CELLULAR MECHANISM UNDERLYING EMBRYO-MATERNAL RELATIONSHIP IN INTRASPECIFIC AND INTERSPECIFIC PREGNANCY

Mekanisme Seluler yang Melandasi Hubungan Embrio-Induk dalam Kebuntingan Intraspesifik dan Interspesifik

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ABSTRACT

Mouse and vole embryos were transferred into pseudopregnant CD-1 and scid female mice. Cellular changes involved in the formation of decidua in the pregnant mouse uterus up to day 8 of pregnancy were examined by histological, electron microscopic, and histochemical techniques. On day 6 of pregnancy, the vole embryos were laid in interstitium of antimesometrial side of the uterus, as well as in intraspecific pregnancy. Compared with intraspesific pregnant mouse, blood vessels were numerous in the decidua around the vole embryos in interspecific pregnancy. Both distribution and dilation of the blood vessels were increased on day 8. A part of cells in the inner cell mass had not nuclei, suggesting damaged vole embryos on day 8. At the implantation site, the uterine decidua was invaded by extravillous trophoblast (EVT) cells whose function is to destroy the walls of the uterine spiral. Moreover, this decidua was infiltrated by a population of natural killer (NK) cells and macrophage. These cells were particularly numerous in the decidua basalis at the implantation site where they come into close contact with invading EVT cells. These results suggest that interaction between NK, macrophage, and EVT provides the controlling relationship of embryo-maternal in intraspecific and interspecific pregnancy.

Key words: extravillous trophoblast, natural killer cells, macrophage, intraspecific interspecific pregnancy

ABSTRAK

Embrio mencit dan vole (Microtus arvalis) ditransfer pada mencit CD-1 and scid pseudopregnant. Perubahan sel-sel yang terlibat dalam pembentukan desidua diamati secara histologis dengan mikroskop elektron dan imunohistokimia sampai hari ke-8 kebuntingan. Pada kebuntingan hari ke-6, embrio vole ditemukan pada interstitium pada sisi antimesometrial uterus seperti pada kebuntingan intraspesifik. Dibandingkan dengan kebuntingan intraspesifik. pembuluh darah lebih banyak ditemukan di area desidua sekitar embrio vole pada kebuntingan interspesifik. Distribusi maupun dilatasi pembuluh darah meningkat pada kebuntingan hari ke-8. Sebagian inner cell mass tidak bernukleus yang menunjukkan rusaknya embrio vole pada kebuntingan hari ke-8. Pada nodus implantasi, desidua uterus diinvasi oleh sel-sel extravillous trophoblast (EVT) yang berfungsi untuk menghancurkan dinding spiral uterus. Lebih lanjut, decidua ini diinfiltrasi oleh sel-sel natural killer dan makrofag. Sel-sel natural killer dan makrofag ini banyak pada area desidua basalis dari nodus implantasi, dan mempunyai kontak dengan EVT yang menginvasi sel-sel uterus. Hasil ini menunjukkan bahwa interaksi antara natural killer, makrofag, dan EVT berperan dalam pengontrolan hubungan antara embrio-maternal dalam kebuntingan intraspesifik dan interspesifik.

Kata kunci: extravillous trophoblast, natural killer cell, makrofag, kebuntingan intraspesifik interspesifik

INTRODUCTION

During early development of eutherian mammals, an embryo comes into contact with the maternal tissue to form a placenta and establishes itself as a successful natural homograft throughout the pregnancy period. The conceptus, which contains half-paternal and half-maternal genome, logically should be rejected by the immune system of the mother. However, not only the maternal rejection does not occur, but also the conceptus success to develop for the full term of pregnancy without any rejection (Edwards, 1972). Thus, from the immunological point of view, pregnancy is a balancing act of tolerance toward the fetus on one hand and functional immunodefence on other hand (Knoeller et al., 2003).

This immune tolerance prevails not only for the progeny of the mother, but also for the transferred embryo, therefore the transferred embryo can develop to full term. However in the case of xenogeneic transferred embryo the immune tolerance does not appear and maternal rejection do not enable the xenogeniec embryo to develop successfully (Widayati et al., 2004). The trophoblast, which is the only fetal cell type that is exposed to the maternal uterine decidua and blood, has been suggested play an immunological role in protection embryo from the maternal rejection (Pavia and Stites, 1981; Rossant et al., 1983).

