NK22 cells in the uterine mid-secretory endometrium and peripheral blood of women with recurrent pregnancy loss and unexplained infertility

Mai Kamoi M.D.[1], Atsushi Fukui M.D. Ph.D.[1], Kohei Fuchinoue M.D.[1], Ayano Funamizu M.D. Ph.D.[1], Hitomi Chiba M.D.[1], Megumi Yokota M.D. Ph.D.[1], Rie Fukuhara M.D. Ph.D.[1], Hideki Mizunuma M.D. Ph.D.[1]

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

Abstract

Problem: We aimed to investigate natural killer 22 (NK22) cells in the peripheral blood and the uterine endometrium of women with unexplained recurrent pregnancy loss (URPL) and unexplained infertility (UI).

Method of Study: Peripheral blood and endometrial samples were collected from women with URPL (n = 43) and UI (n = 38). Intracellular cytokine production, such as IL-22, IFN-γ and TNF-α, and the expression of NKp46 on NK cells were analyzed by three-color flow cytometry.

Results: The percentages of endometrial $CD56^{+}$/$IL\text{-}22^{+}$ and $CD56^{dim}$/$IL\text{-}22^{+}$ cells in women with URPL were significantly higher than those of UI ($P<0.05$ respectively). In addition, the percentage of $CD56^{bright}$/$IL\text{-}22^{+}$ cells in women with RPL was negatively correlated with those of $CD56^{bright}$/IFN-γ^{+}, $CD56^{bright}$/TNF-α^{+} in both peripheral blood and endometrial NK cells. This was not seen in women with UI. The percentage of $CD56^{bright}$/$IL\text{-}22^{+}$ cells was negatively correlated with $CD56^{bright}$/NKp46 expressing NK cells in peripheral blood.

Conclusions: Endometrial NK22 cells are differently regulated in women with URPL and UI. Women with URPL have higher level of NK22 cells with a potential to induce NK2 shift, than women with UI.

Introduction

Natural killer (NK) cells play important roles in innate immune responses. Ninety percent of peripheral blood NK (pNK) cells are $CD56^{dim}/CD16^{+}$ and have high cytotoxic activity. On the other hand, although 90% of uterine NK (uNK) cells are $CD56^{bright}/CD16^{-}$ these cells have low cytotoxic activity and produce more cytokines.[1-3] Several studies have shown that uNK cells play important roles in maintaining a normal pregnancy.[4, 5] Our previous study showed that the increase in cytotoxic pNK cells was associated with poor IVF-ET outcomes.[6] A recent Meta-analysis study showed that pNK cell numbers were significantly higher in infertile women as compared with that of fertile controls.[7] In women with recurrent pregnancy loss (RPL), the number and activity of pNK cells were increased during early pregnancy as compared to those of normal pregnant women.[8, 9] Although the major population of uNK cells comprised $CD56^{bright}/CD16^{-}$ cells, women with RPL had fewer number of these cells than fertile controls. Moreover, the $CD56^{dim}/CD16^{+}$ population was twice the number of fertile controls.[10]

During early pregnancy, uNK cells are major sources of angiogenic factors.[11, 12] Furthermore, uNK cells produce various cytokines however, they tend not to affect cytotoxicity.[12] Human NK cells are classified into NK1 and NK2 on the basis of cytokine production; similar to the classification of Th1 and Th2 cells.[13] Higuma-Myojo et al., firstly demonstrated NK1 and NK2 cells in reproduction. During early pregnancy, decidual NK cell subsets are functionally different from pNK cell subsets in cytokine production pattern.[14] NK1 cells produce type 1 cytokines such as IFN-γ and TNF-α, and NK2 cells

produce a type 2 cytokine such as IL-4. In normal pregnancy, NK cells may shift from type 1 to type 2 immune response.[15, 16] Previously we reported that the percentages of $CD56^{bright}/CD16^{-}$ NK cells were markedly decreased in patients with IVF-ET failure and miscarriage than those who delivered, suggesting that an abnormal population of uNK cells may lead to implantation failure and miscarriage.[6] In addition, in women with RPL and recurrent implantation failures (RIF) after IVF-ET cycles, type 1 shift has been documented.[17] Thus, the NK1/NK2 ratio might predict pregnancy outcomes in these women.

