Article

Interferon-γ Expression in Natural Killer Cells and Natural Killer T Cells Is Suppressed in Early Pregnancy

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Recent study has suggested that innate immune system might play an important role in pregnancy progression. In this study, to investigate whether NK cells and NKT cells, instead of T cells, are the dominant populations of peripheral blood in early pregnancy, flow cytometry was used to detect the percentage and intracellular cytokine expressions of T cells, NK cells, NKT cells in peripheral blood of non-pregnant women and early pregnant women. In our result, the percentages of NK cells and NKT cells were significantly increased in pregnancy compared to non-pregnancy. However, the percentage of T cells was not changed. We did not detect the Th2-dominance of total lymphocytes or T cells in peripheral blood of early pregnant women and there were also no significant changes of type 1 and type 2 cytokines in T cells, but IFN-γ production in both NK and NKT cells was decreased in early pregnancy. These results suggest that the innate immune system including NK cells and NKT cells should play a pivotal role in pregnancy progression. Type 1/type 2 shift mechanisms in innate immune system during the human early pregnancy should be paid more attention. *Cellular & Molecular Immunology*. 2007;4(5):389-394.

Key Words: cytokine, pregnancy, natural killer cell, interferon-γ

Introduction

Since Tom Wegmann first proposed that fetal survival depends on a shift of maternal immune responses towards T-helper (Th)2 immunity (1), for many years, the Th1/Th2 hypothesis has provided a useful framework for investigation of immunology during pregnancy. Type 1 cytokines, including IFN-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-α/β, promote pro-inflammatory immune responses, whereas type 2 cytokines (IL-4, IL-5, IL-6, IL-10) mainly promote anti-inflammatory, antibody dependent immune responses (2, 3). Normally, body is in a balance of type 1/type 2 cytokines. Once the balance is broken, it maybe initiates diseases. Deregulation of type 1/type 2 cytokine production and imbalanced growth of memory Th1 or Th2 subsets, which produce type 1/type 2 cytokines respectively, have been implicated in the proceeding of multiple immune disorders including asthma and tumors (4, 5).

Recently, the study of type 1/type 2 cytokine correspondence in obstetrics and gynaecology has been a hot topic (6). Conventionally, we consider that type 1 and type 2 cytokines are secreted only by $CD4^+$ Th cells. More recently, it is well notably recognized that the two type cytokines are not only produced by $CD4^+$ T cells, but also by $CD8^+$ cytotoxic T (Tc) cells, NK cells and NKT cells (7-9). So, the concept of "type 1/type 2" balance has been extended largely by demonstrating that cytotoxic T cells, NK cells and NKT cells can also play important roles in their cytokine-secretion profiles. Recently, Borzychowski AM et al. used the surface marker of IL-18 receptors for type 1 cells and ST2L for type 2 cells to distinguish all of the T cell and NK cell populations from total lymphocytes. Their study showed that the type 2 shift during pregnancy was predominantly in the NK ($CD56^+CD3^-$) cells and NKT ($CD56^+CD3^+$) cells instead of in the T-helper cells or cytotoxic T cells. So they proposed that innate immune system, involving NK cells and NKT cells may be predominant population of the peripheral blood in pregnancy, which has challenged the concept of Tom Wegmann's first hypothesis (10, 11). Although more and more literatures are concerning on the interactions between innate immune cells and pregnancy, the exact functions of these cells are still unclear. Maybe they are necessary for the maintenance of pregnancy through the production of cytokines.

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There are numerous methods for cytokine assay, such as single and multiplexed ELISA, reverse transcription-PCR, Taqman real-time PCR, and immunohistochemistry. ELISA is most commonly used in studies because of its relative convenience, however, one limitation of ELISA is that they can not identify which cells the sources of the cytokines secreted into plasma or serum are from (12). The recently established method of intracellular cytokine detection in specific cells by flow cytometry could surmount the limitation and have great potential in experimental and clinical research. In this study, we used the method of flow cytometry to detect the percentage and type 1/type 2 cytokine secretion of T cells, NK cells, and NKT cells in peripheral blood of non-pregnant women and women in early pregnancy in order to further certificate whether the innate immune system including NK cells and NKT cells should play a pivotal role in pregnancy progression.

Materials and Methods

Blood samples

Venous blood (2 ml) samples were collected from 18 healthy women (age 24.2 ± 2.0 years) and 18 pregnant women (age 23.0 ± 2.1 years, gestational age 8-10 weeks) directly into Vacutainer tubes containing sodium heparin anticoagulant. There were no statistical differences between the two groups in terms of age. Informed consent was obtained from all subjects prior to blood collection. The study was approved by the Ethic Committee of Anhui Provincial Hospital of China.

