

## Article

# Cell-Mediated Immunity Imbalance in Patients with Intrahepatic Cholestasis of Pregnancy

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Decidual lymphocytes may mediate fetal trophoblast recognition and regulate maternal immune reaction and play an essential role in the maintenance of normal pregnancy. The aim of this study was to compare the percentage of T cells, natural killer (NK) cells and natural killer T (NKT) cells within decidual parietalis of normal pregnant controls (NP) and patients with intrahepatic cholestasis of pregnancy (ICP), and to investigate the production of interleukin-4 (IL-4), interferon- $\gamma$  (IFN- $\gamma$ ) in the culture supernatant of decidual parietalis mononuclear cells (DPMCs). Compared with controls, the decidual parietalis from ICP were characterized with significant increased percentages of CD3<sup>+</sup>CD56<sup>+</sup> cells, CD3<sup>+</sup>CD56<sup>+</sup> cells, CD56<sup>+</sup>CD16<sup>+</sup> cells, CD56<sup>+</sup>CD16<sup>-</sup> cells, CD56<sup>+</sup>NKG2D<sup>+</sup> cells, and the significant decreased percentages of CD3<sup>+</sup> cells, CD3<sup>+</sup>CD4<sup>+</sup> cells. There were no differences found for the percentage of CD3<sup>+</sup>CD8<sup>+</sup> cells, CD56<sup>+</sup>NKG2A<sup>+</sup> cells between control and study group. In addition, the enhanced concentration of IFN- $\gamma$  was presented in culture supernatant of DPMCs from ICP. It was suggested that the increased NK cells, NKT cells and the decreased T cells in the decidual parietalis and over-secretion of IFN- $\gamma$  could be correlated with the pathophysiology of ICP patients. *Cellular & Molecular Immunology*. 2007;4(1):71-75.

**Key Words:** NK cell, IFN- $\gamma$ , ICP

## Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a specific liver disease of pregnancy (1). The main clinical manifestation is skin pruritus and visible jaundice, usually coinciding with a rise in serum glycocholic acid, aminotransferases and bilirubin (2), all of them usually disappears almost immediately after delivery. ICP was described as a puzzling disorder of pregnancy because of its clinical representation, premature labor, the absence of well-understood etiologic factors, and the unexplained perinatal mortality (3). Despite the precise etiology of ICP is still unknown, the importance of immunology in ICP has been recognized (4, 5). Some investigations found imbalance of immune reaction were present in maternal peripheral blood and placenta tissue, and concluded the missing balance of immunization was one of

the reasons to induce ICP.

The immune responsiveness of women is altered during pregnancy in order to retain protective properties against disease and at the same time to allow tolerance of the fetus (6). The maternal immune system tolerates the fetus in the uterus, where the fetal derived tissues are in direct contact with the maternal deciduas. Serving as an immunologically specialized tissue, the deciduas and its components carry out multiple functions and play an essential role in implantation and in the maintenance of pregnancy (7). Because the deciduas is infiltrated by T cells, NK cells and NKT cells being in close active contact with invading trophoblast cells and secreting cytokines which play a pivotal role in implantation, pregnancy maintenance, parturition and some diseases related with pregnancy (8-12). During normal pregnancy, decidual lymphocyte cytotoxic activity is down-regulated and accompanied with over-production of Th2 activity cytokine by different mechanisms (13, 14). Although the factors leading to the specified distribution of lymphocytes among different phenotypes in deciduas are unknown, lymphocytes expressing activating receptors and inhibitory receptors are able to produce immunoregulatory cytokines, even to recognize and kill target cells, so as to

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constitute a distinct immunologic microenvironment inside maternal-fetal interface to maintenance successful pregnancy (15, 16).

