Article

Increased Uterine NK-Derived IFN-γ and TNF-α in C57BL/6J Mice during Early Gestation

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Natural killer (NK) cells are bone marrow-derived lymphocytes. They produce cytokines that regulate the development of acquired immunity. In view of their accumulation at the maternal-fetal interface, uterine natural killer (uNK) cells are also thought to play essential roles during pregnancy. Our results compared the differences of cytokine secretion profile by NK cells in uterine endometrium, liver, spleen and peripheral blood, and focused on the cytokines secretion by uNK cells. It was demonstrated that the expression of IFN-γ and TNF-α in uterine endometrium of pregnant mice are lower than those in liver, but they increase significantly during pregnancy. Our study showed that the number of uNK cells was increased significantly during pregnancy. They produced more IFN-γ and TNF-α than other organ-derived NK cells, and they also secreted minor amount of IL-4 and IL-5. The results indicated that the IFN-γ and TNF-α produced by uNK cells ensured a successful pregnancy progress. *Cellular & Molecular Immunology*. 2006;3(2):131-137.

Key Words: uNK, IFN-γ, TNF-α, IL-4, IL-5, pregnancy

Introduction

Uterine natural killer (uNK) cells are a unique NK cell subset. They resemble human $CD56^{bright}$ NK cell subset with $CD56^{bright}CD16^{-}CD3^{-}$ phenotype. Moreover, similar to $CD56^{dim}$ NK cells, they contain cytotoxic granules (1). In human, peripheral natural killer (pNK) cells are defined by their $CD56^{+}CD3^{-}$ phenotype. They are represented by two different subsets, the $CD56^{dim}CD16^{+}$ NK cell subset and the $CD56^{bright}CD16^{-}$ NK cell subset, constituting 90% and 10% of pNK cells, respectively (2). $CD56^{dim}$ NK cells are granular and known to be cytotoxic. In contrast, $CD56^{bright}$ pNK cells do not contain granules and are noncytotoxic, but have greater cytokine production capacity.

In mice, NK cells are defined by their $NK1.1^{+}$ $CD3^{-}$ phenotype. Functions of uNK cells during gestation are also of tremendous interest because the cells are highly mobile and potentially dangerous to fetal trophoblasts of the implanting blastocytst (humans) and developing placenta (humans and rodents). In pregnant mice, fully differentiated uNK cells are abundant within early decidua basalis, peak on gestation day (gd) 10. By midgestation, the uNK cell population is in decline (3, 4). The literature suggests uNK cells play essential physiological roles in normal gestations and have destructive roles during pregnancy loss. They produce cytokines and hormones that are essential to the regulation of the fetal-maternal unit. Under normal homeostatic conditions, inhibitory receptors are functionally dominant over activation receptors (5, 6). Traditionally it is considered that under normal homeostatic conditions, inhibitory receptors are functionally dominant over activation receptors, Th1 cytokines, such as IFN-γ, TNF-α, have been shown to be embryotoxic, but current opinions support that Th1, as well as Th2 cells, contribute to normal health of the decidua (7).

In order to state the characteristics of uNK cells and study the biological characteristics of NK cells in different tissues, we adopted mechanical dissection approaches to prepare cell suspension of uterine endometrium, liver, spleen and peripheral blood, then performed intracellular staining to analyze IFN-γ, TNF-α, IL-4 and IL-5 secreted by NK cells. ELISA quantifications were also applied to evaluate the production IFN-γ and TNF-α in different tissues, which helped to further investigate the cytokine network on the fetal-maternal interface.

Materials and Methods

Animals

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C57BL/6J mice, eight to ten week-old, were purchased from Shanghai Experimental Animal Center, Chinese Science Academy (Shanghai, China). All mice were maintained under controlled conditions (22°C, 55% humidity, and 12-hour day/night rhythm) in compliance with the regulations of animal care of University of Science and Technology of China. For pregnant mice, selected, estrous females were caged overnight with adult males and the morning on which a vaginal plug was detected was defined as day 0 of pregnancy (gd 0).

Antibodies and main reagents

The phenotype of lymphocytes was analyzed using monoclonal antibody (mAb) in conjunction with immunofluorescence. Fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1, phycoerythrin (PE)-conjugated anti-IFN-γ, anti-TNF-α, anti-IL-4 and anti-IL-5, PE-Cy5-conjugated anti-CD3e mAb, fixation and permeabilization solution were purchased from eBioscience. RPMI 1640 medium were purchased from GIBOCL, and Percoll was purchased from Amersham Bioscience.

