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Demonstration of the Presence of IL-16, IL-17 and IL-18 at the Murine Fetomaternal Interface during Murine Pregnancy

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PROBLEM: To determine if interleukin-16 (IL-16), IL-17, and IL-18 are present at the murine fetomaternal interface during pregnancy as a first step towards investigating their roles in fetomaternal relationship.
METHODS: Expression of IL-16, IL-17, and IL-18, was assessed by immunohistochemistry (IHC) in the BALB/c × BALB/k ($H2^d \times H2^k$), and the CBA/J × BALB/c non-abortion prone, and CBA/J × DBA/2 abortion prone matings. Enzyme-linked immunosorbent assay (ELISA) were performed for the two latter cytokines to compare local production in the abortion prone CBA/J × DBA/2 versus the non-abortion prone CBA/J × BALB/c matings.
RESULTS: Expression of IL-17 was borderline. The anti-IL-16 staining specifically localized in the uterine stroma and glandular epithelium and was rather low in the placenta. IL-18 staining started in the peri-implantation uterus in the basal proliferative stroma, and was also traced, although weaker, in the glandular epithelium. In the immediate post-implantation period, a weak stromal staining persisted but there was a strong labeling of the ectoplacental cone. Interestingly, when the ectoplacental cone differentiates into placenta having a major histocompatibility complex (MHC) class I+ spongiotrophoblast and a (MHC class I–) labyrinth, a very strong transient labeling of uterine natural killer (u-NK) cells was found. Later in gestation, IL-18 was also produced by giant cell and spongiotrophoblast. Finally, we compared by ELISA the production of IL-17/-18 in CBA/J × DBA/2 and CBA/J × BALB/c matings. We detected significantly more IL-18 in the non-abortion prone combination decidua or placenta.
CONCLUSION: The three cytokines IL-16, IL-17, and IL-18 were detected at the fetomaternal interface with a tissue specific, stage-dependent distribution. The predominance of IL-18 secretion in the non-resorption prone matings lead us to question the general validity of the classical T-helper (Th)1/2 paradigm.

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INTRODUCTION

Mammalian pregnancy involves a complex network of cytokines at the fetomaternal interface.[1–3] It has been suggested that successful pregnancy is a T-helper (Th)2-phenomenon, with tolerance of the fetal allograft relying on a local Th2 cytokine bias,[4,5] while optimal placental growth and function would depend on the so called 'immunotrophic' pathway.[6,7] Conversely, pregnancy failure would depend upon too high an expression of the proinflammatory Th1-type cytokines and/or the absence of Th2-type cytokines.[8] The local expression and function of the classical Th1 and Th2 cytokines has been extensively studied,[9,10] but little is known of the expression and role of the more recently discovered cytokines. It is established that newer cytokines play significant roles in other immune responses. They are likely to act similarly at the fetomaternal interface, and

exert regulatory functions on the immune, endocrine, and vascular system functions that are involved in implantation and in the success of established pregnancy.

Therefore, as a prerequisite before launching functional studies, we have begun a systematic catalog-like investigation of the expression of these molecules at the fetomaternal interface throughout murine pregnancy, irrespective of their putative Th1 or Th2 classification.

Interleukin-16 (IL-16), a proinflammatory, chemotactic and immunomodulatory cytokine[12] is also endowed with growth and activating factor capacity for CD4+ T cells, but it can transiently suppress both human mixed lymphocyte reaction (MLR) and anti-CD3 induced proliferative responses.[13] IL-17[14], regulates the production of various proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1-β (IL-1-β) by stromal cells and macrophages and can regulate granulocyte-macrophage colony-stimulating factor (GM-CSF) production.[15] Finally, IL-18[16] in synergy with IL-12 controls Th1 response, inducing interferon-γ (IFN-γ) production by natural killer (NK) cells,[17] CD4+Th1 cells, CD8+TC1 cells,[18,19] B cells,[20] and macrophages. IL-18 is also an inducer of CC and CXC chemokines.[21] Thus, there is a potential for the involvement of such molecules at the interface, where they could for example, participate in the induction of transient T-cell anergy, control Th1 responses and growth factor production, and, most important, act directly or indirectly on the pathways which control proper vascular flow from the mother to the feto-placental unit. We show here that decidua and placenta express these cytokines in a stage-dependent fashion.

We have already obtained data on IL-11, IL-12, IL-13, and IL-15.[11] In this paper, we extend our study by investigation of the expression of IL-16, IL-17, and IL-18 by immunohistochemistry (IHC) on tissue samples obtained at selected stages of pregnancy. To evaluate whether changes of these cytokines were correlated with fetal loss in an abortion prone combination, enzyme-linked immunosorbent assay (ELISA) quantification of IL-17 and IL-18 (IL-16 ELISA being not yet commercially available when these experiments were performed and this manuscript submitted for publication) was performed using tissue explant cultures from the CBA/J × DBA/2 (abortion prone) and the CBA/J × BALB/c (control, non-abortion prone) murine matings.

MATERIALS AND METHODS

Mice

CBA/J, DBA/2 and BALB/c mice were obtained from Iffa Credo (l'Arbresle, France) at the age of 8 weeks, and maintained for a minimum of 15 days in our colony before experimental mating. BALB/k were from our own nucleus colony, originating from a breeding pair obtained in 1992 at Hôpital Cochin, ICGM. The nucleus colony has been maintained since and checked every 2 years by MLR and skin graft rejection tests versus BALB/c, C3H and occasionally obtained retired BALB/k mice. Matings were performed in either the BALB/c × BALB/k ($H2^d \times H2^k$) – which were used for IHC only – or the CBA/J × BALB/c non-abortion prone, and CBA/J × DBA/2 abortion prone matings, which were used for IHC and ELISA studies. Mice were mated in separate cages as 'trios' (one male, two females). The males were removed after overnight caging, dated as day 0.5. The pregnant animals were killed by cervical dislocation on days 6.5, 7.5, 8.5, 10.5, and 12.5.

