Increase of CD69, CD161 and CD94 on NK Cells in Women with Recurrent Spontaneous Abortion and in Vitro Fertilization Failure

Mehri Ghafourian[1], Najmeh Karami[2]*, Ali Khodadadi[3], Roshan Nikbakhat[4]

[1]Department of Immunology, Fertility, Infertility and Perinatology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, [2]Pediatric Infections Research Center, Shahid Beheshti University of Medical Science, Tehran, [3]Department of Immunology, Cancer Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, [4]Fertility, Infertility and Perinatology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Background: Recurrent spontaneous abortion (RSA) and *in vitro* fertilization (IVF) failure with unknown causes are the controversial issues that are probably related to the immune system. **Objective:** To compare circulating NK cells expressing activation and inhibition surface markers between patients with RSA and IVF failure with those of healthy multiparous and successful IVF control women, respectively. **Methods:** In this case-control study peripheral blood samples were collected from 43 patients who included 23 women with RSA and 20 with IVF failure, plus 43 healthy control women comprising of 36 normal multiparous women and seven women with successful IVF. The expression of CD69, CD94 and CD161 surface markers on CD56+NK cells were assessed using specific monoclonal antibodies by flowcytometry. **Results:** The percentage of NK cells increased significantly in patients with RSA and in women with IVF failure in comparison to healthy multiparous and successful IVF control groups (p<0.001). The overall expression of CD69, CD94, CD161 were also increased significantly on NK cells in both patient groups compared to control groups (p<0.001). **Conclusion:** Elevated expression of CD69 and CD161 on NK cells can be considered as immunological risk markers in RSA and IVF failure. However, it is not clear if high expression of CD94 on peripheral blood NK cells is related to abnormal activity of endometrial NK cells.

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*Corresponding author: Najmeh Karami, Pediatric Infections Research Center, Shahid Beheshti University of Medical Science, Tehran, Iran, Tel: (+) 98 21 22907004, Fax: (+) 98 21 22226941, e-mail: najmeh.karami61@yahoo.com

INTRODUCTION

Implantation of embryo into the endometrium is a complex process involving different immunological regulatory mechanisms. Recurrent spontaneous abortion (RSA) is the most common complication of pregnancy. Some couples suffer from infertility of unknown causes. *In vitro* fertilization (IVF) is used for treatment of this type infertility with variable outcomes. Several studies have shown that women with history of RSA and IVF failure may have immunological disorders related to interactions between circulating maternal immune cells with fetus and syncytiotrophoblast. The maternal-fetal systemic immune interactions in human might be related predominantly to natural killer (NK) cells instead of T cells (1-4). These cells represent the first cellular immune defense mechanism and have close contact with the conceptus and placenta. NK cells which comprise 5% to 15% of all lymphocytes in peripheral blood are generally divided into two main populations $CD16^-CD56^{bright}$ and $CD16^+CD56^{dim}$ cells (5). In the peripheral blood, the main population of NK cells is $CD16^+CD56^{dim}$, NK cells that act as the powerful natural cytotoxic cells. On the other hand, $CD16^-CD56^{bright}$ NK cells are the main population in the endometrium potent at cytokine secretion, but with low cytotoxic ability (2). They constitute about 70% of uterine leukocytes in the secretory phase, and their numbers increase further during early pregnancy (5). Many studies have focused on different parameters related to NK cells including, the total number of cells, their functions and activities such as; cytotoxicity, cytokine secretion, receptors and gene expression in peripheral blood NK cells, endometrial or decidual NK cells (3). Despite these findings, the precise role of NK cells in implantation and pathological conditions related to pregnancy are still not fully understood.