NKp46 is a natural cytotoxicity receptor (NCR), which regulates NK cell cytotoxicity and cytokine production through the signal cascade.[18] Our previous study showed that the expression of NKp46 on pNK cells is significantly lower in non-pregnant women with a history of RPL and RIF than women who delivered a live born infant.[19] Moreover, we have reported that in these women, the increase in type 1 cytokine production by pNK cells correlates with lower expression of NKp46.[20] We have also demonstrated a direct relationship between the expression of NKp46 and cytokine production by NK cells.[21] Therefore, NKp46 may play an important role in reproduction via regulation of cytokine production. Recently, a new type of $NKp46^{+}$ NK cell was found in the intestinal mucosa,[22] known as the NK22 cell as it produces interleukin-22 (IL-22).[23] NK22 cells were shown to be present in the uterine mucosa[24]; however, the biological significance of NK22 cells is not yet understood. This study aimed to investigate NK22 cells in the peripheral blood and the uterine endometrium of women with URPL and UI, and the relationship between the production of IL-22 and other cytokines such as IFN-γ, TNF-α, IL-4, IL-10, TGF-β1and

NKp46 expression in NK cell populations.

Materials and Methods

Study Subjects

A total of 81 women with 2 or more URPL (n = 43) and UI (n = 38) were enrolled in this study. Women with anatomic, infectious, endocrine, autoimmune including antiphospholipid antibody syndrome and genetic etiologies for RPL and UI were excluded from the study. Normal fertile controls were not included in this study due to ethical reasons and concerns of performing an endometrial biopsy in healthy fertile women. Patients' characteristics are shown in Table 1. There was no significant difference in age and body mass indices of the two study groups. The peripheral blood and the endometrial samples were obtained during the mid-secretory phase of the normal menstrual cycle. None of the patients had taken oral contraceptive pills or other medications known to interfere with the menstrual cycle. Study subjects were recruited from the Department of Obstetrics and Gynecology at Hirosaki University, Hirosaki, Aomori, Japan from June 1, 2012 to February 28, 2014. All study subjects gave their informed consent prior to entering the study. The study was approved by the Institutional Review Board at Hirosaki University Graduate School of Medicine.

Preparation of NK Cells from Peripheral Blood and Uterine Endometrium

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using HISTPAQUE-1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA). The endometrial (EM) sample was obtained using an endometrial cell sampler (Endosuction; Hakko, Nagano, Japan) and washed in saline to remove the blood cells. Samples were placed into RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Biological Industries Ltd., Beit Haemek, Israel), 2% L-glutamine, 1% penicillin, and 1% streptomycin (Antibiotic-Antimycotic, Gibco, Grand Island, NY, USA). The samples were then mechanically dissociated using the GentleMACS Dissociator (Miltenyi Biotec K.K., Bergisch Gladbach, Germany) to create uterine endometrial single-cell suspensions. The final concentrations of PBMCs and uterine endometrial cells were adjusted to 5×10^6 cells/mL.

Measurement of Natural Cytotoxicity Receptors on NK cells

Multi-color flow cytometric analysis was performed to determine the expression of NCRs on NK cells. Briefly, 100 μL of PBMCs and uterine endometrial cells were labeled with monoclonal antibodies (mAbs) for 20 min at room temperature, lysed (only for EM), fixed, and washed twice in phosphate-buffered saline solution (PBS) as previously described[19]. Anti-CD56-fluorescein isothiocyanate (FITC) (BD, Franklin Lakes, NJ, USA) and anti-CD335 (NKp46)-phycoerythrin (PE) (Beckman Coulter, Inc., Brea, CA, USA) were used to analyze the surface antigens of PBMCs, while anti-CD56-FITC (BD), anti-CD335 (NKp46) PE (Beckman Coulter), and anti-CD45 Pacific Blue (Beckman Coulter) were used to analyze uterine endometrial cells. Appropriate isotype controls

were used for each antibody.

Cytokine Stimulation and Intracellular Staining

In order to determine if phorbol 12-myristate 13-acetate (PMA)/ionomycin could stimulate IL-22 production in NK22 cells, correlations between IL-23-induced and PMA/ionomycin-induced IL-22 production were studied in 11 subjects. Uterine endometrial cells were stimulated either with IL-23 at 40 ng/mL for 24 h or with PMA (25 ng/mL)/ionomycin (1 μM) for 4 h. Brefeldin-A (10 μg/mL) was added for the last 4 h with IL-23 stimulation and simultaneously with PMA/ionomycin stimulation. Stimulation was performed at 37˚C in a 5% CO_2 humidified incubator in RPMI-1640. The cells were then washed in PBS.

These cells were stained for 20 min at room temperature for detection of surface antigens. Anti-CD56-FITC (BD) or anti-CD56-PerCP-Cy5.5 (Beckman Coulter) were used to stain the surface antigens of PBMCs, while anti-CD56-FITC and anti-CD45-Pacific Blue or anti-CD56-PerCP-Cy5.5 and anti-CD45-Pacific Blue were used to stain the surface antigens of endometrial cells. Next, the cells were washed in PBS and fixed with 250 μL of Cytofix/Cytoperm (BD) to permeabilize the cells. After 20 min of permeabilization, cells were washed twice in Perm/Wash Buffer (BD) and stained for 30 min with monoclonal antibodies to the following cytokines: anti-IL-22-PerCP (eBioscience, Hatfield, U.K.), 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, MN, USA), and anti-TGF-β1-allophycocyanin (APC) (R&D Systems) (Table 2). Cells were washed twice

with Perm/Wash Buffer and resuspended in 250 μL of PBS for subsequent flow cytometric analysis. Finally, intracellular cytokine production was measured using multicolor flow cytometry.

