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A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy

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Abstract

Focussing attention on cytokines at the materno-foetal interface represents one of the major advances made in the field. This owes much to the visionary views of Tom Wegmann, and to the changes brought about in the field by immunotrophism and Th1/Th2 paradigms. We review these briefly and also point out some emerging problems.

However, a certain number of newly discovered cytokines do not fit into the classical Th1/Th2 dichotomy. Yet, by their capacity to activate or downregulate NK cells, by their action on adhesion molecules, and by their regulatory effects on the vascularisation process, they are of possible interest within the materno-foetal relationship. Therefore, as a first step, we have undertaken a systematic study of the expression of IL-11, IL-12, IL-13, IL-15, IL-16, IL-17 and IL-18 in the uterus, the peri-implantation embryo, and later on decidual and

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placental tissues throughout pregnancy. These cytokines were detected in every case, with, in each case, a precise localisation, which will be detailed, and which indeed suggests important regulatory functions, especially during implantation. In some cases, as will be shown in the peri-implantation uterus, those cells are perfectly expressed by uterine GMG-NK-like cells. Comparative ELISAs and quantitative RT–PCR have been or are being conducted, but already the expression patterns that are observed, and the very precise window of appearance that is observed for some of the GMG NK-like cells, either around or in the implanting embryo, as well as the complexity of the respective distributions, strongly suggest that, as useful as it certainly was for a while, the Th1/Th2 paradigm must now be considered as an over-simplification. Rather, the existing data point to sequential windows and are suggestive of a system where an extreme complexity is allied to very precise timing and tuning. They also suggest that the materno-foetal relationship is not simply maternal tolerance of a foreign tissue, but a series of intricate mutual cytokine interactions governing selective immune regulation and also control of the adhesion and vascularisation processes during this dialogue.

Keywords: Cytokine expressions; Materno-foetal interface; Th1/Th2 dichotomy

1. Introduction

The field of reproductive immunology has undergone a profound change since Sir Peter Medawar discovered 'the riddle of the foetal allograft' (Medawar, 1953), which Leslie Brent still depicts as 'Nature's (almost) perfect allograft' (Brent, 1997). However, several recent developments seriously question this statement as a purely reductionist perspective that might even obscure, rather than clarify, matters. Some see the foeto-maternal relationship as more akin to a host/tumour or host/parasite relationship than a host/graft relationship (Loke and King, 1995). For ourselves, we would rather tend to believe that such classifications are futile, the foetal-maternal relationship being unique and representing a step-by-step, programmed interactive symbiosis. Furthermore, when these paradigms were described, most of the attention was focused on 'classical' (T) lymphocytes. The more recent developments also point to a hitherto unsuspected role for NK cells (Guimond et al., 1997; Chaouat et al., 1998), gamma delta T cells, (Arck et al., 1997) (although this remains controversial) and to the importance of adhesion (Aplin, 1996) and inflammatory molecules in implantation (Mac Master et al., 1992; Sanford et al., 1992; Simon et al., 1994, 1995; Loke and King, 1995), moving away from the concept of 'tolerance' or unresponsiveness to the conceptus.

At that time as well, the mother's immune system was thought to interact with classical polymorphic MHC class I molecules, whose expression on trophoblasts was seen as a threat to the foetus. There has since been emphasis in primates on the involvement of non-classical MHC class I molecules. The most discussed is HLA-G (Carosella et al., 2000; King et al.,

2000a; Van Der Ven et al., 2000). It should be stated, however, that there is still no convincing evidence of an HLA-G homologue in non-primate species, although candidate molecules exist (Sipes et al., 1996). It should also be pointed out that even the role of MHC-G in primates is disputed (Castro et al., 2000). However, as HLA-C is expressed on human placenta (King et al., 1997a,b, 2000b), and since in fact the resistance of choriocarcinoma to NK lysis is rather mostly HLA-G-independent (Avril et al., 1999; Sivori et al., 2000), the extreme emphasis placed by some (Carosella et al., 2000) (but not all) on this molecule should perhaps be seen in perspective, without ignoring its role: for example, the discovery of HLA-G1 null individuals (Casro et al., 2000; Van Der Ven et al., 2000) does not per se rule out an involvement of sHLA-G2, although the case made from studies in Pongidae may be a stronger indication that HLA-G is not required for successful pregnancy in primates.