The level of peripheral blood $CD56^+$ NK cell declines from the first trimester of normal pregnancy and totally disappears at the end of pregnancy (6). Increase in NK cell activity can lead to placental damage and is down-regulated during normal pregnancy (3). Elevated NK cells and NK cell activity have been reported in women with RSA and IVF failure (7-9). Activation of NK cell cytotoxicity is detrimental to embryo implantation and its inhibition is associated with successful implantation. The cytokine profile of uterine NK cells is unique and plays an important role in the success of pregnancy (10).

Triggering of NK cell effector functions depends on the integration of signals delivered by an array of cell surface inhibitory and activating receptors such as CD69, CD161 and CD94. Some inhibitory receptors are engaged by self-MHC class I molecules preventing NK cell reactivity against normal autologous cells (11). CD69 belongs to C-lectin type superfamily, which is one of the earliest cell surface activation markers expressed and is capable of inducing cytotoxicity and stimulating cytokine production (12). CD161 surface marker is also a C lectin type which may regulate cytotoxicity of NK cells (13). CD94 is part of an inhibitory heterodimer form containing NKG2A and is a sub-group of the C type lectin superfamily (11).

The imbalance between activating and inhibitory receptor on NK cells is reported in women with implantation failure (8,14). Furthermore, imbalance in CD69 and CD94 expression on NK cells in pathological pregnancy is also detected (8,9), but the results are controversial and require more investigations.

In a recent study, we demonstrated an increase in peripheral blood NK cell cytotoxicity and $CD56^{dim}$ cells in women with RSA and those with IVF failure (15). To further understand the role of NK cells in these patients, in the current study we attempted to

evaluate the percentage of CD69, CD94 and CD161 surface markers as activating and inhibitory receptors on CD56^{+}NK cells in success or failure of pregnancy.

MATERIALS AND METHODS

Subjects. A total of 86 women volunteered to participate in this case-control study which comprised 43 individuals as patient group and another 43 individuals in a control group.

The Patient group comprised of two different sub-groups. The first sub-group included 23 women with history of at least two consecutive idiopathic miscarriages (16) with the same partner and a wish for pregnancy. A miscarriage was defined as a spontaneous pregnancy loss before 22 weeks of gestation. Those who had anatomical, genetic (in either partner) and hormonal abnormalities, or infectious and autoimmune diseases were excluded after interviewing by a specialist. The second sub-group included twenty women who had primary and secondary infertility of unknown etiology with at least two times IVF failure, and normal gynecological, hormonal and anatomical state. The cleaving embryos were scored according to equality of size of the blastomeres and proportion of anucleated fragments. Four categories were distinguished within this scoring system. Type A, was defined as equal size embryos without anucleate fragments. Type B was defined as nonequal size embryos and a maximum of 20% of the volume of the embryo filled with anucleate fragments. Type C, was defined as anucleated fragments represented 21-50% of the volume of the embryo and type D, had anucleate fragments present in more than 50% of the volume of the embryo (17). In the current study, the grades of embryos in the IVF cycles were Type A and B.

Women with known causes of infertility, including male factor, presence of endometriosis, tubal factors, uterine polyp and uterine septum were excluded. None of the women had lupus anticoagulant and anti-cardiolipin antibodies. All the selected patients had a normal karyotype.

Thirty-six healthy non-pregnant multiparous women with no history of abortion and at least one live-born infant served as the first control sub-group. The other control sub-group comprised seven women who had one live-born infant via successful IVF treatment.

The number of patients and control groups was calculated by the {$n = \frac{Z^2 \, P(1-P)}{d^2}$} formula and n was 43 for each groups. We divided this number among four groups but since the volunteers for successful IVF were not accessible, we had to used multiparous control woman more than successful IVF treatment.

All women provided a written informed consent before their participation in the study according to the Ethical Committee guidelines. This study has been approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences.

Peripheral blood samples were collected in heparinized tubes from all the patient and control groups and immediately transferred to a laboratory for assessment. All women at sampling time were not pregnant and were at the secretory phase of the menstrual cycle, which was defined by self-reporting. The characteristics of the patient and control groups are shown in Table 1.