Flow Cytometric Analysis

Immunofluorescence and three-color flow cytometric analysis were performed with a FACSCanto flow cytometer (Becton Dickinson; San Jose, CA, USA) with computer interfacing to FACSDiva software (BD) for full-list-mode data storage, recovery, and analysis. Lymphocytes were gated using characteristic forward and side scatter parameters of PBMCs to exclude other cells. The EM was first gated on anti-CD45-positive events and then on the lymphocyte region using characteristic forward and side scatter parameters. For each sample, at least 3×10^4 cells were analyzed.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 20 (IBM, Chicago, IL, USA). Age distribution, number of pregnancies, deliveries, spontaneous miscarriages and BMI of the two groups were analyzed by Mann Whitney *U*-test. The comparison of the number of IL-22 producing-$CD56^+$ NK cells between URPL and UI groups was analyzed by Mann Whitney *U*-test. The data are presented as median with interquartile ranges. A *p*-value of <0.05 was considered to be statistically significant. Correlations between the percentages of $CD56^+$/$IL\text{-}22^+$ and percentages of cytokine-producing NK cells or NKp46 expression on NK cells were analyzed using Pearson's correlation coefficients. Correlations were

considered significant if $r > 0.4$ and $P < 0.05$.

Results

Production of Cytokines by $CD56^+$ NK Cells in the Endometrium Stimulated by IL-23 or PMA/Ionomycin

Representative dot plots of the cytokine producing NK cells are shown in figure 1, including $CD56^+$/Isotype-IgG^+, $CD56^+$/$IL\text{-}22^+$ (NK22 cells), $CD56^+$/IFN-γ or $CD56^+$/TNF-α. Intracellular cytokine producing NK cells were stimulated with either IL-23 or PMA/ionomycin. The percentage of $CD56^+$/$IL\text{-}22^+$ cells in total lymphocytes stimulated with IL-23 **was 2.52 ± 1.44%** and **that with PMA/Ionomycin was 3.19 ± 1.65 respectively (n = 11).**

Figure 2 shows correlation of the percentages of $CD56^+$/$IL\text{-}22^+$ cells between IL-23 and PMA/ionomycin stimulations in women with UI. There was a strong positive correlation between the percentages of $CD56^+$/$IL\text{-}22^+$ cells stimulated with IL-23 and those with PMA/ionomycin ($r = 0.874$, $p < 0.001$). Based on this finding, we determined to use PMA/ionomycin for the stimulation of NK cells to detect intracellular IL-22 secretion in this study.

Percentages of NK22 Cells in Peripheral Blood and Endometrial NK Cells of Women with RPL

The percentages of $CD56^{+}/IL\text{-}22^{+}$, $CD56^{dim}/IL\text{-}22^{+}$ and $CD56^{bright}/IL\text{-}22^{+}$ NK cells amongst pNK and uNK cells are shown in Figure 3 and 4 respectively. There were no significant differences in pNK subsets between women with RPL and UI (Figure 3). However, the percentages of $CD56^{+}/IL\text{-}22^{+}$ (p = 0.036) and $CD56^{dim}/IL\text{-}22^{+}$ (p = 0.043) uNK cells in women with RPL were significantly higher than those in women with UI. The percentage of $CD56^{bright}/IL\text{-}22^{+}$ was higher than that in UI. However, the difference did not reach a statistically significant level (p = 0.055) (Figure 4).

Correlation between $CD56^{bright}/IL\text{-}22^{+}$ Cells and IFN-γ or TNF-α–producing $CD56^{bright}$ NK Cells in Women with RPL

The correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells and IFN-γ or TNF-α producing $CD56^{bright}$ NK cells in women with RPL is shown in Figure 5. In pNK cells, there was a negative correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells and $CD56^{bright}$ NK cells producing IFN-γ (r = -0.343, p = 0.054) (Figure 5a) or TNF-α (r = -0.407, p = 0.020) (Figure 5b). Similarly, in uNK cells, there were significant negative correlations between $CD56^{bright}/IL\text{-}22^{+}$ cells and $CD56^{bright}$ NK cells producing IFN-γ (r = -0.399, p = 0.006) (Figure 5c) or TNF-α (r = -0.347, p = 0.017) (Figure 5d). There was no significant correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells and $CD56^{bright}$ NK cells producing IL-4, IL-10, or TGF-β1 in women with RPL (data not shown).