Preparation of peripheral blood mononuclear cells (PBMC)

Blood samples were diluted 1:1 with 2 ml PBS, and PBMCs were separated by density gradient centrifugation (Ficoll-Paque) and resuspended in RPMI1640 and 10%BSA (both from GIBCO) at about 2×10^6 cells/ml.

Flow cytometry

Directly conjugated monoclonal antibodies (CD3-FITC, CD56-PE-Cy5, BD) were used to label surface antigen markers for T ($CD3^+CD56^-$) cells, NK ($CD3^-CD56^+$) cells and NKT ($CD3^+CD56^+$) cells.

The resuspended cells were stimulated in 24-well plates with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma) and ionomycin (250 ng/ml, Sigma) for 4 hours at 37°C and 5% CO_2 in the presence of GolgiStop™ (2 μM, BD) to inhibit cytokine secretion. Then the cells were harvested and washed twice with staining buffer (1× DPBS, 0.09% NaN_3, 1% FCS) and added 250 μl Fixation/Permeabilization solution (BD) per tube for 20 min at 4°C. These fixed and permeabilized mononuclear cells were then thoroughly resuspended in 50 μl of Perm/Wash™ buffer (BD) and added anti cytokine antibody (IFN-γ-PE, IL-4-PE, BD) for 30 min at 4°C. Finally, the cells were washed with Perm/Wash™ buffer twice and with staining buffer once, and then data were acquired by FACSCalibur (BD). For each sample analyzed, gate was set within the live lymphocyte population, 10,000 events were collected, and all data were saved for subsequent analysis using WinMDI software.

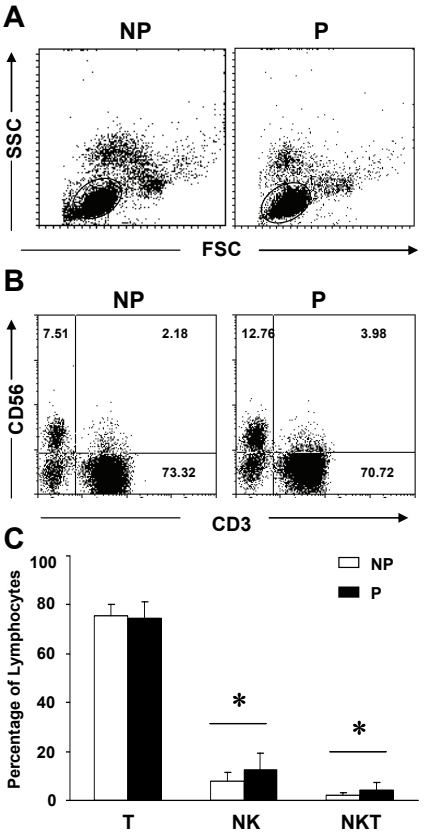

Figure 1. Flow cytometric detection of total T cells, NK/NKT cells within lymphocytes in peripheral blood of non-pregnant women or early pregnant women. (A) In the scatter plot of PBMC, a circular gate was placed around live lymphocyte population based on size (FSC) and granularity (SSC). (B) A sample of flow cytometric detection of total T cells, NK cells and NKT cells. (C) The percentage of T cells had no difference in two groups ($p > 0.05$), however, the percentage of NK cells and NKT cells in pregnancy group was higher than that in non-pregnancy group (*$p < 0.05$). NP, non-pregnancy; P, early pregnancy.

Statistical analyses

Student's *t*-test was used to interpret the significance of differences, which were considered statistically significant for p values < 0.05. Data were shown as mean ± S.D.

Results

The percentages of NK cells and NKT cells were increased in peripheral blood of early pregnant women

In the scatter plots of all PBMC (Figure 1A), an electronic, circular gate was used to select the lymphocyte population based on cellular size (forward scatter) and granularity (side scatter). Within the total lymphocyte population, the percentage of NK ($CD3^-CD56^+$) cells was significantly increased in early pregnancy (12.73 ± 6.40%) compared to non-pregnancy (7.81 ± 3.47%). Like NK cells, NKT ($CD3^+$

