



Expression of Natural Cytotoxicity Receptors and Natural Killer Cells Cytokines Production in Peritoneal Fluid of Women with Endometriosis

Journal:	<i>American Journal of Reproductive Immunology</i>
Manuscript ID:	AJRI-10-13-185.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Funamizu, Ayano; Hirosaki University, Obstetrics and Gynecology Fukui, Atsushi; Hirosaki University, Obstetrics and Gynecology; Kamoi, Mai; Hirosaki University, Obstetrics and Gynecology Fuchinoue, Kohei; Hirosaki University, Obstetrics and Gynecology Yokota, Megumi; Hirosaki University, Obstetrics and Gynecology Fukuhara, Rie; Hirosaki University, Obstetrics and Gynecology Mizunuma, Hideki; Hirosaki University, Obstetrics and Gynecology
Keywords:	cytokine, endometriosis, natural killer cell, NKp46, peritoneal fluid

SCHOLARONE™
Manuscripts

NCR and cytokine in PF of Endometriosis

**Expression of Natural Cytotoxicity Receptors and Natural Killer Cells
Cytokines Production in Peritoneal Fluid of Women with Endometriosis**

Ayano Funamizu, Atsushi Fukui, Mai Kamoi, Kohei Fuchinoue, Megumi Yokota,
Rie Fukuhara, Hideki Mizunuma

Department of Obstetrics and Gynecology, Hirosaki University Graduate School
of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan.

E-mail: f-ayano@cc.hirosaki-u.ac.jp

Corresponding Author

Atsushi Fukui

Department of Obstetrics and Gynecology, Hirosaki University Graduate School
of Medicine

5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

TEL: 81-172-39-5107

FAX: 81-172-37-6842

Email: a.fukuipon@mac.com

Running head: NCR and cytokine in PF of Endometriosis

NCR and cytokine in PF of Endometriosis

Abstract

Problem: To investigate the relationship between the expression of Natural Cytotoxicity Receptors (NCRs) on peritoneal fluid NK (pfNK) cells and cytokine production by pfNK cells in women with endometriosis.

Method of Study: Peritoneal fluid was collected from women with endometriosis undergoing laparoscopic surgery (n=21) and controls without endometriosis (n=28). The expression of NK cell surface antigens such as CD16 and NCRs (NKp46, NKp44 and NKp30) on pfNK cells and cytokines production by pfNK cells (TNF- α , IFN- γ , IL-4, IL-10, GM-CSF and TGF- β_1) were measured using multicolor flow cytometry.

Results: The percentages of CD56⁺/NKp46⁺ cells and CD56^{dim}/NKp46⁺ cells in severe endometriosis group were significantly lower than that in controls. TNF- α and IFN- γ production by pfNK cells in severe endometriosis group was significantly higher than those in controls.

Conclusions: The differential expression of NKp46, TNF- α and IFN- γ on pfNK cells in women with severe endometriosis may allow the proliferation and angiogenesis of endometriotic cells.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

NCR and cytokine in PF of Endometriosis

Key words

cytokine, endometriosis, , natural killer cell, NKp46, peritoneal fluid

For Peer Review Only

NCR and cytokine in PF of Endometriosis

Introduction

Endometriosis is a common gynecological disease in women in reproductive age and characterized by the presence and growth of **endometrial** tissue outside the uterus.¹ The details of the development of endometriosis have not been clarified yet. However, it is suggested that immune factors and genetic factors are involved in the development of endometriosis.²⁻⁵ As for Natural Killer (NK) cells, it has been reported that the function of peritoneal fluid lymphocytes is decreased and implantation of endometrial cells would occur in abdominal cavity of women with endometriosis.⁶⁻⁹ The function of NK cells is mediated by the balance of activating and inhibiting receptors on the NK cell surface. It has been reported that the increase of inhibitory Killer Immunoglobulin-like Receptor (KIR) expression on NK cells indicated decreased NK cell cytotoxicity in women with endometriosis.¹⁰⁻¹² However, there are only a few reports that show the relationship between activating receptors of NK cells and endometriosis.

We have previously reported on the roles of Natural Cytotoxicity Receptors (NCRs: NKp46, NKp44, and NKp30), which have been known as one of the

NCR and cytokine in PF of Endometriosis

activating receptors of NK cells, in women with recurrent pregnancy loss (RPL), implantation failure, and preeclampsia.¹³⁻¹⁶ Specifically, we have reported that the expression of NKp46 on peripheral blood NK (pNK) cells is significantly lower in non-pregnant women with a history of RPL and implantation failures.¹³ We have also reported that the lower expression of NKp46 on pNK cells is associated with abnormal cytokine production by pNK cells in those women.¹⁴ In pregnant women with preeclampsia, the expression of NKp46 on pNK cells was significantly lower compared with pregnant women without preeclampsia.¹⁵ However, the expression of peritoneal fluid NK (pfNK) cells in women with/without endometriosis has not been clarified yet.

In addition, numerous cytokines play an important role in the development of endometriosis. Several studies have reported that increase of various kinds of inflammatory cytokines induced chronic inflammation in the abdominal cavity of women with endometriosis.¹⁷⁻²¹ Furthermore, it has been reported that cytokines may act directly on ectopic endometrium and stimulate implantation and proliferation of endometriotic cells.²² NK cells also produce various kinds of proinflammatory and immunoregulatory cytokines such as tumor necrosis

1 NCR and cytokine in PF of Endometriosis

2
3
4
5
6 factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-4, IL-10, granulocyte
7
8
9
10 macrophage colony-stimulating factor (GM-CSF), and transforming growth
11
12
13 factor (TGF)- β_1 .