In women with UI, there was no correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells and cytokine producing $CD56^{bright}$ NK cells, such as IFN-γ, TNF-α, IL-4, IL-10, or TGF-β1 (data not shown).

Correlation between the Percentages of $CD56^+/IL\text{-}22^+$ Cells and $CD56^+/NKp46$ Cells

There was no correlation between the percentages of $CD56^+/IL\text{-}22^+$ pNK cells and $CD56^+/NKp46$ cells in peripheral blood (r = -0.113, p = 0.279) (Figure 6a). However, there was a significant negative correlation between $CD56^{bright}/IL\text{-}22^+$ cells and $CD56^{bright}/NKp46^+$ cells in peripheral blood (r = -0.271, p = 0.008) (Figure 6b).

Discussion

NK22 cells are functionally different from conventional NK cells because they express low levels of granzyme and perforin, have decreased killing activity,[25-27] and produce IL-22 in response to local micro environmental signals. During bacterial infection, for example, IL-23 produced by dendritic cells,[28] macrophages[29] and monocytes,[30] activates NK22 cells to secrete IL-22.[31] IL-22 is indispensable for the early phase of bacterial infection.[31] In addition, IL-22 was suggested to be important for viral infection. IL-22 was reported to promote accumulation of neutrophils into murine cytomegalovirus-infected tissues, such as liver and lung.[32] IL-22, first described in 2000,[33] belongs to the IL-10 cytokine family. Various types of lymphocytes, including those of the innate and adaptive immune systems, produce IL-22.[34] The main biological role of IL-22 is in immunological defense by promoting the production of antimicrobial peptides from epithelial cells and stimulating their active regeneration. NK22 cells target certain tissue cells from the skin, liver, and

kidney in addition to organs of the respiratory and gastro-intestinal systems.[35] Recently, accumulated evidence has indicated that IL-22 also plays an important role in the pathogenesis of many autoimmune diseases such as psoriasis, rheumatoid arthritis (RA), hepatitis, graft versus host disease, and allergic diseases.[36-38] Moreover, NK22 cells are present in the human decidua and endometrium during the proliferative and secretory phases,[24] although their roles in reproductive organs are not well understood.

In this study, we report that women with RPL have higher percentages of $CD56^{+}/IL\text{-}22^{+}$ and $CD56^{dim}/IL\text{-}22^{+}$ NK cells than those of women with UI. Since these women were screened for infectious etiologies for RPL and UI, the differences in NK22 cell subsets between women with RPL and UI may not be related to infectious etiologies. The data however, should be carefully interpreted since other evidence of infection such as endometrial neutrophil accumulation or viral infection was not investigated in study population.

With regards to activators of NK22 cells, Cella et al.,[22, 39] showed that IL-23 is a direct and acute trigger of IL-22 secretion and proposed that IL-23 is a physiological activator of IL-22 production by NK22 cells. However, at least a 24-h IL-23 stimulation is needed to obtain a significant increase in IL-22 production *in vitro*. Therefore, an alternative stimulation method was required in order to conduct *in vitro* studies on NK22 cell function. As shown in figure 2, there was a strong positive correlation between the percentages of $CD56^{+}/IL\text{-}22^{+}$ NK cells - stimulated with IL-23 and PMA/ionomycin ($r = 0.874$, $p < 0.001$). This finding suggested that conventional stimulation using PMA/ionomycin was acceptable to study NK22 cells, and thus, we performed our studies using PMA/ionomycin

stimulation.

Presence of NK22 cells in the endometrium has been demonstrated previously,[24] and this study confirms the previous findings. In addition, the present study showed for the first time that the proportion of NK22 cells in the mid-secretary endometrium was higher in women with RPL than that in women with UI, suggesting that NK22 cell regulation of women with RPL are different from that of women with UI. Wang et al.,[40] demonstrated that IL-22 promotes proliferation and inhibits apoptosis of the human trophoblastic cell line HTR8/SVneo. In addition, trophoblast from unexplained miscarriage patients expressed significantly lower levels of IL-22R1, which might be involved in the occurrence of disease by limiting the growth and proliferation of trophoblast.[40] Therefore, IL-22 might have a major role in RPL and UI since the IL-22 secreted by the decidual NK (dNK) cells, decidual stromal cells (DSCs) and trophoblasts at the maternal-fetal interface promotes the proliferation, enhances the cell variability and represses the apoptosis of trophoblasts in paracrine and autocrine manners through the cross-talking between dNK cells, DSCs and trophoblasts.[40]