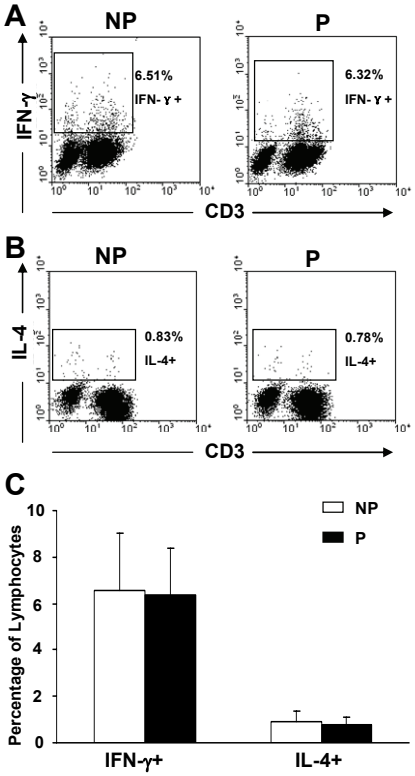

Figure 2. Flow cytometric detection of total type 1/type 2 cytokine expressions in PBMC of non-pregnant or early pregnant women. PBMC were stimulated with PMA/Iono, and stained with cytokine-specific antibodies (IFN-γ-PE for type 1 cells and IL-4-PE for type 2 cells) and CD3-FITC for surface label. (A) Cells stained positive for IFN-γ were classified as type 1 cells. (B) Cells stained positive for IL-4 were classified as type 2 cells. (C) There was no difference in expression of IFN-γ and IL-4 in two groups ($p > 0.05$). NP, non-pregnancy; P, early pregnancy.

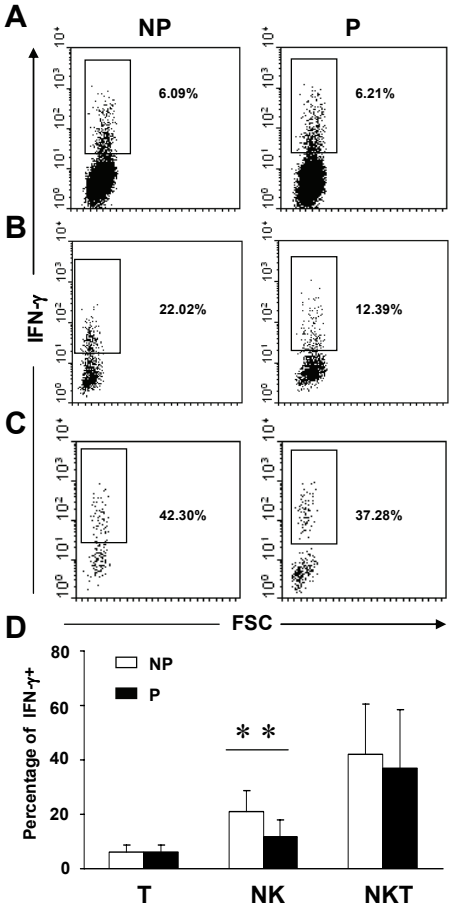

Figure 3. Flow cytometric detection of intracellular IFN-γ expression in T cells, NK cells and NKT cells of non-pregnant women or pregnant women. PBMC were stimulated with PMA/Iono, and stained with cytokine-specific antibodies. The expression of IFN-γ was analyzed in T cells (A), NK cells (B) and NKT cells (C). (D) There was no difference of IFN-γ expression within T cells in two groups ($p > 0.05$). The percentage of IFN-γ positive cells in NK cells was significantly decreased (**$p < 0.01$). IFN-γ production of NKT cells was also decreased in early pregnancy, but there was no statistical significance. NP, non-pregnancy; P, early pregnancy.

$CD56^+$) cells were also significantly increased in early pregnancy (4.10 ± 3.02%) compared to non-pregnancy (2.25 ± 0.87%). However, there was no difference of T ($CD3^+$ $CD56^-$) cells between the two groups of non-pregnancy and early pregnancy, which was 75.59 ± 4.67% and 74.35 ± 6.78% respectively (Figures 1B and 1C).

No difference of the IL-4 and IFN-γ expression in total lymphocytes between non-pregnancy and early pregnancy women

Within the total lymphocyte population, the percentage of type 1 cells did not differ between non-pregnancy (6.55 ± 2.45%) and early pregnancy (6.38 ± 1.99%) groups. Also there was no change in type 2 lymphocytes between the two groups, which was 0.89 ± 0.47% and 0.76 ± 0.37% respectively (Figure 2).