Isolation of mononuclear cells from uterine endometrium, liver, spleen and peripheral blood

Mice at specified days after pregnancy were anesthetized with ether and were sacrificed by total bleeding of the incised axillary artery and vein. Peripheral blood was collected from the orbital sinus of each mouse and suspended in phosphate-buffered saline (PBS) containing 100 U/ml heparin. Then the cells were treated with red blood cell (RBC) lysis solution twice on ice, washed once by PBS, and peripheral blood mononuclear cells were prepared. The mononuclear cells from uterine endometrium, liver and spleen, were prepared by mechanical dissection method as previously described (8).

Flow cytometry analysis

The cell-surface staining of all samples were analyzed by FACScalibur™ (Becton Dickinson) as previously described (8). For intracellular cytokine staining, mononuclear cells were incubated in the presence of monensin (5 μg/ml, Sigma), ionomycin (1 μg/ml, Sigma) and phorbol myristate acetate (PMA, 20 ng/ml, Sigma) for 4 h, and then stained with FITC-conjugated anti-NK1.1 mAb and PE-Cy5-conjugated anti-CD3e mAb. After fixation with fixation solution and permeabilization with permeabilization solution (eBioscience), intracellular cytokine staining was performed using PE-conjugated anti-IFN-γ, anti-TNF-α, anti-IL-4 or anti-IL-5 mAb. To prevent nonspecific binding, respective isotype antibodies were used as control. Stained cells were acquired by FACSCalibur and analyzed with WinMDI 2.8 (9).

ELISA

Before analysis, uterine endometrium, liver and spleen were removed and homogenized in PBS. Homogenates were centrifuged to remove debris and then passed through 1.2 μm filters, and the filtrates and serum were analyzed for cytokine concentrations by ELISA. The levels of IFN-γ and TNF-α were quantified by commercial available ELISA kits from Bender MedSystems Inc.

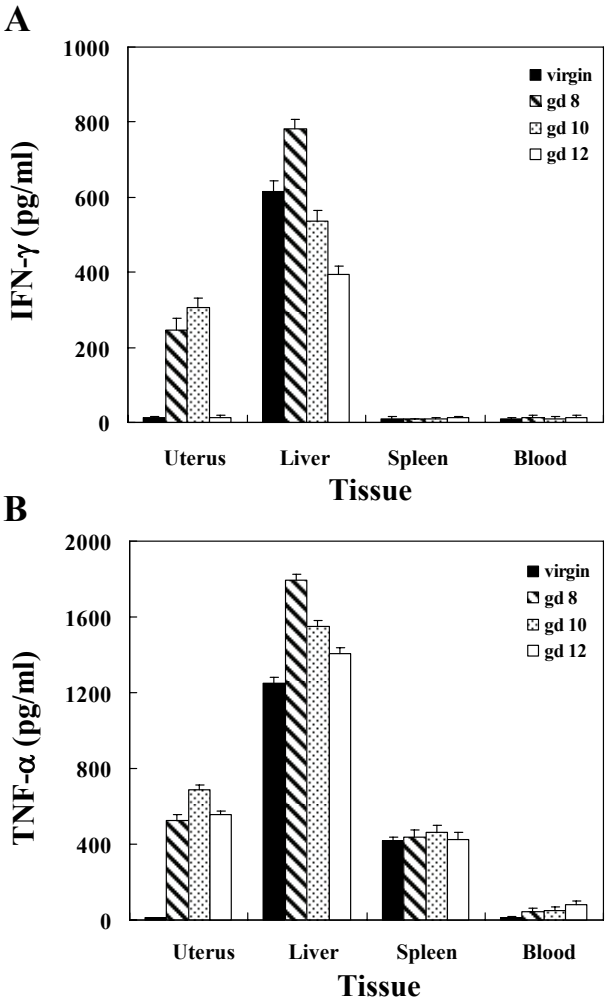

Figure 1. Expression of IFN-γ and TNF-α in different tissues during pregnancy. The production of (A) IFN-γ and (B) TNF-α were detected by ELISA assay. Uterine endometrium, liver and spleen were removed and homogenized in 200 μl PBS on ice. All data were collected to pg/ml of per gram tissue weight. The results were representative of five experiments.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using the Student's *t* test. Difference between the groups was considered statistically significant when *p* value was less than 0.05.