Phytohaemagglutinin Blasts

Spleen was dissected from a virgin BALB/k mouse, and tissue was homogenized in a Kontes P22 glass homogenizer (Poly Labo, Strasbourg, France). Lymphocytes were washed three times in RPMI 1640 and adjusted, after enumeration using trypan blue dye exclusion, to 5×10^5 viable cells/mL in culture medium, e.g. RPMI 1640-Glutamax supplemented with 1% Penicillin-streptomycin, 1% sodium bicarbonate, all from Gibco Biocult (Paris, France), and 10% heat inactivated fetal calf serum (FCS) (Gibco Biocult, France). After 48 hr of culture at 37°C in a 5% CO_2 humid incubator, lymphocytes were washed, and 10^4 lymphocytes were smeared on Super Frost microscope slides (Poly Labo) which were then processed as for tissue sections (see below).

Preparation of Decidual Uterine Tissues and Placentae

Each implantation site was treated individually. We kept the whole site (full uterine horn) for days 6.5 to 8.5. Later on, the feto-placental unit was peeled from uterine tissues, and then the placentae were separated from the embryo and annexes. In such cases, deciduae were also peeled off individually from the respective implantation sites. In a few cases, especially at days 6.5, 7.5, and 8.5, we kept the complete uterine chambers, e.g. embryo, proliferating uterine stroma, and uterine walls for examination of a complete site whenever possible. The tissue samples were immediately placed in embedding medium (Tissue-Tek OCT Compound, Miles Inc., via Tebu, Le Perray, France; 4583), snap frozen and stored at −80°C until further use. Tissue sections (6 μ) were then cut in a cryostat at −22°C and collected on slides (Super Frost Plus, Menzer Glaser, via Polylabo, Paris, France; 041300). They were then dried for 2 hr at room temperature before being stored at −80°C until use for IHC.

A minimum of three female mothers was used at each point for IHC in each mating combination. The data presented here are representative of the experiments.

Immunohistochemistry

For the detection of IL-16 we used the EnVision™kit (Dako EnVision™ System) with alkaline phosphatase, (Mouse/rabbit Fast Red, Ref. K1396), a two-step system which uses a labeled dextran polymer conjugated to the secondary antibody, resulting in a brightly red colored precipitate. For the detection of IL-17 and IL-18, we used the Vectastain Elite ABC kit (ref. PK-1605, Vector Laboratories, Burlingame, CA, USA) goat immunoglobulin G (IgG) avidinin-biotin three-step system as described in Zourbas *et al.*[11] where the diaminobenzidine substrate (DAB) reacting with horseradish peroxidase forms a dark brown precipitate at the antigen site.

Antibodies and Slides Mounting

The primary antibodies were, respectively, an anti IL-16 [(rabbit antimouse/rat/human IL-16 neutralizing antibody), (ref. H-110, Santa Cruz Inc, USA); (original concentration 200 μg/mL)], an anti IL-17 (goat antimouse IL-17 neutralizing antibody, ref. AF-421-NA, R&D Systems, Abingdon, UK; original concentration 100 μg/mL) and an anti IL-18 (goat antimouse IL-18 neutralizing antibody), (ref. AF-422, R&D Systems, UK), (original concentration 100 μg/mL)]. In every manipulation we have included a negative control with normal rabbit IgG [(normal rabbit serum, immunoglobulin fraction), (ref. X0903; Dako, France)] for IL-16, and normal goat IgG antibody [(normal goat IgG), ref. AB-108-C; R&D Systems, UK)] for IL-17 and IL-18. The sites were also pre-saturated by incubation with 1.5% normal rabbit serum [(normal rabbit serum, immunoglobulin fraction), (ref. X0903; Dako, France)] and normal human IgG [(human IgG), (ref. G-5009; Sigma, Saint Quentin Fallavier, France)] (for the saturation of the Fc fragments). Final counterstaining was by Mayer's hematoxylin solution, and final mounting in Glycergel® (Dako, France).

ELISA Assays

We used the Quantikine IL-17 M-mouse ELISA kit (ref. M1700; R&D System) and the Quantikine M-mouse IL-18 ELISA kit (ref. M1800; R&D System) according to the instruction of the manufacturer. Cytokine standards were those included in the kits. 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 RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Gibco BRL, Paris, France) and 1% sodium bicarbonate as previously described.[22–28] After individual harvesting, each supernatant was centrifuged at 12,000 ***g*** for 3 min and stored at −20°C till the ELISA analysis. For each point in ELISA analysis, we performed duplicates. It must be acknowledged that albeit we tested in the CBA/J × DBA/2 mating combination most of the resorption sites, there were in each of the two experiment a few sites (2 or 3), which were already so partially necrotic we could not culture them.

Labeling of NK Cells by Immunocytochemistry

Uterine NK (u-NK) cells were specifically labeled with Dolichos biflorus (DBA) lectin as recently described elsewhere [personal communication to C. Truyens, and[29]]. DBA selectively binds to glycoconjugates present on the u-NK surface and in the cytoplasm. Briefly, sections were successively incubated with phosphate-buffered saline/bovine serum albumin (PBS/BSA) 1% for 30 min, H_2O_2 3% in PBS for 30 min to inhibit endogenous peroxidase, biotinylated DBA (Vector, Burlingame, CA, USA) 25 μg/mL in PBS/BSA 1% overnight, peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) 2.5 μg/mL PBS/BSA 1% for 30 min, and PBS containing the substrate H_2O_2 0.01% and the chromogen diaminobenzidine 0.5 mg/mL for 10 min (Sigma, St Louis, MO, USA). Sections were mildly counter stained with hematoxylin. For each section, the specificity of DBA labeling was controlled on an adjacent section, by adding N-acetyl-galactosamine 0.1 M to the DBA lectin solution, the sugar which specifically inhibits DBA fixation.