The expression of IFN-γ in NK cells and NKT cells was suppressed in early pregnant women

In peripheral blood of non-pregnant women, T cells of 6.11 ± 2.51% expressed type 1 cytokines (IFN-γ), which were similar to those of pregnant subjects (6.24 ± 2.33%). However, the percentage of IFN-γ^+ NK cells was significantly decreased in early pregnancy (12.03 ± 6.15%) compared to non-pregnancy (21.05 ± 7.89%). Like NK cells, IFN-γ production of NKT cells was also decreased in early pregnancy (36.86 ± 21.78%) compared to non-pregnancy (42.02 ± 18.51%), but there was no statistical significance between two groups (Figure 3).

No difference of IL-4 expression in NK cells and NKT cells between non-pregnancy and early pregnancy women

In peripheral blood of non-pregnant women, T cells of 0.78 ± 0.41% expressed type 2 cytokines (IL-4), which were similar to those of pregnant subjects (0.74 ± 0.40%). In this experiment, NK cells in both non-pregnancy and pregnancy

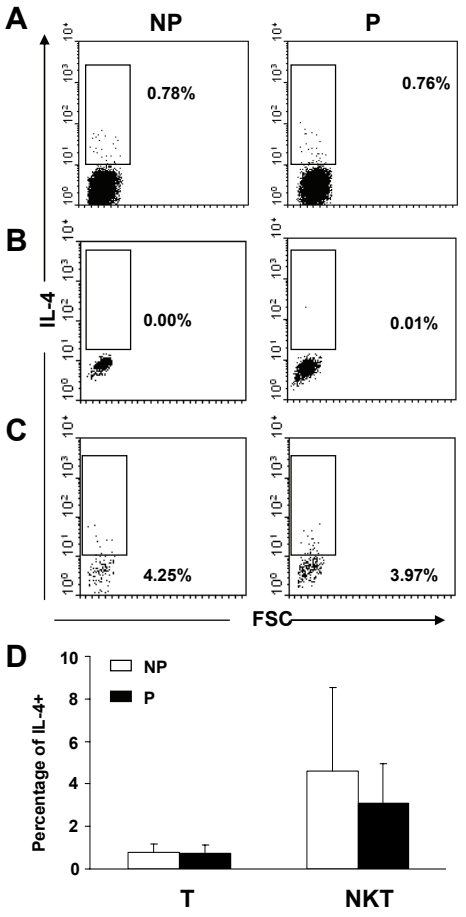

Figure 4. Flow cytometric detection of intracellular IL-4 expression in T cells, NK cells and NKT cells of non-pregnant women or pregnant women. PBMC were stimulated with PMA/Iono, and stained with cytokine-specific antibodies. The expression of IL-4 was analyzed in T cells (A), NK cells (B) and NKT cells (C). (D) There was no difference of IL-4 expression within T cells and NKT cells in two groups ($p > 0.05$). NP, non-pregnancy; P, early pregnancy.

groups almost did not express any IL-4 cytokine. However, the expression of IL-4 within NKT cells was relatively high compared to T cells and NK cells in both non-pregnancy (4.62 ± 3.9%) and early pregnancy (3.07 ± 1.9%) groups, but there was also no difference in the two groups (Figure 4).

Discussion

There are two different immunological interfaces in pregnancy (Figure 5). Interface 1 is between decidual immune cells and the extravillous trophoblast invading into the decidua and interface 2 is between blood maternal immune cells from uterine spiral artery and the syncytiotrophoblast that forms the villous surface of the placenta (13). Villous syncytiotrophoblast does not express any classical MHC class I antigens and thus it is presumably immunologically inert to T-cell-mediated responses (14).