14
15
16 Therefore, in order to reveal the participation of NCRs, we aimed to investigate
17
18
19 the relationship between expression of NCRs in pfNK cells and endometriosis.

20
21
22 We also aimed to investigate the difference of cytokines production by pfNK
23
24
25 cells in women with/without endometriosis and the relationship between the
26
27
28 expression of NCRs and the cytokines production by pfNK cells.

29 30 31 32 33 34 35 **Materials and methods**

36 37 38 Study subjects

39
40 Women with endometriosis undergoing laparoscopic surgery at Hirosaki
41
42
43 University Hospital were enrolled for this study (n=21). The controls were
44
45
46 women without endometriosis undergoing laparoscopic surgery for benign
47
48
49 diseases such as uterine myoma and ovarian cyst (n=28). The patients were
50
51
52 divided into three groups: severe endometriosis group (R-ASRM stage III and
53
54
55 IV) (n=18), mild endometriosis group (R-ASRM stage I and II) (n=3), and

NCR and cytokine in PF of Endometriosis

controls (n=28). Number of mild endometriosis group was very low because they were found accidentally during benign disease operation. Therefore, we excluded mild endometriosis group from this study. Severe endometriosis group included women with (n=6) / without (n=12) hormonal treatment such as GnRH agonist, low dose estrogen progestin (LEP) or dienogest. Controls included women with (n=9) / without (n=19) hormonal treatment (GnRH agonist). The phase of menstrual cycle of women without hormonal treatment at the time of surgery are as follows; proliferative phase (severe endometriosis group: n=7, controls: n=6), secretory phase (severe endometriosis group: n=3, controls: n=8), and anovulatory menstrual cycle (severe endometriosis group: n=2, controls: n=5). Patient characteristics were shown in Table I. There were no differences in age, obstetrical history, distribution of collection time and with/ without hormonal treatment between severe endometriosis group and controls. All women had given informed consent prior to entering the study, and the study was approved by the institutional review board.

The expression of NK cell surface antigens such as CD16 and Natural

NCR and cytokine in PF of Endometriosis

Cytotoxicity Receptors (NCRs) on pfNK cells

Total amount of peritoneal fluid was collected by aspiration using Nelaton catheter before any other operating procedure. Peritoneal fluid (PF) cell suspension was placed into phosphate-buffered saline (PBS) and washed twice. Then, the concentration of PF cell suspension was adjusted to 5×10^6 cells/ml. Then, 100 μ l of this cell suspension (5×10^6 cells/ml) was labeled by monoclonal antibodies for 20 minutes at room temperature. The following combinations of monoclonal antibodies were used to analyze the surface antigens of peritoneal fluid leukocytes: anti-CD45 PerCP-Cy5.5 (Beckman Coulter, Inc., Brea, CA, USA)/ anti-CD56 phycoerythrin (PE) (Beckman Coulter)/ anti-CD16 fluorescein isothiocyanate (FITC) (Beckman Coulter), anti-CD45 PerCP-Cy5.5/ anti-CD56 FITC (BD, Franklin Lakes, NJ, USA)/ anti-CD335 (NKp46) PE (Beckman Coulter), anti-CD45 PerCP-Cy5.5/ anti-CD56 FITC/ anti-CD336 (NKp44) PE (Beckman Coulter), and anti-CD45 PerCP-Cy5.5/ anti-CD56 FITC/ anti-CD337 (NKp30) PE (Beckman Coulter). After that, cells were lysed and fixed, and the cells were washed twice in PBS. Finally, the expression of these surface antigens was measured using multicolor flow cytometry.

NCR and cytokine in PF of Endometriosis

Cytokines production by pfNK cells

Peritoneal fluid cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (25ng/ml) and ionomycin (1 μ mol/l) in the presence of brefeldin-A (10 μ g/ml) for 4 hour at 37° C in a 5% CO2 humidified incubator to investigate intracellular cytokines. These cells were stained for 20 minutes at room temperature with anti-CD45 Pacific Blue (Beckman Coulter) and anti-CD56 PerCP-Cy5.5 (Beckman Coulter), or anti-CD45 Pacific Blue and anti-CD56 FITC. These cells were washed and fixed with 250 μ l of Cytofix/Cytoperm (BD) to allow anticytokine antibodies to enter the cells. After permeabilization, cells were washed twice in Perm/Wash Buffer (BD) and stained for 30 minutes with monoclonal antibodies to cytokines: anti-TNF- α PE (Beckman Coulter), anti-IFN- γ FITC (Beckman Coulter), anti-IL-4 PE (Beckman Coulter), anti-IL-10 FITC (R&D Systems, Minneapolis, MK, USA), anti-GM-CSF PE (R&D Systems), and anti-TGF- β_1 allophycocyanin (APC) (R&D Systems). Cells were washed twice with Perm/Wash Buffer and resuspended in 250 μ l of PBS for subsequent flow cytometric analysis. Finally, the intracellular cytokine

NCR and cytokine in PF of Endometriosis

production was measured using multicolor flow cytometry.

Flow cytometric analysis

A gate was set on the anti-CD45-positive events at first and then on the lymphocyte region by characteristic forward and side scatters parameters. For each sample, at least 1×10^4 cells were evaluated.

Statistical analysis

SPSS 20 (IBM, Chicago, IL, USA) was used for statistical analysis. Age distribution, number of pregnancies, deliveries and spontaneous abortions of two groups were analyzed by Mann-Whitney U test. The data are presented as median (interquartile range). Number of treated patients, and untreated patients and their menstrual cycle at the time of operation were analyzed by χ^2 test. The significance of differences of laboratory data between two groups was determined using Mann-Whitney U test. The laboratory data are presented as median (interquartile range). Significance was set at P value < 0.05 .

