there was often (75% of multiparous women) an emergence of anti-paternal alloantibody, there was, in variance with other types of ‘allograft’, *never* an induction of CTLs.

(2) NK cell-mediated activity as estimated then with the classical ^{51}Cr release test was generally referred to as statistically lower in pregnant women than in nonpregnant controls. (This will also make him focus on progesterone-mediated immunosuppression, expression of progesterone receptors on allopregnancy-activated lymphocytes, and their subsequent secretion of PIBF, all phenomena which were beginning to be investigated by Szekeres-Bartho.)

(3) TNF and interferon- γ , Th1-associated cytokines, and in general induction of an inflammatory response by LPS were associated with fetal demise.

(4) Some parasite diseases associated with a Th1 immune response also underwent remission during pregnancy, while there were scattered reports of exacerbation of some others, supposedly Th2-mediated ones.

He and his colleagues [97] then wrote their second hypothesis: ‘successful pregnancy is a Th2 phenomenon’ and began to test the predictions of the model (see below). But the model also drew NKs out of the backstage and into the limelight.

NK Cells and HLA-G

In the meantime, Ellis [55] had discovered HLA-G, its unique characteristics, and she and simultaneously Kovatts et al. [56] had demonstrated, using unique transfectants, that HLA-G transfection could protect otherwise NK-sensitive cells from NK cell-mediated lysis.⁴ This was important for us, because I [98] had discovered that in contrast to all our predictions, resorbing embryos in the CBA $\times$ DBA/2 murine combination were first attacked and infiltrated by NK cells, not CTLs.

In a series of very elegant experiments, Baines and colleagues [99–101] demonstrated that the implantation sites of would-be resorbed embryos, and not the other sites, in a murine uterine horn, were infiltrated by asialo-

⁴ It is particularly painful when some French authors are trying to promote themselves as the ‘discoverers of HLA-G and their function’. This is simply *not* true.

GM1 + cells, e.g. NK cells, whereas treatment with anti-asialo-GM1 significantly reduced both these infiltrates and, more importantly, resorption rates. Key experiments were the ultimate demonstration by this group [100, 101] and Kinsky et al. [102] that poly IC treatment, a double-stranded RNA and a well-known NK activator boosted CBA × DBA/2 resorption rates. Even more importantly, Kinsky et al. [102] showed that transfer of poly IC-activated spleen cells or direct injection of the less toxic poly IC12u resulted in the induction of abortion (sometimes,⁵ as seen in India, to very high levels), and Hamilton and Hamilton [103] showed that CBA × DBA/2 abortion rates depended on environmental pressure.

In that period, NKs were seen merely as cells which could, in the presence of IL-2, become LAKCs, and thus a potential threat to the placenta.

'Charlie' (Y.W.) Loke was the first to envisage that the role of NKs was more complex than purely potentially detrimental, and the recent data at the end of this review amply confirm this. He was the first to point out the importance for the maternal relationship of the lack of expression of HLA-A, HLA-B, and by then perhaps C, on the human trophoblasts. He emphasized that, because of this peculiar MHC expression, the human placenta was unique, and could not be (well) studied using murine models. But he went so far as not only to point out the importance of the 'missing self' theory, ultimately developed by Karre [104].

Indeed, his laboratory and several other British labs, including Judith Bulmer's [105], proved that in humans uterine NK cells (uNKs) were of a different phenotype than peripheral ones [see for a review 57, 105]. For example, and most often quoted, uNKs and PBLs are CD56 dim versus CD56 bright. A very recent study by Strominger and colleagues [106] confirms this by an extensive microarray assay: the set of genes expressed by uNKs and peripheral NK cells are strikingly different. By then, the cells were first termed uterine large granular lymphocytes.

In mice, Croy et al. [107] devoted special interest to the granulated metrial gland cells, and she was the first to

show interest in the important NK accumulation in the peri-implantation decidua and in the metrial gland which also intrigued Tom Wegmann. Hence the initial term of granulated metrial gland cells was soon replaced with uNK. Both Croy and Bulmer realized that these cells could represent up to 70% of the uterine stroma in a peri-implantation uterus.

Not only did Charlie Loke begin very early to look at NK function at the interface, suggesting it was more complex than envisaged by the Th1/Th2 paradigm, but he [108, 109] even wrote prophetically in 1991 that uNK 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'. He also moved away from the Medawar paradigm, and suggested that we were nearer a tumor/host than an allograft/recipient one.

These concepts, being developed from 1991 to 2003, would be the key in the recent reassessment of the Th1/Th2 paradigm.

Reign of the Th1/Th2 Paradigm

As soon as it was announced, the original Th1/Th2 paradigm proved to be very useful in mainstream immunology, though it needed very quick readjustment with the introduction of Th0 and Th3 cells. As early as 1995, however, it was challenged by Kelsö [110]. There continued to be repeated challenges, the more recent ones being rather aggressive [see 111].

The First Studies

As far as pregnancy is concerned, the Th1/Th2 paradigm was quickly considered as verified because of the results obtained in mice.

First, Lin et al. [112] demonstrated the presence and secretion of the main Th2 cytokines IL-3, IL-4 and IL-10 at the fetomaternal interface. Except for one study by Delassus et al. [113], the very presence of the classical Th1 and especially Th2 cytokines – Th1 were already known – and of their receptors was traced at the interface in mice and humans and was demonstrated by a variety of techniques [ELISA, immunohistochemistry (IHC), in situ hybridization, PCR] and this was taken to be highly suggestive of autocrine-paracrine interactions [114–118], and a support for the paradigm.

We [119] performed serial longitudinal IHC studies in mice, which confirmed that localization in uterine tissues and placenta.

⁵ The 'environment', in fact the local flora, can regulate and controls the TNF level in the uterovaginal system. Indeed, Hamilton and Hamilton [103] were the first to demonstrate that gnotobiotic CBA had no high resorption rates when mated with DBA/2, while conventionally reared control CBA did (10"). At the NII and AIMS in Delhi, 'conventional' rearing was done in another environment different than in the European and even more so Canadian facilities. Here, resorption rates of 70–90% were not uncommon, and the poly poly C12 U system was very difficult to manipulate.

In parallel, in a series of elegant experiments conducted in C57Bl/6 mice, Krishnan et al. [120, 121] first demonstrated that infection with *Listeria monocytogenes*, well known to be abortifacient, occurred by inducing an excess Th1 response and there was a correlative defect in local Th2 activity.

In contrast, later on, in 'mirror experiments', she and her colleagues [122] had found that *Nippostrongylus brasiliensis* whose rejection is Th2 (cellular immune response)-dependent and which indeed induces a Th2 profile was in fact better rejected during pregnancy than outside it (unpubl. data, presented at the 7th ASRI Meeting, Vancouver, Canada) [122 for abstracts]. Similar interesting results have been reported since by Buendia et al. [123] in humans. This is one of the few sets of data dealing with Th2 bias per se.

In parallel, Tangri et al. [124], indeed, confirmed our [95] earlier ELISA studies showing in a very elegant series of studies of experiments that the placentas and deciduas of CBA mice undergoing fetal resorption (when mated with DBA/2) had more TNF and interferon- γ (two classical Th1 cytokines) than the ones from a nonresorbing combination, e.g. mated with BALB/c [124]. This was verified using the 'mixed lymphocyte placenta reaction', where splenic lymphocytes are made to react with placental fragments, a model which was thought to be representative of the in vivo in situ situation.