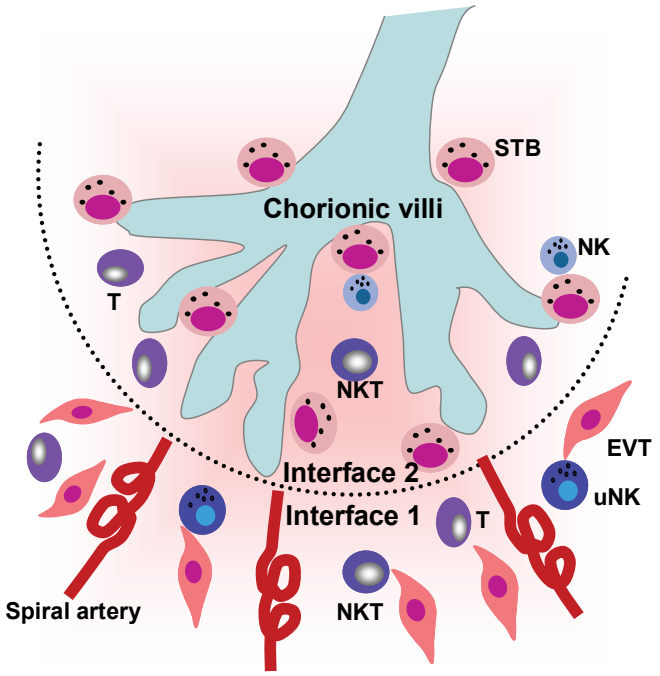

Figure 5. There are two different immunological interfaces in human pregnancy. The first (interface 1) is between decidual immune cells such as uterine NK cells (uNK), T lymphocytes (T), NKT cells (NKT) and the extravillous trophoblast (EVT) invading into the decidua. The second immune interface (interface 2) is anatomically distinct from the first, involving interactions between blood maternal immune cells from uterine spiral artery and the syncytiotrophoblast (STB) that forms the villous surface of the placenta.

However, Lucia Mincheva-Nilsson, et al. recently found that syncytiotrophoblast expressed MHC class I chain (MIC)-related proteins A and B (MICA/B) molecules, which are the ligands of the NK-cell activating receptor NKG2D (15). Furthermore, those extravillous trophoblast cells do express a unique combination of HLA-C, HLA-E and HLA-G, which interact with uterine natural killer (uNK) cells (16, 17). Recently, Boyson et al. reported that CD1d molecule, receptor of NKT cell, was expressed on extravillous trophoblast (18). Taken together, we argue that fetal-maternal immune interactions in humans might be predominantly mediated by NK cells and NKT cells instead of T cells.

As we all know, the most distinctive feature of the decidua during early pregnancy is the presence of a large population of NK cells (19, 20). Up to 70% of decidual lymphocytes are $CD56^+$ cells (21, 22). The percentage of NKT cells was also increased in decidua as compared with the peripheral blood (23). In this study, we demonstrated that more NK cells and NKT cells were present in peripheral blood of early pregnant women compared to non-pregnant women as well as in decidua, however, there was no distinguished change of T cells. These results raise the possibility that NK cells and NKT cells might play important roles in immunomodulator in pregnancy. A considerable

aspect of their immunomodulatory roles in infection, cancer and transplantation have been found through the production of cytokines (24, 25). So, further studies are required to clarify the nature of type 1/type 2 cytokine secretions in NK cells and NKT cells.

When all lymphocytes were considered together, previous studies showed that there was a shift to type 2 in normal pregnancy. This shift was significantly less in preeclampsia and recurrent miscarriage (26, 27). In many studies, cytokines were detected by ELISA after PBMCs were stimulated in culture (28). With this technique, it is impossible to identify which cells the source of the cytokines secreted into supernatant is from, so the presumption has been made that the changes are due to Th1 and Th2 cells. In our study, we surprisingly found no distinguished change of total type 1 and type 2 lymphocytes in peripheral blood of human early pregnancy compared to non-pregnancy, which is inconsistent with previous reports on Th1/Th2 immunity in pregnancy. Whether these differences were caused by different measuring condition or not needs to be further studied. Furthermore, there is strong evidence that the levels of particular type 1 cytokines are raised instead of lowered in normal pregnancy compared with the non-pregnant state (29, 30). No matter rise or no change in type 1 cytokines, the traditional concept of a bias of maternal immune responses towards Th2 immunity requires re-evaluation.

We also found that there were no significant changes of type 1/type 2 cytokines in T cells, but IFN-γ production of both NK cells and NKT cells was decreased in early pregnancy although there was no statistical significance in NKT cells. These data further confirm the previous studies that NK cells and NKT cells are involved in the type 1/type 2 dichotomy except T cells. In this study, we also found that human NK cells produced more IFN-γ than T cells, but the production of IL-4 was very minor even no. NKT cells produced a significant amount of both IFN-γ and IL-4 compared with NK cells and T cells. Although total amount of NK cells and NKT cells in peripheral blood is much less than T lymphocytes, they are characteristic by rapid responsiveness, extensive activation and strong potentials of cytokine production; in contrast, T lymphocytes are antigen-specifically, clonal selectively activated, only a relatively small part of the cells will be involved in the immune response. So, the innate immune system including NK cells and NKT cells might play a pivotal role in pregnancy progression.

In conclusion, we found no change of total type 1 and type 2 lymphocytes, however, IFN-γ was decreased in NK cells and NKT cells in human early pregnancy. Type 1/type 2 shift mechanisms in innate immune system underlying human early pregnancy should be paid more attention.

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