Results

Productions of IFN-γ and TNF-α in uterine endometrium were increased during the normal pregnancy of mice

The IFN-γ and TNF-α contents in uterine endometrium, liver, spleen and peripheral blood were examined by ELISA. The

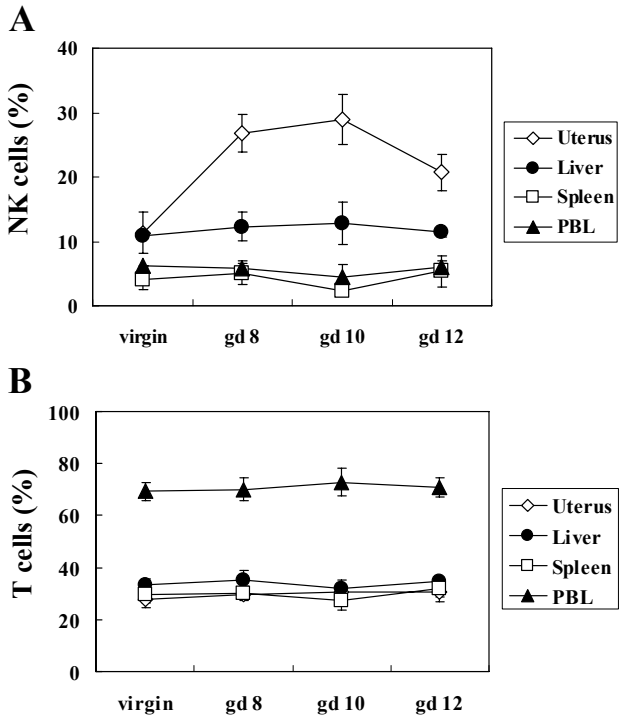

Figure 2. The percentage of NK and T cells in different tissues during pregnancy. The percentages of NK (A) and T (B) cells were analyzed by FACS. Lymphocytes from uterine endometrium, liver, spleen and peripheral blood of pregnant mice on gd 8, gd 10, gd 12 (5 mice per group) were isolated with mechanical dissection, labeled with FITC-conjugated anti-NK1.1 mAb and PE-Cy5-conjugated anti-CD3e mAb, then analyzed by FACS. The uterus of virgin mice was also tested as control. Five independent experiments were performed. Data were shown as mean ± SD.

results showed that the content of IFN-γ from uterine endometrium was low in virgin mice, increased significantly to 246 ± 31 pg/ml in pregnant uterus at gd 8 ($p < 0.05$), and peaked at gd 10 (305 ± 27 pg/ml), then decreased after gd 12. The expression of IFN-γ in liver was fluctuated during normal pregnancy, but the production in spleen and serum remained in a stable value, either from virgin, gd 8, gd 10 or gd 12 mice (Figure 1A).

Meanwhile, the results showed that the level of TNF-α in uterine endometrium was lower in virgin mice than that in pregnant uterus on gd 8 (528 ± 31 pg/ml) ($p < 0.05$), and peaked at gd 10 (690 ± 21 pg/ml), then decreased on gd 12 (558 ± 20 pg/ml), whereas production of TNF-α in spleen and peripheral blood was similar among virgin and pregnant mice on different gestation day. The expression of TNF-α in liver was higher than any other tissue in virgin mice or pregnant mice (Figure 1B).

Percentages of NK and T cells in pregnant mice

There were large number of NK cells located in the fetal-maternal surface during the rodent pregnant process, and herein we applied NK1.1, a mature NK cells surface marker, to investigate the NK population in the mesometrial decidua areas, as well as the liver, spleen and peripheral blood. The distribution of NK cells in various tissues was also analyzed. The number of uterine NK cells was significantly increased at gd 8, peaked at gd 10 (28.88 ± 3.90%), and declined from gd 12 (20.69 ± 2.80%). There were no considerable changes in the NK cell number of other organs (e.g., liver, spleen, and peripheral blood) on different gestation day (Figure 2A). While the number of uterine T cells had no significant increase during pregnancy (27.76 ± 3.23% in virgin mice versus 30.72 ± 1.58% at gd10). In other organs, the T cells had no significant increase either (Figure 2B). The present data showed that uNK cells are a unique NK cell subset, and reconstitution of uNK cells was different from other NK cells during pregnancy. So we further studied the cytokines profile of NK population in various tissues at different gestation stage.