NCR and cytokine in PF of Endometriosis

Correlations between the percentage of NCRs and percentage of cytokines producing NK cells were analyzed by Pearson linear correlation. Correlations were considered significant if $r > 0.4$ and P value < 0.05 .

Results

Expression of NK cell surface antigens such as CD16 and NCRs on pfNK cells

Representative dot plots of the expression of NK cell subpopulation and NCRs in peritoneal fluid for severe endometriosis group and controls are shown in Fig. 1(A).

The percentage of NKp46⁺ NK cells on CD56⁺ NK cells in severe endometriosis group was 22.84 (11.58 – 46.34) and significantly lower than that in controls (30.20 (18.72 – 51.20)) ($P < 0.05$) (Fig. 2(A) and Table II). Similarly, the percentage of CD56^{dim}/NKp46⁺ NK cells in CD56^{dim} cells in severe endometriosis group was 16.67 (8.95 – 33.09) and significantly lower than that in controls (26.12 (14.60 – 41.14)) ($P < 0.01$) (Fig. 2(B)). On the other hand, there was no significant difference in the percentage of CD56^{bright}/NKp46⁺ cells in CD56^{bright} cells between two groups. For the expression of CD16, NKp44 and

1 NCR and cytokine in PF of Endometriosis

2
3
4
5
6 NKp30 on NK cells (CD56⁺, CD56^{bright}, CD56^{dim}), there were no significant
7
8
9 differences between two groups (Table II).

10
11
12 We also analyzed the difference of the expression of NKp46 on pfNK cells
13
14 among patients in proliferative phase, those in secretory phase and those with
15
16 hormonal treatment for both severe endometriosis group and controls. However,
17
18 there were no significant differences for the comparison of each group (data not
19
20 shown).
21
22
23
24
25
26
27
28
29
30

31 Cytokines production by pfNK cells (TNF- α , IFN- γ , IL-4, IL-10, GM-CSF and
32
33 TGF- β_1)
34
35

36
37 Representative dot plots of intracellular cytokines expression by pfNK cells for
38
39 severe endometriosis group and controls are shown in Fig. 1(B).
40
41

42
43 The percentage of TNF- α producing NK cells in severe endometriosis group
44
45 was 33.87 (25.21 – 60.73) and significantly higher than that in controls (25.42
46
47 (17.56 – 30.43)) ($P < 0.05$) (Fig. 3(A) and Table III). Similarly, the percentage of
48
49 IFN-g producing NK cells in severe endometriosis group (50.04 (26.98 – 71.33))
50
51 was significantly higher than that in controls (19.41 (6.01 – 38.37)) ($P < 0.05$)
52
53
54
55
56
57
58
59
60

NCR and cytokine in PF of Endometriosis

(Fig. 3(B) and Table III). There were no significant differences in the IL-4, IL-10, GM-CSF and TGF- β_1 productions by NK cells between two groups.

We also analyzed the differences of cytokines production by pfNK cells among patients in proliferative phase, those in secretory phase and those with hormonal treatment for both severe endometriosis group and controls. However, there were no significant differences for the comparison of each group (data not shown).

Correlation between the expression of NKp46 on NK cells and cytokines production by NK cells

Correlation between the expression of NKp46 on NK cells and cytokines production by NK cells in two groups is shown in Fig. 4 and Table IV. The percentage of CD56⁺/NKp46⁺ cells and TNF- α producing NK cells showed a significant negative correlation in severe endometriosis group ($r = -0.708$, $P < 0.05$), while no significant correlation between the percentage of CD56⁺/NKp46⁺ cells and TNF- α -producing NK cells in controls. There were no significant correlations in the other cytokines between two groups.

NCR and cytokine in PF of Endometriosis

Discussion

We hereby show lower expression of NKp46 on pfNK cells and higher production of TNF- α and IFN- γ by pfNK cells in women with severe endometriosis. Wu et al. reported that the percentage of NK cells in PF and their activation markers, such as CD69, CD25, and HLA-DR, were similar in women with endometriosis compared with women without endometriosis despite the decrease in NK cell cytotoxicity noted in PF of women with severe endometriosis.⁹ However, there are no reports yet about the relevance of endometriosis and the expression of NCRs. NCRs regulate NK cell cytotoxicity and cytokine production through the signal cascade.^{15,23} The NKp46 receptor is a 46-kDa type I membrane glycoprotein characterized by two C2-type Ig-like domains in the extracellular portion and is associated with CD3 ζ and Fc ϵ RI γ that become tyrosine phosphorylated. The NKp44 receptor is a 44-kDa type I membrane glycoprotein characterized by a single V-type Ig-like domain in the extracellular portion and is associated with KARAP/CAP12 molecules. The NKp30 receptor is a 30-kDa type I membrane glycoprotein characterized by a

NCR and cytokine in PF of Endometriosis

single V-type Ig-like domain in the extracellular portion and associated with CD3 ζ polypeptides.²³ NKp44 receptor is expressed on the surface of activated NK cells. But NKp46 and NKp30 receptors are expressed on the surface of activated and non-activated NK cells. In addition, NKp46 and NKp30 play a central role in the cytotoxic activity and cytokine production of NK cells.^{15,24 25}