In close cooperation with Tom Wegmann, we [125] were able to decrease resorption rates in the abortion-prone mating CBA $\times$ DBA/2 by injection of anti-interferon- γ or pentoxifylline, and it was proven by simultaneous neutralization of the two cytokines that they were required in synergy for maximal abortion rates. Conversely, purified IL-10 decreased resorption rates [125], and this in a much better fashion than the immunotrophic cytokines had done in the previous 'immunotrophism' experiments we [87] had conducted earlier. In keeping with this protective effect of IL-10, anti-IL-10 injection boosted resorption rates in the CBA $\times$ DBA/2 mating.

With the group of Martal and of course Tom Wegmann [125] we wanted to investigate which were the signals which could trigger IL-10 production. Interestingly, while no Th2 cytokines were detected in the reproductive tract of CBA $\times$ DBA/2 mice (it does not mean there were none; they were only below the limit of detection of ELISA tests), alloimmunization by BALB/c (H-2^d) splenocytes increased the levels to detectable ones, in fact above the IL-3, IL-4, and IL-10 levels of the organs of the CBA $\times$ BALB/c control mating. Interestingly (unpubl. data) in keeping with the original immunogenetic studies carried

out in this model, immunization of CBA/J mice was effective only in this respect if carried out with BALB/c splenocytes, not with congenic BALB/b (H-2^b on BALB background), BALB/k (H-2^k), or B10.D2 (also H-2^d but on B10 background) ones.

In the meantime, Martal and colleagues [126–128] had described a molecule which replaces hCG in the maintenance of the corpus luteum in sheep and bovine species, oTP-trophoblastin, and found that it was a new family of interferons, interferons- τ , which are endowed with endocrine activity.

We tested the effects of the recombinant interferon- τ in the system. As expected from the concept that interferon excess induces a Th2 feedback response in Th2 cells, the injection of interferon- τ quickly corrected the Th1/Th2 imbalance in the CBA $\times$ DBA/2 matings, and this prevented resorption rates [125]. This interferon is nontoxic for trophoblasts (which secrete it and which do so a few days/hours prior to implantation), and does *not* activate the coagulation pathway in the presence of TNF.

In contrast, interferon- γ was abortifacient. Trophoblasts cannot distinguish between the two types of interferon (albeit, for some, there are perhaps two distinct receptors, one being tau specific but their properties are different) and therefore react to the huge amounts secreted by the pre- and peri-implantation trophoblasts as if they had to suppress a potent ongoing Th1 response, e.g. by 'trying' to suppress/downregulate it by Th2 cytokine production. Thus, a common 'early pregnancy' signal was conceivable, endowed with immunoendocrine activity and prior to implantation [125].

This explained why, in preimmunized animals, the IgG1/IgG2 ratio in an anti-SRBC response was observed to be altered as early as on day 4 especially in a secondary response in anti-SRBC preimmunized mice, e.g. well before there could be MHC-mediated allorecognition of the fetus [12], and before MHC class I antigens (K, D, and L) appear at detectable levels at the trophoblast surface –day 9.5 [129], representing in the mouse the beginning of the differentiation of the placenta into two zones: the external spongiotrophoblast, MHC class I+, and the inner part of the placenta, the labyrinth MHC class I–.⁶

⁶ There are studies, however, which convincingly show a very low expression of MHC class I molecules on the early preimplantation embryo, but whether these can interact with NK or T cells has not been convincingly demonstrated [130]. Also, a candidate class Ib to fulfill HLA-G analogue functions at this stage has been described [131].

Further studies which we [132, 133] conducted determined that, surprisingly, the alloimmunization effect in inducing IL-10 production by trophoblasts was not T cell-, but NK-mediated, and using MHC mutants we confirmed that the NKs could 'see' MHC mutations the TCR did not detect, moving already slightly away from a T cell-mediated only placental recognition.

These experiments nevertheless also linked 'classical' reproductive immune events (immunosuppression) and endocrine events since interferons- τ , besides being antiviral, as bona fide ones, *and* endowed with an endocrine activity on the corpus luteum, are cytostatic for PHA or mixed lymphocyte reaction (MLR)-activated lymphocytes as shown first by Fillion et al. [134].

Interestingly, Soos et al. [135] reported after experiments conducted partly on the basis of these observations that interferons- τ were orally given with success in the treatment of autoimmune encephalomyelitis. Another proof of the entwinement of both immune and endocrine systems was offered when the group of Szekeres-Bartho [136] demonstrated that the progesterone-dependent production of PIBF was indeed able to shift lymphocyte cytokine production towards a Th2 bias. Simultaneously, in mice TNF was even implied by Arck et al. [137, 138] in the stress-induced abortion process. Finally, the group of Clark and us [139] also showed in the CBA $\times$ DBA/2 matings that TNF + interferon- γ were not acting by a cytostatic effect on trophoblast cells, as expected, but, rather surprisingly at the start, in maternal vessels on the fgl2 prothrombinase-mediated pathway.

The involvement of coagulation events in the final pathway of abortion is very interesting, because it unifies the process with autoimmune disease-mediated abortion, where a procoagulant effect is well known to be associated with the elicited autoantibodies. This could explain the rather high efficiency of aspirin heparin treatments in RSA, which in several double-blind studies have been reported to be effective in the treatment of the RSA syndrome, and in most cases in fact much more effective than reported by most double-blind studies carried out on the effect of the treatment of RSA by lymphocyte alloimmunization. (This is so very true that even lymphocyte immunization promoters such as Cadavid et al. [140] have acknowledged that it was a simpler and more effective treatment of recurrent spontaneous abortions than lymphocyte alloimmunization of the women.)

As Th1 and Th2 were joined by Th0 and Th3 cells, and while new cytokines were classified (sometimes more and more painfully) as either Th1 or Th2, some of the 'newer' Th1- or Th1-like cytokines were embedded in the original

Th1/Th2 pregnancy concept, and indeed the interferon inducer and coinducer cytokines IL-12 and IL-18 are abortifacient mice, when given in synergy, as shown by Muranaka et al. [141], whereas Hayakawa et al. [142] observed that incubation of splenocytes with IL-12 and their subsequent injection proved to be both embryo- and nephrotoxic.

A similar, albeit slightly different incubation protocol was used later on by Zenclussen et al. [143, 144] in mice and was proven to be indeed abortive and to induce similar renal lesions as in the report of Hayakawa et al. [142], but only during pregnancy, thus being presented as a murine model of preeclampsia.

The Human Studies

In humans, in parallel, there soon was a shift towards the use of the new paradigm. It is fair to recall that even before the original paper of the Edmonton group Anderson and Hill [145], in keeping with the studies carried out in mice, had shown the role of cellular immunity in female sterility. They subsequently showed that TNF and interferon- γ were the likely key mediators involved and coined the term 'immunodystrophism' to name such pathologies in both in vitro fertilization failures [33] and RSA [see 145–147].

In a series of elegant experiments, the group of Raghu-pathy et al. [148, 149] used the very same mixed lymphocyte placental reaction concept/technique in the human as they had previously used in mice, and showed here again a Th1 bias in the response of spontaneous aborters towards the placenta. In a series of cohort studies following recurrent spontaneous aborters and fertile women, the same group [149] was able to demonstrate a correlation between a Th1 bias and RSA; the studies of Jenkins et al. [150] extended these findings to IL-12.

Piccinni et al. [151] used a very elegant method of cloning decidual T cells and they also found a Th2 bias in those clones. Interestingly, this bias is associated with two other important 'non-Th' cytokines, macrophage colony-stimulating factor, an 'immunotrophic one' (see above) and leukemia inhibitory factor (LIF), the key in implantation (see below).