Statistical Analysis

Analysis of variance (ANOVA) was used to compare ELISA values between cytokine production by placentas and deciduas in both mating models, using the Fisher's exact test (*t*-test). All *P* values were considered to be significant at least at the 95% level of confidence. All data are given as mean ± S.E.

RESULTS

Immunohistochemistry

We show the data obtained either in the BALB/c × BALB/c or BALB/c × BALB/k matings. There was no gross qualitative nor quantitative differences between those syngeneic and allogeneic combinations. Within the limit of sensitivity of the technique, there was also no gross qualitative alteration or detectable quantitative alteration between those matings, the non-abortion prone CBA/J × BALB/c, and the abortion prone CBA/J × DBA/2 abortion prone murine mating combination.

TABLE I. Qualitative distribution of the interleukines-16 (IL-16), IL-17 and IL-18 in different periods of pregnancy

	Period of pregnancy (days)								
	8.5 (peri-implantation)			9.5			12.5		
Localization of interleukins	IL-16	IL-17	IL-18	IL-16	IL-17	IL-18	IL-16	IL-17	IL-18
Decidua									
Uterine stroma	+++	−/+	++				+/−	−	++
Uterine stroma – basal				++	+	++			
Uterine stroma – distant				++	+/−	+			
Glandular epithelium	++++	−/+	++	++++	+	+	+	+/−?	+/−
Uterine NK candidate cells				−	−	++++			
Placenta									
Spongiotrophoblast				+	−	++	−	−	+++
Labyrnthe				+	−	−	+/−	−	−
Giant cells				−	−	++			
Reichert membrane							−	−	++

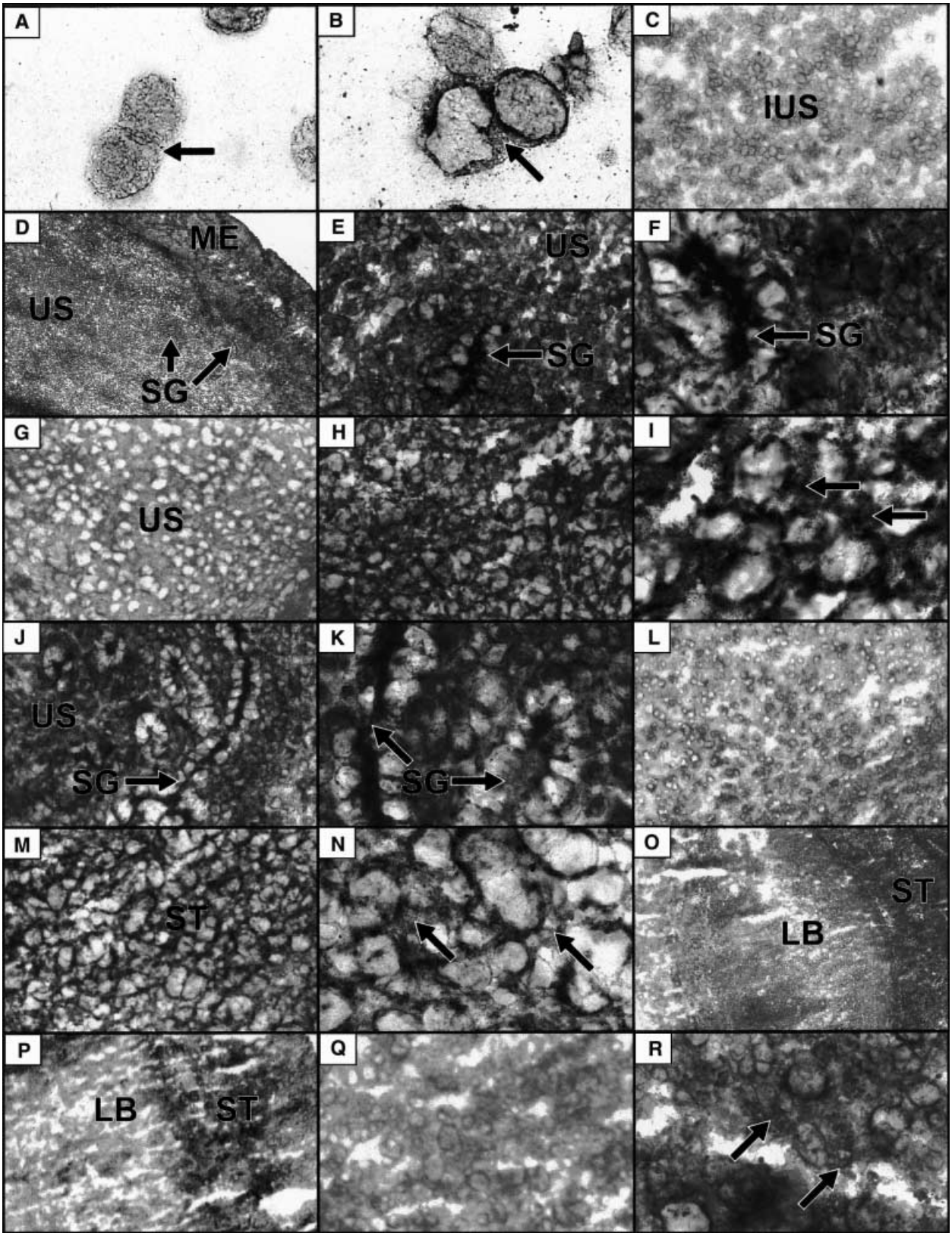
Table I summarizes the qualitative distribution of the IL-16, IL-17 and IL-18 at different periods of pregnancy.