In this study, the percentages of CD56⁺/NKp46⁺ and CD56^{dim}/NKp46⁺ cells in severe endometriosis group were significantly lower than that in controls. It has been reported that CD56^{dim}/CD16⁺ cells had a function in strong cytotoxic activity.²⁶ As mentioned above, however, NKp46 receptor has a function in cytotoxic activity and cytokine production of NK cells. In addition, CD56^{dim} NK cells are considered to be cytotoxic, while CD56^{bright} NK cells have a higher ability of producing cytokines but have lower cytotoxicity.²⁷ Therefore, it is considered that CD56^{dim}/NKp46⁺ cells show the cytotoxicity. The percentage of CD56^{dim}/CD16⁺ was not significantly different between two groups, but the decrease in CD56^{dim}/NKp46⁺ may be a cause of reduction of cytotoxicity in women with endometriosis. Furthermore, we have previously reported that CD56^{dim}/NKp46⁺ cells are also involved in cytokines production as well as

1 NCR and cytokine in PF of Endometriosis

2
3
4
5
6 CD56^{bright}/NKp46⁺ cells.²⁵ Abnormal expression of NKp46 on CD56^{dim} NK cells
7
8
9
10 may be related to abnormal production of cytokines by NK cells as well as
11
12
13 cytotoxicity.
14

15
16 The percentage of TNF- α and IFN- γ production by pfNK cells was significantly
17
18
19 higher in women with severe endometriosis compared with controls. TNF- α is
20
21
22 one of the inflammatory cytokines. It has been reported that TNF- α is related to
23
24
25 inflammation in peritoneal cavity, as well as angiogenesis²⁸ and proliferation of
26
27
28 endometriotic stromal cells in women with endometriosis.^{20, 22} And they
29
30
31 reported that TNF- α concentration in PF was increased in women with
32
33
34 endometriosis. These data support the notion that TNF- α is an important
35
36
37 cytokine for endometriosis development. Similarly, IFN- γ is related to
38
39
40 inflammation and angiogenesis.^{14,15} IFN- γ has been reported to have an
41
42
43 essential role in implantation especially in vascular remodeling process. Genetic
44
45
46 absence of IFN- γ in NK cells in mice results in absence of pregnancy-induced
47
48
49 spiral artery modification. NK cell-derived IFN- γ modifies the expression of
50
51
52 genes in the uterine vasculature and stroma, which initiates vessels instability
53
54
55 and facilitates pregnancy-induced remodeling of decidual arteries.²⁹ Therefore,
56
57
58
59
60

NCR and cytokine in PF of Endometriosis

it seemed that angiogenesis of endometriotic cells was promoted by increase of TNF- α and IFN- γ producing peritoneal fluid NK cells.

Because we found that expression of NKp46 on pfNK cells was lower and TNF- α and IFN- γ productions of pfNK cells were higher in women with severe endometriosis, we analyzed the correlation between expression of NKp46 on NK cells and cytokines production by NK cells to understand their mutual relationship. There was significant correlation between the percentages of CD56⁺/NKp46⁺ cells and the percentage of TNF- α producing NK cells in severe endometriosis group. In women with recurrent pregnancy losses, we have reported that there is significantly negative correlation between the expression of NKp46 on NK cells and the percentage of TNF- α producing NK cells¹⁵. In those women, NK cells may be activated and the correlation may possibly reflect to activation status of NK cells. In controls, there was no significant correlation between CD56⁺/NKp46⁺ and TNF- α producing NK cells. In peritoneal cavity in controls, CD16⁺/CD56^{dim} cytotoxic NK cells may attack endometrial cells in the menstrual blood. So, these NK cell may not need to produce various kinds of cytokines such TNF- α and IFN- γ that are important for

1 NCR and cytokine in PF of Endometriosis

2
3
4
5
6 angiogenesis. Therefore, the number of TNF- α producing NK cell might be low.
7
8
9
10 Consequently, no correlation was indicated in controls. Moreover, NK cell
11
12 cytotoxicity in peritoneal fluid has not fully elucidated yet. Further analysis is
13
14 needed to clarify the pathophysiology of endometriosis.
15
16
17

18
19 Endometriosis affects various parts of a woman's reproductive system: ovarian
20
21 function, oocyte quality, embryo development and implantation, uterine
22
23 function and the endocrine system choreographing the reproductive process.³⁰
24
25
26
27 High concentration of TNF- α in PF can activate caspase-dependent signaling
28
29 pathways to increase embryo apoptosis.³¹ In addition, we have reported that the
30
31 expression of NKp46 is significantly lower in peripheral blood of women with a
32
33 history of RPL and implantation failures and that the lower expression of
34
35 NKp46 is associated with higher TNF- α production by peripheral blood NK cells
36
37 in such women.^{14, 15} These results suggest that the lower expression of NKp46
38
39 and higher TNF- α production by NK cells in endometriosis may account for a
40
41 part of the infertility process of endometriosis.
42
43
44
45
46
47
48
49
50
51

52
53 In this study, we collected peritoneal fluid at a various phase of menstrual
54
55 cycle and some patients had a hormonal treatment such as GnRH agonist, LEP
56
57
58
59
60

NCR and cytokine in PF of Endometriosis

or dienogest because of difficulty for harmonize the phase of menstrual cycle for the operation. Therefore, we analyzed the differences of NK cell surface antigens and cytokines production by NK cells in each phase of menstrual cycle. However, there were no significant differences. The number of each group was very low. Therefore, further analysis is needed to clarify the differences of NK cell surface antigens and cytokines production by NK cells in each phase of menstrual cycle in future.

In conclusion, this study has demonstrated that the expression of NKp46 on pfNK cells was lower and TNF- α and IFN- γ production by pfNK cells was higher in women with severe endometriosis. Lower expression of NKp46 on pfNK cells is likely to be involved in the decrease of NK cytotoxicity and higher TNF- α and IFN- γ production by pfNK cells. As a result of higher TNF- α and IFN- γ environment in the abdominal cavity, proliferation and angiogenesis of the endometriotic cells may be promoted.