Also, in keeping with the original Wegmann hypothesis, most of the studies find a statistically lower PBL NK activity with the chromium release test in healthy pregnant women than in nonpregnant women, aborting individuals showing an enhanced NK activity when compared to the pregnant controls, but also in some studies to the nonpregnant women. These studies have been extended to infertility of unknown etiology, and even more

recently by Ntrivalas et al. [152] to implantation failures with, apparently, similar results [153, 154].

It is on that basis that LIT or IVIG has recently been proposed by Ng et al. [154] to be extended to implantation failures. Finally, there are numerous reports suggesting an alteration of Th1/Th2 bias in preeclampsia [see for example 155].

The expression of cytokines at the interface as early as the peri-implantation period seemed to be a confirmation [see above 115–119, 153–161]; Th2 chemokines were even described by Tsuda et al. [159], as important in the phenomenon so that according to most reviews [160–165], the Th1/Th2 seemed to reign supreme. It was often expressed in its (over)simplified form as done by Tom Wegmann for didactic reasons (but it was *not*, I can testify, having had discussions with him in Goa during the last days before his stroke, his ultimate thought!):

(1) Th1 cytokines are ‘bad guys’ mediating fetal loss.

(2) Th2 cytokines are ‘good guys’ mediating fetal protection.

(3) Fetal death is mediated by activated cytotoxic NKs, the activation being dependent on the Th1 cytokines.

It is important to remember that ‘NK triggering’ was a very remote interest in that period, even for mainstream immunologists. Hence it was not attracting much more interest in the field of reproductive immunology, except in a few groups, amongst which were those of Charlie Loke and B.A. Croy. But I can testify that Tom was very intrigued by NK functions, and had already been so earlier [107]. Indeed, the role of NK as suggested on the basis MHC mutant experiments carried out by myself [132] with the help of the group of P. Kourilsky had also been discussed at length with him.

Limits of the Th1/Th2 Paradigm in Pregnancy

Croy [166] wrote recently: ‘Where now for the Th1/Th2 paradigm of the gestational uterus?’ There were also discrepant data, however, in the midst of the apparent triumph of the paradigm.

Discrepant Data

First, Tom Wegmann was perfectly aware of those data when we discussed and wrote the final version of our last cosigned paper in Majorda, Goa, India, a few days before his death; there had been *no* effect of anti-IL-10 in non-abortion-prone murine mating combinations [125], which he very quickly attributed to the original environmental effects outlined by Hamilton and Hamilton [103];

these Clark et al. [167] would in fact reexplore later on in more detail in their studies of the role of bacterial flora in cooperation with Knackstedt et al. [168], and its use of the ‘danger signal’⁷ hypothesis by Matzinger [169].⁸

In other words, one possible explanation would be that the high preexistent TNF levels in CBA were too high to be corrected by placental recognition of CBA × DBA/2 placenta-induced production of IL-10, especially since experiments of the group of Raghupathy [124], above all the ‘mixed lymphocyte placental reaction’, confirmed that DBA/2 antigens bearing (placental) cells are bad Th2 promoters compared with BALB/C ones, a bias towards preferential Th1 induction instead of Th2 also previously observed in premating experiments with DBA/2 and BALB/c males as well as in preimmunization experiments [94, 95, 168].

The original model of Clark [170] in mice and humans, for a while, attributed much more importance to TGFβ2-producing cells, whose defect was linked to abortion than to the Th1/Th2 imbalance, and thus the proponents of the Th1/Th2 model had to use – as Clark himself – the ‘extension’ in the murine species to the Th0 and Th3 cells, before finally shifting to the role of gamma delta T cells and NKs more than the one of CD4 subsets [170–172].

In the prevention of stress-induced abortion, Arck et al. [171, 172] observed that CD8+ T cells appeared more important than CD4+ ones and placental suppression, which must not be forgotten, appeared in experiments of Brooks-Kaiser and Hoskin [173] and Nagaeva et al. [174] to involve also TGFβ1 and not solely IL-10 and low molecular weight factors as discussed by us [175].

⁷ According to the original Matzinger hypothesis, the overreaching interpretation of the danger theory is that an embryo, insuring the reproductive success of the species, is not ‘dangerous’ and thus is not rejected. B10 × B10.A, however, exhibit higher resorption losses than controls (though the differential is much lower than in the CBA system) and this even in gnotobiotic or germ-free conditions (at least in our hands). What is then for a B10 the ‘danger’ of a B10 × B10A embryo versus a B10 × B10Br or B10 × B10D2 one? This was never explained to me, despite questioning on my part, nor why alloimmunization could, in ‘the donkey in horse system’, prevent abortion by restarting the process of trophoblast invasion. This is ultimately associated in the equine species as well as in other species with limited but real risks of retroplacental hemorrhages, death in utero, or maternal death close to delivery, while stopping pregnancy at these very early stages presents no danger.

⁸ The theory says in fact that during normal pregnancy ‘there is *no* alarm signal to initiate an immune response’ [169a], whereas the presence of antipaternal antibodies in ‘producer strains’, the ‘recall flare’ phenomenon, in draining lymph nodes in a second pregnancy testifies that there is one.

The system (re)appeared more complex; 'forgotten' cells and pathways resurfaced, moving away from the simpler description of the Th1/Th2 paradigm. Much more worrisome was the fact, known almost since 1995, that IL-10 KO mice had normal pregnancies, the delivered litters thriving perfectly well. This was further confirmed by Svensson et al. [176] dealing with two classical cytokines of the original paradigm.

In such a case, one could still say that redundancy in the cytokine network could have ensured pregnancy success, other members of the Th2 cytokine family and of the IL-10 families of cytokines fulfilling the role otherwise accomplished by the knocked-out IL-10. A severe blow to this alternative can be found in recent experiments of Fallon et al. [177] where a quadruple Th2 KO in mice does not affect in any way the reproductive performance. In all strictness, one can evoke the substitute role of such Th2 cytokines such as IL-13, known to be present like a shield on trophoblast as early as the first postimplantation period in mice [178] and in the decidua in humans [179]. Also, even other members of the IL-10 family of cytokines whose properties are reviewed by Fickenscher et al. [180] could eventually be involved but that would anyway not fit in with the original Th1/Th2 concept.

Th1/Th2 in Humans?

Indeed, in humans, the very existence of a Th2 bias in pregnancy had been very quickly challenged by Vince and Johnson [181]. However, the main criticism was aimed first at the principal support of the paradigm in humans, e.g. the RSA and miscarriage studies. First, it was pointed out that the studies were conducted in populations of aborters who had already experienced an abortive event, which could be of any origin (including genetic, infectious) but which might have 'primed' the system towards a Th1 bias, as seen in mice with DBA/2 immunization or mating [175], analogue to the Th1 bias seen in women with ongoing endometriosis. Interestingly, in fact, and incidentally, in a murine model system, the 'Th1' cytokine IL-12 was shown by Somigliana et al. [182] not to be causative, but to prevent endometriosis.

In the case of miscarriages, the critics pointed out that in many cases – but not all [see for reviews 160] – blood samples are taken either during the abortion process or immediately during or shortly after expulsion. In such cases they pointed out, the Th1 bias would be a consequence, but not the cause, of the initial abortive event.

