Expression of Natural Cytotoxicity Receptors on and Intracellular Cytokine Production by NK Cells in Women with Gestational Diabetes Mellitus

（妊娠糖尿病における末梢血 NK 細胞 NCR 発現と細胞内サイトカイン産生に関する研究）

弘前大学大学院医学研究科　成育科学領域　周産期医学教育研究分野

氏名　千葉仁美

指導教授　水沼英樹

Introduction

NK cells are the major lymphocytes in the uterus during early pregnancy.[1] The number of uterine NK cells increases during the late secretory phase of the menstrual cycle and increases rapidly during early pregnancy.[2, 3] NK cells are important for the regulation of extravillous trophoblast cell invasion and spiral artery remodeling in the pregnant decidua.[4-7] Maximum extravillous trophoblast cell invasion occurs during the first trimester of gestation, peaking at around 10–12 weeks.[8-10] NK cells with abnormal expression of surface receptors and cytokine production have been reported to be related with reproductive failure such as infertility, implantation failures, recurrent pregnancy loss, and preeclampsia (PE). [11-15] Therefore, the function of NK cell and pregnancy are strongly correlated with each other.

Gestational diabetes mellitus (GDM) is characterized by glucose intolerance that is caused by metabolic changes during pregnancy, and is considered a risk factor for the occurrence of DM in future. There are studies that reported changes in the cytokine in women with GDM. The concentration of serum IFN-γ and adiponectin decreased, while serum leptin, IL-6, IL-10, and TNF-α increased in GDM women when compared to that in healthy pregnant women.[16 17] Placenta and adipose tissues from patients with GDM released higher amounts of TNF-α in response to high glucose than that from normal pregnant women.[18] Plasma TNF-α were reported to be low during the first and second trimester, but it increased during late pregnancy, and was inversely correlated with the insulin sensitivity.[19, 20]

However, the relationship between GDM and the functions of NK cells in pregnant women has not been clarified so far. The purpose of this study was to evaluate the expression of peripheral blood NK (pNK) cell surface markers (CD16, NKp46, and NKp30) and the percentage of cytokine (IFN-γ, TNF-α, TGF-β, and VEGF) producing pNK cells in women with / without GDM at 12 weeks of pregnancy.

Materials and Methods

Study Subjects

We designed a prospective study and patients from Hirosaki University Hospital were recruited for this study between July 2014 and March 2015. This study was approved by the local ethics committee, and signed informed consent forms were obtained from all study subjects. Blood samples were collected during routine blood tests for prenatal check-up at 12 weeks of gestation.

Thirty-four Japanese women (GDM (n=7), non-GDM (n=27)) with singleton pregnancy were included. All GDM patients were diagnosed at 12 to 16 weeks of gestation. Pregnant women with multi-fetal gestations, pre-gestational diabetes, and overt diabetes were excluded, while pregnant women with impaired glucose tolerance before the onset of pregnancy were included in this study. Maternal and neonatal characteristics are shown in Table 1. Two-hour-glucose level of 75-g OGTT in GDM group was significantly higher than that in the non-GDM group. There were no complications reported in the newborn infants (such as macrosomia, shoulder dystocia, and hypoglycemia) in both the GDM and non-GDM groups and there were no pregnant women suffering from PE in both the GDM and non-GDM groups.

Screening of GDM

All pregnant women were subjected to a two-step screening method for GDM. This screening method is based on the Guideline for Obstetrical Practice in Japan 2014.[21] Briefly, women who had a random plasma glucose level of ≥95mg/dl at 12 weeks of gestation were subjected to 75-g OGTT after fasting overnight. Diagnosis of GDM was based on the criteria of one or more abnormal values [fasting ≥92mg/dl (5.1mmol/l), such as 1 hour ≥180mg/dl (10.0mmol/l) or 2 hour ≥153mg / dl (8.5mmol/l)] reported for the subjects. Pregnant women, not diagnosed as GDM at 12 weeks of screening, were again subjected to random blood glucose measurement at

28 weeks of gestation. Women with random plasma glucose ≥100mg/dl were subjected to 75-g OGTT (the same GDM criteria).