Aside from those new perspectives, a key role is now accorded to cytokines at the foeto-maternal interface. These new appraisals, as well as some of the re-formulations mentioned above, are due to a large extent to the two seminal proposals formulated by the late Tom Wegmann: (a) the immunotrophic theory (Wegmann, 1984), and (b) the more recent proposal that 'allo pregnancy is a Th2 phenomenon' (Wegmann et al., 1993).

However, we review here some facts that are, in our view, incompatible with those classical paradigms, as useful as they might have been in our understanding of the materno-foetal relationship.

2. Inflammation and implantation

The first indication that the paradigm 'pregnancy is a Th2 phenomenon' (Wegmann et al., 1993) might not be universally applicable came with the discovery of the involvement in implantation of a pro-inflammatory-like environment. The key roles of LIF (leukaemia inhibitory factor) (Stewart et al., 1992) and IL-11 (Robb et al., 1998) were not predicted by any models.

For LIF, in mice, Stewart has elegantly shown that the maternal production of LIF is mandatory for successful implantation: LIF-deficient mice, obtained by 'gene knockout', are fertile: the embryos are normal, but they do not implant at all. Successful implantation of embryos in HILDA (human interleukin for DA cells), best known as LIF-deficient mice, can be observed via supplementation by recombinant HILDA/LIF via an osmotic pump (Stewart et al., 1992).

In humans, we have obtained evidence that uterus-specific LIF production defects could be involved in human sterility (Delage et al., 1995), and

this has been confirmed by others (Laird et al., 1997). The question appears now rather more complex than initially suspected, since others have recently reported the discovery of tissue-specific regulation of LIF isoform production, some isoforms being much more active than others (Haines et al., 1999). Also, others have described pinpoint LIF gene mutations in a sterile population (Giess et al., 1999). Some women might therefore exist who have quantitatively normal LIF levels in the uterus, but a qualitatively deficient isoform expression leading to excess local production of the hypo-active molecule, while some women with low LIF production might still be fertile because they would express predominantly the most active part.

The effects of such a local inflammatory response have now been linked to optimal expression on placental trophoblast and uterine decidual cells of adhesion molecules, which are necessary for implantation to occur. Indeed, early and first trimester trophoblasts express both laminin and fibronectin receptors, and specifically the α 1 β 1, α 5 β 1, α 6 β 1 and α 6 β 4 integrin heterodimers, while their receptor molecules (laminin and fibronectin) are particularly abundant in the peri-implantation as well as the first trimester uterus. Trophoblast cell themselves downregulate the expression of the β 4 integrin and upregulate the α 1, α 5 , β 1 subunits during the invasion process (Jokhi, 1994; Loke and King, 1995). We have now been able to link (Lapprée et al., in preparation) LIF deficiency and abnormally low levels of integrins in the pre-implantation human uterus.

This role of inflammation in implantation means that pregnancy is by no means solely Th2, and already points to the existence of several 'windows' with selected cytokine profiles, the so-called implantation window being one of these.

3. New insights on NK cells and MHC class I

The Th1/Th2 paradigm, which most researchers still say predominates, states that established pregnancy is characterised by low levels of Th1 cytokines (TNF, γ IFN), which are known to be abortifacient in a variety of animal models (see, for example, Parand and Chedid, 1964; Chaouat et al., 1990, 1995; Tangri and Raghupathy, 1993; Clark et al., 1997) and are likely to be so in humans (Hill, 1995; Piccinni and Romagnani, 1996; Raghupathy et al., 1999).

Conversely, Th2 cytokines, including IL-10, appear to play a protective role (Chaouat et al., 1990).

Both the Th1/Th2 and the 'immunotrophic' model (Wegmann, 1984; Wegmann et al., 1993) paradigms were described at a time when we saw confrontation or dialogue with the mother as mediated mostly by T cells, which were supposed to be the main producers of regulatory interleukins.

However, it was soon apparent that this scheme was a simplification since the vast majority of interleukins and 'immune cytokines' appeared to be synthesised by cells of the reproductive tract of NON- immune origin. An example is IL-10 secretion by trophoblasts (see Chaouat et al., 1999 for mice; Roth et al., 1996 for the case in humans).

Also, at that time, the role of NK was not fully understood, as it is now.