Interleukin 16. A representative collection of the data is shown in Fig. 1, together with a lymphocyte [phytohemagglutinin (PHA) blasts] control (1A). We always observed a positive anti-IL-16 staining in the uterus, throughout pregnancy. This staining was rather homogeneous in the proliferative uterine stroma as early as the peri-implantation stage (Fig. 1D,E), with a rather strong labeling in the cells of the glandular epithelium, whose expression was polarized at the apical pole of the cells (Fig. 1E,F). The expression was strongest around day 6.5. On day 9.5, the staining, although still present, was fainter in the stroma (Fig. 1H,I), while a stronger labeling could still be traced in the glandular epithelium (Fig. 1J,K). There is an apparently time-dependent decrease of the intra-uterine production of IL-16, because by day 12.5 the labeling of the decidua, although still positive, was much weaker than at pregnancy onset.

As far as the placenta is concerned, we observed a faint staining in both labyrinthine (Fig. 1P) and spongiotrophoblast areas (Fig. 1M,N,P) on 9.5 and 10.5 days of pregnancy. The staining was much lower later on, and by day 12.5 the labyrinthine staining traced was very faint, while the staining of the spongiotrophoblast remained relatively strong. Fig. 1R shows such a staining in a positive granules like structures in some cells in the labyrinth, at day 12.5.

Interleukin 17. The expression of IL-17 was very low, as was later confirmed using the ELISA (see below). When positive, anti-IL-17 staining was localized predomin-

Fig. 1. Anti IL-16 labeling of Murine uterus and placentae at various stages of gestation. (envision system): **(A)** a couple of dividing control PHA blasts on a cytospin smear, **(B)** anti IL-16 labeled dividing PHA blasts showing positive labeling of the cytoplasm, **(C)** day 6.5: inner proliferative uterine stroma (IUS), negative control, **(D)** day 6.5: a complete implantation site (whole uterine chamber) labeled by anti IL-16. Note the positivity of the uterine stroma (US) and the much fainter positivity of the cytoplasm of the cells from the uterine secretory glands (SG). The mesometrium is negative (me), **(E)** focus on day 6.5: uterine stroma (US) and the uterine secretory glands (SG). Note the apical positivity and the positivity of the secretion (see below), **(F)** day 6.5: uterine secretory glands, with the glandular epithelium showing a mostly negative cytoplasm, and a possible positivity of the apical pole. However, for the strong positivity of the secretory mucus, the labeling of the secretions themselves, often encountered with the envision kit, a labeling artifact cannot be completely ruled out, **(G)** day 9.5 uterine stroma, negative control, **(H)** day 9.5: uterine stroma, labeled with anti IL-16. Note that when comparing with day 6.5 uterine stroma (Fig. 1D–f) the labeling is much decreased, **(I)** day 9.5: high magnification of the area shown in Fig. 1H. Note that the labeling is definitely localized in the cytoplasm, **(J)** day 9.5: uterine stroma (US) and secretory glands (SG), as in Fig. 1J, **(K)** higher magnification of the area shown in Fig. 1J, **(L)** day 9.5, placenta: negative control of the spongiotrophoblast and labyrinthine area, **(M)** day 9.5: spongiotrophoblast (40× magnification) labeling by anti IL-16, **(N)** day 9.5: spongiotrophoblast cells, 100×, showing intra cytoplasmic positivity by anti IL-16; **(O)** day 12.5, placenta; negative control, showing on the left side the labyrinth (LB) and on the right hand side the more intensely colored, but still with no red positive anti IL-16 label, spongiotrophoblast (ST), **(P)** day 10.5: same, with same orientation, at a slightly higher magnification, but this time using anti IL-16. Note the faint, but positive staining, on the right, of the spongiotrophoblast (ST) and the overall negativity, on the left, of the labyrinth (LB), **(Q)** day 10.5, placenta; negative control, **(R)** day 12.5; positive granules like structures in some cells in the labyrinth.



antly in the glands and in the basal proliferative stroma at days 6.5, 8.5, 9.5, and 10.5. Occasionally reactive cells were nevertheless in scattered areas (Fig. 2D). By day 12.5, the deciduae were totally negative. No anti-IL-17 labeling could be detected in the placenta, or Ecto Placental Cores, at any time point studied.

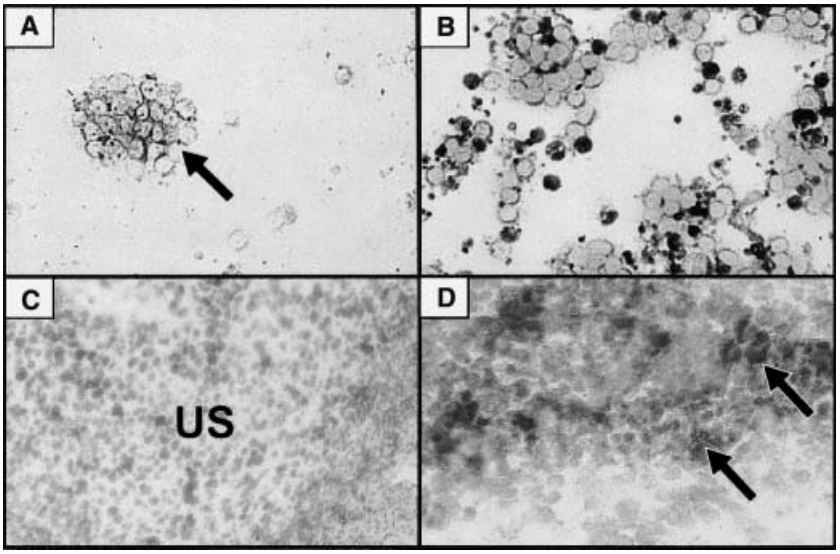


Fig. 2. Anti IL-17 labeling of murine uterus at two stages of gestation (vectastain avidinin-biotin). **(A)** cytospin smear of lymphocytes controls, showing here a complete clone, **(B)** anti IL-17 labeled lymphocytes. Note that, interestingly, not all the blasts within a clone are labeled, suggestive of a stage dependent secretion, **(C)** day 10.5: uterine stroma, negative control, **(D)** day 10.5, some anti IL-17 positive cells scattered in the uterine stroma.