NCR and cytokine in PF of Endometriosis

References

- 1 Strathy JH, Molgaard CA, Coulam CB, Melton LJ, 3rd: Endometriosis and infertility: a laparoscopic study of endometriosis among fertile and infertile women. *Fertility and sterility* 1982;**38**:667-672.
- 2 Wu Y, Strawn E, Basir Z, Halverson G, Guo SW: Aberrant expression of deoxyribonucleic acid methyltransferases DNMT1, DNMT3A, and DNMT3B in women with endometriosis. *Fertility and sterility* 2007;**87**:24-32.
- 3 Bianco B, Andre GM, Vilarino FL, Peluso C, Mafra FA, Christofolini DM, Barbosa CP: The possible role of genetic variants in autoimmune-related genes in the development of endometriosis. *Human immunology* 2012;**73**:306-315.
- 4 Paskulin DD, Cunha-Filho JS, Souza CA, Bortolini MC, Hainaut P, Ashton-Prolla P: TP53 PIN3 and PEX4 polymorphisms and infertility associated with endometriosis or with post-in vitro fertilization implantation failure. *Cell death & disease* 2012;**3**:e392.
- 5 Xu S, Wu W, Sun H, Lu J, Yuan B, Xia Y, De Moor B, Marchal K, Wang X,

NCR and cytokine in PF of Endometriosis

Xu P, Cheng W: Association of the vascular endothelial growth factor gene polymorphisms (-460C/T, +405G/C and +936T/C) with endometriosis: a meta-analysis. *Annals of human genetics* 2012;**76**:464-471.

6 Oosterlynck DJ, Meuleman C, Waer M, Vandeputte M, Koninckx PR: The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. *Fertility and sterility* 1992;**58**:290-295.

7 Wilson TJ, Hertzog PJ, Angus D, Munnery L, Wood EC, Kola I: Decreased natural killer cell activity in endometriosis patients: relationship to disease pathogenesis. *Fertility and sterility* 1994;**62**:1086-1088.

8 Ho HN, Chao KH, Chen HF, Wu MY, Yang YS, Lee TY: Peritoneal natural killer cytotoxicity and CD25+ CD3+ lymphocyte subpopulation are decreased in women with stage III-IV endometriosis. *Human reproduction* 1995;**10**:2671-2675.

9 Wu MY, Chao KH, Chen SU, Chen HF, Yang YS, Huang SC, Ho HN: The suppression of peritoneal cellular immunity in women with endometriosis could be restored after gonadotropin releasing hormone

1 NCR and cytokine in PF of Endometriosis
2
3
4
5

6 agonist treatment. *American journal of reproductive immunology*
7
8
9
10 1996;**35**:510-516.

11
12 10 Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T:
13 Increased killer inhibitory receptor KIR2DL1 expression among natural
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

10 Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T:
Increased killer inhibitory receptor KIR2DL1 expression among natural
killer cells in women with pelvic endometriosis. *Fertility and sterility*
2002;**77**:297-302.

11 Zhang C, Maeda N, Izumiya C, Yamamoto Y, Kusume T, Oguri H,
Yamashita C, Nishimori Y, Hayashi K, Luo J, Fukaya T: Killer
immunoglobulin-like receptor and human leukocyte antigen expression
as immunodiagnostic parameters for pelvic endometriosis. *American
journal of reproductive immunology* 2006;**55**:106-114.

12 Kitawaki J, Xu B, Ishihara H, Fukui M, Hasegawa G, Nakamura N,
Mizuno S, Ohta M, Obayashi H, Honjo H: Association of killer cell
immunoglobulin-like receptor genotypes with susceptibility to
endometriosis. *American journal of reproductive immunology*
2007;**58**:481-486.

13 Fukui A, Ntrivalas E, Gilman-Sachs A, Kwak-Kim J, Lee SK, Levine R,

NCR and cytokine in PF of Endometriosis

Beaman K: Expression of natural cytotoxicity receptors and a2V-ATPase on peripheral blood NK cell subsets in women with recurrent spontaneous abortions and implantation failures. *American journal of reproductive immunology* 2006;**56**:312-320.

14 Fukui A, Ntrivalas E, Fukuhara R, Fujii S, Mizunuma H, Gilman-Sachs A, Beaman K, Kwak-Kim J: Correlation between natural cytotoxicity receptors and intracellular cytokine expression of peripheral blood NK cells in women with recurrent pregnancy losses and implantation failures. *American journal of reproductive immunology* 2009;**62**:371-380.

15 Fukui A, Funamizu A, Yokota M, Yamada K, Nakamua R, Fukuhara R, Kimura H, Mizunuma H: Uterine and circulating natural killer cells and their roles in women with recurrent pregnancy loss, implantation failure and preeclampsia. *Journal of reproductive immunology* 2011;**90**:105-110.

16 Fukui A, Kwak-Kim J, Ntrivalas E, Gilman-Sachs A, Lee SK, Beaman K: Intracellular cytokine expression of peripheral blood natural killer cell subsets in women with recurrent spontaneous abortions and implantation failures. *Fertility and sterility* 2008;**89**:157-165.