3.1. Emerging role of NK cells and non-classical MHC molecules

Initially, the NKs were regarded solely as a potential threat to the foetus, since it was thought that their activation might lead to the destruction of the foeto-placental unit. Indeed, in mice, the involvement of NK cells was demonstrated by both the correction/prevention and the induction of abortion in the murine CBA × DBA/2 model (Baines and De Fougerolles, 1988; Kinsky et al., 1990; Gendron and Baines, 1988). But it was also shown to be dependent on local IL-10 production. Surprisingly, IL-10 production by trophoblasts was shown to be controlled by NKs (Chaouat et al., 1998 and Samama M., Chaouat G. et al., unpublished data and manuscript in preparation), demonstrating for the first time a dual involvement of NK cells.

3.2. 'Immunological events' needed therefore to be reconsidered

The first important step was the aforementioned discovery of the role of gamma delta receptors bearing T cells in the decidua (Arck et al., 1997), which, it is fair to say, still remains controversial.

The discovery by the group of Anne Croy that placentae of NK-deficient mice were grossly hypotrophic (Guimond et al., 1997, 1998), leading to premature fœtal death, shifted the 'immunotrophic' paradigm from T cells to NK cells, definite proof being provided by the recent reconstitution experiments (Guimond et al., 1998). The data showing that the production of immunotrophic cytokines might be in fact under the direct control of NK cells do indeed suggest that 'allorecognition' of pregnancy is in fact exerted by NK rather than by T cells, and these data might also be true for humans.

3.3. Cytokine regulation by HLA-G?

As a consequence, a hitherto little-suspected role could be envisaged for HLA-G in humans: control of the production of immunotrophic cytokines, and not solely the local defusing of NK mediated cytolytic functions. Such a control of uterine NK cytokine production by HLA-G was forecasted by Y.W. Loke as early as his invited lecture at the 15th World Congress on

Fertility and Sterility at Montpellier in September 1995 (see also his now classic book with Ashley King, Human implantation, Cell Biology and Immunology; Loke and King, 1995). Data already exist to support this (Maejima et al., 1997)

4. New cytokines and the Th1/Th2 concept

The Th1/Th2 paradigm is further complicated by our own recent studies (Chaouat et al., 2000; Zourbas et al., in press, Ostojic et al., submitted for publication), which forms the body of our presentation at this Delhi meeting.

We have undertaken a systematic study of the expression of IL-11, IL-12,IL-13,IL-14, IL-15, IL-16,IL-17 and IL-18 in mice in the uterus, the peri-implantation embryo, and later on decidual and placental tissues throughout pregnancy.

All these cytokines were found at the foeto-maternal interface, and were mostly produced by cells of non-immune origin.

We will briefly summarise here the immuno-histochemistry data at the peri-implantation period only (although we have longitudinal data that were presented at the New Delhi meeting).

We performed these immuno-histochemistry studies on frozen tissue sections using commercial polyclonal goat anti-mouse cytokine antisera that were, respectively, anti-IL-11 (Anti-mouse IL-11 Neutralizing Antibody, Ref. AF-418-NA, R and D Systems, UK); an anti-IL-12 (Anti-mouse IL-12 Neutralizing Antibody, Ref. AF-419-NA, R and D Systems); anti-IL-13 (Anti-mouse IL-13 Neutralizing Antibody, Ref. AF-413-NA, R and D Systems); anti-IL-15 (anti-IL-15 Goat Affinity Purified, Santa Cruz Biotechnology Inc., via Tebu; Ref. SC-1296); anti-IL-16 (Rabbit anti-mouse/rat/human IL-16 neutralizing antibody, ref. H-110, Santa Cruz 7902, USA); anti-IL-17 (Goat anti-mouse IL-17 neutralizing antibody, ref. AF-421-NA, RandD Systems; UK); and finally an anti-IL-18 (Goat anti-mouse IL-18 neutralizing antibody, ref. AF-422, R&D Systems; UK).

Antibodies of the same origin were used as negative controls to prove the absence of non-specific binding.