Flowcytometric Analysis. The following mouse anti-human monoclonal antibodies were used for flowcytometry (Partec, Germany), including fluorescein isothiocyanate (FITC)-conjugated anti-human CD69 (Clone FN50), phycoerythrin (PE)-conjugated

anti-human CD56 (Clone B159), FITC-conjugated anti-human CD94 (Clone HP-3D9), FITC-conjugated anti-human CD161 (Clone DX121), and the relevant isotypics controls (IgG1 FITC, IgG1 PE- Clone X40).

Table 1. Characteristics of the patient and control groups.

	RSA	Normal	IVF failure	Successful IVF
Number of cases	23	36	20	7
Median age (range)	30 (24-42)	30 (19-45)	31.5 (27-42)	31.5 (28-34)
Median number of miscarriages (range)	3.5(2-5)	0	0	0
Median IVF failure (range)	0	0	4 (2-5)	2 (1-3)
Median number of children (range)	0	3 (2-6)	0	1 (1-2)

All the reagents were produced by Becton Dickenson (BD Biosciences, USA). The examination was performed on the fresh blood on the day transfer. 100 µL of blood was placed in flowcytometric tube and then treated with the appropriate monoclonal antibodies. Erythrocytes were lyzed using Lysing solution (Dako cytomation, Germany) and washed twice with phosphate buffer saline (PBS). A total of 15,000 events were acquired for each sample. The different physical properties of granulocytes, monocytes and lymphocytes allowed us to distinguish them from each other and from cellular contaminants. Lymphocyte gates set using linear forward angle light scatter and 90°/side scatter. In the current study, Two-color flow cytometric analysis was conducted to assess the expression of CD69, CD94 and CD161 on CD56$^+$NK cells within a lymphocyte gate in terms of percent using WinMDI 2.8 software.
Statistical Analysis. Data analysis was carried out using SPSS 17.10 software. The Mann-Whitney U test was used to detect statistical differences in mean percentage of NK cell populations between patient and control groups. Data was presented as median (range) and $p<0.05$ was considered significant.
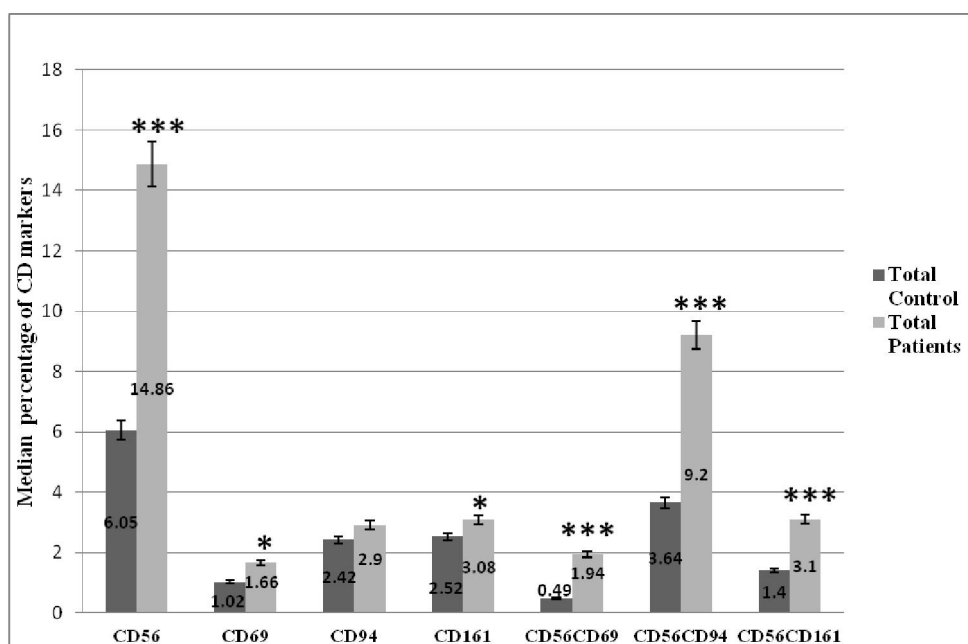
RESULTS