A recent study by Kruse [183] with a limited number of patients and the other (serious) limitation that a certain number of women received treatments such as IVIG sup-

ports this hypothesis: in a longitudinal follow-up study before the abortion, there is no clear bias towards a Th1 profile and a lack of or decreased Th2 activity in the recurrent miscarriage group, except that 'with regard to the PBMCs TNF- α production the intercept coefficients were significantly different between patients who miscarried and those with successful pregnancies ($p < 0.05$), meaning that the TNF- α measurements were generally higher in the miscarrying patients'.

It must in fact be admitted that so far there has been no satisfactory large-scale prospective study of a cohort of women sampled before their first positive hCG test (pregnancy), in order to try to individualize in a prospective fashion the profiles of those who will become primary or secondary recurrent aborters.

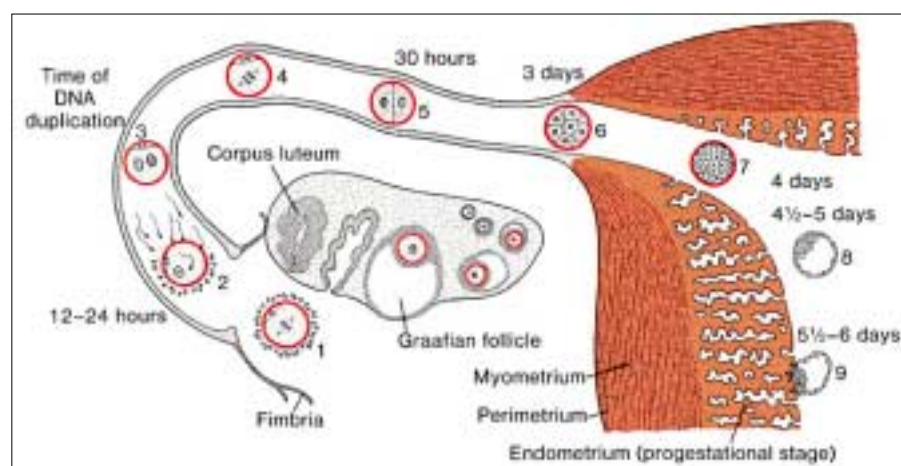
It is even more troublesome that there are groups which not only do not detect a Th2 bias in human pregnancy, but who fail to detect the main Th2 cytokine, IL-10, in their assays and particularly in trophoblasts, its supposed main source of production [pers. commun., 184]. Indeed many others have found IL-10 in trophoblasts since the original description by Roth et al. [185].

My belief is that since several members of the IL-10 family have been cloned by sequence analogy, some of the first anti-IL-10 reagents might have been directed against such members rather than IL-10 itself, but this does not explain why most groups detect IL-10 in purified trophoblasts by real-time PCR with IL-10-specific primers. Another important criticism is that one of the main criteria to sustain the Th1/Th2 paradigm in humans is, in keeping with the original hypothesis, a reduced NK activity in PBL of pregnant women, while there is 'activation' in case of RSA.

The critics claim that peripheral NKs are *not* representative of uNK cells, and recent extensive data by Strominger and his group [106, 186] offer important new arguments to further extend this hypothesis/criticism, since the microarray data they presented definitely prove that uNKs and peripheral blood NK cells are very different subsets. This is well known in mice and has been discussed by Croy et al. [187].

Indeed, the data obtained when looking at decidua cytokine profiles and decidual NK count status of the RSA patients represent a far from universal agreement of the Th1/Th2 concept: one can summarize the results by saying that some studies find such a bias, *but* only in a fraction of biopsy samples as the studies of Kwak et al. [188], while others do not fit in at all with the hypothesis of an altered Th1 bias in RSA, be it by cytokine profiles such as Plevyak et al. [189] or cell counts as in the report

Fig. 1. Implantation steps of the human embryo.



of Michimata et al. [190], though results from the same group may look confusingly contradictory [see also 191].

Again, it is possible here that technical bias is important, such as time after expulsion or depth of the biopsy samples. For example, Th1/Th2 cells undergo differential death in utero with the Fas/Fas ligand system as suggested by Zhang et al. [192], but it seems very unlikely that this is a universal explanation.

Further problems are the lack of abnormal polymorphism of IL-10 promoters in RSA as reported by Karhukorpi et al. [193], though the data on Th1 genes by Reid et al. [194] are slightly more convincing. But even in this case, it is surprising for the tenets underpinning the concept (but in keeping with new knowledge, see below) that the 'Th1' cytokine IL-12 levels are decreased in PBLs of miscarriage patients when compared to controls as in the study of Zenclussen et al. [195]. For some, such as Check [196], the Th1/Th2 imbalance might reflect only the progesterone balance though I also find this unlikely.

Finally, and Tom Wegmann was perfectly aware of that, the concept of a Th1/Th2 bias assumes that there is a steady state throughout the established pregnancy. Somehow, microarray studies conducted in mice seem to support this concept, since the profiles are relatively homogeneous in the second half of gestation [197], but far from what a simple Th1/Th2 hypothesis would predict. Such a steady state is not really the case in human studies conducted for an HIV mother to child transmission project revealing unexpected 'surges' of certain cytokines in human pregnancy PBLs [198]. But such a steady state has certainly not been observed in the first half of murine pregnancy.

Indeed, studies conducted in mice have shown an important quantitative and qualitative distribution of classical Th1 and Th2 cytokines in the postimplantation period, be it for the classical Th2 cytokines or, more importantly, for the 'newer' cytokines, which are found to be expressed in the reproductive tract, and which do not fit easily into the paradigm classification, even when renewed with Th0 and Th3 cells [178]. That such a steady state does not exist is manifest in the preparation of labor, with enhanced levels of TNF, all involved in labor induction, and for some of them in the triggering of premature delivery in the case of infection. Interestingly, at that stage, TNF does not induce coagulation, highlighting the need for simultaneous high interferon- γ production so that such a phenomenon occurs.

Implantation Does Not Fit within the Th1/Th2 Paradigm

Implantation, itself, is *totally* opposite to the necessity of an absence of inflammatory cytokines (see fig. 1 and 2 for implantation steps, fig. 3 for the human placenta, and fig. 4 for involvement of cytokines in implantation steps).

In contrast, it has been known since 1992 that a preimplantation cellular influx is observed. This is mostly due to activated macrophages (likely phagocytosing dying spermatozoa and other cellular debris, but also T cells) [199, 200]. This is incidentally one of the gateways of entry for AIDS. It is those T cells which are likely to be one of the targets of sperm-related immunoregulation demonstrated by the elegant studies of Robertson et al.

Fig. 2. Preparation and transformation of the uterine decidua during implantation. Note the very important role of the transformation of the spiral arteries (freeware pictures found on the Net).