GDM screening method in Japan was modified by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) recommendations.[22] For GDM screening, IADPSG recommends the measurement of fasting plasma glucose (FPG), HbA1c, or random plasma glucose in all or only high-risk women during the first prenatal visit and to perform 75-g OGTT at 24-28 weeks of gestation after overnight fasting. This test has been suggested for all women not previously diagnosed with overt diabetes or GDM in earlier tests.[22] The cut-off value for random plasma glucose level has not been determined, however a random plasma glucose level of ≥95mg/dl at 12 weeks of gestation and ≥100mg/dl at 28 weeks of gestation are easier and more cost-effective for GDM screening in multi-organizations studies conducted in Japan (unpublished data). Therefore, in the Guidelines for Obstetrical Practice in Japan 2014, a random plasma glucose of ≥95mg/dl at 12 weeks of gestation and ≥100mg/dl at 28 weeks of gestation are recommended as cut-off values for GDM screening.[21] In our study, all GDM women were diagnosed between 12 to 16 weeks of gestation. Based on the IADPSG criteria, the total incidence of GDM was 17.8%.[22] On the other hand, the percentage of GDM in our study was 20.5% (7 women with GDM in 34 pregnant women).

Expression of NK cell surface receptors

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using a Histopaque®-1077 (Sigma-Aldrich, Co., St Louis, MO, USA). Isolated mononuclear cells were washed twice with phosphate-buffered saline solution (PBS), and adjusted to 5×10^6 cells / ml.

Using 100μl of PBMCs, we evaluated the expression of pNK cell surface markers. For the NK cell labeling, monoclonal antibodies (mAbs) were used. The combinations of antibodies

were as follows; CD56 phycoerythrin (PE) (Beckman Coulter, Inc., Brea, CA, USA) / CD16 fluorescein isothiocyanate (FITC) (BD, Franklin Lakes, NJ, USA), CD56 FITC (BD) / NKp46 PE (Beckman Coulter), and CD56 FITC / NKp30 PE (Beckman Coulter). The cells were incubated in dark with mAbs for 15 minutes at room temperature. Then cells were washed twice in PBS, lysed and fixed. Two more washes were carried out with PBS. Finally, immunofluorescence and multicolor flow cytometric analysis were performed using a FACSCanto flow cytometer (Beckton Dickinson, Son Jose, CA, USA) with computer interfacing to BD FACSDiva for full-list-mode data storage, recovery, and analysis. The gate was set on the lymphocyte region by characteristic forward and side scatter parameters. For each sample, 3 x10^4 peripheral blood lymphocytes were evaluated.

Expression of intracellular cytokine production by NK cells

PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA) (25ng / ml) and ionomysin (1μmol / l) in the presence of brefeldin A (10μg / ml) for 4 hours at 37℃ in a 5% CO_2 humidified incubator, after washing. These cells were stained for 15 minutes at room temperature in the dark with CD56 FITC or CD56 Phycoerythrincyanin 5.1 (PC5) (Beckman Coulter) and washed twice with PBS. Next, 200μL of Cytofix/Cytoperm® (BD) were added to the stimulated cells and washed twice with the Perm/Wash Buffer (BD). After fixation and permeabilization, the cells were incubated for 30 minutes in the dark with IFN-γ FITC (Beckman Coulter), TNF-α PE (Beckman Coulter), and TGF-β allophycocyanin (APC) (R&D systems, Inc., Minneapolis, MN, USA). Appropriate isotype controls were used for each antibody. Cells were washed twice in Perm/Wash Buffer and resuspended in 250μL of PBS for subsequent flow cytometric analysis. Intracellular VEGF (R&D systems) expression was also evaluated using methods similar to that of the cytokine staining without stimulation in PMA, ionomysin, and brefeldin A.

Immunofluorescence and multicolor flow cytometric analysis were performed using FACSCanto flow cytometer (Beckton Dickinson, Son Jose, CA, USA) with computer interfacing to BD FACSDiva for full-list-mode data storage, recovery, and analysis. The gate was set on the lymphocyte region by characteristic forward and side scatter parameters. For each sample, 3 x10^4 peripheral blood lymphocytes were evaluated

Statistical Analysis

Patient characteristics were presented as means ± SD, and laboratory data were presented as median (interquartile range). Age, BMI, gestational age at delivery, and birth weight were analyzed by Student's t-test, and a family history of diabetes mellitus were analyzed by χ^2-test. Statistical differences between GDM and non-GDM groups were evaluated using Mann-Whitney U non-parametric analysis. Differences were considered significant for a probability of <0.05. All statistical analysis were performed using SPSS version 22.0 (IBM, Chicago, IL, USA).