The IFN-γ *and TNF-α productions by NK cells were increased during early pregnancy*

IFN-γ is a proinflammatory cytokine and critical for the defense against a variety of pathogens. IFN-γ production by $NK1.1^+$ cells has also been shown to be important for the restructuring of arteries in the murine placenta during pregnancy. The IFN-γ-positive $NK1.1^+$ cells at designated gestation day were analyzed. The virgin mice were also tested as control. The percentage of IFN-γ- positive uterine $NK1.1^+$ cells of pregnant mice on gd 8 was higher (17.35%) than that of the virgin ones (10.75%). IFN-γ-positive uterine $NK1.1^+$ cells were peaked at gd 10 (21.43%), and declined from gd 12 (17.99%) (Figures 3A and 3B).

TNF-α production by $NK1.1^+$ cells on each designated gestation day was analyzed by FACS. TNF-α secreted by uterine $NK1.1^+$ cells of virgin mice was lower (4.09%) than pregnant mice at gd 8 (10.84%). On gd 10, TNF-α production by uterine $NK1.1^+$ cells peaked (17.26%) and declined after gd 12 (9.69 %) (Figures 3C and 3D). However, $NK1.1^+$-cell-derived IFN-γ and TNF-α of liver, spleen and peripheral blood had no remarkable change in pregnant mice at different gestation days, and there was no change between pregnant and virgin mice (Figures 3).

The IL-4 and IL-5 productions by NK cells during early pregnancy

IL-4 and IL-5 produced by uterine $NK1.1^+$ cells of pregnant and virgin mice were also analyzed by FACS. IL-4 or IL-5 positive $NK1.1^+$ cells was not significantly different in uterine (IL-4: 1.63% in virgin mice versus 1.61% at gd10; IL-5: 1.16% in virgin mice versus 1.53% at gd 10).Similarly, there was no remarkable change detected in the IL-4 or IL-5 production by $NK1.1^+$ cells from liver, spleen and peripheral blood (Figure 4).

Discussion

During the pregnancy, fetal cells invade the maternal decidua but remain spared from attack by the maternal immune system,