Interspecific pregnancy has been pursued as an interesting theme in the field of life science since establishment of embryo transfer technique. Combined with embryo manipulation techniques including nuclear transfer, interspesific pregnancy will enable to recreate extinct animals or proliferate endangered animals, if the accompanying problems are overcome (Anderson, 1988). An experimental model for interspecific pregnancy, in which allogeneic mouse and xenogeneic vole embryos were transferred bilaterally into uterine horns of female mice have been presented (Widayati et al., 2003). In this allogeneic and xenogeneic combination transfer, all the vole embryos were rejected after day 11 of pregnancy, although they implanted and developed without hindrance until day 7 of pregnancy. While the mouse embryos could

develop normally irrespective of the abortion of the vole embryos in contralateral horns. The findings suggest that disorder of embryo-maternal interaction might induce appearance of numerous granulated metrial gland cells and rejection of the vole embryos. In the present study, we used severe combined immunodeficiency (scid) mice as recipients for combined transfer of allogeneic mouse and xenogeneic vole embryos and examined the cellular changes involved in the formation of decidua in the pregnant mouse uterus up to day 8 of pregnancy by histological, electron microscopic and histochemical techniques.

MATERIALS AND METHODS

Animals

As embryo donors, common field voles (*Microtus arvalis*) and transgenic mice with enhanced green fluorescent protein gene (GFP-Tg mice) (Okabe et al., 1997; Ikawa et al., 1998) were used, so that mouse embryos could be distinguished from the vole embryos by green fluorescence. As recipients, female mice of CD-1 strain (Japan SLC, Hamamatsu, Japan) and scid mice (CLEA Japan, Tokyo, Japan) were used. All the animals were kept under controlled lighting of 12 L (6:00-18:00) and 12 D (18:00-6:00) and temperature of 25±3° C, and all animals procedures are performed according to the guidelines for care and use of experimental animals of Nagoya University.

Embryos Collection and Transfer

Super-ovulation was induced by the intraperitoneal injection of 7.5 IU PMSG and 7.5 IU hCG (Teikoku Zouki, Tokyo, Japan) in female GFP-Tg mice, and 30 IU PMSG and 30 IU hCG in female common field voles with an interval of 48 h. The mouse and vole females were mated overnight with the males of GFP-Tg mice and field voles, respectively, and examined next morning. The female mice that showed a vaginal plug were defined as day 0 of pregnancy and age of embryos was counted as day 0. In vole, copulation was confirmed by the presence of sperms in the vaginal smear, and the age of embryos was similarly defined.

To collect embryos, the females were sacrificed at day 3 of pregnancy by cervical dislocation. The uterine horns were removed and flushed with the media M16 (Hogan et al., 1994) for mice and mKRB (Toyoda and Chang, 1974) for voles. The blastocysts of mice and voles were incubated briefly in M16 and mKRB, respectively, at 37° C in 5% CO_2 and 95% air until transfer to recipient females.

To induce pseudopregnancy, female CD-1 and *scid* mice of 10-12 weeks of age were mated with vasectomized male mice (B6C3F1) in parallel with the donor animals as mentioned above. The pseudopregnant mice were anesthetized by intraperitoneal injection of 0.5% sodium pentobarbital solution (diluted Nembutal, Dinabot, Chicago, IL, USA) with a dosage of 0.15 ml/10 g body weight. The embryos (day 3 of embryonic age) were transferred into uterine horns of a recipient; 3-5 vole embryos to the right horn and 3-8 mouse embryos to the left horn.

Necropsy and Sampling

On days 6-8 of pregnancy, some recipients were perfused with Ringer's solution followed by the fixative of 4% paraformaldehyde in 0.1 M phosphate buffer through the left ventricle under sodium pentobarbital anesthesia, and abdominal wall was opened. And then uterine swellings (implantation sites) were counted and measured for their diameters (the distance from mesometrial end to anti-mesometrial end), investigated macroscopically, and dissected ones were observed using a fluorescent stereomicroscope (Olympus SZX12). Thereafter, the uterine horns were dissected out and immersed in the same fixative overnight at 4° C.