The secondary antibody was most often a biotinylated rabbit anti-goat IgG (Ref. E466, Dako). Use was then made of the Vectastain Elite ABC Kit (Goat IgG, Ref. PK-6105, Vector Laboratories, Burlington, USA), and revelation was via addition of DAB tetra hydrochloride (DAB Substrate Kit for Peroxydase, Ref. SK-4100, Vector Laboratories), which forms a dark brown precipitate at the antigen site. For the detection of IL-16, we used the EnVision™ kit (Dako EnVisionTM System with alkaline phosphatase;

mouse/rabbit Fast Red, Ref. K1396), resulting in a bright red coloured precipitate. Counter-staining was by Mayer's haematoxyllin solution, and final mounting in Glycergel® (Dako, France).

IL-11 was strongly expressed in the early decidua, where the strongest expression was detected around the implantation site. On the foetal side, we could detect slight production by the ectoplacental cone and later on the giant cells.

Fig. 1a shows a complete uterus, and Fig. 1b the embryo differentiated already with an inner cell mass, icm, the ecto placental cone, ec, and some positive giant cells, gc, whose presence indicates that ec differentiation into spongiotrophoblast and labyrinth is just about to take place.

The anti-IL-12 staining is far more heterogeneous in the uterus at that stage, the positive areas in the proliferative stroma being still localized around the peri-implantation embryo. Fig. 2a shows the whole stroma, whereas Fig. 2b shows the aforementioned area around the embryo.

Anti-IL-13 labeling is also dispersed in various patches of the uterine stroma. However, all the cells around the implantation site are labelled, as if a shield of the anti-inflammatory IL-13 existed that would protect the embryo against the action of the inflammatory cytokines. Fig. 3a shows the whole uterine chamber and Fig. 3b shows IL-13 shielding the embryo.

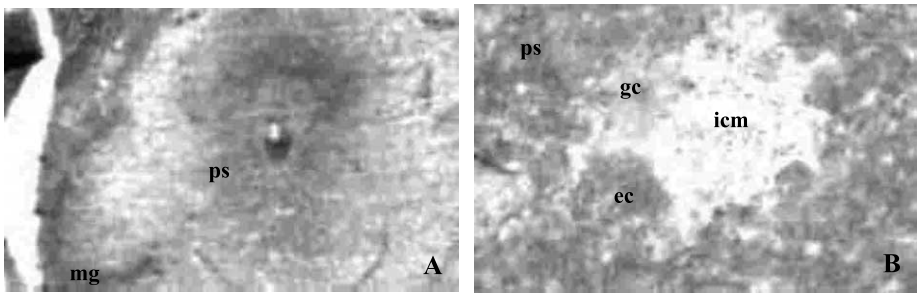

Fig. 1. (a) Localisation by polyclonal goat anti-IL-11 (Ref. AF-418-NA, R and D Systems, UK) of IL-11 in a peri-implantation uterine chamber. The secondary antibody is a biotinylated rabbit anti-goat IgG (Ref. E466, Dako). Black and white rendering from Zourbas et al. (in press). (a) shows at day 8.5 the whole uterus, with the inner proliferative stroma (ps) intensively labelled. The darker area corresponds to the implantation site, and the ectoplacental cone is very intensively stained in the uterine lumen. Mg denotes the uterine metrial glands. The isolated black dots are lymphocytes in the stroma, much more intensively stained than the proliferative stroma itself. One is arrowed as an example. (b) Higher magnification centred on the embryo itself. The inner cell mass (Icm) is totally negative. Cells from the ecto-placental cone (EC) and cells from the proliferative stroma are highly positive. The giant cells (giant cells) that begin to differentiate are much less stained. ec, ectoplacental cone; icm: inner cell mass. A negative control was performed with normal goat serum as the primary antibody.