1 NCR and cytokine in PF of Endometriosis
2
3
4
5

6
7 17 Keenan JA, Chen TT, Chadwell NL, Torry DS, Caudle MR:
8

9
10 Interferon-gamma (IFN-gamma) and interleukin-6 (IL-6) in peritoneal
11
12 fluid and macrophage-conditioned media of women with endometriosis.
13
14

15
16 *American journal of reproductive immunology* 1994;**32**:180-183.
17

18
19 18 Harada T, Yoshioka H, Yoshida S, Iwabe T, Onohara Y, Tanikawa M,
20

21
22 Terakawa N: Increased interleukin-6 levels in peritoneal fluid of infertile
23
24 patients with active endometriosis. *American journal of obstetrics and*
25
26
27
28 *gynecology* 1997;**176**:593-597.
29

30
31 19 Barcz E, Kaminski P, Marianowski L: Role of cytokines in pathogenesis of
32

33
34 endometriosis. *Medical science monitor : international medical journal of*
35
36
37
38 *experimental and clinical research* 2000;**6**:1042-1046.
39

40
41 20 Harada T, Iwabe T, Terakawa N: Role of cytokines in endometriosis.
42

43
44 *Fertility and sterility* 2001;**76**:1-10.
45

46
47 21 Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR: Transforming
48

49
50 growth factor-beta activity is increased in peritoneal fluid from women
51
52 with endometriosis. *Obstetrics and gynecology* 1994;**83**:287-292.
53
54

55
56 22 Iwabe T, Harada T, Tsudo T, Nagano Y, Yoshida S, Tanikawa M,
57

NCR and cytokine in PF of Endometriosis

Terakawa N: Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. *The Journal of clinical endocrinology and metabolism* 2000;**85**:824-829.

23 Biassoni R, Cantoni C, Marras D, Giron-Michel J, Falco M, Moretta L, Dimasi N: Human natural killer cell receptors: insights into their molecular function and structure. *Journal of cellular and molecular medicine* 2003;**7**:376-387.

24 Bryceson YT, March ME, Ljunggren HG, Long EO: Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 2006;**107**:159-166.

25 Yokota M, Fukui A, Funamizu A, Nakamura R, Kamoi M, Fuchinoue K, Sasaki Y, Fukuhara R, Mizunuma H: Role of NKp46 expression in cytokine production by CD56-positive NK cells in the peripheral blood and the uterine endometrium. *American journal of reproductive immunology* 2013;**69**:202-211.

26 Cooper MA, Fehniger TA, Caligiuri MA: The biology of human natural

1 NCR and cytokine in PF of Endometriosis

2
3
4
5
6 killer-cell subsets. *Trends in immunology* 2001;**22**:633-640.

7
8
9
10 27 Nagler A, Lanier LL, Cwirla S, Phillips JH: Comparative studies of
11
12 human FcRIII-positive and negative natural killer cells. *Journal of*
13
14 *immunology* 1989;**143**:3183-3191.

15
16
17
18
19 28 Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir
20
21 N: Macrophage-induced angiogenesis is mediated by tumour necrosis
22
23 factor-alpha. *Nature* 1987;**329**:630-632.

24
25
26
27
28 29 Ashkar AA, Di Santo JP, Croy BA: Interferon gamma contributes to
29
30 initiation of uterine vascular modification, decidual integrity, and uterine
31
32 natural killer cell maturation during normal murine pregnancy. *The*
33
34 *Journal of experimental medicine* 2000;**192**:259-270.

35
36
37
38
39 30 Stilley JA, Birt JA, Sharpe-Timms KL: Cellular and molecular basis for
40
41 endometriosis-associated infertility. *Cell and tissue research*
42
43
44
45
46 2012;**349**:849-862.

47
48
49 31 Hu X: Proteolytic signaling by TNFalpha: caspase activation and
50
51 IkappaB degradation. *Cytokine* 2003;**21**:286-294.

52
53
54
55
56 **Figure Legends**

NCR and cytokine in PF of Endometriosis

Figure 1: Representative dot plots of the peritoneal fluid NK (pfNK) cells in a woman with/without severe endometriosis.

(A) The expression of NK cell subpopulation and Natural Cytotoxicity Receptors on pfNK cells. (B) The expression of cytokines in pfNK cells.

Figure 2: The percentages of NCRs positive cells on pfNK cells in severe endometriosis group and controls.

(A) The percentage of NKp46⁺ cells on CD56⁺ cells in severe endometriosis was significantly lower than that in controls. (B) The percentage of CD56^{dim}/NKp46⁺ cells in severe endometriosis was significantly lower than that in controls. There was no significant difference in the percentage of CD56^{bright}/NKp46⁺ cells between two groups. Box and plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Differences between the two groups were analyzed by Mann-Whitney *U* test.

Figure 3: The percentage of TNF- α and IFN- γ producing NK cells in severe endometriosis and controls.

NCR and cytokine in PF of Endometriosis

(A) $\text{TNF-}\alpha$ producing NK cells in severe endometriosis were significantly higher than that in controls. (B) $\text{IFN-}\gamma$ producing NK cells in severe endometriosis were significantly higher than that in controls. Box and plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Differences between the two groups were analyzed by Mann-Whitney *U* test.

Figure 4: Correlation between the percentages of $\text{CD56}^+/\text{NKp46}^+$ cells and $\text{TNF-}\alpha$ producing NK cells in severe endometriosis group and controls.

There was a significant correlation between the percentages of $\text{CD56}^+/\text{NKp46}^+$ cells and $\text{TNF-}\alpha$ producing NK cells in severe endometriosis group. Correlations between the percentage of $\text{CD56}^+/\text{NKp46}^+$ cells and percentage of cytokines producing pFNK cells were analyzed by Pearson linear correlation.