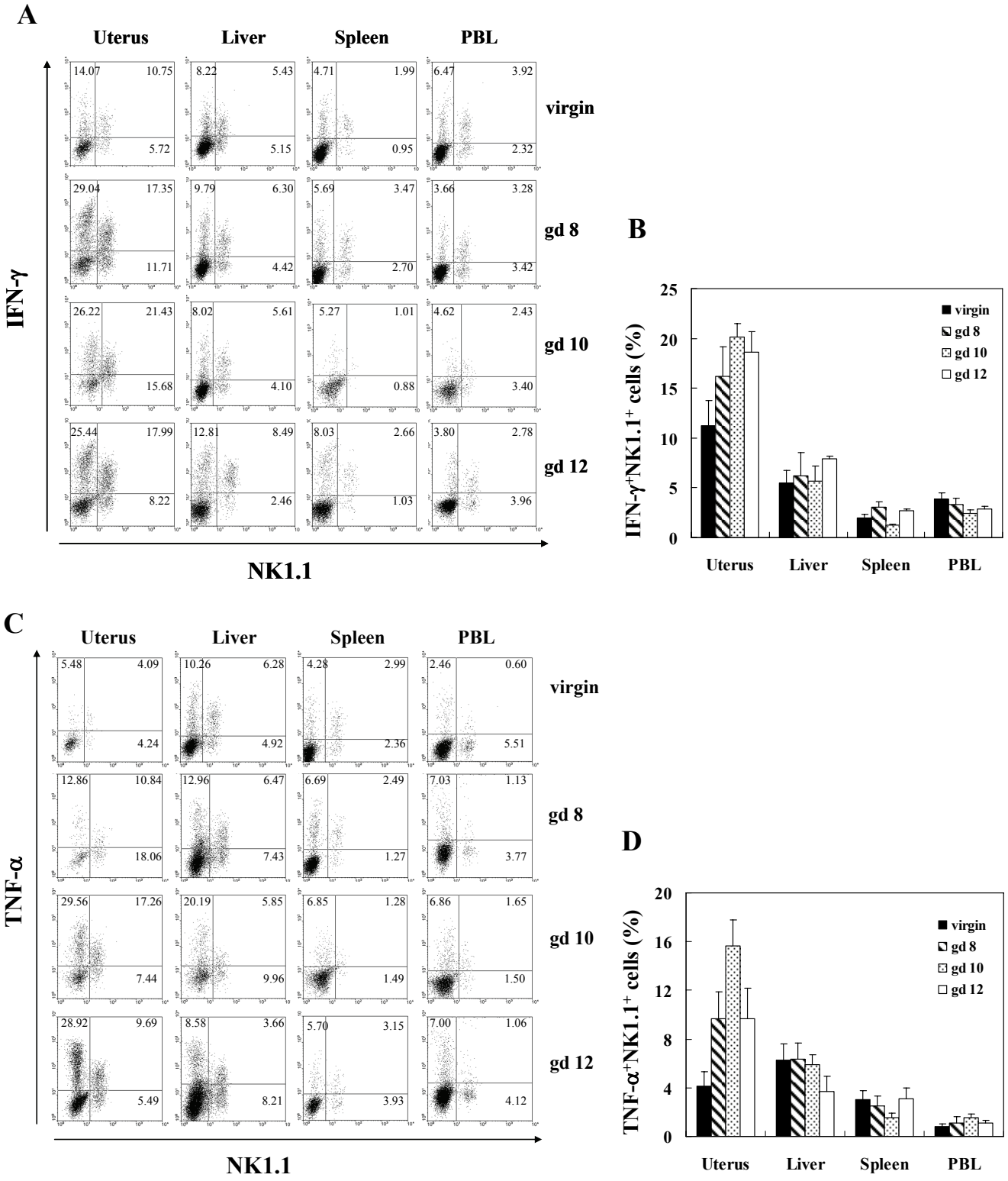

Figure 3. Dynamics of IFN-γ and TNF-α secreted by murine uterine NK cells. Lymphocytes from uterine endometrium, liver, spleen and peripheral blood of pregnant mice on gd 8, gd 10, gd 12 (5 mice per group) were isolated with mechanical dissection and then analyzed by FACS. Membrane CD molecules and intracellular cytokine of lymphocytes were labeled with FITC-conjugated anti-NK1.1 mAb and PE-conjugated anti-IFN-γ or anti-TNF-α mAb. The uterus of virgin mice was also tested as control. (A) One representative result from five independent experiments of intracellular IFN-γ analysis by FACS, (B) The percentages of intercellular IFN-γ positive cells in $NK1.1^+$ cells; (C) One representative result from five independent experiments of intracellular TNF-α analysis by FACS, (D) The percentages of intercellular TNF-α positive cells in $NK1.1^+$ cells. Data were shown as mean ± SD.

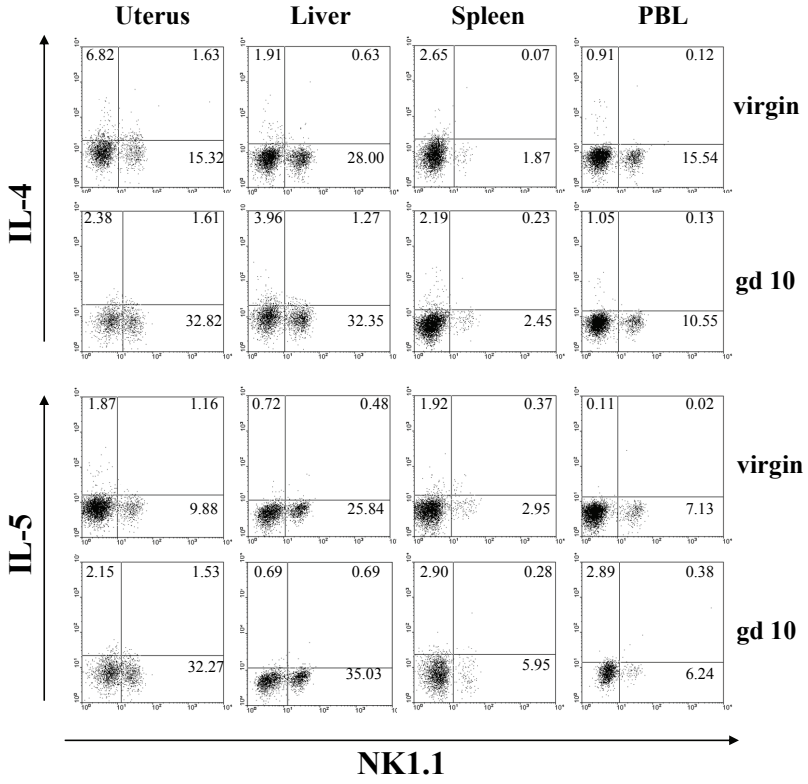

Figure 4. The production of IL-4 and IL-5 by uNK cells. Lymphocytes isolation and staining, experiment group were same as Figure 3. Intracellular cytokine staining was performed using PE-conjugated anti-IL-4 or IL-5 mAb. Five experiments were performed and the results shown were from one representative experiment.

posing a great unsolved paradox of immunology (10, 11). Although several proposes have been raised to explain maternal tolerance, understanding of the immune-biology of normal pregnancy and its implications for pregnancy-related pathologies is still limited (12, 13).