By late pregnancy, there are two distinct regions of maternal deciduas anatomically: one is the decidua basalis which come into direct contact with the placenta, the other is named as the decidua parietalis which connect with the amniochorion directly. We hypothesize that immune maladaptation in deciduas may be correlated with the pathophysiology of ICP. Therefore, the aim of the study was to measure the precise composition of T cells, NK cells and NKT cells in the decidua parietalis and detect cytokine production of decidua parietalis mononuclear cells (DPMCs) from normal pregnancy or ICP in order to investigate whether disturbed immunological state was present in decidua parietalis.

## Materials and Methods

### Inclusion criteria

The study population consisted of 18 pregnant women subjected to elective Caesarean section from the Anhui Provincial Hospital undergoing delivery between October 2004 and April 2006. To eliminate possible clinical situations which could strongly influence the lymphocytic composition in decidua parietalis, none of the women included had underlying diabetes, renal diseases, chronic hypertension, chronic liver disease, metabolic diseases, skin, symptomatic infectious diseases or the onset of labor. All women received spinal anesthesia prior to cesarean section. Informed consent was obtained from all patients. Eight patients complicated with ICP were selected as study group, and 10 healthy term pregnancy were selected as control group. All patients with ICP suffered from skin pruritus associated with elevated fasting serum levels of glycocholic acid and glutamate-pyruvate transaminase observed. Ultrasound examination of liver and biliary tract was normal in all patients. Serological markers of viral hepatitis were negative. The reasons for elective operative delivery in the control group (n = 10) were: pelvic deformations (n = 2), breech position of the fetus (n = 5), presentation of umbilical cord (n = 2), shoulder presentation (n = 1). We did not find any supposition that any of these complications could possibly influence lymphocytic composition of deciduas (17).

### Group characteristics

Some of the parameters from interviews, clinical examinations and laboratory tests characterizing two groups of pregnant women and the differences existing between them are presented in Table 1.

### Tissue specimens

Fetal membranes were macroscopically collected from patients with ICP and from the controls delivered during elective caesarean section by eye scissors. The blood clots and fragments of the fetal membranes were removed macroscopically from the samples in the operating theater. Samples were then placed into bottles containing PBS and

**Table 1.** Different parameters of healthy and ICP patients

Parameters	Normal (n =10)	ICP (n = 8)
Age (years)	28.9 ± 2.8	27.3 ± 3.3
Primigravidae (%)	80.0	87.5
Gestational age (weeks)	35.5 ± 0.3	38.0 ± 1.5*
Serum glycocholic acid (mg/L)	2.5 ± 0.6	48.6 ± 10.9*
ALT (IU/L)	55.5 ± 10.6	189.6 ± 50.9*
AST (IU/L)	33.5 ± 8.6	79.6 ± 45.9*
Serum conjugated bilirubin (μmol/L)	0.6 ± 0.3	12.3 ± 7.6*

\**p* < 0.05

delivered immediately to the laboratory where the remaining steps of the procedure were performed.

### Cell isolation

Mechanical disaggregation was used to process the deciduas and to isolate the decidua lymphocytes. Decidua tissue was collected by removing the amnion and delicately scraping the tissue from the chorion in PBS, areas of ischemia or necrosis were avoided. Decidua slices were rinsed three times with PBS to remove residual blood and then mechanically disaggregated to fragments of approximately 5 mm<sup>3</sup> volume. The resultant suspension was filtered through a 100 μm metal sieve washed in PBS. Decidua mononuclear cells were layered on an equal volume of Ficoll-Hypaque at room temperature for density gradient centrifugation (20 min, 2,400 rpm). Mononuclear cells were collected from the interface, washed twice and resuspended in PBS for cell staining. The viability of the cells in suspension was 95% as confirmed by trypan blue.