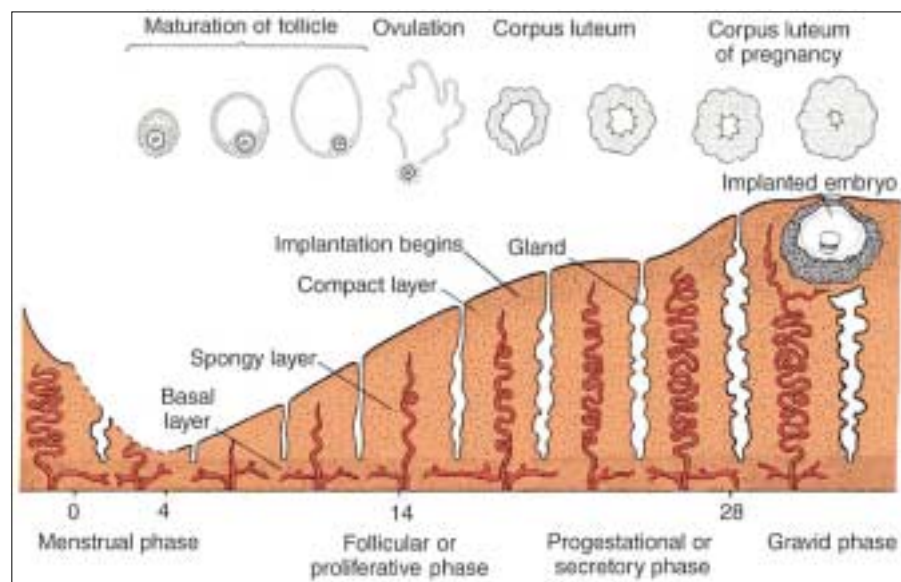
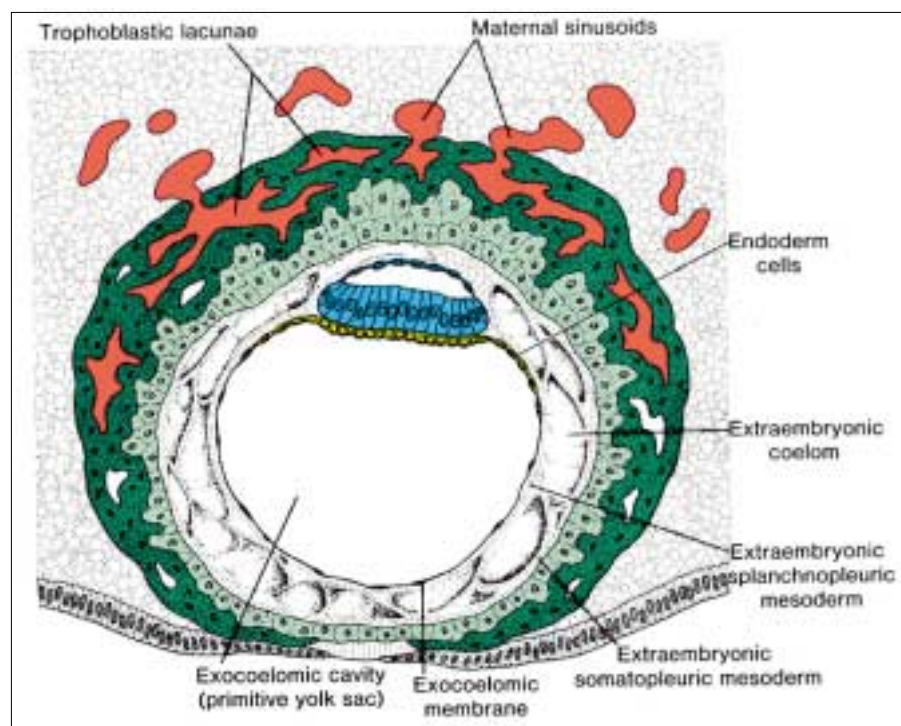


Fig. 3. The human embryo at early implantation.



[94]. Such an influx is necessary for a successful pregnancy because a blockade of such an afflux in one uterine horn would result in a localized implantation defect, the contrauterine, untreated, control horn showing a successful implantation and normal term pregnancy [200]. Subsequent Northern analysis of a preimplantation murine uterus confirmed the importance of an ‘inflammatory-like

reaction’ in the preparation of implantation, and indeed several Th1 cytokines were seen as upregulated by then [201, 202].

An important inflammatory component in implantation – but also subsequent pregnancy – is the presence of CSFs [90, 203–205], partly under progesterone regulation as shown by Kariya et al. [206].

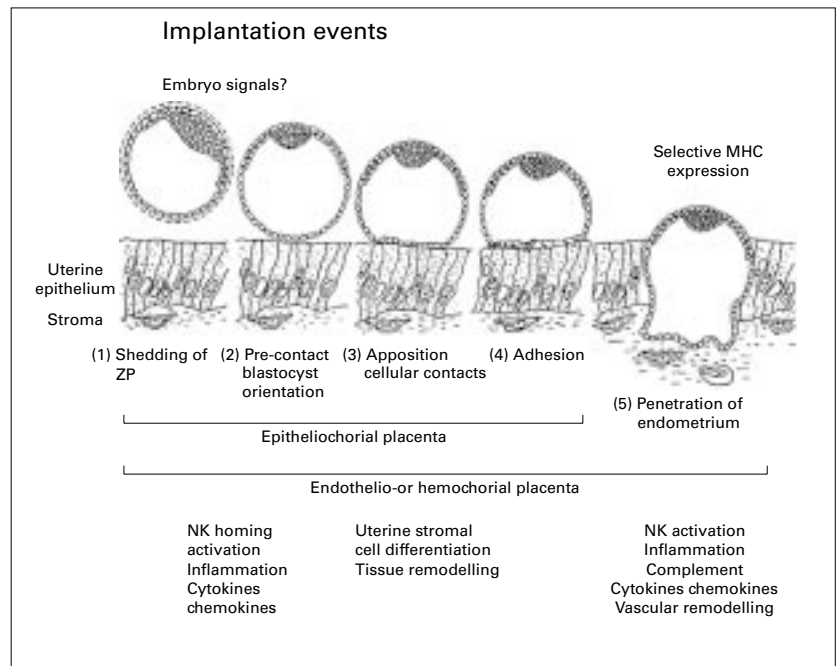


Fig. 4. Key events during the implantation window.

The absolute necessity for CSF-1 deduced from initial experiments by the group of Pollard et al. [207] was revised when it was realized by Cohen et al. [208] that the main defects were in fact in the male.

Since these early findings, implantation has been described quasi universally as an ‘inflammation-like process’. For many, those cytokines are correlated with a further expression of important molecules such as adhesion molecules (notably integrins and adhesins) important for the adhesion stages (see fig. 4), e.g. for trophoblast cells to adhere to – and in some species fuse with! – the uterine stroma cells, and a series of enzymes such as MMPs, which are very important components of the invasion stage [57, 209–214], permitting trophoblast migration by degradation and permeation of the stromal cellular and extracellular matrix. Chemokines might partly regulate homing of trophoblasts in the uterus (the decidua basalis at the implantation site).

Role of Inflammatory Cytokines

Indeed, while there is no report of implantation defects in Th2 KO mice, this is not the case for inflammatory ones; IL-1 seems to be rather important. Though the total block of implantation by blocking the IL-1 receptor as originally described by Simon et al. [215] is very controversial, he and his group [216–220] have convincingly

described an IL-1 system in humans, which is very suggestive of autocrine-paracrine regulation as suggested as early as 1990 by Kauma et al. [221] and Frank et al. [222].

There are at least two well-known cases where KO of inflammatory cytokines do result in implantation failure.⁹

(1) Recently, it has been shown by Robb et al. [223] or Bilinski et al. [224] that IL-11R KO mice have abnormal decidualization, uterine stroma remodelling, and implantation defects. Our own studies so far have not found such defects in humans, but the series is still perhaps a bit limited.