uNK cells produce variety of cytokines such as IFN-γ, TNF-α, IL-4, IL-10, and TGF-β_1. Of these cytokines, IFN-γ inhibits trophoblast invasion,[41] while TNF-α has diverse roles, including stimulation of trophoblast cell growth, cell differentiation, angiogenesis, and trophoblast apoptosis.[42] In general, it is presumed that NK2 shift is present when the levels of IFN-γ and TNF-α positive NK cells are decreased in the early stages of pregnancy in order to maintain an endometrium that is suitable for implantation. As shown in figure 5, there was a negative correlation between IFN-γ and TNF-α producing $CD56^{bright}$ NK cells

and $CD56^{bright}$/IL-22^{+} NK22 cells, suggesting that $CD56^{bright}$/IL-22^{+} NK22 cells might be involved in an NK2 shift. This negative correlation, however was not detected in women with UI. Thus, we inferred that this NK2 shift mechanism was dysregulated in women with UI however, still maintained in women with RPL. Our previous study on intracellular cytokine expression of pNK cells[17] showed that a NK1 shift occurs in pNK cells obtained from women with RPL and RIF. The NK1 shift in pNK cells was more severe in women with RIF than RPL.[17] It is speculated that women with UI may have more severe NK1 shift via deregulated and decreased NK22 cells, than women with RPL. However, the cautious interpretation of the data is needed, since IL-22 concentration or NK22 functional studies have not been performed in this study.

NKp46 is a natural cytotoxic receptor, and its expression is decreased in pNK and uNK cells of women with a history of RPL.[19] Moreover, lower expression of NKp46 is associated with abnormal cytokine production.[21] It is important to understand the role of NKp46 in the cytokine production of NK22 cells. As shown in figure 6, there was no significant correlation between NKp46 and IL-22 production by $CD56^{+}$ NK cells. However, when examining $CD56^{bright}$ NK cells, there was a significant negative correlation between NKp46 and IL-22 producing $CD56^{bright}$ NK cells. This finding is consistent with our previous studies that women with RPL have decreased NKp46 expression in pNK cells[19]; and decreased NKp46 expression in peritoneal fluid NK cells, both $CD56^{bright}$ and $CD56^{dim}$ was associated with increased TNF-α and IFN-γ in women with endometriosis.[43] Actually, in this study the percentage of $CD56^{dim}$/IL-22^{+} cells were higher in women with RPL than women with UI, and type 1 cytokines producing NK cells were negatively correlated with

$CD56^{bright}$/IL-22^{+} NK22 cells in women with RPL. Therefore, endometrial NK22 cells in women with RPL may have a different functional capacity than those in women with UI.

Generally, cytokine-producing NK cells are mainly $CD56^{brights}$ NK cells. When we investigated NKp46 expression in cytokine-producing NK cells, we showed that $CD56^{dim}$ cells also produced various cytokines even though they were not main producers.[21] In this study, $CD56^{dim}$/IL-22^{+} NK cells were significantly higher in women with RPL than UI. Similarly, the percentage of $CD56^{bright}$/IL-22^{+} was higher in women with RPL than UI however, the difference did not reach a statistically significant level. This finding is somehow contradictory to a fact that the main cytokine-producing NK cells are known to be $CD56^{bright}$ NK cells.[2, 14, 17, 44, 45] Further characterization of IL-22 producing NK cells are needed.

It is proposed that the $CD16^{-}$ pNK cells migrate to the uterus and TGF-β may promote conversion of $CD16^{+}$ pNK cells into $CD16^{-}$ NK cells to acquire the uNK phenotype.[2, 46] Alternatively, hematopoietic progenitor cells have the potential to differentiate into NK cells when stimulated with IL-15.[47] The presence of $CD34^{+}$ hematopoietic precursors in human decidua was recently demonstrated and they differentiate into functional (IL-22-producing) NK cells upon interaction with stromal cells.[48] The origin of uNK cells is still largely unknown.[49, 50] IL-22 producers were reported to be essential for mucosal integrity in the face of cyclical breakdown and renewal of the mucosa, whereas NK progenitors are required for regenerating the cycling mature NK cell population.[24] In this study, we did not find any difference in IL-22 producing pNK cell subsets between women with RPL and UI, but differences in uNK cells. Whether these findings suggest that the

women with UI has a limited recruitment of IL-22^{+} NK cell from peripheral blood to endometrium, or repressed maintenance/proliferation of recruited IL-22^{+} NK cells in the endometrium are questions need to be answered.

The results of the present study showed that NK22 cells were present in the endometrium and peripheral blood, and the percentages of NK22 cells are higher in the endometrium of women with RPL than that of women with UI. Interestingly, IL-22 expression was mainly in CD56^{dim} NK cells, not CD56^{bright} cells. Of interest, the percentage of NK22 cells was negatively correlated with the expression levels of IFN-γ and TNF-α in women with RPL, but not in women with UI. The percentage of NK22 cells was negatively correlated with NKp46^{+} NK cells. These results suggest that women with RPL and UI have different endometrial NK22 cell regulations. Perhaps, women with UI have more severe NK22 cell immune pathology than women with RPL, which may partially explain the clinical manifestation of UI, e.g., implantation failure. Further exploration of NK22 cell function and receptor expression may shed light on understanding the role of NK22 cells in human reproduction.