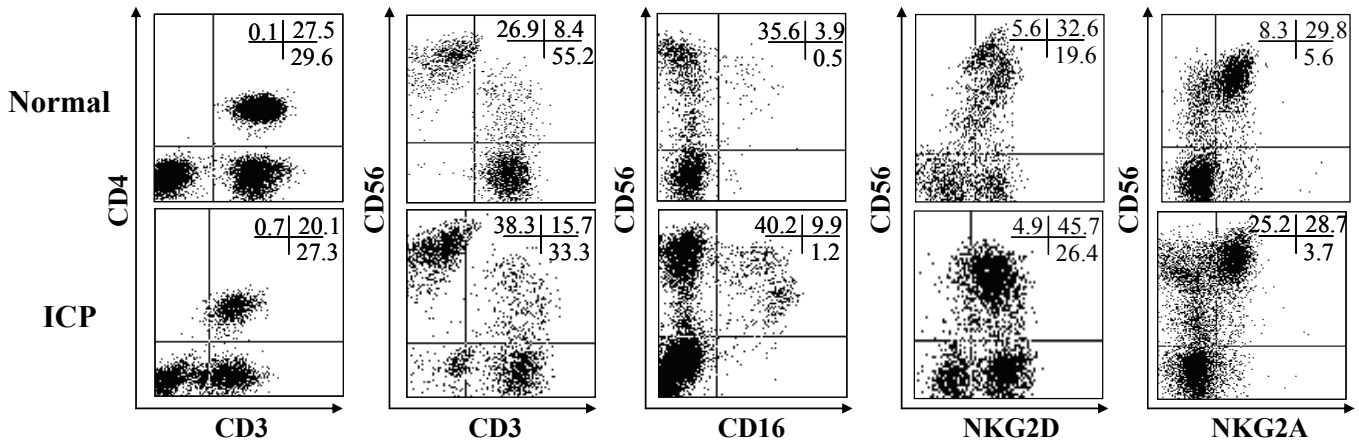
### Antibodies and cell staining

FITC-conjugated CD4, CD16 antibodies, PE-conjugated CD56 antibodies, PE-CY5-conjugated CD3, CD56 antibodies were obtained from BD Pharmingen. PE-conjugated NKG2A, NKG2D antibodies were purchased from R&D.

Cells were stained with monoclonal antibodies at a density of 5 × 10<sup>6</sup> cells/ml in PBS (100 μl/sample) and incubated for 30 min at 4°C in the dark. Cells were resuspended and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACSCalibur flow cytometer with 488 nm argon laser. No less than 1 × 10<sup>4</sup> cells were measured in each analysis. The acquired data were then analyzed using WinMDI 2.9 software. The results were given as (%) of positive cells in the sample.

### Cytokine secretion

The isolated DPMCs were obtained by density gradient centrifugation and resuspended in DMEM culture (with 10% fetal calf serum, 100 U/ml of penicillin, 10 μg/ml of streptomycin) at 1 × 10<sup>6</sup> cells/ml. Cultures were conducted in 96-well tissue culture plates using 1 × 10<sup>5</sup> cells/200 μl of



**Figure 1.** The analysis of T cells, NK cells and NKT cells and their subtypes in DPMCs from normal pregnancy and ICP by flow cytometry. Cells isolated from decidua parietalis of normal pregnancy and patients with ICP were stained with monoclonal antibodies CD3, CD4, CD56, CD16, NKG2D, NKG2A, respectively, to distinguish the different subsets of lymphocytes. Typical dot plots were showed with percentage of each population.

medium for 72 h, at 37°C, in 5% CO<sub>2</sub> and each experiment carried out in triplicate. To stimulate lymphocyte proliferation, PHA in a concentration of 10 mg/ml of culture medium was used. Culture supernatants were collected 72 h later and frozen at a temperature of -80°C, ELISA was used for estimation of IL-4 and IFN-γ concentrations.

*Statistical analysis*

Results are presented as the mean ± SD. Statistical analysis was made by student *t*-test. *p* < 0.05 was considered to be statistically significant.