Results

Expression of surface receptors on pNK cells in pregnant women with/without GDM

Representative dot plots of the expression of NK cell surface receptors such as CD16 and natural cytotoxicity receptors (NKp46 and NKp30) on pNK cells for pregnant woman with GDM and pregnant woman without GDM is shown in Fig.1. The percentage of $CD56^{dim}/CD16^{+}$ NK cells in GDM group [77.81% (56.67-86.31), Median (interquartile range)] was higher than that in the non-GDM group [58.94% (51.96-72.00)] (Fig. 2 (A)), however the difference was not significant. The percentage of $CD56^{bright}/CD16^{-}$NKcells in GDM group [1.86% (1.06-3.02)] was significantly lower than that in the non-GDM group [3.39% (2.20-4.58), $p<0.05$] (Fig. 2 (B)). The percentage of $CD56^{+}/NKp46^{+}$ NK cells was not different between the GDM and

non-GDM group. Further analysis of the CD56 fluorescence intensity revealed that the percentage of $CD56^{bright}/NKp46^{+}$ NK cells in the GDM group [2.57% (2.00-2.77)] was significantly lower than that in the non-GDM group [5.98% (5.20-9.14), $p<0.05$] (Fig. 2 (C)). There was no significant difference in the expression of NKp30 between the two groups.

Cytokines Production by pNK Cells in pregnant women with/without GDM

Representative dot plots for the percentage of cytokine (IFN-γ, TNF-α, TGF-β and VEGF) producing NK cells and NK cell subpopulation in pregnant woman with GDM and without GDM is shown in Fig. 3. NK cells were double stained for IFN-γ and TNF-α. We classified NK cells as $IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$, $IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$, $IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$, and $IFN\text{-}\gamma^{-}/ TNF\text{-}\alpha^{-}$NK cells. The percentage of IFN-γ producing NK cells (the sum of $CD56^{+}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$ and $CD56^{+}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$ cells) was significantly higher in the GDM group [73.90% (68.46-78.95)] than that in the non-GDM group [53.14% (11.80-68.88), $p<0.05$] (Fig. 4 (A)). The percentage of TNF-α producing NK cells (the sum of $CD56^{+}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$ and $CD56^{+}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$ cells) was significantly higher in the GDM group [59.91% (52.90-70.24)] than that in the non-GDM group [42.98% (18.20-60.77), $p<0.05$] (Fig. 4 (B)). The percentage of both IFN-γ and TNF-α producing NK cells ($CD56^{+}/ IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$ cells) was significantly higher in the GDM group [54.74% (48.29-64.71)] than that in the non-GDM group [37.41% (8.22-53.92), $p<0.05$] (Fig. 4 (C)). On the other hand, the percentage of TGF-β producing $CD56^{+}$ cells was significantly lower in the GDM group [3.28% (2.68-5.98)] than that in the non-GDM group [12.92%(6.19-37.59), $p<.05$] (Fig4 (D)). Similarly, the percentage of VEGF producing $CD56^{bright}$ cells was also significantly lower in the GDM group [1.46%(1.29-1.51)] than that in the non-GDM group [3.88%(2.99-6.31), $p<0.05$] (Fig. 4 (E)).

Discussion

GDM is characterized by hyperglycemia during pregnancy. Women with GDM have a high risk of type 2 DM in future than the women with normal glucose tolerance during pregnancy. [23] [24] It was reported that women diagnosed as GDM have islet cell antibodies. [25, 26] Whether positive islet cell antibodies can predict type 1 diabetes in future is debatable. [26] [27] However, it is obvious that GDM women have a risk of DM in future.

The etiology of DM is associated with multiple factors such as genetic factors, diet, obesity ,and reduced exercise. [28] Immunological abnormalities such as abnormal expression of NK cell surface markers and abnormal cytokine production may also be associated with the pathogenesis of DM. In fact, some researchers had reported decreased expression of NKp46 and changed levels of serum cytokines such as IFN-γ, TNF-α, TGF-β, and VEGF in DM patients.[29-47] Recently, the relationship between immune response and metabolic diseases such as obesity, type 2 diabetes, and GDM was reported. [48]