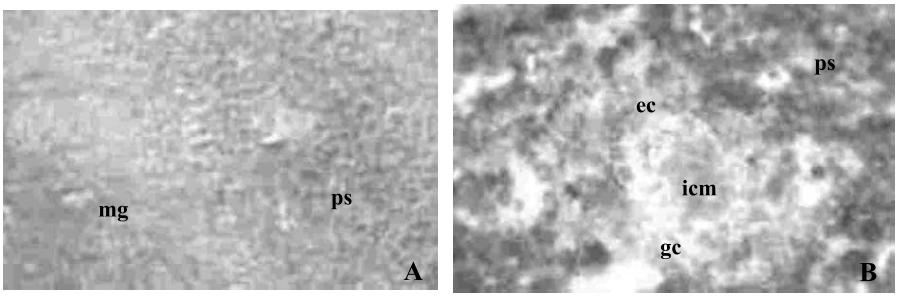

Fig. 2. Same uterine chamber as that in Fig. 1, except this time, the primary antibody is an anti-IL-12 (Ref. AF-419-NA, R and D Systems, again a goat IgG). Again, black and white rendering from Zourbas et al. (in press). Note the differential pattern when compared with anti-IL-11 staining (an unrelated control per se). The labeling within the proliferative stroma is much weaker and patchy, with more positive areas surrounding the implantation site (a). At this stage, some weak staining is seen for the whole inner cell mass, but the giant cells are almost negative (in fact not differentiated from background staining, not shown).

The staining of uterine stroma with anti-IL-15 staining was rather low, but again, as for IL-13, the inner stroma around the embryo appeared positive, circling the embryo just as was described with anti-IL-13 (Fig. 4 A).

In all cases, we always detected IL-16 production by the whole proliferative uterine stroma, and a much stronger labelling was observed for the cells of the glandular epithelium. The production seemed to be at a maximum on around day 6.5. Fig. 5a shows a positive lymphocyte control for the kit, at a high magnification on a cytofuge PHA lymphoblast smear, whereas Fig. 5b shows the peri-implantation uterus itself.

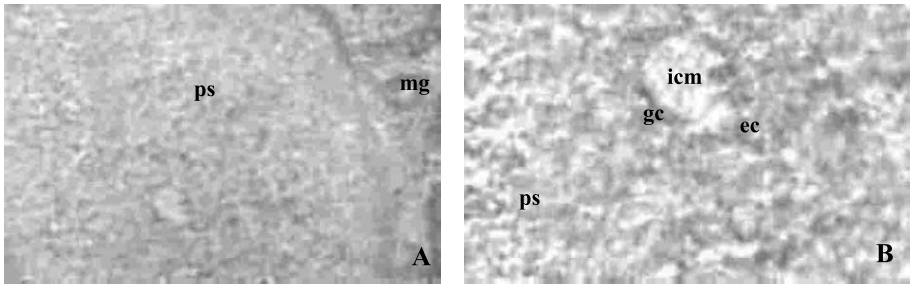

Fig. 3. Again, the very same uterine chamber as in Figs. 1 and 2, also BW rendering from Zourbas et al. (in press), but here, the staining is with AF-413-NA, R and D Systems anti-IL-13 goat antibody. Note that the staining is much weaker in the uterine PS, and that those positive scattered areas are more numerous around the implantation site. At higher magnification, one can see that the inner cell mass is negative, whereas the future placenta and annexes are positive, as if shielding the embryo itself.

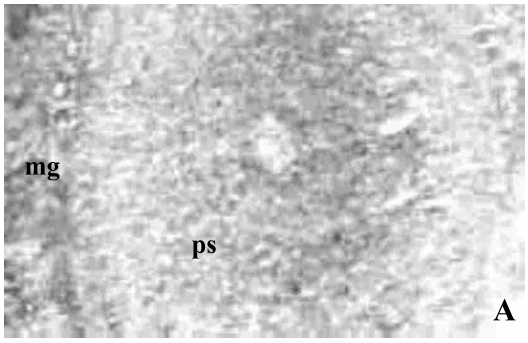

Fig. 4. Whole uterine chamber on day 8.5 stained by Goat Affinity Purified anti-IL-15, Santa Cruz Biotechnology Inc. SC-1296. The negative control (not shown) is also goat. Uterine chamber stained by anti-IL-15. Again, the staining of the proliferative stroma is very patchy, but no staining was observed for embryonic cells.

The expression of IL-17 was the only one we found debatable. If it did exist, we localised IL-17 secreting cells in the in glands and the basal proliferative stroma at days 6.5, 8.5 and 10.5.

IL-18 production started as early as day 6.5 in the basal proliferative stroma, but a strong, localized and temporary production was noted elsewhere: on days 9.5/10.5, we found a very strong labelling by anti-IL-18 of cells of lymphocytic lineage. Using the DBA lectin marker, these cells were definitely identified as uterine NK cells. The cells were very abundant all along the implantation sites, as well as in the adjacent uterine epithelium. However, those cells, which were relatively distant from the implantation site, were negative, whereas only those localized in the very basal decidua were (very strongly) labelled. Fig. 6 depicts the anti-IL-18 positivity of such uterine NK cells. Table 1

For the foetal side, anti-IL-18 staining was observed in the ectoplacental cone, and then in the differentiating spongiotrophoblast, while the inner part, differentiating as the future labyrinthine area, was negative, as was the inner embryonic cell mass. Later on, only the giant cells and the spongiotrophoblast were labelled, with no expression in the labyrinth itself.