Procedures for Immunohistochemistry

The fixed samples were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and freezed for cryostat sectioning. Cryostat sections of 10 μm thickness were subjected to immunohistological staining for uNK cells and macrophages according to the standard method (Marriott and Reeves, 1982). In order to discriminate the three types of cells, the following primary antibodies were used: rat anti-mouse LY-49G2 (Pharmingen, San Diego, CA, USA) for uNK cells and rat anti-mouse macrophages (Cedarlane, Ontario, Canada) for macrophages. FITC conjugated goat anti-rat IgG (Jackson Lab., Bar Harbor, ME, USA) was used as secondary antibody for uNK cells and macrophages (Jackson Lab., Bar Harbor, ME, USA). Normal goat serum (Dako, Japan) was adopted as the negative control for the primary antibodies. The sections were mounted with fluoroguard antifade reagent (BIO-RAD, Hercules, CA, USA) and examined under the confocal laser scanning microscope (Carl Zeiss, Germany). Some tissues from pregnant uteri were fixed in Bouin's solution for 48 h and processed for paraffin embedding. Serial sections of 5-6 μm thickness were stained by hematoxylin-eosin, Giemsa and periodic acid-Schiff (PAS) reaction to detect neutrophils, lymphocytes and granulated metrial gland (GMG) cells. The sections were observed under a light microscope.

Procedures for Electron Microscopy

The fixed uteri were excised and transversely sliced. The tissue pieces were postfixed for 2 hr in 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer, pH 7.4. After passing through an ethyl alcohol series, the specimens were trimmed and embedded in araldite resin (TAAB Co., England). Thin sections were stained with saturated uranyl acetate lead citrate. Observations were made in a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis

The survival rate of embryos and the density of UNK cells and macrophage were analyzed using Chi-square test and Student's t-test, respectively. The difference was considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Development of Vole and Mouse Embryos in mouse Uteri

As the implantation in mice occurs on day 4 of gestation, the remaining embryos after day 6 of pregnancy were considered to be implanted. The vole embryos were recognized in both CD-1 and scid uteri at laparotomy on days 6-8 of pregnancy. Compared with the results of xenogeneic and allogeneic transfer in CD-1, the survival of vole embryos in combined transfer was not affected by the presence of normal developing mouse embryos. Also the presence and abortion of xenogeneic vole embryos did not affect the development of mouse embryos. The survival rates of mouse embryos were relatively constant during days 6-8 of pregnancy in both CD-1 and scid recipients. While the survival rate of vole embryos decreased in the same periode, but tended to remain higher in the scid than in the CD-1 recipients. However, no statistically significant differences were found between CD-1 recipients with mouse embryos and those with vole embryos, and between CD-1 and scid recipients with vole embryos (Table 1).

Implantation of Xenogenic Vole Blastocyst

On day 6 of pregnancy, the vole embryos were laid in interstitium of antimesometrial side of the uterus, as well as in intraspecific pregnancy. Compared with intraspesific pregnant mouse, blood vessels were

Tabel 1. Survival rate of mouse and vole embryos transferred into mouse uteri

Day of gestation	Embryo Recipient	Mouse		Vole	
		CD-1	scid	CD-1	scid
6		78.3 ± 2.9* (3)**	80.8 ± 4.0 (6)	75.0 ± 8.3 (3)	83.3 ± 14.4 (3)
7		71.0 ± 4.2 (3)	80.6 ± 17.3 (3)	64.4 ± 3.8 (3)	77.8 ± 19.2 (3)
8		86.9 ± 12.5 (3)	86.7 ± 11 (3)	58.3 ± 11.8 (2)	62.2 ± 3.9 (3)

*Survival rate: no of uterine swellings/no. of embryos transferred (percentage).
**No. of recipients examined. Due to the collected vole embryos was very few, some recipients received mouse embryos only