Interleukin 18. A representative collection of the data is shown in Fig. 3. We found a weak emerging anti IL-18 staining in the uterine tissues as early as day 6.5 in the basal proliferative stroma and some weak staining of the cells of the glandular epithelium, which again was polarized and thus strongly suggestive of a secretion. The labeling remained strong later on in the uterine stroma (Fig. 3D shows the uterine stroma at day 8.5) but declined in the glands. Interestingly, on days 9.5 to 10.5, we found a very strong labeling of cells of lymphocytic lineage, which are NK cells, but only at the implantation site at relatively short distance of the spongiotrophoblast/uterus junction (Fig. 3I,K). Indeed, cells of lymphocytic lineage were abundantly scattered all along this interface, as well as in the adjacent, but more profound, uterine stroma. It is noteworthy that those u-NK cells which were at some distance of the utero-placental junction appeared negative, just as those in the mesometrial axis, which were deeper in the uterine stroma, external to the circular smooth muscle. Only the u-NK cells of very basal deciduae proximat to trophoblast were strongly labeled (Fig. 3I,K). Expression was transient, and was not traced at any other stage of pregnancy. By the DBA method, these cells were almost (it is a correlative, not a double-labeling study) unequivocally ascribed as being u-NK cells (Fig. 3E–G). The labeling of u-NK cells was not seen after day 10.5. By days 11.5 and 12.5, the uterine stroma remained positive, but fainter than before.

As far as embryonic tissues were concerned, an anti-IL-18 staining was observed in the ectoplacental cone (gestation day 6.5) (future invasive trophoblast), but not in the inner part of the differentiating trophoblast (future labyrinthine area), nor in the inner cell mass. Indeed, later on (days 8.5 to 10.5), positive labeling was observed in the giant cells area and the spongiotrophoblast, whereas the labyrinth was almost negative.

Later (days 11.5 to 12.5), a strong positive staining was observed only in the outer part of spongiotrophoblast area, while the labyrinth was negative. We also found labeling of cells within the Reichert membrane.

ELISA

Detection of cytokine does not *per se* predict functional effects. As a first insight toward understanding the putative role of these cytokines, we quantified when ELISA was available, in two independent experiments, the levels of those cytokine supernatants of placentae and deciduae, cultured in individual culture wells, and prepared from tissues collected from mice at the apparent peak of expression as determined by IHC, e.g. on day 9.5. In each plate, we compared a total of 42 placentae/deciduae coming from the CBA/J × DBA/2 mating versus 42 of the non-abortion prone CBA/J × BALB/c mating. IL-17 production was very low. In fact we were at the very limit of sensitivity of the assay as defined by the manufacturer, and therefore differences in production levels, if any, would have been of dubious significance. However, no significant variation could be traced between the two mating combinations (Fig. 4), nor in each mating combination between decidua and placenta. However, differences were noted for IL-18 production. Despite the placenta being a rather bigger organ than decidua, the production of IL-18 was higher in the deciduae than in the placenta in each mating combination. This indicates a much more important production per cell, which is attributed to u-NK cells of the decidua. When we compared the same organs for IL-18 secretion between