We believe that such a complex pattern of expression is difficult to reconcile with a pure Th1/Th2 paradigm. This impression is further strengthened by the additional complexity unravelled in the placenta and decidua at further stages of pregnancy, which, for the sake of space in this survey, we will not detail here (Ostojic et al., submitted for publication, Zourbas et al., in press). Also, one must add that the paradigms do not take into account the discrete balance that may exist between various isoforms of each cytokine, which allow very fine tuning. IL-12 expression at the

utero-placental interface, for example, displays a great level of complexity, the ratio of IL-12 p 40 homodimer/heterodimers/bioactive IL-12 being somehow different in the CBA × BALB/c and CBA × DBA/2 mating combinations (Zourbas et al., in press). Thus, as is the case for LIF (see above), the existence of multiple isoforms has now to be taken into account.

Another example of difficulties encountered by the models is exemplified by IL-18.IL-18, given in synergy with IL-12, is abortifacient in mice (Muranaka et al., 1998), and such may be the case in humans (Ida et al., 2000). The Th1/Th2 paradigm would thus predict more IL-18 in the abortion prone CBA × DBA/2 matings than in the non-abortion prone CBA × BALB/c mating.

To test such a hypothesis, we therefore performed ELISA for IL-18 (as well as for other cytokines) using commercially available kits (mostly, but not always, quantikine R&D System).

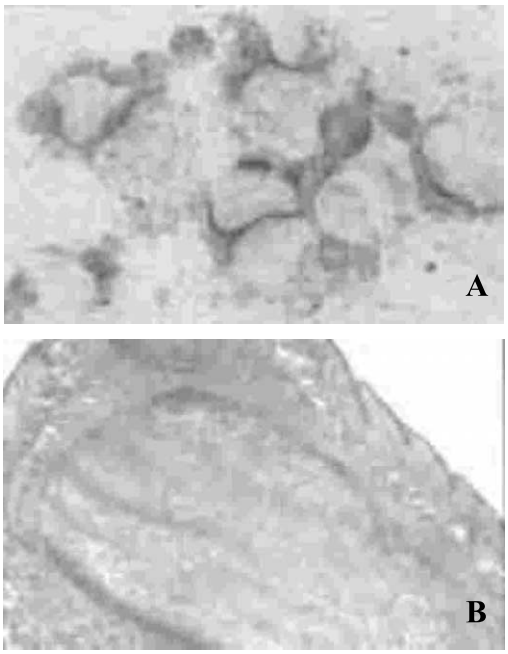

Fig. 5. (a) (From Ostojic et al., submitted for publication) Staining of PHA Blast aggregates with a rabbit anti-mouse/rat/human IL-16 antibody, ref. H-110, Santa Cruz Inc. The revelation here is made by the Dako EnVisionTM System with alkaline phosphatase and mouse/rabbit Fast Red. The nuclei of the blasts appear in white on this rendering, while most of the cytoplasmic areas of the blasts are intensively coloured in red (grey or black areas surrounding the nucleus). (b) Ref. K13967902intra cytoplasmic control staining of PHA blast cytofuge smears with anti-Il-16 (a) and (b) staining of the uterine chamber.

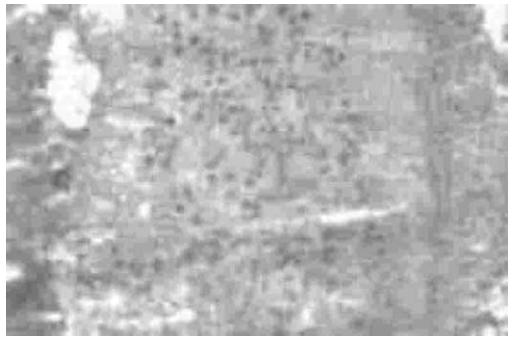

Fig. 6. Staining by anti-IL-18 (Goat anti-mouse IL-18. AF-422, R&D Systems; UK) of uNK cells in the murine uterus at day 9.5, near the placenta/uterus interface. The spongiotrophoblast area is on the right, the uterus on the left. The secondary antibody is as in Fig. 1 a biotinylated rabbit anti-goat IgG (Ref. E466, Dako). The black dots are cells (identified as uNK cells by the DBA lectin method) stained very intensively by the anti-IL-18. Such labelling is restricted to this time window (from Ostojic et al., submitted for publication).