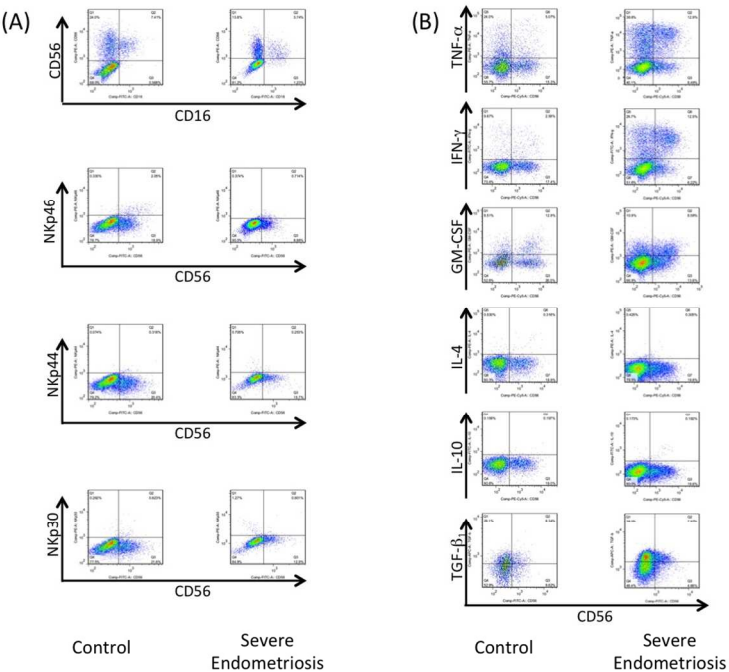


Fig.1

Figure 1: Representative dot plots of the peritoneal fluid NK (pfNK) cells in a woman with/without severe endometriosis.

(A) The expression of NK cell subpopulation and Natural Cytotoxicity Receptors on pfNK cells. (B) The expression of cytokines in pfNK cells.
508x381mm (72 x 72 DPI)

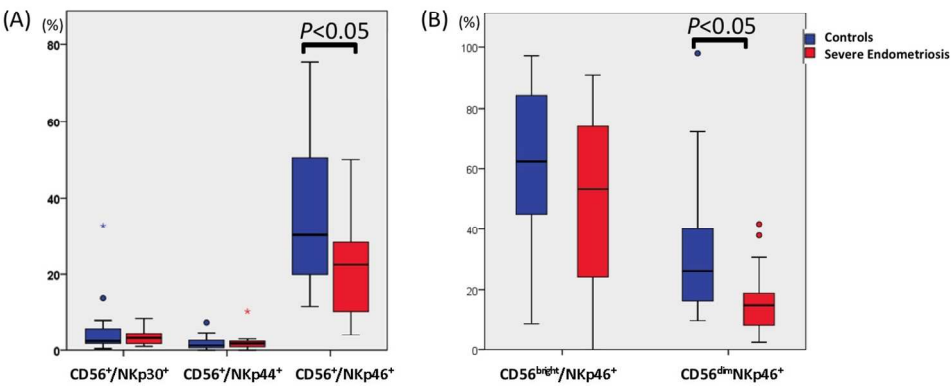


Fig.2

Figure 2: The percentages of NCRs positive cells on pFNK cells in severe endometriosis group and controls. (A) The percentage of NKp46+ cells on CD56+ cells in severe endometriosis was significantly lower than that in controls. (B) The percentage of CD56dim/NKp46+ cells in severe endometriosis was significantly lower than that in controls. There was no significant difference in the percentage of CD56bright/NKp46+ cells between two groups. Box and plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Differences between the two groups were analyzed by Mann-Whitney U test.

508x381mm (72 x 72 DPI)

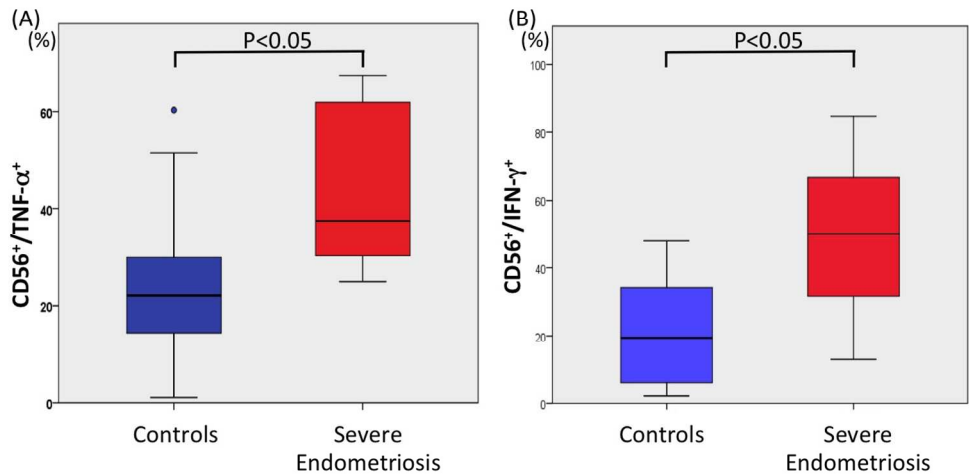


Fig.3

Figure 3: The percentage of TNF-α and IFN-γ producing NK cells in severe endometriosis and controls. (A) TNF-α producing NK cells in severe endometriosis were significantly higher than that in controls. (B) IFN-γ producing NK cells in severe endometriosis were significantly higher than that in controls. Box and plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Differences between the two groups were analyzed by Mann-Whitney U test. 508x381mm (72 x 72 DPI)

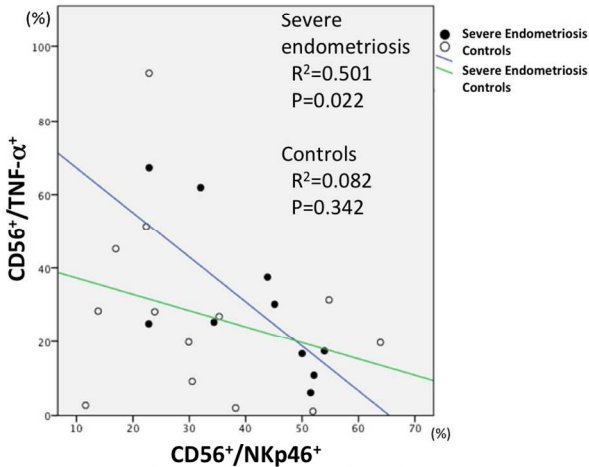


Fig.4

Figure 4: Correlation between the percentages of CD56+/NKp46+ cells and TNF- α producing NK cells in severe endometriosis group and controls. There was a significant correlation between the percentages of CD56+/NKp46+ cells and TNF- α producing NK cells in severe endometriosis group. Correlations between the percentage of CD56+/NKp46+ cells and percentage of cytokines producing pNK cells were analyzed by Pearson linear correlation.