In type 1 DM, circulating peripheral blood NK cells play an immunoregulatory role. [34, 38] NK cells were not only capable of killing islet cells, but also seemed to be important in the development of diabetes [32]. For the onset of type 1 DM, NK cell activity was diverse. [45] On the other hand, NK cell cytotoxicity decreased in long-standing type 1 DM group than that in the non-DM healthy group. [33, 42] [45] Type 2 diabetes mellitus (DM) and impaired glucose tolerance (IGT) patients had also significantly lower levels of NK cell activity compared to that of the normal controls in both men and women. [43, 44, 47] These reports prompted us to investigate the expression of pNK cell surface markers and the percentage of cytokine producing pNK cells in women with GDM. We found that the percentages of $CD56^{bright}$ / $CD16^{-}$ NK cells were significantly lower while $CD16^{dim}$ / $CD16^{+}$ were increased in GDM women than that in normal pregnant women, suggesting that cytotoxicity of NK cells is increased in GDM women. In general, it is clarified that cytotoxicity of NK cell are decrease during normal pregnancy, [49] therefore, it is suggested that increased NK cell activity shown in this study may play some

roles in the etiology or pathophysiology of GDM progression.

In addition, we found that the percentage of $CD56^{bright}/NKp46^{+}$ NK cells was decreased in GDM patients. The physiological function of NKp46-positive NK cells include cytotoxicity and cytokine production. [50] In type 1 DM patients with long-standing disease, NKp46 and NKp30 levels in peripheral NK cells were reduced.[45, 51] However, in healthy blood donors, type 2 diabetes patients also showed a profound decrease in NKp46-positive NK cells. [29]

Moreover, we found that the percentage of IFN-γ producing $CD56^{+}$ cells and TNF-α producing $CD56^{+}$ cells were significantly higher in GDM women than those in non-GDM women. IFN-γ plays important roles in the progressive loss of beta-cell mass and function during the development of both type 1 and type 2 diabetes. [52] In recent-onset type-1 diabetes patients (within one month after diagnosis of T1DM) strong IFN-γ expression was observed in the peripheral NK cells. [45] However in long-standing group (duration > 1 year), the expression of IFN-γ in peripheral NK cells was reduced. [45] Hayashi et al. suggested that IFN-γ could cause pancreatic islet inflammation leading to islet cell destruction. [37] TNF-α has been implicated in the pathogenesis of insulin resistance in type 2 DM [53]. Patients with type 2 DM had high levels of TNF-α than that in the healthy volunteers and the increase in the level was dependent on the patients' degree of obesity. [54] TNF-α has been also demonstrated to be the most significant predictor of pregnancy-induced insulin resistance. [17]

Interestingly, the results of this study showed that the percentage of TGF-β producing $CD56^{+}$ cells and VEGF producing $CD56^{bright}$ cells were significantly lower than that in non-GDM women. TGF-β is one of the anti-inflammatory and immunosuppressive cytokines. [55] Serum TGF-β level was shown to increase in type 2 DM with poor glycemic control (HbA1c > 7.6%) compared to the patients with good glycemic control (HbA1c 5.5-7.6%). [56] There are significant linear relations between plasma TGF-β1 and fasting insulin level. [57] VEGF is an angiogenic protein which contributes to the angiogenesis and endothelial cell proliferation. [58] Serum

VEGF-A levels was significantly higher in uncontrolled type 2 DM group than well-controlled DM group and healthy volunteers [59]. Kubisz et al reported serum VEGF could be a sensitive predictor of endothelial dysfunction in type 2 DM [60]. Subjects enrolled in this study were not overt DM and had only slight abnormality in glucose intolerance accounting for the discrepancy between previous studies and the present one. Although NK cells can produce TGF-β1 and VEGF, it is not clear how NK cells regulate the serum cytokine levels and the present study could not clarify whether low serum levels of these cytokines were dependent on the lower cytokine producing activity of NK cells. Further studies are required to answer these questions.

In this study, we have only collected blood samples from pregnant women at 12 weeks of gestation. Further studies, which compare the difference in the function of NK cells among DM, GDM, and normal glucose tolerance or at other gestational weeks are required, however our results may contribute to explain one of the pathological mechanisms for GDM and how women with a history of GDM has the risk of developing DM.