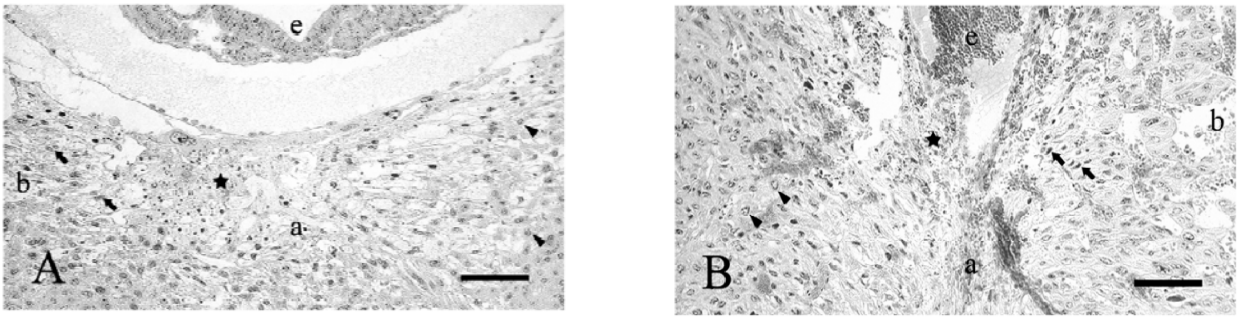

Figure 1. Portions of antimesometrial decidua near the embryo (e) on day 8 of gestation. (A) Mouse decidua of normal gestation. (B) Mouse decidua of xenogeneic transferred gestation. Arrows show examples of trophoblast invaded into endometrium. Decidual cells situated close to the embryo are degenerating (∗) and contain pyknotic nuclei. Arrowheads show examples of stromal cells differentiated into large and rounded decidual cells. In the decidual area around implantation site of vole embryo showed more numerous blood vessels than decidual area of mouse embryo. a = antimesometrial decidua; e = blood vessel. Bars = 100 μm. Paraffin section at 5 μm, hematoxylin and eosin staining.

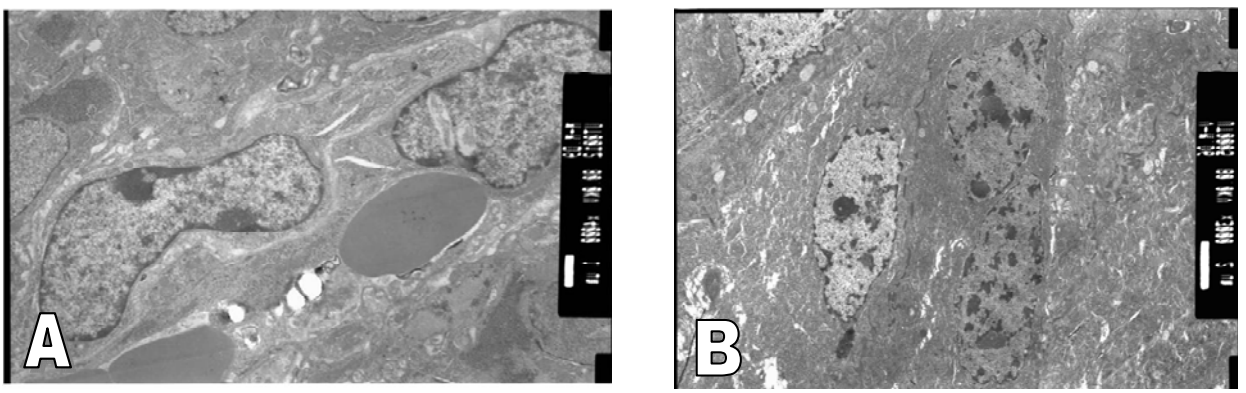

Figure 2. Part of implantation site on day 8 of gestation. (A) Mouse decidua of normal gestation. (B) Mouse decidua of xenogeneic transferred gestation. The tropoblast cells are shown in direct with stromal cells (arrows). Polymorphonuclear leucocytes were seen incontact with, or within the foreign trophoblast cells (arrows heads). X 20,000.

numerous in the decidua around the vole embryos in interspecific pregnancy. Both distribution and dilation of the blood vessels were increased on day 8. A part of cells in the inner cell mass had not nuclei, suggesting damaged vole embryos on day 8. At the implantation site, the uterine decidua was invaded by extravillous trophoblast (EVT) cells whose function is to destroy the walls of the uterine spiral (Figure 1 and 2).