508x381mm (72 x 72 DPI)

Table I : Age, obstetrical histry and patient characteristics of severe endometriosis group (n=18) and controls (n=28).

	Severe Endometriosis (n=18)	Controls (n=28)	P value
Age (years)	35 (33.75 - 42.00)	38 (33 - 41)	NS
No. of pregnancies	0 (0 - 2)	1 (0 - 3)	NS
No. of deliveries	0 (0 - 1)	0 (0 - 1)	NS
No. of spontaneous abortions	0 (0 - 0)	0 (0 - 0)	NS
No. of treated patient	6/18	9/28	NS
No. of untreatd patient	12/18	19/28	NS
proliferative phase	7/12	6/19	NS
secretory phase	3/12	8/19	NS
anovulatory	2/12	5/19	NS
Stage of endometriosis			
Stage III	3/18	–	
Stage IV	15/18	–	

Age and obstetrical history were analyzed by Mann-Whitney U test. Values are expressed as median (Interquatile range). No. of treated patient, untreated patient and menstrual cycle were analyzed by χ^2 test.

Table II : Expression of NK cell surface antigens of peritoneal fluid NK cells in severe endometriosis group and controls.

	Severe Endometriosis		Controls		<i>P</i> value
	Median (%)	Interquartile range	Median (%)	Interquartile range	
CD16 ⁺ /CD56 ⁺	16.36	(1.17 - 20.93)	11.66	(2.06 - 18.26)	NS
CD16 ⁺ /CD56 ^{bright}	8.65	(0.00 - 12.50)	4.43	(0.29 - 10.85)	NS
CD16 ⁺ /CD56 ^{dim}	16.44	(1.01 - 21.81)	12.10	(2.25 - 19.47)	NS
CD56 ⁺ /NKp46 ⁺	22.84	(11.58 - 46.34)	30.20	(18.72 - 51.20)	< 0.05
CD56 ^{bright} /NKp46 ⁺	66.75	(26.55 - 79.97)	62.37	(45.02 - 84.80)	NS
CD56 ^{dim} /NKp46 ⁺	16.67	(8.95 - 33.09)	26.12	(14.60 - 41.14)	< 0.01
CD56 ⁺ /NKp44 ⁺	1.53	(0.78 - 2.62)	1.21	(0.61 - 2.60)	NS
CD56 ^{bright} /NKp44 ⁺	1.40	(0.00 - 3.02)	0.47	(0.00 - 2.48)	NS
CD56 ^{dim} /NKp44 ⁺	1.09	(0.88 - 2.47)	1.16	(0.51 - 2.58)	NS
CD56 ⁺ /NKp30 ⁺	3.77	(1.84 - 5.44)	2.38	(1.67 - 5.88)	NS
CD56 ^{bright} /NKp30 ⁺	1.49	(0.00 - 4.90)	2.59	(1.36 - 5.28)	NS
CD56 ^{dim} /NKp30 ⁺	3.54	(1.47 - 5.91)	2.30	(1.60 - 5.81)	NS

Difference between severe endometriosis group and controls was analyzed by Mann-Whitney U test. Values are expressed as median (Interquartile range).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table III : Cytokine producing CD56⁺ peritoneal fluid NK cells in severe endometriosis group and controls.

CD56 ⁺	Severe Endometriosis		Controls		P value
	Median (%)	Interquartile range	Median (%)	Interquartile range	
TNF- α	33.87	(25.21 - 60.73)	25.42	(17.56 - 30.43)	< 0.05
IFN- γ	50.04	(26.98- 71.33)	19.41	(6.01 - 38.37)	< 0.05
IL-4	1.85	(1.40 - 6.58)	3.05	(1.56 - 5.88)	NS
IL-10	1.09	(0.64 - 8.70)	0.71	(0.45 - 3.86)	NS
GM-CSF	31.73	(7.63 - 57.44)	18.69	(8.28 - 33.48)	NS
TGF- β_1	14.93	(7.45 - 26.23)	22.14	(8.10 - 33.20)	NS

Difference between severe endometriosis group and controls was analyzed by Mann-Whitney U test. Values are expressed as median (Interquartile range).

Table IV : Correlation between the percentage of CD56⁺/NKp46⁺ cells and cytokine producing CD56⁺ peritoneal fluid NK cells in severe endometriosis group and controls.

Cytokine producing CD56 ⁺ NK cells	Severe Endometriosis		Controls	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
TNF- α	-0.708	<0.05	-0.292	NS
IFN- γ	-0.438	NS	-0.287	NS
IL-4	0.353	NS	-0.148	NS
IL-10	0.301	NS	-0.150	NS
GM-CSF	-0.149	NS	-0.254	NS
TGF- β_1	-0.171	NS	-0.271	NS

Correlations between the percentage of CD56⁺/NKp46⁺ cells and percentage of cytokines producing CD56⁺ peritoneal fluid NK cells were analyzed by Pearson linear correlation. Correlations were considered significant if $r > 0.4$ and $P < 0.05$.