There are more and more evidence indicating that cytokines play a very important role in the maintenance of pregnancy by modulating immune and endocrine systems (14, 15). Because of the expression of paternal genes, the fetus and placenta have traditionally been considered to be analogous to an allograft and, therefore, subject to the laws of contemporary transplantation immunology (16). The Th1/Th2 paradigm, which most researchers still say predominates, states that established pregnancy is characterized by low levels of Th1 cytokines (IFN-γ and TNF-α), which are known to be abortifacient in a variety of animal models and are likely to be so in humans. Though Th1/Th2 hypothesis was a useful one, in the present context, it now appears to be an oversimplification. The maternal-fetal relationship is not simply maternal tolerance to 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 (17, 18).

Some studies suggested that in pregnant women, cytokines produced by Th2 cells predominate over those produced by Th1 cells, resulting in the maintenance of pregnancy (19, 20). Here, we demonstrated that Th1 cytokines could not be ignored. In our result, pregnant mice permit uNK cell production IFN-γ and TNF-α. Furthermore, it had been reported that uNK cells of IFN-γ-deficient mice failed to initiate pregnancy-induced remodeling of spiral arteries (5). TNF-α is another uNK cell-derived regulatory cytokine. The terminal differentiation (not initial activation) and population size regulation of uNK cells are mediated by low concentration of IFN-γ. Normal levels of TNF-α combined with high doses of IFN-γ are compatible with healthy pregnancy. Low levels of endogenous (non uNK derived) IFN-γ are required to maintain decidual integrity. High levels of IFN-γ, normally derived from uNK but substituted for by daily infusions of IFN-γ, are needed for vascular remodeling. An intact IFN-γ signal transduction pathway is required in donor cells for normal uNK development. The low levels of non-lymphoid uterine endometrium IFN-γ present in pregnant uNK cell deficient mice are sufficient to drive uNK cells through their complete differentiation and senescence cycle, this levels, however, are inadequate to correctly support decidual development or to modify the decidual arteries. Only higher levels of IFN-γ, derived from uNK cells or inoculated daily in the absence of any lymphocytes, gave correct development of the decidua basalis and its major arteries in mice genetically uNK cell

deficient (21). However, unregulated IFN-γ production may be detrimental to successful pregnancy. Both Th1 pro-inflammatory and Th2 anti-inflammatory cytokines determined success or failure of pregnancy. Th1 activity during early peri-implantation period, premature and term labour not only accompanies but even predominates over Th2 activity. Th1 activity plays an important role in promotion of Th2 response, regulation of placentation process, defense against infections and initiation of delivery. Together with Th2 activity it is a necessary component of immunological reactions during pregnancy, so paradigm of "Th1-Th2 cooperation" is much closer to reality than "Th2 phenomenon" (22, 23). In the placenta, the Th1 cytokines IFN-γ and TNF-α are also synthesized in response to this pathogen, without fetal loss. It shows a requirement for Th1 cytokines during pregnancy for effective immunity and indicates that a bias away from Th1 cytokine synthesis is not a necessary prerequisite of pregnancy (24).

Uterine NK cell was a novel subset that migrate from peripheral to the mesometrial areas during the onset of pregnancy, but they exert distinct property besides gene backgrounds (10). We can find out that, after stimulated by ionomycin and PMA, IFN-γ and TNF-α secreted by uNK cells were higher than those NK cells in liver, spleen and peripheral blood at same gestation period. They began to increase on about gd 8, and reached the peak when uNK cell numbers peaked on about gd 10 and declined in number after gd 12 (7). Levels of IFN-γ and TNF-α in uterine endometrium were lower than those in liver on same gestation day. It was out of our expectation that uNK cells had limited ability to produce IL-4, IL-5, either in uterine mesometrial tissue or liver, spleen and peripheral blood, indicating that uNK cells have limited cytotoxic potential and can produce numerous cytokines and "cytokines storm" was watched during the normal pregnancy of C57BL/6J mice, suggesting that Th1 cytokines dominate the normal pregnant process. Actually, under normal homeostatic conditions, uNK cells could produce IFN-γ to guaranteed process of decidualization and vasculation of spiral artery under the normal physiological conditions (25, 26).

Acknowledgements

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