(2) LIF KO as in the original experiments of Stewart et al. [225] results in total implantation failure, and even LIF+/LIF+ embryos (they do secrete LIF at early stages) do not implant in a LIF–/LIF– mother. LIF– embryos do implant in LIF+ mothers. This defect is highly suggestive of the key role of maternal LIF and this was proven by the correction of the defect via an osmotic pump administra-

⁹ Outside the scope of this review there are several KOs such as prolactin, studied by Kelly et al. [225a].

tion of recombinant LIF; thus a *Nature* editorial asks: can there be life without LIF? [226]. These data have been confirmed independently; Conquet et al. [227] in the group of Brulet designed LIF KO's too with another technique for developmental studies, which harbor the same reproductive defects.

There were many reasons to think that some sterile women might harbor qualitative or quantitative LIF deficiencies, including the synchronized expression of LIF, LIF mRNA, in the endometrium, and LIF receptor on the embryo [228–230]. Indeed, quantitative defects have been spotted by Delage et al. [231] in our group, perhaps under the influence of a lack of CSF-1, or in keeping with observations of Simon et al. [220], IL-1, since both appear to be LIF inducers (with progesterone) [232]. Similar data have been obtained by Laird et al. [233] using uterine flushing media, and qualitative defects have been spotted too: Giess et al. [234] found putatively inactive LIF mutations in some sterile women.

However, care must be taken: first, and to some extent other than Laird et al., we [235] find that an excess of luminal LIF is correlated with high decidual NK counts, and an enhanced TNF and interferon- γ production. This would fit in better with the Th1 hypothesis, sterility in the latter case being linked with an overall inflammation persisting abnormally after the adhesion steps, where all cellular fluxes [198–200] and most inflammatory cytokines return to normal basal levels [201, 202]. This is not true for all cytokines: CSF-1 levels continue to rise, for example [202, 203]. Normally, as stated, a more Th2-dominated balance, for some similar to the so-called 'tolerance', is established, for many, under the influence of embryonic signals (interferon- τ is an example) [125–128, 236]. α -Interferons are present in humans at low doses as shown by Chard et al. [237], and the search continues for interferon- α expression [238].

Anyhow, LIF is a complex cytokine, and as described by Haines et al. [239] there are three isoforms. Only a fraction of it is important, the soluble isoform, and in our hands it seems to be necessary to see also about the localization: stromal LIF and perhaps a moderate amount of luminal LIF are useful, excess stromal LIF might be deleterious or reflect another reaction. The antibodies available at present do not distinguish the three isoforms, and moreover most of the commercial antibodies do not detect glycosylated LIF. Thus, the interpretation of studies using recombinant LIF in sterile women with those and as and all out without sorting the women deficient in soluble LIF production might be difficult.

On the embryonic side, inflammatory cytokines are also important: using the delayed implantation system, Basak et al. [240] for the first time demonstrated that this was under hormonal control. Interestingly, the secretion of HLA-G by only those embryos which could implant as shown in the pioneering study of Fuzzi et al. [241] (it is a necessary, not a sufficient condition) also suggests that the embryo could modulate early cytokine secretion, since as stated above human embryos are as the murine ones susceptible to LAK-mediated lysis [242], and thus need to control the secretion of both harmful and trophic cytokines; this function is perhaps, as suggested by Le Bou-teiller and Blaschitz [243], exerted by HLA-G.

Beyond the Th1/Th2 Paradigm

As stated, the simplified version of the concept (see fig. 5, 6) was that both NK and Th1 cytokines should be downregulated and neither studies in humans nor mice support such an oversimplification any longer.

We have already stated that this is not fully compatible with IL-12 production observed in the pre-, peri-, and immediate postimplantation periods in mice, and indeed in this respect, it should be noted that mice with a mutation towards IL-12 or components of its signal transduction showed a vascular pathology at the uteroplacental interface in the first studies of Croy et al. [244]. IL-12 is thought to act not only as an NK cytotoxicity activator, and interferon inducer, but also on angiogenesis [245]. It seems pathological in that way at high doses, but necessary at low doses [Sargent, pers. commun.] (see also below).

Let us examine this. The classical prediction of the paradigm, used as a rationale by most lymphocyte immunization therapists, is that both IL-12 and IL-18 are interferon inducers, and interferon is a 'bad guy' for the paradigm, and so is NK activation.

Where Are We Now?

Since the Delhi meeting IL-18 has been known to be abortifacient if given with IL-12, and this was in keeping with the fact that for some it is a 'dual cytokine with both Th1 and Th2 activities' [142, 143]. Thus, as regards most people, it should be predicted to be expressed to a greater extent in the CBA $\times$ DBA/2 abortion-prone murine mating combination than in a non-abortion-prone one.

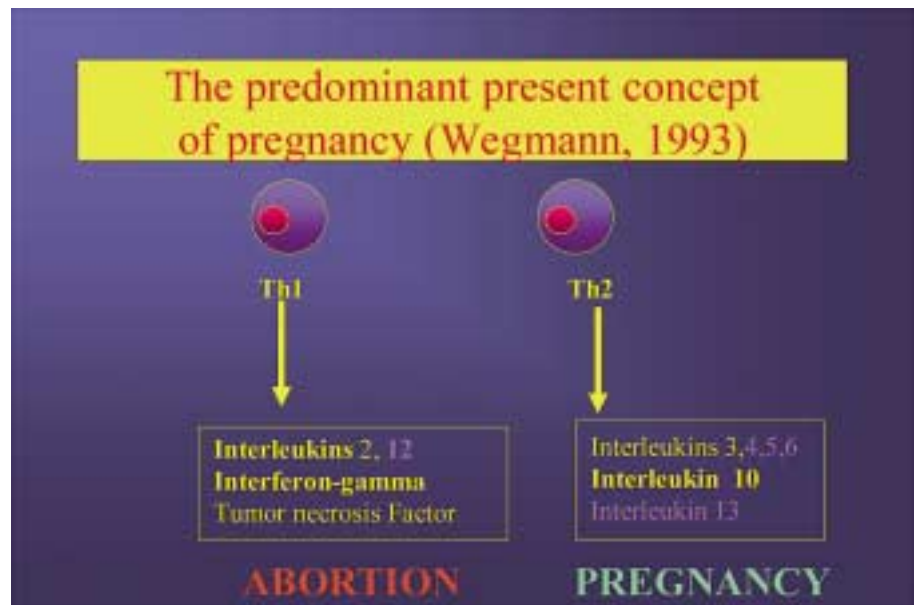


Fig. 5. The 1993 Th1/Th2 concept as updated in 2002.



Fig. 6. Immunopathology of pregnancy as predicted by the Th1/Th2 standard model.

The data from Ostojic et al. [246] by ELISA, soon confirmed by real-time PCR, represented the exact opposite of this prediction: the more IL-18-expressing mating combination was the CBA $\times$ BALB/c non-abortion-prone combination, not the abortion-prone CBA $\times$ DBA/2 mating. Furthermore, IHC studies confirmed that the peri-implantation uterine stroma is literally filled with NK cells (70%).

At days 7–9.5, concomitant to the lack of expression of MHC molecules on the pre- and peri-implantation embryos, uNKs are activated (expression of granzyme, perforin; remember that the trophoblast and cells are resistant to such cell-mediated lysis). Recent studies by Ostojic et al. [246] revealed that these cells are activated to produce high levels of IL-18 [197].

In the meantime, Croy and her group extended their original observations. They first demonstrated that NK

cell-deficient TgE26 mice had profound reproductive defects, notably a reduced placental size and profound vascular anomalies, which in their KO system ultimately led to a high percentage of fetal deaths as first observed by Guimond et al. [247]. The vascular anomalies (thick vascular walls, absence of transformation) are confirmed in the KO of Barber and Pollard [248] but not Miyazaki et al. [249].¹⁰

Both the vascular defects and reduced placental size were cured by appropriate NK reconstitution in the original series of experiments by Guimond et al. [250]. Reminiscent of the fact that T cell-depleted or nude mice show a limited reduction of placental size, Anne Croy said at a meeting: 'This is immunotrophism as predicted by Tom Wegmann, but you both until the IL-10 inducing experiments looked at the wrong (T) lymphocytes.'