The expression of surface markers including, CD56, CD69, CD94 and CD161 in the lymphocyte gate was compared in all patient and normal control groups. Flow cytometric dot plot analysis of all normal samples in comparison with all patient samples are shown in Figure 1. The results in Table 2 showed that all the markers on the peripheral blood lymphocytes increased in RSA group compared to control group. However, it was significant for CD56 ($p<0.001$) and CD161 surface markers ($p<0.01$).

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Table 2. Comparison of mean percentages of CD56, CD69, CD94 and CD161 expression on peripheral blood lymphocytes among RSA (n=23) and control (n=36) groups.

Markers	Groups	Median (range)	*P* Value
CD56	Normal RSA	5.98 (2.42-8.69) 13.10 (9.24-24.24)	<0.001
CD69	Normal RSA	0.64 (0.21-3.96) 0.89 (0.23-4.79)	NS
CD56CD69	Normal RSA	0.45 (0.11-1.57) 1.46 (0.78-3.91)	<0.001
CD94	Normal RSA	2.18 (0.56-5.42) 2.07 (0.37-8.16)	NS
CD56CD94	Normal RSA	4.04 (0.81-7.08) 8.98 (1.46-21.42)	<0.001
CD161	Normal RSA	2.52 (0.4-5.37) 3.42 (0.40-7.88)	<0.01
CD56CD161	Normal RSA	1.26 (0.29-3.61) 2.86 (0.50-10.93)	<0.001

NS: difference not statistically significant

Figure 1. Comparison of mean percentage of CD56, CD69, CD94 and CD161 expression between RSA and IVF failure patients (n=43) with control (n=43) groups.
* *P <0.05*
*** *P< 0.001*

The expression of CD94, CD161, CD69 and CD56 increased in IVF failure compared to successful IVF women (Table 3). However, the difference was significant only for CD56 ($P<0.001$) and CD69 ($p<0.01$) surface markers. While, the mean percentage of CD69CD56, CD94CD56 and CD161CD56 cells increased significantly in IVF failure patients in comparison with successful IVF group, the mean percentages of CD56CD69 and CD56CD94 surface markers was highly elevated ($p<0.001$).

Table 3. Comparison of mean percentage of CD56, CD69, CD94 and CD161 expression on peripheral blood lymphocytes among IVF failure (n=20) and control group (n=7).

Markers	Groups	Median (range)	P Value
CD56	Successful IVF IVF Failure	5.95 (4.47-7.82) 14.01 (8.50-22.87)	< 0.001
CD69	Successful IVF IVF Failure	0.59 (0.18-1.12) 1.68 (0.55-6.76)	<0.01
CD56CD69	Successful IVF IVF Failure	0.25 (0.14-0.98) 1.68 (0.79-8.85)	<0.001
CD94	Successful IVF IVF Failure	2.30 (1.83-2.64) 2.40 (0.84-7.12)	NS
CD56CD94	Successful IVF IVF Failure	3.39 (2.04-4.65) 7.62 (2.43-15-46)	<0.001
CD161	Successful IVF IVF Failure	2.16 (0.95-3.02) 2.79 (0.28-4.93)	NS
CD56CD16 1	Successful IVF IVF Failure	1.31 (1.02-2.43) 2.47 (0.20-6.65)	<0.01

NS: difference not statistically significant.