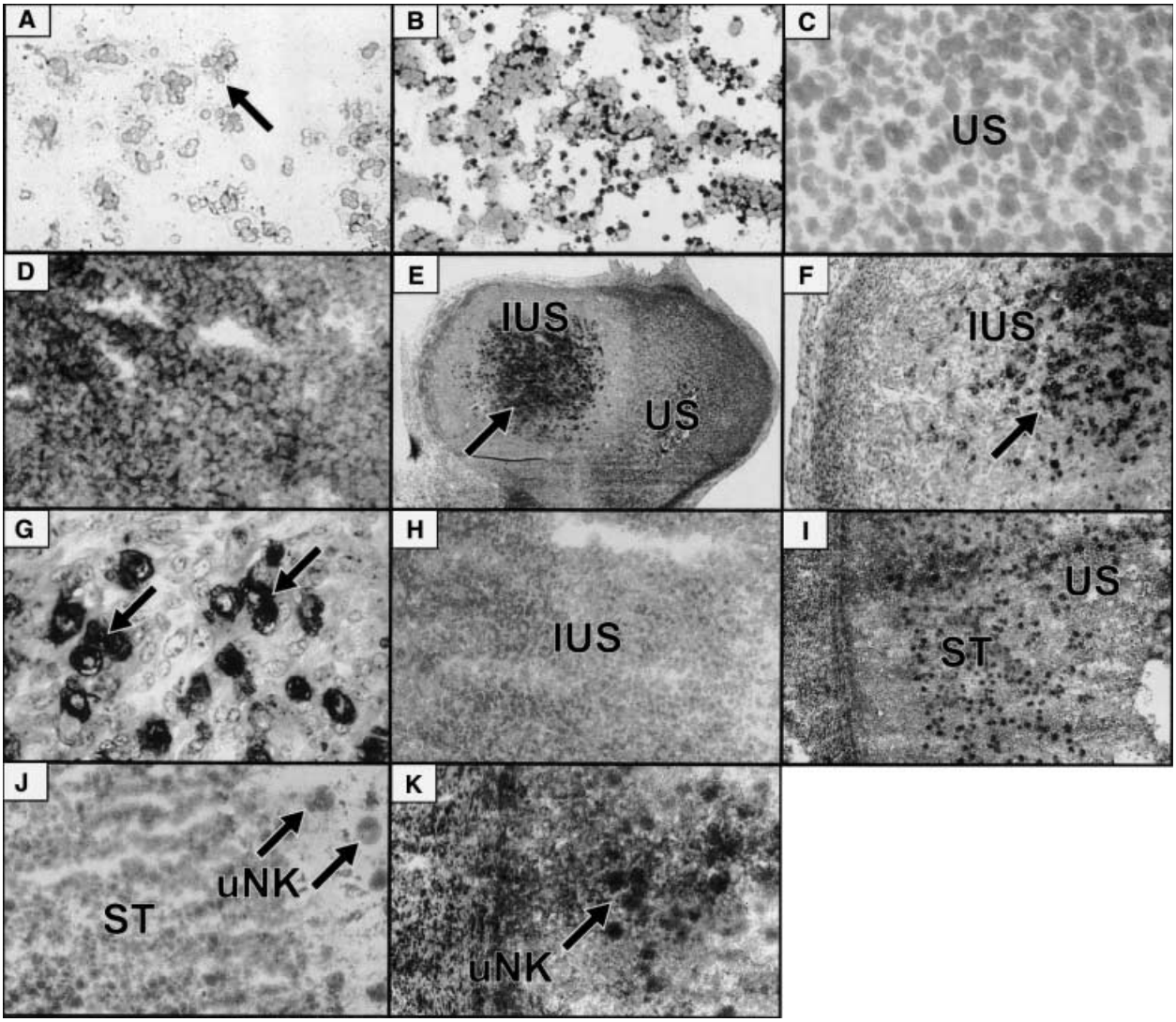


Fig. 3. Anti IL-18 labeling of murine uterus and placentae at various stages of gestation (envision system): **(A)** cytospin smear of PHA blasts, negative control, **(B)** cytospin smear of PHA blasts, labeled by anti IL-18. Note again that as for anti IL-17, interestingly, not all the blasts within a clone are labeled (this pattern is not seen with, for example, an anti IL-2 (data not shown), **(C)** day 8.5; uterine stroma (US); negative control, **(D)** day 8.5, uterine stroma, labeled by anti IL-18, **(E)** whole implantation site labeled by DBA (dolichos biflorus lectin). Note the accumulation of DBA + cells (NK cells) in the uterine stroma (US) and note the position all around the inner part of the stroma (IUS), undergoing decidualization, **(F)** inner uterine stroma showing the DBA + (NK) cells, **(G)** uterine stroma; DAB + (NK) cells, high magnification, **(H)** day 10.5: inner uterine stroma (IUS), mesometrial area of the implantation site; negative control, **(I)** day 10.5: anti IL-18 labeling of cells of the lymphocyte lineage in the uterine stroma (US) at the implantation site. The spongiotrophoblast (ST) is on the left side of the photography, **(J)** day 10.5: uterine stroma, higher magnification, negative control. The NK cells (u-NK) are the large round cells on the right. On this photo they are just near the spongiotrophoblast (ST) on the left side of the photo, **(K)** day 10.5: similar area, showing a positive anti IL-18 labeling of NK cells at near the uterus–placenta junction.

the two combinations, the titer of IL-18 production was significantly lower ($P < 0.01$) in the decidua 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 placenta from resorption prone mating (2.17 ± 0.05 pg/mL) than in the non-resorption prone mating (5.44 ± 0.18 pg/mL) (Fig. 5).

DISCUSSION

The present study was undertaken to localize the secretion of IL-16, IL-17, and IL-18 as a preliminary clue indicating putative functions. Expression of the three cytokines IL-16, IL-17, and IL18, varied according to the stage of pregnancy (Table I). The need to use staining of frozen sections resulted from the fact that the monoclonal antibodies (MoAbs) used in this study

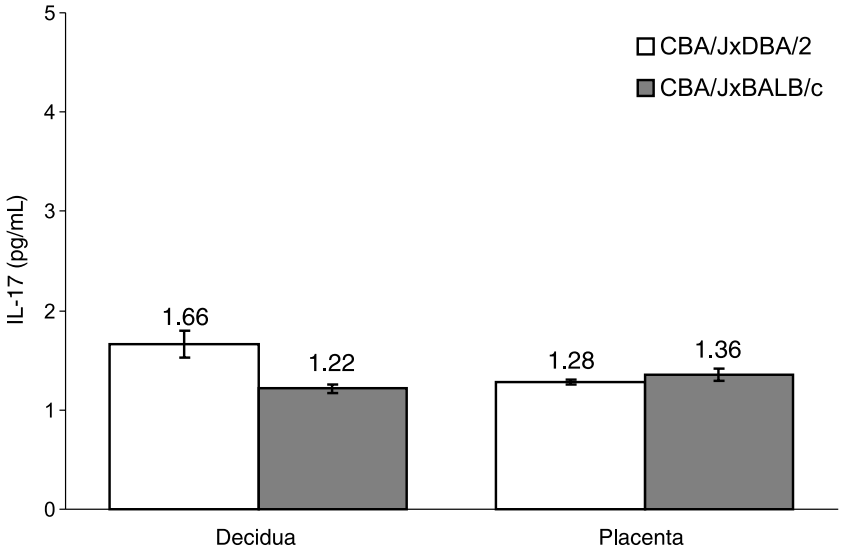


Fig. 4. Comparison on day 9.5 of decidual and placental IL-17 production ($p < 0.01$) by CBA/J × DBA/2 and CBA/J × BALB/c murine matings (±SEM).