Totally opposed to the simple classification of the original Th1/Th2 paradigm, subsequent experiments proved that interferon- γ was not solely a 'bad guy', but convincingly showed a key role for an interferon- γ -dependent pathway in vascular bed remodelling. Studies by Ashkar et al. [251] revealed that interferon- γ receptor KOs have implantation defects and vascular wall anomalies. Most importantly in this respect, uNKs have been shown to secrete the crucial vascular growth factor, angiopoietin 2, in mice [252] and also in humans [253].

Thus, Croy et al. [252] proposed that the uNKs were key regulators of the initiation of preeclampsia, an hypothesis which has been gaining more and more support; the paternal specificity at NK of the T cell level is possibly linked to the recognition patterns of HLA-C, which since the studies of King et al. [254] has been known to be coexpressed with HLA-G on invading extravillous trophoblasts. Recent data presented by Robertson et al. [255] in Australia appears to show exactly that.

Finally, Sacks et al. [184] have recently shown that: 'Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy'. Priming of the innate immune system and production of a Th1 lymphocyte are considered heretical within the paradigm! In their conception, IL-12, as IL-18, is required for a successful pregnancy. Only their absence or too high levels of one or both would have immediate (abortion) or delayed (preeclampsia)

tic syndrome) consequences. This fits in both with the data (paradoxical for the paradigm) of Zenclussen et al. [195] and those of Jenkins et al. [150].¹¹

On the basis of murine studies we have also postulated an important role for the IL-12/IL-18 system, and we have, therefore, studied infertile women. The data obtained were from microbiopsy fragments taken on a fixed day of the cycle for automatized NK counting on IHC slides and real-time PCR cytokine quantification, while echographic studies of the uterine arteries were simultaneously performed.

We [256] found four distinct profiles of expression in infertile women compared to a (very much more than expected) standard profile in fertile control women: a first profile, characterized by an abnormal localization (stromal instead of glandular) and too high a production there (dense IHC labelling) of IL-18, a second profile shows a high glandular secretion of IL-12, more intense than the one of IL-18, a third profile which is characterized by high to very high counts of uNK cells; this can be regrouped eventually in a 'cytokine excess' profile, and a last profile which we name total cytokine depletion shows no detectable cytokines at all (it is being verified whether this does or does not correlate with LIF deficiencies).

This last cytokine defect might have an effect as early as the adhesion stage, but might also later on affect vasculogenesis, whereas the high IL-12/IL-18 profile might affect rather, though not exclusively, the latter invasion stage, for it is very important that in all abnormal cytokine profiles, whether they are defects or excess, vascular anomalies are traced with echography. Thus, as in the murine systems, a lack of IL-18 production and/or IL-12 secretion could be linked to an absence of proper transformation of uterine arteries into spiral arteries, and as in Jenkins et al. [150], too high IL-12 levels, but also high IL-18, are associated with a vascular pathology (of interest is the fact that Jenkins et al. find high IL-12 levels in preeclampsia, as do Sacks et al. [184]).

In keeping with the concept that the Th1/Th2 paradigm is an oversimplification, and as suggested from earlier studies in mice [178], not only quantitative defects,

¹⁰ It is important to mention that there are discrepant reports: Saito's group [249] (IL-2 common receptor γ chain KO) reports a normal pregnancy. Barber and Pollard [248] with another NK KO confirm that a lack of NKs results in profound vascular alterations, but does not hamper fetal survival. The simplest (?) interpretation would be that KOs do not target all NKs, or alternatively they do but TgE26 have another associated but yet unknown cellular defect.

¹¹ IL-12 exists as two isoforms: only the heterodimer is bioactive. Thus, a proper measurement should be for both IL-12 p40 and p70. Indeed, the active heterodimer is up regulated during the resorption process in the CBA $\times$ DBA/2 vs. the nonresorbing CBA $\times$ BALB/c mating within the total 'pool' of IL-12, which is grossly unaffected. There is in fact slightly more p40 in the CBA $\times$ BALB/c than in the CBA $\times$ DBA/2 system at that time [197]. In addition, the placenta contains IL-27 EBI-3, an IL-12 p40-related molecule, which could also play a key role in the regulation of the system [197a].

but also changes in the distribution pattern will determine whether a cytokine would exert positive or negative effects, reminiscent of the aforementioned observations in the LIF system.

IL-15 is widely expressed at the murine interface [178] and Ye et al. [257] have shown that it regulates granulated metrial gland cells, where it controls NK granzyme and perforin content [258]. Kitaya et al. [259] and Okada et al. [260] have shown that it is also expressed at the human interface and it is an important growth and differentiation factor not only for transiting NK cells, but more specifically as shown by Verma et al. [261] for uNK cells. Since the experiments of Zygmunt et al. [262] it has been thought to be involved in such a way in the control of invasion but, paradoxically, could protect from apoptosis as suggested by Bulfone-Paus et al. [263], which shows the need for more studies; however, it can in fact synergize with IL-2 to generate LAK in fresh human (PBL!) cells as, for example, reported by DeBlaker-Hohe et al. [264]. To the best of our knowledge no such studies on uNK isolated from the decidua have so far been conducted.

In our studies on infertile women, recent data [255] correlate IL-15 levels with high IL-18 levels, but not with excess NK counts, suggesting that the main effect of IL-15 would be on NK activation rather than replication, and there would possibly be an effect on non-uNK cells.

In the Croy studies [244, 250, 252] as well as in recent ones of Barber and Pollard [248], IL-15 is necessary for uNK cells. The recent data of Croy's group collected in NK + T cell-deficient mice prove that in their system NK reconstitution only is not sufficient. A T cell component, and likely 'activation' of NK by IL-15, is indeed necessary to correct NK deficiency-associated placental growth and vascular defects [265].¹² The (main) discrepancy between Croy and Pollard concerns fetal survival: both find vascular defects. Surprisingly also, but this is another topic, Barber and Pollard [248] find NK-independent defenses against *Listeria*.

These data go well beyond the Th1/Th2 paradigm and sometimes refute it *totally*. They show the new importance of the IL-15, then IL-12/IL-18 tripod in controlling implantation, *but* beyond that placental growth and vascular remodelling.

Recent data show that NK cytokine production is necessary, hence there is a need for 'proper' activation. They

are no longer compatible with a 'universal' concept of NK 'activation', but on the contrary suggest different pathways under the control of discrete KIR/KAR MHC interactions, cytokine/chemokine recruitment, and other events. They show that some cytokines classified as 'bad guys' by the paradigm are necessary for successful implantation and successful pregnancy!

However, IL-12 is also involved in preterm labor [266]. In this context, it is worth recalling that even TNF is not solely a bad guy, but as pointed out by Hunt et al. [267] pivotal to pregnancy [see also 268, 269] (not to mention here its role in key events in fetal development).¹³

The data suggest new avenues of research. But they also show that the so-called rationales for lymphocyte alloimmunization as a treatment of RSA, which have 'evolved' to fit in with the 'in the mood paradigm' while the double-blind data became more and more thin, if existing at all, must be seriously reconsidered even by the present 'believers'. If they pursue, they should be warned of hitherto unsuspected side effects!