**Results**

*The changes of T cells, NK cells and NKT cells in DPMCs between control and ICP group*

The percentages of T cells, NK cells, NKT cells and their subtypes within decidua parietalis lymphocytes from normal

pregnant controls are presented in Figure 1 and Table 2. Compared with control group, patients complicated with ICP were characterized with significantly increased percentages of CD3<sup>+</sup>CD56<sup>+</sup> cells, CD3<sup>+</sup>CD56<sup>-</sup> cells, CD56<sup>+</sup>CD16<sup>-</sup> cells, CD56<sup>+</sup>CD16<sup>+</sup> cells and CD56<sup>+</sup>NKG2D<sup>+</sup> cells, and decreased percentages of CD3<sup>+</sup> cells, CD3<sup>+</sup>CD4<sup>+</sup> cells. Whereas the changes of the percentages of CD56<sup>+</sup>NKG2A<sup>+</sup> cells and CD3<sup>+</sup>CD8<sup>+</sup> cells were not significant.

*PHA-stimulated cytokine secretion from cultured DPMCs of normal pregnancy and patients with ICP*

The concentrations of cytokines secreted by PHA-stimulated DPMCs between normal group and study group are presented in Table 3. In contrast to normal late pregnancy, the level of IFN-γ was significantly higher in the ICP group, whereas the change of IL-4 concentration was not significant.

**Discussion**

Our results demonstrated that the percentages of T cells (CD3<sup>+</sup>), NK cells (CD3<sup>-</sup>CD56<sup>+</sup>), NKT cells (CD3<sup>+</sup>CD56<sup>+</sup>) in term deciduas parietalis from healthy pregnant women is 58.7 ± 4.2%, 29.3 ± 2.6%, 7.9 ± 1.7%, respectively, suggesting that T cells are the most abundant lymphocytes (18), that NK cells account for a substantial proportion, and

**Table 2.** The percentage of T cells, NK cells and NKT cells

Cell type	Normal (% , n=10)	ICP (% , n=8)
CD3 <sup>+</sup>	58.7 ± 4.2	47.2 ± 2.8*
CD3 <sup>+</sup> CD4 <sup>+</sup>	27.9 ± 2.4	20.5 ± 3.0*
CD3 <sup>+</sup> CD8 <sup>+</sup>	29.2 ± 3.2	28.4 ± 3.6
CD3 <sup>-</sup> CD56 <sup>+</sup>	29.3 ± 2.6	38.3 ± 3.3*
CD56 <sup>+</sup> CD16 <sup>-</sup>	35.6 ± 2.4	41.4 ± 3.4*
CD56 <sup>+</sup> CD16 <sup>+</sup>	4.6 ± 1.3	8.8 ± 1.7*
CD56 <sup>+</sup> NKG2D <sup>+</sup>	33.4 ± 3.5	45.0 ± 3.8 *
CD56 <sup>+</sup> NKG2A <sup>+</sup>	33.3 ± 3.7	29.9 ± 5.2
CD3 <sup>+</sup> CD56 <sup>+</sup>	7.9 ± 1.7	14.8 ± 1.9*

\**p* < 0.05

**Table 3.** PHA-stimulated cytokine secretion from cultured DPMCs of normal pregnancy and patients with ICP

Cytokine (pg/ml)	Normal (n = 10)	ICP (n = 8)
IL-4	22.5 ± 12.3	33.9 ± 9.7
IFN-γ	490.3 ± 202.2	1558.8 ± 535.9*

\**p* < 0.05

that NKT cells comprise a minor lymphocytes in term deciduas parietalis. This result indicated that the presence of uterine NK cells was not only restricted to early pregnancy, contrary to current belief (19, 20). Additionally, the NK cells express activating receptors, inhibitory receptors and IgG Fc receptor (CD16), which induce NK cells to kill or modulate NK cell cytotoxicity (21). However, within the CD3<sup>+</sup> population, there was a similar pattern of CD3<sup>+</sup>CD4<sup>+</sup> cells and CD3<sup>+</sup>CD8<sup>+</sup> cells present in term deciduas parietalis. Thus, these decidual lymphocytes might regulate balanced network of local interactions, produce immunoregulatory cytokines, and play an important role in maintaining pregnancy by elimination of infected cells and protection against excessive lymphocyte response (22). Additionally, we analyzed the features of lymphocyte subsets accumulated in the third trimester deciduas parietalis of healthy pregnant subjects, allowed us to create our own reference values.