Distribution Patterns of Uterine Natural Killer Cells and Macrophage

Natural killer cells in the pregnant uteri

Anti LY-49G2 immunoreactive uNK cells were observed in all the pregnant CD-1 and scid uteri. At day 6 of pregnancy, immunoreactive uNK cells were present in the inner area of decidua surrounding an embryo. At day 7 and 8, the uNK cells were mostly distributed in the antimesometrial decidua and lateral decidua. There is no difference in the distribution pattern of uNK cells between CD-1 and scid recipients having either mouse or vole embryos.

Table 2 shows the density of LY-49G2 immunoreactive uNK cells and GMG cells in the implantation sites on days 5-7 of pregnancy. The scid uteri with vole embryos had significantly fewer uNK cells than the CD-1 uteri with vole embryos. Although the uNK cells were more numerous in the scid uteri with vole embryos than in those with mouse embryos on day 5 and 6, no significant differences were seen on days 7. This finding suggests that increment of the uNK cells responding to xenogeneic vole embryos is suppressed in the scid mouse.

Macrophages in the pregnant uteri

Immunoreactive cells to anti-macrophage antibody were distributed throughout the uteri including metrial triangle, myometrium and outer decidual area on day 5-7 (Figure 3). The macrophages were scarce in the decidua surrounding embryos. As shown in Table 3, the density of macrophages was almost similar in all five kinds of pregnancy and showed a tendency to decrease slightly parallel with decidualization. The decrease in the density may be due to the expansion of the uterine wall.

In our previous study, common field vole embryos were recognized in CD-1 mouse uteri on day 6 to 10 of pregnancy as well as GFP-Tg mouse embryos. However, at day 8 of pregnancy, internal hemorrhage was observed in the implantation sites. Thereafter, survival rate of vole embryos decreased markedly, and the embryos were excluded completely after day 11. On the contrary, GFP-Tg mouse embryos were alive through day 6 to 14 of pregnancy and developed well in spite of the abortion of the vole embryos (Widayati et al., 2003). In the present study, no internal hemorrhage was observed in the gravid scid uteri containing vole embryos, and the survival rate of the vole embryos was better than that in CD-1 recipients.

Table 2. Density of natural killer cells in the implantation sites with allogeneic and xenogeneic embryos (cells/mm^2)

Day of gestation	Embryo Recipient	Mouse		Vole		Normal Pregnancy*
		CD-1	Scid	CD-1	scid	
6		122.7 ± 34.9 (8)**	106.2 ±31.7 (12)	194.7 ± 45.8[a] (8)	153.0 ± 42.6[b] (8)	155.3 ± 40.8 (5)
7		178.0 ± 52.9 (8)	174.9 ± 39.5 (8)	320.3 ± 73.9[a] (7)	174.0 ± 36.3[c] (7)	187.0 ± 42.0 (5)
8		319.9 ± 51.5 (8)	304.9 ± 63.0 (8)	503.0 ± 84.2[a] (4)	308.7 ± 46.1[c] (8)	215.9 ± 78.5 (5)

* Natural mating of CD-1 males and females. Values are mean ± standard deviation. Superscript letters, a, b and c exhibit significant difference ($P < 0.05$) from CD-1 uteri with mouse embryos (a), from scid uteri with mouse embryos (b), and from CD-1 uteri with vole embryos (c).
*No. of implantation sites examined.

Table 3. Density of macrophages in the implantation sites with allogeneic and xenogeneic embryos (cells/mm^2)

Day of gestation	Embryo Recipient	Mouse		Vole		Normal Pregnancy*
		CD-1	scid	CD-1	scid	
6		105.2 ± 8.0 (8)**	106.1 ± 14.2 (12)	106.0 ± 14.1 (8)	104.6 ± 14.8 (8)	103.4 ± 9.5 (5)
7		101.2 ± 10.3 (8)	102.0 ± 23.8 (8)	109.0 ± 21.9 (7)	106.5 ± 15.7 (7)	105.5 ± 18.2 (5)
8		99.6 ± 8.4 (8)	79.5 ± 7.5 (8)	81.2 ± 9.5[a] (4)	83.0 ± 9.2 (8)	95.8 ± 4.0 (5)

Values are mean ± standard deviation. Superscript letter, a exhibits significant difference (P< 0.05) from CD-1 uteri with mouse embryos. For other footnotes, see Table 2.