As it is shown in Figure 1, in contrast to CD94 marker, the differences in the expression of CD69 and CD161 were significant ($p<0.05$) on lymphocytes. The expression of CD56CD69, CD56CD94 and CD56CD161 on CD56^{+}cells increased significantly on both patient groups compared to both control groups ($p<0.001$). The mean percentage of CD56, CD69, CD161 and CD94 positive cells in the control and patient groups are compared in Figures 2, 3, 4 and 5, respectively.
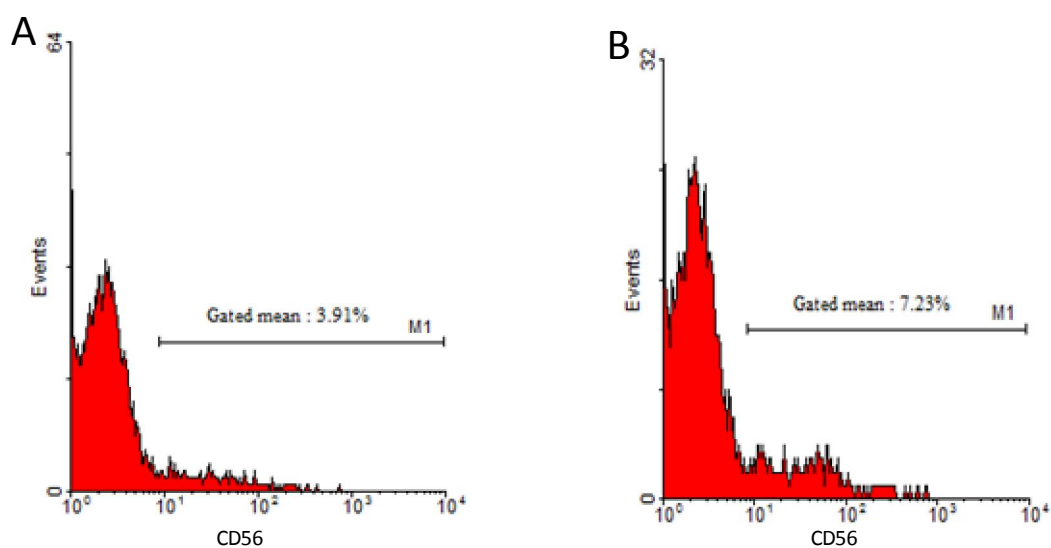
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Figure 2. A) Mean of CD56+ cells in one of the control group (with WinMDI 2.8 software), B) Mean of CD56+ cells in one of the patient group**.**
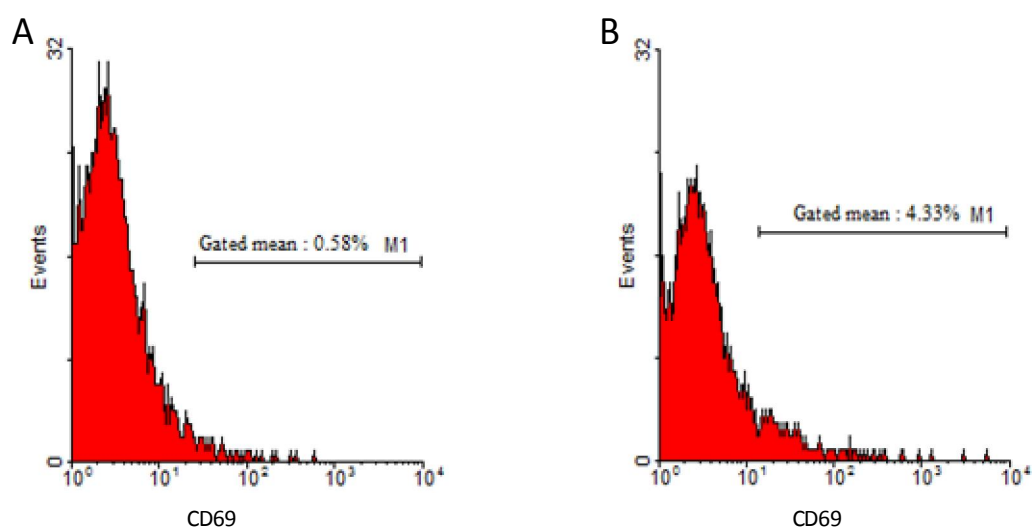