Comparison with healthy pregnancy, the deciduas parietalis with ICP contained increased percentage of CD3<sup>+</sup>CD56<sup>+</sup>NK cells, CD3<sup>+</sup>CD56<sup>+</sup> NKT cells, and decreased percentage of CD3<sup>+</sup> T cells. Inside population of CD56<sup>+</sup> lymphocytes of ICP, the percentages of CD56<sup>+</sup>CD16<sup>+</sup> NK cells of high cytolytic activity and CD56<sup>+</sup>NKG2D<sup>+</sup>NK cells with activating receptors were significantly higher, while the percentage of CD56<sup>+</sup>NKG2A<sup>+</sup> NK cells with inhibitory receptors was decreased. Our results suggested that during ICP, increased over-activity of NK cells and co-existence of lower CD3<sup>+</sup> cells number together in deciduas parietalis could induce imbalance of local interactions, and be responsible for cytokine disbalance (23).

As far as cytokine was concerned, the IFN- $\gamma$  over-secretion was present in ICP group. Though we described the method of isolation of cell suspension was crude, at the same time IFN- $\gamma$  was secreted by a number of different cells inside maternal-fetal interface, in that it did not permit us to sorting the concrete lymphocyte subset and could not examine their intracellular cytokine patterns, but it did allow the assessment of the condition of DPMCs in the third trimester pregnancy. The increased secretion of IFN- $\gamma$  in the ICP group might reflect a Th1 bias in women with ICP. Some investigations had been shown that Th1 type cytokines were adverse to pregnancy. IFN- $\gamma$  could bring about killing of trophoblastic cells and inhibit proliferation of human trophoblastic cells *in vitro* (24). So we concluded that cytokine imbalance might participate in an important pathological change – excessive apoptosis of trophoblast in patients with ICP *in vivo*.

In summary, we found that changes in lymphocyte subsets followed with IFN- $\gamma$  over-secretion were present inside maternal-fetal interface with ICP, however, we could not conclude the changes are associated with pathomechanism or were only a phenomenon. It was sure that those changes in deciduas parietalis with ICP might be correlated with the pathophysiology of ICP. So further study were required to clarify the function of lymphocyte subsets (especially increased percentage of NK cells and NKT cells) and their affection on trophoblast cells in the third trimester deciduas with ICP.