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Figure legend

Figure 1

Representative dot plots of the expression of CD16 and natural cytotoxicity receptors (NCRs) on peripheral blood NK ($CD56^{+}$) cells and NK cell subpopulation in GDM and non-GDM group. A gate is set on the lymphocyte. Lymphocytes are classified into $CD56^{+}$ and $CD56^{-}$ cells. In addition, $CD56^{+}$ cells are classified into $CD56^{bright}$ and $CD56^{dim}$. Co-expression of CD56 and CD16 or NCRs is evaluated.

Figure 2

The percentage of the expression of natural cytotoxicity receptors (NCRs) on peripheral blood NK cells and NK cell subpopulation in GDM and non-GDM group.

(A) The percentage of $CD56^{dim}/CD16^{+}$ NK cells in GDM group and non-GDM group.

(B) The percentage of $CD56^{bright}/CD16^{-}$ NK cells in GDM group and non-GDM group.

(C) The percentage of $CD56^{bright}/NKp46^{+}$ NK cells in GDM group and non-GDM group.

Figure 3

Representative dot plots of intracellular cytokine production on peripheral blood NK cells and NK cell subpopulation in GDM group and non-GDM group. A gate is set on the lymphocyte. Lymphocytes are classified into $CD56^{+}$ and $CD56^{-}$ cells. In addition, $CD56^{+}$ cells are subdivided into $CD56^{bright}$ and $CD56^{dim}$. The percentage of cytokines producing NK cells is evaluated.

Figure 4

The percentage of intracellular cytokine production on peripheral blood NK cells and NK cell subpopulation in GDM group and non-GDM.

(A) The percentage of IFN-γ producing $CD56^{+}$ cells in GDM group and non-GDM group.

(B) The percentage of TNF-α producing $CD56^{+}$ cells in GDM group and non-GDM group.

(C) The percentage of both IFN-γ and TNF-α producing $CD56^{+}$ cells in GDM group and non-GDM group.

(D) The percentage of TGF-β producing $CD56^{+}$ cells in GDM group and non-GDM group.

(E) The percentage of VEGF producing $CD56^{bright}$ cells in GDM group and non-GDM group.

Table 1 maternal characteristics, oral glucose tolerance test results and maternal and neonatal outcomes

	GDM (n=7)	Non-GDM (n=27)	P-value
Maternal age (years)	36.4±5.0	32.4±4.4	NS
Pre-gestational BMI (kg/m²)	22.5±2.3	21.6±2.8	NS
A family history of diabetes mellitus	2(28%)	10(37%)	NS
[in first degree relationship]	[2(100%)]	[4(40%)]	
HbA1c at diagnosis (%)	5.50±0.16	–	
Glucose levels of 75-g OGTT (mg/dL)			
Fasting glucose	91.0±4.0	85.2±4.1	NS
1-hour	140.0±37.1	122.0±22.8	NS
2-hours	143.7±26.1	119.6±26.7	$p<0.05$
Gestational age at delivery (week)	39.2±1.2	39.7±1.2	NS
Birth weight (g)	3052.0±430.7	3038.3±289.4	NS

BMI: body mass index, OGTT: oral glucose tolerace test

Figure 1 Representative dot plots of the expression of CD16 and natural cytotoxicity receptors (NCRs) on peripheral blood NK ($CD56^{+}$) cells and NK cell subpopulation in GDM and non-GDM group