Some kind of NK activation, not 'dampening', is required for successful pregnancy.

As long as we do not know the precise molecular mechanisms of the pathology of each specific case, an all-out immunization approach totally lacks any scientific basis for implantation failure, if it remains one for RSA. It is interesting indeed to see that even Clark [268, 269] adopts a cautious approach as regards implantation.¹⁴

The additional complexity of the uterine network is also reflected by the microarray data in our first longitudinal microarray study in the CBA $\times$ BALB/c versus the CBA $\times$ DBA/2 mice, which we will not outline here in detail; however, while this technique allows to monitor extremely rapidly the activation of a whole panel of 'newer' cytokines, including as we expected many cytokines of the IL-10 family, which would have required a much greater effort with other techniques, the detailed pattern is incompatible with the simpler Th1/Th2 scheme.

¹² As many inflammatory cytokines, IL-15 [266] is enhanced in preterm labor and labor. The involvement of IL-6 and TNF at parturition is outside the scope of this review, but parturition *is useful*; thus these cytokines are no 'bad guys' there either.

¹³ This means that I consider the proposal to use Remicade in blind studies for implantation failure on the basis that 'implantation failure is like rheumatoid arthritis' totally unjustified, foolhardy, and unethical.

¹⁴ This does *not* negate that prereproductive cycle therapies are *not* recommended, some of them involving immunomodulation; see for example the approach of Ledée-Bataille et al. [276] to the thin mucosa syndrome.

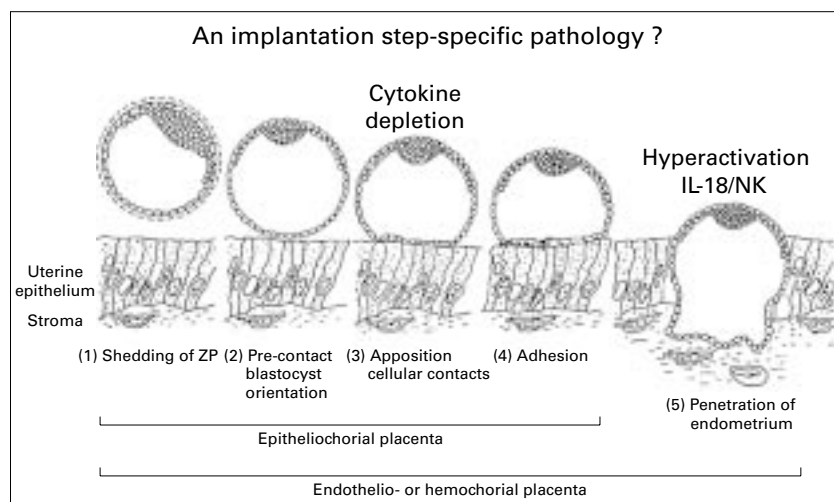


Fig. 7. Possible involvement of cytokine defects in implantation.

Discussion and Conclusions

The 1980s to 1990s saw a shift in mainstream immunology: the field moved from a single lymphocyte, responsible for antibody production, to the specificities of lymphocyte receptors, the discovery of cytokines and their pleiotropic functions, chemokines, and then to NKs, KIRs, and KARS.

Hence, immunological malfunction disequilibrium could be very localized in time and place and affect a discrete cytokine-producing organ or even a single cytokine, redundancy and chaotic activation being as pointed out by Callard et al. [270] another element of the system! In the 1990s there was another revolution: the component of immunity, the 'innate' one, and the concept of the 'missing' self. In a pregnancy, all these concepts have to be integrated, and after immunotrophism, Tom Wegmann wrote that 'the embryo was immersed in and bathed by a sea of cytokines'.

While the Th1/Th2 classification, initially developed for mainstream immunology, appeared to him in 1993 to be a satisfactory depiction of the ongoing maternofetal relationship when he wrote the sentence above, he already knew that in implantation some Th1 molecules were crucial for some steps of the process.

I am sure that he would now be fascinated by the complexity of the network, the untying of which will certainly lead to the discovery of multiple specific deregulations (see fig. 7 for an oversimplified overview), each one very precise and with its own therapy.

In the meantime, simple concepts have to be considered as an oversimplification, which have been as useful

as the Th1/Th2 paradigm has been. Some of its consequences, like the need for a 'lack of NK activation or downregulation of NK activity' or 'interferon is a bad guy', are now known not to be valid in the framework of what we know about what an NK cell 'sees', how it 'reacts', and the pathways it can choose. As useful as it has been, the paradigm can no longer be used as a general explanation, and is particularly irrelevant for implantation, but it is now also evident that it does no longer explain the ongoing pregnancy.

In this context, deciphering the proper molecular basis of each kind of implantation defect and abortion is paramount to choose treatments. It is also important for basic research and for our further understanding of early human development.

At best, recent data of Christiansen et al. [271] show that IVIG is effective only in a small percentage of women and data of Thomas et al. [272] suggest that this may in fact be due to antiviral effects rather than immunomodulation.

In consensus double-blind studies, LIT is effective in only 1/11 of all treated women, but there is no positive selection criterion to identify her [30, 31]. The percentage might for some be higher if one looks at primary aborters only, but this is heavily challenged.

At worst, for many studies which are objective, it does not work. Some are simply computer meta-analyses of published data by the practitioners themselves [273].

Alloimmunization is known to have side effects some long-term, such as a higher incidence of some cancers, albeit this is known for all transplant studies, not for pregnancy failures, because a proper follow-up was not ini-

tiated. Some are short-term: in animal models, there is evidence that it can induce occult pregnancy losses [197, 269], possibly in a strain-dependent fashion (strain-dependent effects are seen with IL-12 [274]).¹⁵

This, and the reconsideration of the Th1/Th2 paradigm, and particularly of the role of NK, should lead to a reconsideration of such therapies and their abandoning, not to an all-out extension to implantation. There was nothing wrong in 'trying out' such treatments, especially when we had a feeling that it might work from open studies and one double-blind study. Now that we know it works at best in 1/11 women, while more and more studies argue that it does not work, all-out extensions of 'NK dampening and TNF neutralization' should be abandoned because of a lack of proper scientific justification.

We work towards further deepening our understanding of the cytokine networks at implantation and in pregnancy, using the results obtained with the use of the Th1/Th2

paradigm as milestones. The Th1/Th2 paradigm has been immensely useful in reproductive immunology. Using it in the present context would make it a 'dangerous dogma' as stated by Allen and Maizels [275]. Rather, we prefer to design syndrome-specific therapies. The tocopherol Torental treatment (which somehow addresses TNF) in the thin mucosa syndrome is an example [276].

Had Tom Wegmann been here, he would undoubtedly have chosen the option to develop new concepts rather than clinging to older ones.

And he would certainly miss Charlie Loke in the field.

'Et penchés à l'avant des blanches caravelles
Ils regardaient monter en un ciel ignoré,
Du fond de l'océan, des étoiles nouvelles.'

José Maria de Heredia, Les Conquérants

This is also valid for cytokine research!

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¹⁵ Abortifacient effects are also strain dependent: for example, Reina et al. [274] report that IL-12 is not effective in inducing abortion in CRL:CD1(ICR) BR outbred mice.

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Addendum

Three recent papers by the Croy group [1–3] confirm the key role of IL-15, IL-18, IL-12, interferon- γ and possibly IL-27 in controlling the development of spiral arteries and that NK cells need to be activated.

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