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## References

1. Ropponen A, Sund R, Riikonen S, Ylikorkala O, Aittomaki K. Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based. *Hepatology*. 2006;43:723-728.
2. Kenngott S, Lohse P, Beuers U. Intrahepatic cholestasis in pregnancy. What to consider in jaundice and pruritus. *MMW Fortschr Med*. 2002;144:41-44.
3. Germain AM, Carvajal JA, Glasinovic JC, et al. Intrahepatic cholestasis of pregnancy: an intriguing pregnancy-specific disorder. *J Soc Gynecol Investig*. 2002;9:10-14.
4. Qin L, Liu SY, Xing AY, et al. Study of fetal lymphocyte of intrahepatic cholestasis of pregnancy. *Zhonghua Fu Chan Ke Za Zhi*. 2006;41:445-448.
5. Shi Q, Liu S, Peng Z, et al. Effects of TNF- $\alpha$  and dexamethasone on the expression of intercellular cell adhesion molecule-1, P-selectin in patients with intrahepatic bile duct obstruction. *Zhonghua Gan Zang Bing Za Zhi*. 2002;10:125-128.
6. Pereira L, Maidji E, McDonagh S, Genbacev O, Fisher S. Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity. *J Virol*. 2003;77:13301-13314.
7. Hanna J, Goldman-Wohl D, Hamani Y, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*. 2006;12:1065-1074.
8. Chao KH, Wu MY, Chen CD, Yang JH, Yang YS, Ho HN. The expression of killer cell inhibitory receptors on natural killer cells and activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the decidua of normal and abnormal early pregnancies. *Hum Immunol*. 1999;60:791-797.
9. Wilczynski JR, Tchorzewski H, Banasik M, et al. Lymphocyte subset distribution and cytokine secretion in third trimester decidua in normal pregnancy and preeclampsia. *Eur J Obstet Gynecol Reprod Biol*. 2003;109:8-15.
10. Tsuda H, Sakai M, Michimata T, Tanebe K, Hayakawa S, Saito S. Characterization of NKT cells in human peripheral blood and decidual lymphocytes. *Am J Reprod Immunol*. 2001;45:295-302.
11. King A, Allan DS, Bowen M, et al. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur J Immunol*. 2000;30:1623-1631.
12. Matthiesen L, Berg G, Ernerudh J, Ekerfelt C, Jonsson Y, Sharma S. Immunology of preeclampsia. *Chem Immunol Allergy*. 2005;89:49-61.
13. Vince GS, Johnson PM. Leucocyte populations and cytokine regulation in human uteroplacental tissues. *Biochem Soc Trans*. 2000;28:191-195.
14. Raghupathy R, Makhseed M, Azizieh F, Hassan N, Al-Azemi M, Al-Shamali E. Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. *Cell Immunol*. 1999;196:122-130.
15. Kusumi M, Yamashita T, Fujii T, Nagamatsu T, Kozuma S, Taketani Y. Expression patterns of lectin-like natural killer receptors, inhibitory CD94/NKG2A, and activating CD94/NKG2C on decidual CD56<sup>bright</sup> natural killer cells differ from

- those on peripheral CD56<sup>dim</sup> natural killer cells. *J Reprod Immunol.* 2006;70:33-42.
16. Wilczynski JR, Malinowski A, Szpakowski M. Immunophysiology of normal pregnancy. Part I. Immunoregulatory mechanisms inside the maternal-fetal interface. *Ginekol Pol.* 2004;75:482-495.
  17. Sindram-Trujillo AP, Scherjon SA, van Hulst-van Miert PP, Kanhai HH, Roelen DL, Claas FH. Comparison of decidual leukocytes following spontaneous vaginal delivery and elective cesarean section in uncomplicated human term pregnancy. *J Reprod Immunol.* 2004;62:125-137.
  18. Sindram-Trujillo AP, Scherjon SA, van Hulst-van Miert PP, et al. Differential distribution of NK cells in decidua basalis compared with decidua parietalis after uncomplicated human term pregnancy. *Hum Immunol.* 2003;64:921-929.
  19. Sindram-Trujillo A, Scherjon S, Kanhai H, Roelen D, Claas F. Increased T-cell activation in decidua parietalis compared to decidua basalis in uncomplicated human term pregnancy. *Am J Reprod Immunol.* 2003;49:261-268.
  20. Delgado SR, McBey BA, Yamashiro S, Fujita J, Kiso Y, Croy BA. Accounting for the peripartum loss of granulated metrial gland cells, a natural killer cell population, from the pregnant mouse uterus. *J Leukoc Biol.* 1996;59:262-269.
  21. Vacca P, Pietra G, Falco M, et al. Analysis of natural killer cells isolated from human decidua: Evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood.* 2006;108:4078-4085.
  22. Rukavina D, Podack ER, Rubesa G, Spanjol-Pandelo S, Randic L. Down-regulated expression of perforin-positive/CD16t cells in the peripheral blood lymphocytes in the first trimester of pregnancy and up-regulation at the end of pregnancy. *Am J Reprod Immunol.* 1997;38:189-196.
  23. Chaouat G, Tranchot-Diallo J, Volumenie JL, et al. Immune suppression and Th1/Th2 balance in pregnancy revisited: a (very) personal tribute to Tom Wegmann. *Am J Reprod Immunol.* 1997;37:427-434.
  24. Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. *APMIS.* 1995;103:161-179.