References

1 King A, Balendran N, Wooding P, Carter NP, Loke YW: CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Developmental immunology* 1991;**1**:169-190.

2 Moffett-King A: Natural killer cells and pregnancy. *Nature reviews Immunology* 2002;**2**:656-663.

3 Caligiuri MA: Human natural killer cells. *Blood* 2008;**112**:461-469.

4 Tang AW, Alfirevic Z, Quenby S: Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Hum Reprod* 2011;**26**:1971-1980.

5 Moffett A, Regan L, Braude P: Natural killer cells, miscarriage, and infertility. *BMJ* 2004;**329**:1283-1285.

6 Fukui A, Fujii S, Yamaguchi E, Kimura H, Sato S, Saito Y: Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilization. *Am J Reprod Immunol* 1999;**41**:413-422.

7 Seshadri S, Sunkara SK: Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Human reproduction update* 2014;**20**:429-438.

8 Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I: Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Hum Reprod* 2000;**15**:1163-1169.

9 Lee SK, Na BJ, Kim JY, Hur SE, Lee M, Gilman-Sachs A, Kwak-Kim J: Determination of clinical cellular immune markers in women with recurrent pregnancy loss. *Am J Reprod Immunol* 2013;**70**:398-411.

10 Lachapelle MH, Miron P, Hemmings R, Roy DC: Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *J Immunol* 1996;**156**:4027-4034.

11 Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, Robson SC, Bulmer JN: Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *Journal of leukocyte biology* 2006;**80**:572-580.

12 Zhang J, Chen Z, Smith GN, Croy BA: Natural killer cell-triggered vascular transformation: maternal care before birth? *Cellular & molecular immunology*

2011;**8**:1-11.

13 Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G: Differentiation of human NK cells into NK1 and NK2 subsets. *J Immunol* 1998;**161**:5821-5824.

14 Higuma-Myojo S, Sasaki Y, Miyazaki S, Sakai M, Siozaki A, Miwa N, Saito S: Cytokine profile of natural killer cells in early human pregnancy. *Am J Reprod Immunol* 2005;**54**:21-29.

15 Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, Faas MM: Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle. *Fertility and sterility* 2002;**77**:1032-1037.

16 Borzychowski AM, Croy BA, Chan WL, Redman CW, Sargent IL: Changes in systemic type 1 and type 2 immunity in normal pregnancy and pre-eclampsia may be mediated by natural killer cells. *European journal of immunology* 2005;**35**:3054-3063.

17 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.

18 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. *J Cell Mol Med* 2003;**7**:376-387.

19 Fukui A, Ntrivalas E, Gilman-Sachs A, Kwak-Kim J, Lee SK, Levine R, 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. *Am J Reprod Immunol* 2006;**56**:312-320.

20 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. *Am J Reprod Immunol* 2009;**62**:371-380.

21 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. *Am J Reprod Immunol* 2013;**69**:202-211.

22 Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC,

Colonna M: A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 2009;**457**:722-725.

23 Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S, Ribeiro VS, Mandelboim O, Renauld JC, Vosshenrich CA, Di Santo JP: The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. *J Immunol* 2009;**183**:6579-6587.

24 Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A: Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. *J Immunol* 2010;**185**:3913-3918.

25 Luci C, Reynders A, Ivanov, II, Cognet C, Chiche L, Chasson L, Hardwigsen J, Anguiano E, Banchereau J, Chaussabel D, Dalod M, Littman DR, Vivier E, Tomasello E: Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nature immunology* 2009;**10**:75-82.

26 Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A: RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nature immunology* 2009;**10**:83-91.

27 Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, Mention JJ, Thiam K, Cerf-Bensussan N, Mandelboim O, Eberl G, Di Santo JP: Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 2008;**29**:958-970.

28 Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA: Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000;**13**:715-725.

29 Pirhonen J, Matikainen S, Julkunen I: Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. *J Immunol* 2002;**169**:5673-5678.

30 Tchatalbachev S, Ghai R, Hossain H, Chakraborty T: Gram-positive pathogenic bacteria induce a common early response in human monocytes. *BMC microbiology* 2010;**10**:275.

31 Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W: Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature medicine* 2008;**14**:282-289.

32 Stacey MA, Marsden M, Pham NT, Clare S, Dolton G, Stack G, Jones E, Klenerman P,

Gallimore AM, Taylor PR, Snelgrove RJ, Lawley TD, Dougan G, Benedict CA, Jones SA, Wilkinson GW, Humphreys IR: Neutrophils recruited by IL-22 in peripheral tissues function as TRAIL-dependent antiviral effectors against MCMV. *Cell host & microbe* 2014;**15**:471-483.