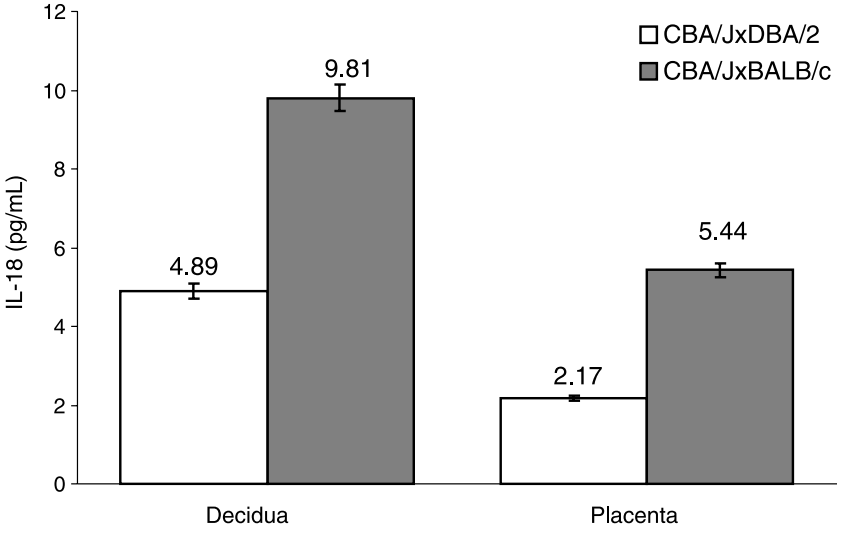


Fig. 5. Comparison on day 9.5 of decidual and placental IL-18 production ($p < 0.01$) by CBA/J × DBA/2 and CBA/J × BALB/c murine matings (±SEM).

were not validated for use in paraffin embedded sections. As a consequence, the slides could not be focused as satisfactorily as what is the case for paraffin embedded material.

There were no qualitative differences within the BALB/c × BALB/c or BALB/c × BALB/k matings by IHC. There was also no gross qualitative variations by the IHC technique between the CBA/J × DBA/2 and the CBA/J × BALB/c matings. Variability is expected between specimens using IHC and quantitative assays must be used for definitive data, such as ELISA (a first result being given here) alone or in conjunction with reverse transcriptase-polymerase chain reaction (RT-PCR) as for other cytokines.[10,11]

Interleukin-16 was produced in relatively large amounts at the interface. One site of secretion is the glandular epithelium (Fig. 1E,F). It is also present in early proliferative uterine stroma (Fig. 1D,E), which strongly suggests a role in early pregnancy. One obvious interpretation would be that it acts at this stage on IFN-γ production and NK cells. Later on, one observes in the placenta a predominant expression by spongiotrophoblast (Fig. 1M,N). Again, this localization, coupled with a lack of labyrinthine expression favors a role in proliferating tissue.

Interleukin-17 is produced at only a low level when assessed by immunohistochemistry. In this respect, it should be stated for IL-17 that the photograph on Fig. 2d was taken with an automatic exposure which somehow seems to compensate for the low staining, and thus the staining may appear rather intense on photographs, and thus much stronger than it is in reality, albeit there is definitely a positivity when comparing with controls. Analysis of IL-17 ELISA values were at

the limit of the commercially guaranteed sensitivity of the assay. As the standards of the assay showed linearity after being diluted twice 1 : 2 (1 : 4) when incorporated in the plate, this suggests that the data are real. Nevertheless, other methods of detection, such as *in situ* hybridization, and quantitative RT-PCR, are definitely needed to ascertain the cellular origin of IL-17.

Interleukin-18 showed distribution (Fig. 3d) similar to IL-16, although in the mature placenta the expression of IL-18 within the spongiotrophoblast area was in fact limited to the outer spongiotrophoblast.

As for IL-16, the pattern of expression, coupled with a lack of labyrinthine expression favors a role at the fetomaternal interface. This assumption is further strengthened by the peak expression of this cytokine in u-NK cells (Fig. 3i,k) known to play an important role in pregnancy[30,31] at the very period where major histocompatibility complex (MHC) class I expression on the spongiotrophoblast is generally agreed to occur. Identification of IL-18 producing cells as u-NK is only correlative here, the DBA lectin method rendering difficult by its intense staining double labeling experiments. Yet, the co-localization is nevertheless striking, and it is unlikely, as there are no other abundant lymphocytes at the implantation site, that the cells could be non-NK cells. We believe that such transient expression of IL-18 on u-NK cells when MHC class I appears on spongiotrophoblasts is unlikely to be merely coincidental. It remains, however, necessary to definitely rule out the presence of T-cell receptor-γ, δ (TCR-γ, δ) bearing cells.[31–34]

Function of these cytokines at the fetomaternal interface is at present purely speculative. IL-16 was originally described as a T-cell specific chemoattractant factor and therefore named lymphocyte chemoattractant factor (LCF). It plays a role in the trafficking of dendritic cells (DCs) toward themselves and toward T cells.[35] Thus, IL-16 may play a role in decidualization and regulation of the local remodeling events that regulate trophoblast invasion. In addition, as T cells responding to IL-16 (e.g. by migration, secreting cytokines and proliferation) are unresponsive to subsequent activation by antigen, IL-16 might be as important as IDO (and other suppressor factors) to induce local CD4 tolerance. It is noteworthy that while the initial experiments blocking IDO resulted in abortion,[36] more recent studies show that such a disruption does not always lead to abortion, suggesting that other mechanisms than IDO are indeed involved.[37] IL-16 could be one of these.

IL-17 is a T-cell derived hematopoietic cytokine like IL-3, IL-4, IL-5 and GM-CSF and could thus be involved in immunotrophic like phenomena, provided the detected concentrations are sufficient for such an effect, which might well not be the case.