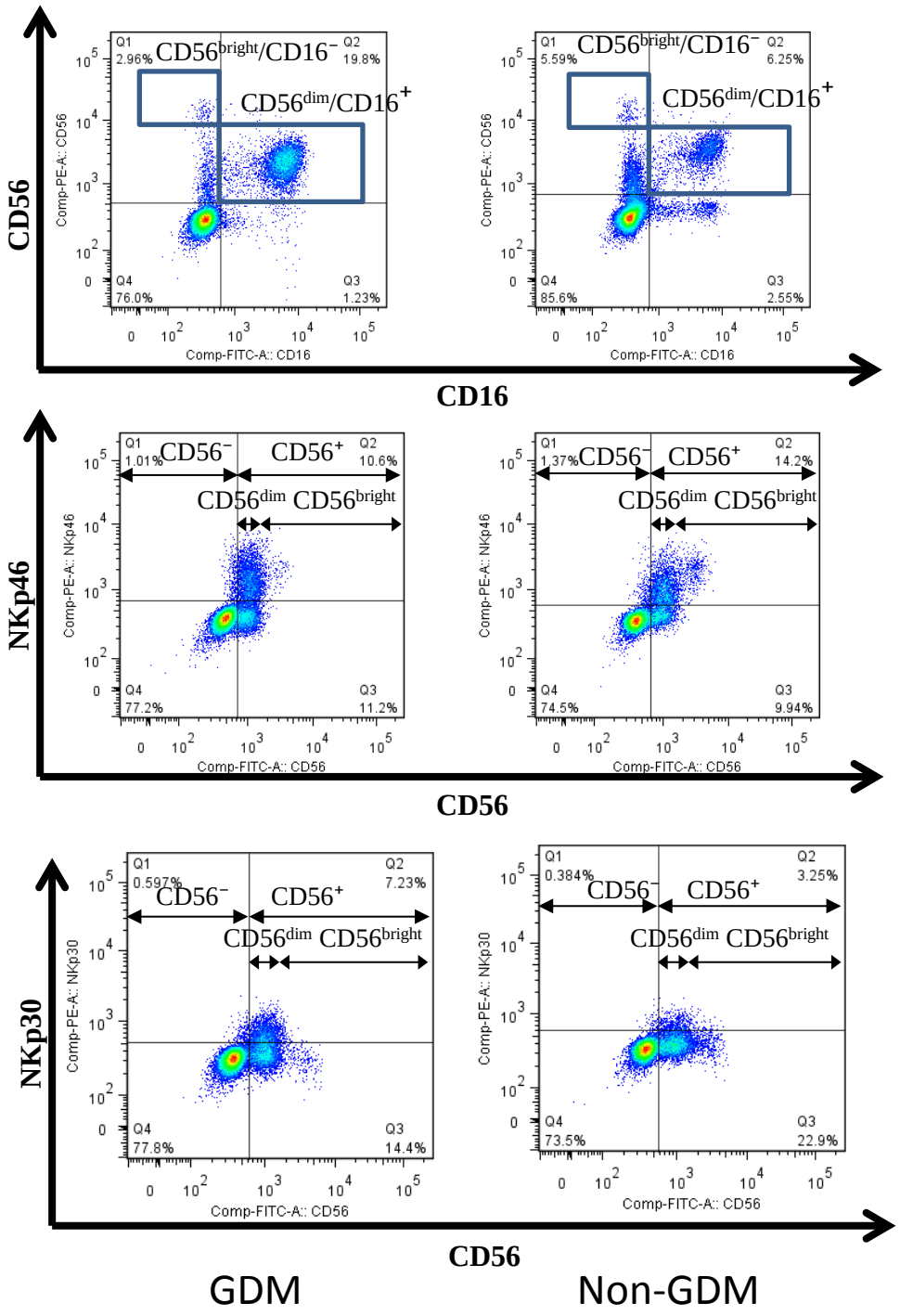


Figure 2 The percentage of the expression of natural cytotoxicity receptors (NCRs) on peripheral blood NK cells and NK cell subpopulation in GDM and non-GDM group