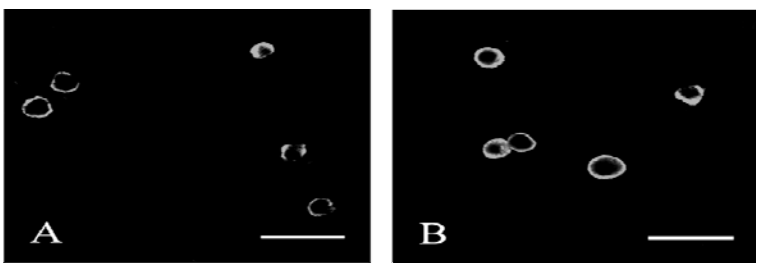

Figure 3. Macrophages in the myometrium of pregnant CD-1 mouse uterus on day 7 of pregnancy. (A) Implantation site with a mouse embryo and (B) implantation site with a vole embryo. Densities of macrophages are almost the same in both transfers. Bars = 25 µm. (Widayati et al., 2004, with permission from Journal of Anatomy)

Our present observations demonstrated at ultrastructural level of vole blastocyst transferred to the uterus of mouse successfully undergo the stages of ovum implantation from the early attachment to the initial phase of trophoblastic invasion of the endometrium. However, none of xenogeneic vole blastocyst survived beyond 96 hr after transfer. Species differences in the composition of cellular surface was suggested induced abnormal interaction in this interspesific pregnancy and the vole trophoblast may fail to protect vole embryos from maternal immune rejection.

The PAS positive GMG cells which increased markedly in the uteri with vole embryos were considered to be NK cells (Widayati et al., 2003). In the present study, the NK cells were detected by anti LY-49G2 antibody which was reported to be a marker of GMG cells (Kiso et al., 1992b). They are distributed in the inner areas of decidua near the conceptus on day 5-6 of pregnancy. At days 7 of pregnancy, however, most uNK cells were present in the mesometrial triangle and outer areas of deciduas. This distribution pattern coincides with the findings by Stewart and Peel (1978), Peel (1989) and Kiso et al. (1992a).

The anti LY-49G2 immunoreactive cells increased with progress of pregnancy from days 5-7. They were the most numerous in the CD-1 uterus with the vole embryos, but their density in the scid uteri with vole embryos was almost the same as in the scid and CD-1 uteri with mouse embryos. King et al. (1996) reported that decidual NK become potent lymphokine-activated killer (LAK) cells when activated by interleukin-2 (IL-2) which could kill trophoblast. It has been reported the importance of trophoblast in maintenance of mice interspecific pregnancy. They enhanced survival of *M. caroli* embryo in *M. musculus* uteri by surrounding *M. caroli* embryos with *M. musculus* trophoblast (Rossant et al., 1983). From this finding, trophoblasts play an immunological role in protecting embryo from rejection as foreign by mother. Therefore, the remarkable increase in NK cell number in the CD-1 uteri with vole embryos seems destructive to the implanted embryos because of the suppressive effect on trophoblast proliferation and their normal activity. The absence of uNK cell increments in the scid uteri seems to improve the survival rate of vole embryos. Despite of suppression in uNK cell number, the survival rate of the vole embryos in scid uteri was lower than that of the mouse embryos in CD-1 and scid uteri. From these findings, the survival of the vole embryos transferred into mouse uteri seems to be deteriorated by increase in number of uNK cells.

In mammalian pregnancy, usual immune response does not function in the uterus, where some macrophages are present. In the present study macrophages were found at myometrium, metrial triangle and decidua. Changes were detected in the distribution of macrophage during the period of pregnancy studied. Macrophages were detected in decidua but fewer than was apparent in the myometrium at day 7 of pregnancy. In pregnancy progress to term macrophages continue to be excluded from primary decidua as reported by Hunt (1990). The reduction in number of macrophage cells in decidua is probably mainly due to failure of this cell population to increase in number while other cell populations present are undergoing extensively proliferative activity. However, no significant differences were seen in the distribution and density of the macrophages among the allogeneic and xenogeneic transfer and normal pregnancy.

CONCLUSION

In summary, our findings did demonstrate, (i) disorder of embryo-maternal cellular interaction induce rejection of the xenogeniec vole, (ii) the increment of uNK responding to xenogeneic embryo is suppressed in scid mice, and the suppression may contribute partly to survival of the embryo, and macrophage play a minor role in early pregnancy.

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