The placentae and deciduae from each individual implantation site were cultured individually in a single well of a Costar 24-well culture plate, filled with 2 ml/well of RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Gibco BRL) and 1% sodium bicarbonate for 48 h in a 5% $C0_2$ incubator. Each individual supernatant was stored at -20 °C to await the ELISA analysis. Given the ELISA kits, we could compare supernatants from 42 placentae (or 42 deciduae) from the resorption-prone CBA × DBA/2 mating vs. 42 placentae (or 42 deciduae) from the non-abortion-prone CBA × BALB/c mating.

We find for IL-18 exactly the opposite pattern to that which the Th1/Th2 paradigm would predict. IL-18 production was *significantly lower* ($P < 0.01$ using the Fisher exact test) in the deciduae from the *resorption prone* mating (4.89 ± 0.19 pg/ml) than in the non-resorption prone mating (9.81 ± 0.34 pg/ml). Similarly, the placental production of IL-18 was significantly lower ($P < 0.01$) in placentae from resorption prone matings (2.17 ± 0.05 pg/ml) than in the non-resorption prone mating (5.44 ± 0.18 pg/ml) (Ostojic et al., submitted for publication).

5. Discussion

These data, and similar data obtained from comparative ELISA analysis between CBA × BALB/c and CBA × DBA/2 matings, as well as the expres-

sion patterns that are observed (Zourbas et al., in press), the very precise window of appearance that is observed for some cytokines in the GMG NK-like cells, and finally the complexity of the respective distributions, strongly suggest that, as useful as it certainly was, the Th1/Th2 paradigm must now be considered as an oversimplification. In addition, the localisation of several cytokines does indeed suggest that they do not solely play an immunological function, but are involved in such events as vascular remodelling, etc.

As a consequence, it is no longer possible to classify a cytokine as a 'bad guy' or a 'good guy' as the Th1/Th2 paradigm would suggest. Indeed, as an example, the recent data from Anne Croy and her group suggest a critical role for low doses of interferon and thus lead to a further reconsideration of the Th1/Th2 paradigm: NK cells, possibly acting directly by their own secretion of Angipoietin 2 (Ang 2) or controlling its local production, as well as the production of other cytokines, are also involved in local vascular events (King et al., 1997a,b; Chaouat et al., 1998; Croy et al., 2000; Samama and Chaouat, in preparation).They might also act on trophoblasts, just as they appear to control IL-10 trophoblast secretion (Chaouat et al., 1998).Therefore, they could be involved as major regulators of local angiogenesis and as such play a major role in pre-eclampsia (Croy et al., 2000). Recent data show also that interferon might be involved in the control of VEGF secretion by trophoblasts (Chung et al., 2001). And interferon, for example, though abortifacient at high doses (Chaouat et al., 1990, 1995; Clark et al., 1997), appears also to be required for successful vascularisation (Ashkar et al., 2000).

Altogether, the Th1/Th2 hypothesis was a useful one. But in the present context, it now appears to be an oversimplification. Rather, the existing data point to sequential windows and are suggestive of a system where an extreme complexity is mixed with very precise timing and tuning. They also suggest that the materno-foetal relationship is not simply maternal tolerance to a foreign tissue, but a series of intricate mutual cytokine interactions

Table 1
Secretion of IL-18 by the murine decidua and placenta as assessed by ELISA in explant culture supernatants of murine tissues

Mating combination	*n*	Mean values ± S.E.M. (pg/ml)	
		Decidua	Placenta
CBA/J × DBA/2	42	4.89 ± 0.19^{a}	2.17 ± 0.05^{b}
CBA/J × BALB/c	42	9.81 ± 0.34^{c}	5.44 ± 0.18^{d}

[a–b, c–d, a–c, b–d]Significally different ($P < 0.01$).

governing selective immune regulation and also control of the adhesion and vascularisation processes during this dialogue.

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