Figure 3. A) Mean of CD69+ cells in one of the normal group (by WinMDI 2.8 software)
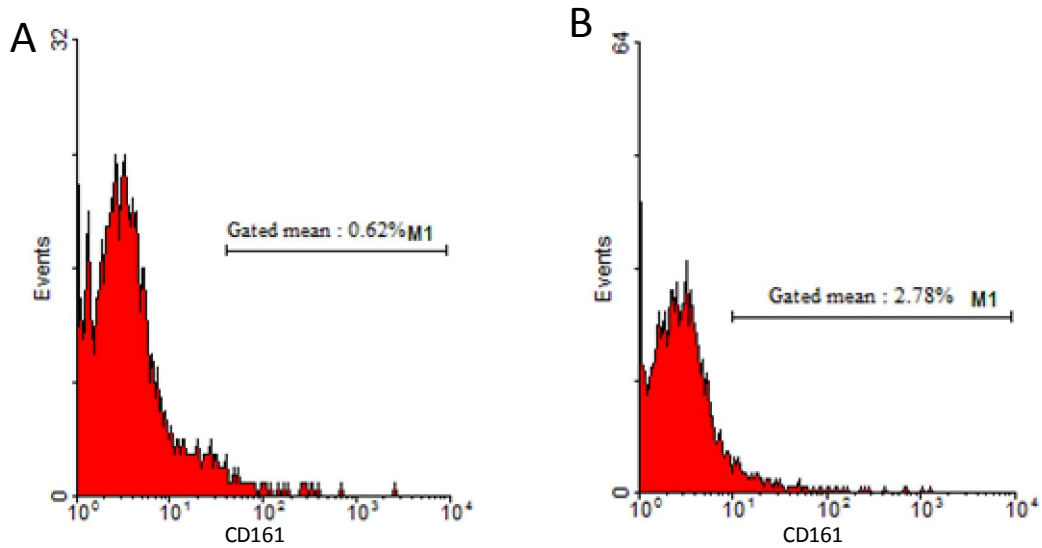B) Mean of CD69+ cells in one of the patient group.

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Figure 4. A) Mean of CD161$^+$ cells in one of the normal group (with WinMDI 2.8 software) B) Mean of CD161$^+$ cells in one of the patient group.
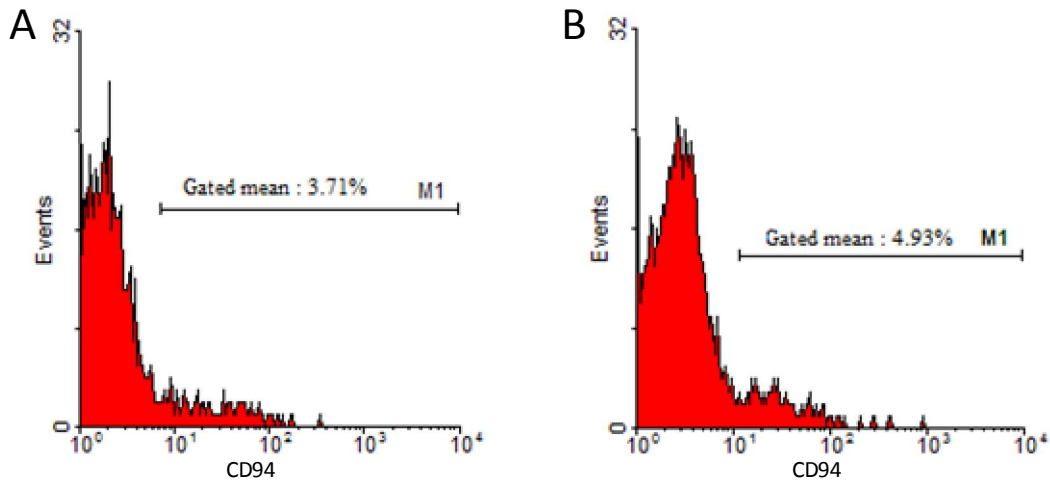

Figure 5. A) Mean of CD94$^+$ cells in one of the normal group (by WinMDI 2.8 software) B) Mean of CD94$^+$ cells in one of the patient group.