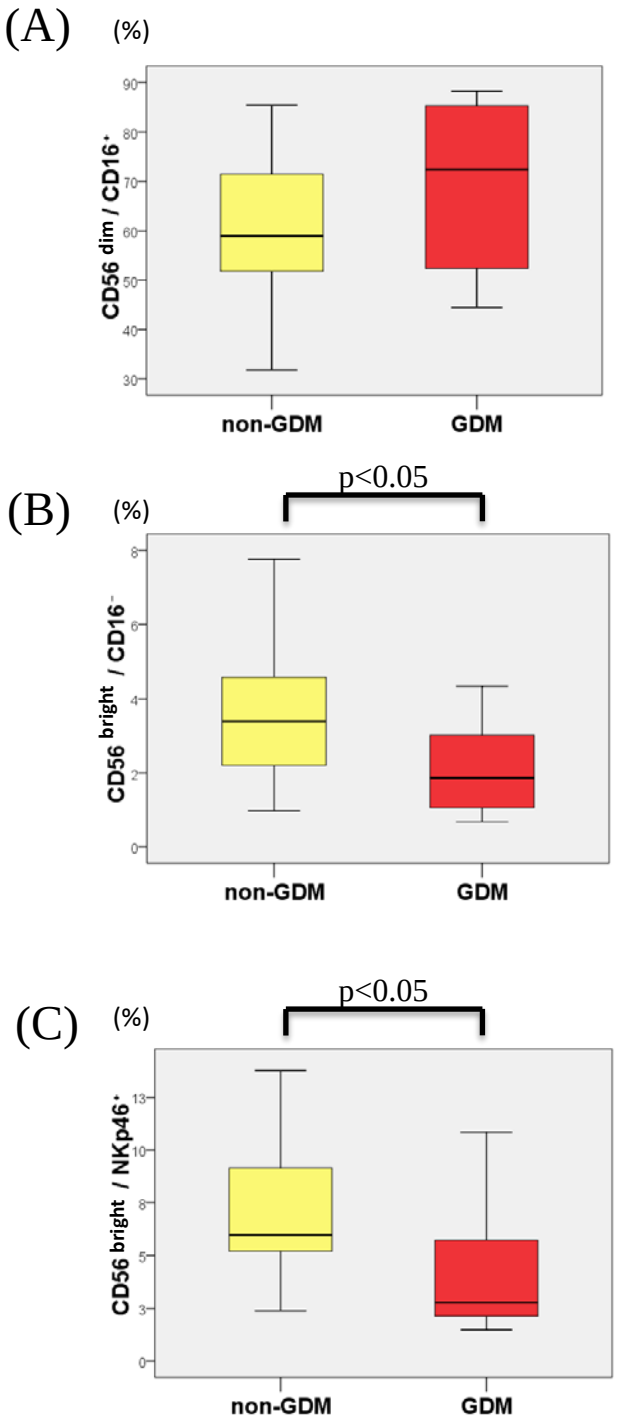
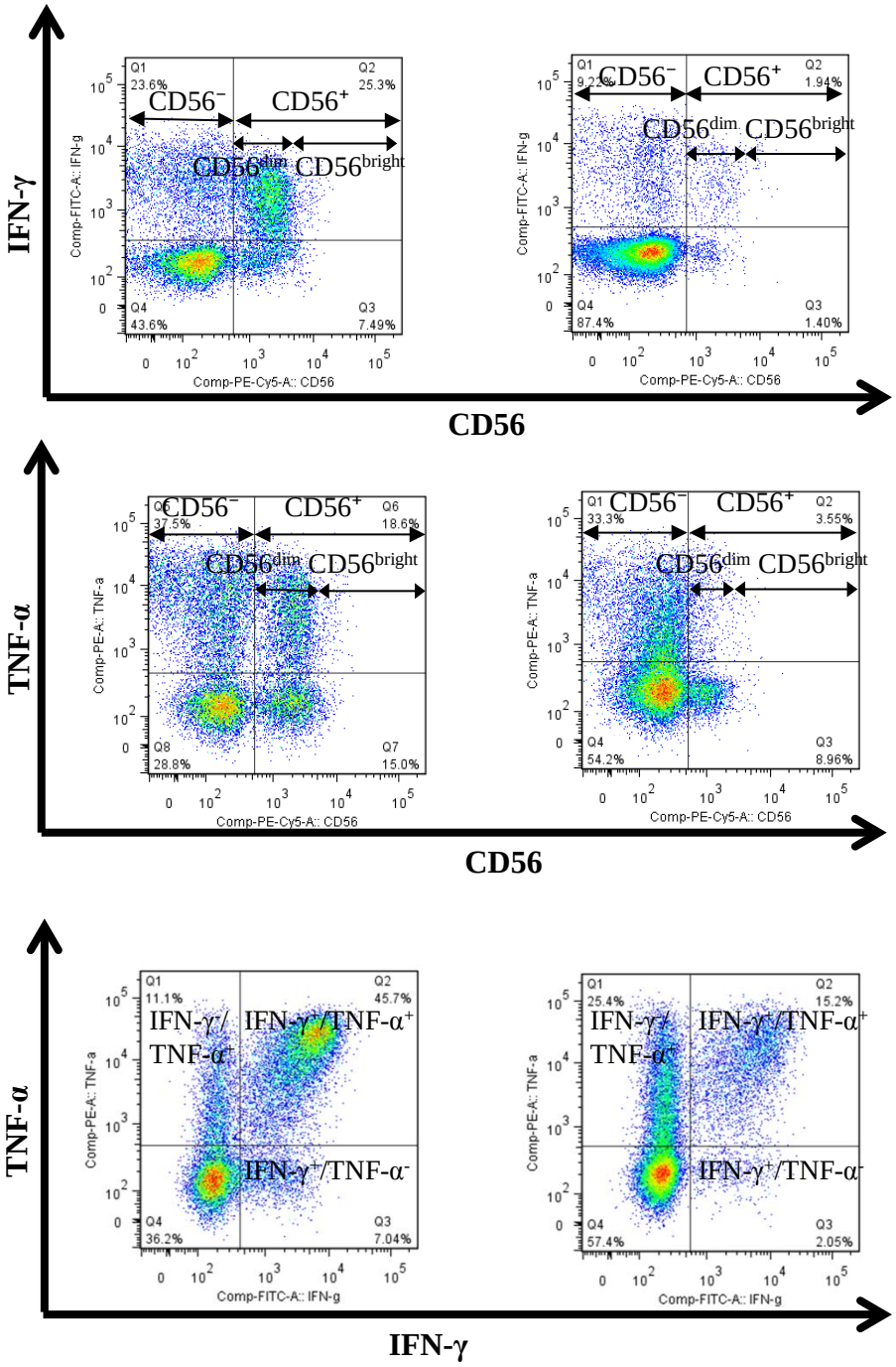
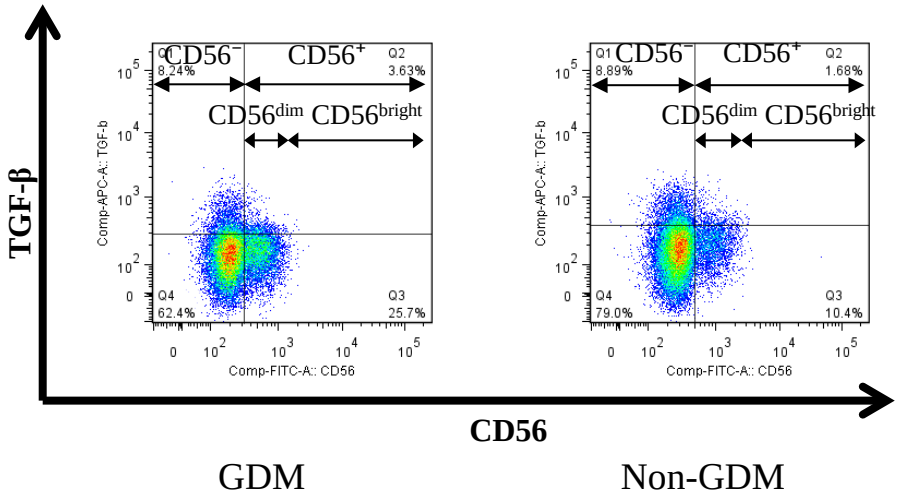
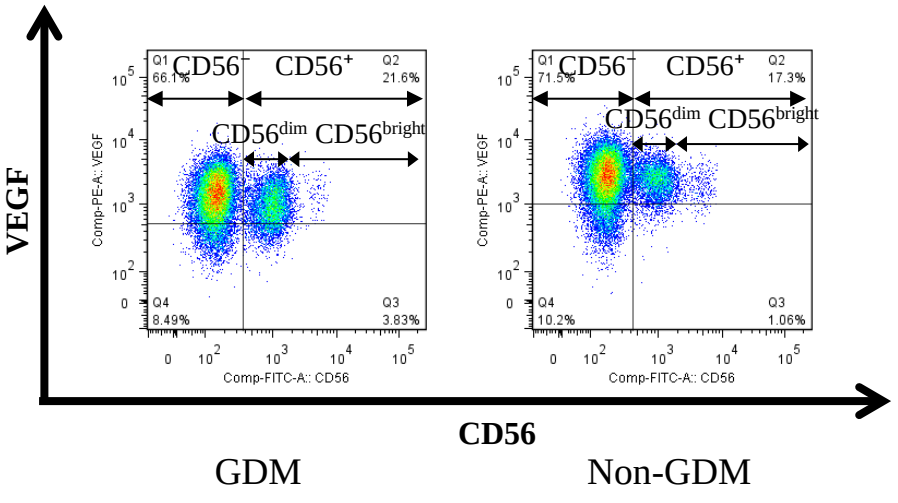


Figure 3 Representative dot plots of intracellular cytokine production on peripheral blood NK cells and NK cell subpopulation in GDM group and non-GDM group





GDM Non-GDM



GDM Non-GDM

Figure 4 The percentage of intracellular cytokine production on peripheral blood NK cells and NK cell subpopulation in GDM group and non-GDM.