Injection of IL-18 plus IL-12, but not IL-12 or IL-18 alone, is abortifacient in DBA $\times$ CBA mice.[38] A more recent report indicated that much higher doses of IL-12 alone induce abortions.[39] There is a significant elevation of human IL-18 levels in sera from the first trimester until the onset of labor, and there are reports of high levels of IL-18 in sera from women with complicated pregnancies.[40] The report by Kruse *et al.*[41] correlating reduced IL-18 levels in the first and second trimester in pregnancy compared with non-pregnant women, with changes in IL-18 messenger-RNA (mRNA) expression correlating inversely with serum values for human choriogonadotropin (HCG) suggests that a low level of IL-18 might be necessary for vascular effects (see below), too high a level of IL-18, or too high levels of IL-12 with normal levels of IL-18 would compromise pregnancy.

But, most important, if one views pregnancy as a continuum, the Th1/Th2 paradigm, should not predict such a scheme. IL-18 being classified (with some difficulties) as 'Th1-like', the tenants of the paradigm of Th1/Th2 balance would thus predict higher IL-18 values in the aborting than in the non-abortive combination. Hence the importance of ELISA quantification. It might have been better to quantify IL-18 in cytosol extracts of the organs rather than by measuring the production in culture. However, the quantitative limits of the ELISA kits for these cytokines do not permit such an evaluation on cytosols from individual implantation sites, but would require using tissues from the whole litter, which does not take into account individual variations which might be very important in the CBA/J $\times$ DBA/2 especially in the resorption window. At this point, it must be stated that we do not believe that not culturing a maximum of two or three resorbing implantation sites which we discarded as being already in advanced state of necrosis has influenced the results, but we nevertheless think it is fair to mention that reservation. Notwithstanding that, we believe that the results of explant cultures are meaningful, because we know from kinetic experiments conducted in mice and humans that we observe a continuous accumulation of cytokine or chemokines in the culture medium till at least 72 hr[22–28] as well as for such viability markers as placenta specific proteins and hormones such as, in human, HCG. This testifies that indeed the explants remain viable, and selectively blocking protein synthesis also confirmed that we indeed measure protein secretion rather than tissue necrosis related release, at least in the first 48 hr which is the period for which the plates were cultured in a 5% CO_2 incubator.

Why might the data obtained by ELISA show an exactly opposite pattern as what one would expect

from the classical Th1/Th2 paradigm (Figs 4 and 5)? A possible explanation could lie in the necessary role of IFN-γ,[42] which is abortifacient at high doses, in synergy with TNF,[22,42] but which seems required at physiological doses for optimal local vascularization at the fetomaternal interface.[42] Indeed, recent work[42,43] emphasizes the role of vascular events during normal,[42] and compromised[42] pregnancies, which show abnormally high levels of decidual TNF and IFN-γ.[44,45] Too high an IFN-γ production and enhancement of TNF levels is the result of an IL-18/IL-12 synergic effect,[46] and thus higher levels of IL-18 in the absence of high IL-12 levels might indeed be beneficial. In addition, high levels of IL-12 have been reported recently to induce defects in vascular protection by decreasing local heme oxygenase production.[39] Indeed we trace in the resorption windows more bioactive IL-12 (heterodimer) than inactive (homodimer) in the CBA/J × DBA/2 compared with the CBA/J × BALB/c combination (Zourbas *et al.*, unpublished data, and in preparation).

However, IL-18 is clearly more than an inducer of IFN-γ.[46] This multifunctional cytokine possesses several biological properties such as regulation of GM-CSF production, which is an important cytokine at implantation.[47] It also activates synthesis of TNF-α, a key pivotal cytokine in pregnancy,[43–45,48] as well as IL-1, IL-2, IL-8, and other chemokines.[21] It also upregulates Fas ligand mediated apoptosis on NK cells and Th1 cells,[49] an event which may be relevant to local tolerance. Finally, IL-18 dependent stimulation of the intracellular adhesion molecule-1 (ICAM-1) expression on monocytic cell lines, and its ability to induce CC and CXC chemokines,[21] might be of importance in regulating trophoblast invasion and adhesion. Thus, IL-18 function needs to be explored in more detail. As stated above, this will require *in vivo* neutralization of the molecule, because injection of the cytokine *per se* has proved not to affect pregnancy (with the reservation that this study has been conducted in non-abortion prone mice, which might be less sensitive to such excess IL-18 injection).[38] However, IL-18 KO mouse deliver apparently normal litters, as do IL-12/IL-18 double KO mice.

Whichever the final function of these cytokines, the data, especially those obtained by ELISA, are among the accumulating evidence that in the past 2 years severely challenge the classical Th1/Th2 paradigm.[11,30,42,43,50] We believe that as useful as it certainly has been, it must now, in our opinion be considered as obsolescent as we have already pointed,[11] partly because it far too simple to account for the emerging complexity and fine tuning, stage-dependent, status of the cytokine networks at the fetomaternal interface. The data obtained here and those reported in other papers[11] suggest a very complex and stage-dependent pattern of events. Some Th1 cytokines, that the paradigm would class as 'bad guys', such as interferon-γ, might have very different effects at high doses[43–45] and low doses.[41,42] Some others, such as IL-18, might have different effects alone or with high doses of others (in this case IL-12).

The clinical consequences of such a network is that one must now be ready to accept the idea that there are likely multiple causes of early pregnancy failure, distinguishing between implantation defects and later on, early fetal damage. The mechanisms might even for those two steps be of different origin: for example, implantation defects correlated with Leukaemia Inhibitor Factor (LIF) deficiency[26,27] or abnormal IL-11 production or defects in IL-11 receptor,[51] are unlikely to be cured by the same treatment. The precise elucidation of this network clearly requires further work, such as selective local neutralization be it by antibodies or promoter directed site specific gene inactivation.

The determination of the cellular site of production of the cytokines was a prerequisite for such future investigations, but must only be taken as a first step toward this direction.

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