DISCUSSION

Maternal immune system has different ways to suppress NK cells, and dysregulation of this system, is likely to be in favor of pregnancy (18). For example, in pregnancy, maternal uterine lymphocytes produce Antipaternal Leukocyte Antibodies (APLA)

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against the father's HLA. APLA have been identified as a protection mechanism for fetus from maternal NK cells that are capable of rejecting the fetus (19).

Several reports have shown changes in the number and in the function of peripheral blood NK and uterine NK (uNK) cells which may be associated with reproductive failure. Despite the mismatch of immunophenotyping between peripheral blood NK cells and uNK cells, most studies conducted are based on peripheral blood sampling (3-5). In order to gain a better view on the role of uNK, few studies have succeded in taking biopsies taken from the endometrium of recurrent miscarriage patients (20). Thus, despite the differences between peripheral NK and uNK and due to problems of taking uterine biopsy, this study conducted on peripheral blood sampling.

Activated NK cells, with CD69 expression on their cell surface, play an important role in the control of trophoblast growth and placental development (21). In the current study, the level of CD69 NK cells was significantly higher in patients with RAS and IVF failure than in healthy control. This finding supports and confirms a previous study (1), showing that activated NK cells increas in women with spontaneous recurrent miscarriage and IVF failure. However, Baczkowski *et al.* reported no difference in CD69 expression on peripheral blood lymphocyte subpopulations including T and B and NK cells among fertile control group, infertile women who achieved and those who did not achieve a pregnancy after intracytoplasmic sperm injection (ICSI) (1). In this study, they used pregnant women within the control group instead of non-pregnant healthy women as control group. In agreement with our study, Chernyshov *et al.* have reported that immune mechanisms of IVF failure include not only elevated NK cells but also some other factors, such as elevated expression of CD69, CD8 and CD158a on NK cells, T lymphocyte activation and diminished T helper 2 parameters (7).

CD69 represents a functional triggering molecule on activated NK and T cells, and cross-linking of CD69 induces cytotoxic activity and cytokine production (12). Women with multiple implantation failures and recurrent pregnancy losses have increased peripheral blood T cell activation (22). Significantly elevated Th1 cytokine secretion by $CD4^+T$ cells has been reported in women with RSA and infertility due to implantation failures (23). Certain Th1 cytokines such as; IL-2, IL-12 and IFN-γ have immunoregulatory roles on NK cells, and can activate these cells (24). Women with failed IVF show unfavorable Th1-oriented changes of NK and NKT-like cells (25). Therefore, it seems that CD69 expressing activated peripheral blood NK cells in the current study, may result from dominant Th1 cytokine expression (TNF-α and IFN-γ) over Th2 cytokines (IL-4 and IL-10) in women with RSA and IVF failure.

Cytotoxic effect of CD69 on NK cells is blocked by CD94 inhibitory receptor (35). CD94/NKG2A expressed by decidual and peripheral blood NK cell is the predominant inhibitory receptor for HLA-G expressed on trophoblastic cells (27). The CD94 subunit also plays a pivotal role in the interaction between CD94/NKG2A and HLA-E/peptide complex (11). This recognition leads to inhibition of NK cell cytotoxicity, and may provide a mechanism to render maternal NK cells tolerant towards the fetus.