33 Dumoutier L, Van Roost E, Colau D, Renauld JC: Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. *Proceedings of the National Academy of Sciences of the United States of America* 2000;**97**:10144-10149.

34 Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP: gammadelta T cells protect against lung fibrosis via IL-22. *The Journal of experimental medicine* 2010;**207**:2239-2253.

35 Witte E, Witte K, Warszawska K, Sabat R, Wolk K: Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection. *Cytokine & growth factor reviews* 2010;**21**:365-379.

36 Yang X, Zheng SG: Interleukin-22: a likely target for treatment of autoimmune diseases. *Autoimmunity reviews* 2014;**13**:615-620.

37 Zenewicz LA, Flavell RA: Recent advances in IL-22 biology. *International immunology* 2011;**23**:159-163.

38 Kumar P, Rajasekaran K, Palmer JM, Thakar MS, Malarkannan S: IL-22: An Evolutionary Missing-Link Authenticating the Role of the Immune System in Tissue Regeneration. *Journal of Cancer* 2013;**4**:57-65.

39 Cella M, Otero K, Colonna M: Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proceedings of the National Academy of Sciences of the United States of America* 2010;**107**:10961-10966.

40 Wang Y, Xu B, Li MQ, Li DJ, Jin LP: IL-22 secreted by decidual stromal cells and NK cells promotes the survival of human trophoblasts. *International journal of clinical and experimental pathology* 2013;**6**:1781-1790.

41 Lash GE, Otun HA, Innes BA, Kirkley M, De Oliveira L, Searle RF, Robson SC, Bulmer JN: Interferon-gamma inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2006;**20**:2512-2518.

42 Terranova PF, Hunter VJ, Roby KF, Hunt JS: Tumor necrosis factor-alpha in the female

reproductive tract. *Proc Soc Exp Biol Med* 1995;**209**:325-342.

43 Funamizu A, Fukui A, Kamoi M, Fuchinoue K, Yokota M, Fukuhara R, Mizunuma H: Expression of natural cytotoxicity receptors on peritoneal fluid natural killer cell and cytokine production by peritoneal fluid natural killer cell in women with endometriosis. *Am J Reprod Immunol* 2014;**71**:359-367.

44 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. In *Journal of reproductive immunology*, 2011/06/03 edn, 2011, 99 105-110.

45 Saito S, Nishikawa K, Morii T, Enomoto M, Narita N, Motoyoshi K, Ichijo M: Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. *International immunology* 1993;**5**:559-563.

46 Keskin DB, Allan DS, Rybalov B, Andzelm MM, Stern JN, Kopcow HD, Koopman LA, Strominger JL: TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proceedings of the National Academy of Sciences of the United States of America* 2007;**104**:3378-3383.

47 Colucci F, Caligiuri MA, Di Santo JP: What does it take to make a natural killer? *Nature reviews Immunology* 2003;**3**:413-425.

48 Vacca P, Vitale C, Montaldo E, Conte R, Cantoni C, Fulcheri E, Darretta V, Moretta L, Mingari MC: CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proceedings of the National Academy of Sciences* 2011;**108**:2402-2407.

49 Manaster I, Mandelboim O: The unique properties of human NK cells in the uterine mucosa. *Placenta* 2008;**29 Suppl A**:S60-66.

50 Jabrane-Ferrat N, Siewiera J: The up side of decidual natural killer cells: new developments in immunology of pregnancy. *Immunology* 2014;**141**:490-497.

Figure Legends

Figure 1: Representative dot plots of cytokines, such as IL-22, IFN-γ or TNF-α producing NK cells in total uterine endometrial NK cells. A gate was set on the lymphocyte region by characteristic forward and scatter parameters. Lymphocytes were classified into $CD56^+$ and $CD56^-$ cells. Q1 = $CD56^-$/$cytokine^+$ NK cells, Q2 = $CD56^+$/$cytokine^+$ NK cells, Q3 = $CD56^+$/ $cytokine^-$ NK cells and Q4 = $CD56^-$/$cytokine^-$ NK cells.

(a): The ordinate indicates isotype matched IgG-Per CP positive NK cells.

(b): A representative dot plots of a woman with unexplained infertility (UI). The ordinate indicates IL-22 producing NK cells.

(c): A representative dot plots of recurrent pregnancy losses (RPL). The ordinate indicates IL-22 producing NK cells.

(d): The ordinate indicates IFN-γ producing NK cells.

(e): The ordinate indicates TNF-α producing NK cells.

The abscissa of all dot plots indicates $CD56^+$ NK cells.