Various studies have been done on CD94 receptor, but the results are controversial. No significant difference was seen in CD94 expression on peripheral blood NK cells between 22 infertile women undergoing IVF/ET and 26 healthy pregnant women (8), in women with successful and failed IVF treatment (19), and in women with recurrent spontaneous abortion of unexplained etiology (28). In another study which was performed on peripheral blood mononuclear cells from women with RSA or infertility of unknown etiology, CD94 expression significantly decreased in both RSA and

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infertile women in comparison with that of controls (9). The results of this study leaded to the conclusion that NK inhibitory activity may play a role in successful pregnancy a litter later than the beginning of the implantation period.

In contrast to the above studies, our study showed significantly increased expression of CD94 on peripheral blood NK cells in women with RSA and IVF failure as compared with those of normal fertile controls. Masilamani *et al.* reported that CD94/NKG2A is a potential activating receptor and could induce a response to normal bystander cells (29). In an *in vitro* study, the effect of mifepristone as a drug for endometrium contraception, showed the increase expression of CD94/NKG2A on peripheral blood NK cells and the level of NK cell cytotoxicity, which may be exerted its anti-implantation function (30). CD94/NKG2A is also transiently inducible in peripheral blood NK cells under the influence of IL-12 stimulation in another *in vitro* study (31). Enhanced secretion IL-12 in infertile women correlates with the increased absolute numbers of NK cells in peripheral blood (32). Our study showed increased significantly NK cells in abnormal women compared with the control group. Therefore, high levels of IL-12 and Th1 type cytokines which are observed in pathological pregnancies may affect the expression of CD94 on peripheral blood NK cells. The increase in CD94 marker may incline balance toward an activating state in NK cells, which requires more investigations.

In contrast to our results, a decrease in expression of CD94 and CD158a as an inhibitory receptor on NK cells of decidua in sporadic miscarriage with normal chromosome karyotype. Moreover, differences in expression of CD94 in both peripheral blood and endometrium of infertile women also reported (33). The differences among the results may be related to patient selection criteria, timing of sample collection, specimen type, number of sample group, and NK markers.

Few studies have been performed on CD161 marker on reproductive in comparison to CD69 and CD94 in fertility. The functional role, as well as its ligand, is not well defined. It is mostly known as an activating receptor. An *in vitro* study showed blocking of CD161 receptor on NK cells lead to suppression of their cytotoxic properties (13). A significant increase in CD161 activating receptor by CD56/CD3 NKT cells in women with implantation failure was reported (14). Moreover, higher expression of CD161 receptor on NK cells has been shown in blood samples taken from 22 infertile women undergoing IVF/ET than in fertile women (8). However, CD161-activating receptor expressing CD56^{+}NK cells significantly decreased in women with RSA (34). In another report, the percentage of NK cells showing CD161 was relatively, but non-significantly, higher in RSA women in comparison with controls (27). No difference in expression of CD161 on NK cells in the peripheral blood of 11 women with IVF failure is reported (9). This discrepancy among the results may be partly related to few numbers of samples or the time of sampling. In our with larger sample size, we found high expression of CD161 receptor on NK cells in peripheral blood of 23 RSA and 20 IVF failure patients in comparison to 43 normal non-pregnant women with a history of successful pregnancy. This finding is supported by previous studies, which demonstrated peripheral blood NK cells in women with RSA and IVF failure are in activating status (7,15). Resistance of trophoblast to NK-mediated cytotoxicity was reported to be the result of insufficient activating interactions between the various triggering NK receptors and their target cells (35). Therefore, it seems that an imbalance between activating and inhibitory receptors in women with reproductive failures in comparison with normal, as shown in the current study, may partly explain the adverse reproductive outcomes.

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The results of the present study support the concept that immunological mechanisms are involved in implantation and that NK cells play a role in these mechanisms. Elevated expression of CD69 and CD161 on NK cells can be added to immunological risk markers in RSA and IVF failure. Further studies should attempt to determine whether high peripheral CD94 on NK cells and possibly inclined balance of that towards an activating state as found during this study is related to endometrial NK cell abnormality, and derived from a common pathogenic factor.

ACKNOWLEDGEMENTS

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