Figure 2: Correlation of the percentages of IL-22 producing NK cells in total uterine endometrial NK cells stimulated with IL-23 or PMA/ionomycin stimulation in women with unexplained infertility. The ordinate indicates the percentage of IL-22 producing NK cells in total NK cells stimulated by IL-23. The abscissa indicates the percentage of IL-22 producing NK cells in total NK cells stimulated by PMA/ionomycin. There was a significant positive correlation between two stimulation methods ($p < 0.001$).

Figure 3: The percentage of $CD56^{+}$/IL-22^{+}, $CD56^{dim}$/IL-22^{+} and $CD56^{bright}$/IL-22^{+} NK cells in the peripheral blood stimulated by PMA/ionomycin in women with recurrent pregnancy loss (RPL) and unexplained infertility (UI). The ordinate indicates the percentage of $CD56^{+}$/IL-22^{+} (a), $CD56^{dim}$/IL-22^{+} (b) and $CD56^{bright}$/IL-22^{+} (c) NK cells in total NK cells. In the peripheral blood, there were no significant differences between RPL and UI. Box and whisker plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Blue box shows the percentage of NK22 cells in UI. Red box shows the percentage of NK22 cells in RPL. Differences between the two groups were analyzed by Mann Whitney *U*-test.

Figure 4: The percentage of $CD56^{+}$/IL-22^{+}, $CD56^{dim}$/IL-22^{+} and $CD56^{bright}$/IL-22^{+} NK cells in the endometrium stimulated by PMA/ionomycin in women with recurrent pregnancy loss (RPL) and unexplained infertility (UI). The ordinate indicates the percentage of $CD56^{+}$/IL-22^{+} (a), $CD56^{dim}$/IL-22^{+} (b) and $CD56^{bright}$/IL-22^{+} (c) NK cells in total NK cells. In the endometrium, the percentage of $CD56^{+}$/IL-22^{+} (a) and $CD56^{dim}$/IL-22^{+} (b) NK cells of women with RPL was significantly higher than that of women with unexplained infertility (UI). Box and whisker plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. Blue box shows the percentage of NK22 cells in UI. Red box shows the percentage of NK22 cells in RPL. Differences between the two groups were analyzed by Mann Whitney *U*-test.

Figure 5: Correlation between the percentage of $CD56^{bright}$/IL-22^{+} cells and IFN-γ or

TNF-α producing $CD56^{bright}$ NK cells stimulated by PMA/ionomycin in women with RPL. The ordinate indicates the percentage of IFN-γ or TNF-α producing $CD56^{bright}$ NK cells in total NK cells. The abscissa indicates the percentage of $CD56^{bright}/IL\text{-}22^{+}$ NK cells in total NK cells.

(a) Correlation between $IL\text{-}22^{+}$ and IFN-γ producing $CD56^{bright}$ pNK cells.

(b) Correlation between $IL\text{-}22^{+}$ and TNF-α producing $CD56^{bright}$ pNK cells.

(c) Correlation between $IL\text{-}22^{+}$ and IFN-γ producing $CD56^{bright}$ uNK cells.

(d) Correlation between $IL\text{-}22^{+}$ and TNF-α producing $CD56^{bright}$ uNK cells.

Both in pNK and uNK cells, there were significant negative correlations between $CD56^{bright}/IL\text{-}22^{+}$ cells and IFN-γ or TNF-α producing $CD56^{bright}$ NK cells. Correlation between the percentage of $CD56^{bright}/IL\text{-}22^{+}$ cells and $CD56^{bright}$/cytokines cells was analyzed by Pearson linear correlation. pNK; peripheral blood natural killer. uNK; uterine natural killer.

Figure 6: Correlation between the percentage of IL-22 producing NK cells and NKp46 expressing NK cells in peripheral blood. The ordinate indicates the percentage of NKp46 expressing $CD56^{bright}$ NK cells in total NK cells. The abscissa indicates the percentage of $CD56^{+}/IL\text{-}22^{+}$ or $CD56^{bright}/IL\text{-}22^{+}$ producing NK cells in total NK cells.

(a) Correlation between $CD56^{+}/IL\text{-}22^{+}$ cells and $CD56^{+}/NKp46^{+}$ cells.

(b) Correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells and $CD56^{bright}/NKp46^{+}$ cells.

There was a significant negative correlation between $CD56^{bright}/IL\text{-}22^{+}$ cells, and $CD56^{bright}/NKp46^{+}$ cells. Correlation between the percentage of $CD56/IL\text{-}22^{+}$ cells and

CD56/NKp46^{+} cells was analyzed by Pearson linear correlation.

Figure 1: Representative dot plots of cytokines producing NK cells in total uterine endometrial NK cells.

(a) Representative dot plot of CD56-FITC and isotype matched IgG-Per CP

(b) Representative dot plot of IL-22 producing NK cells from UI

(c) Representative dot plot of IL-22 producing NK cells from RPL

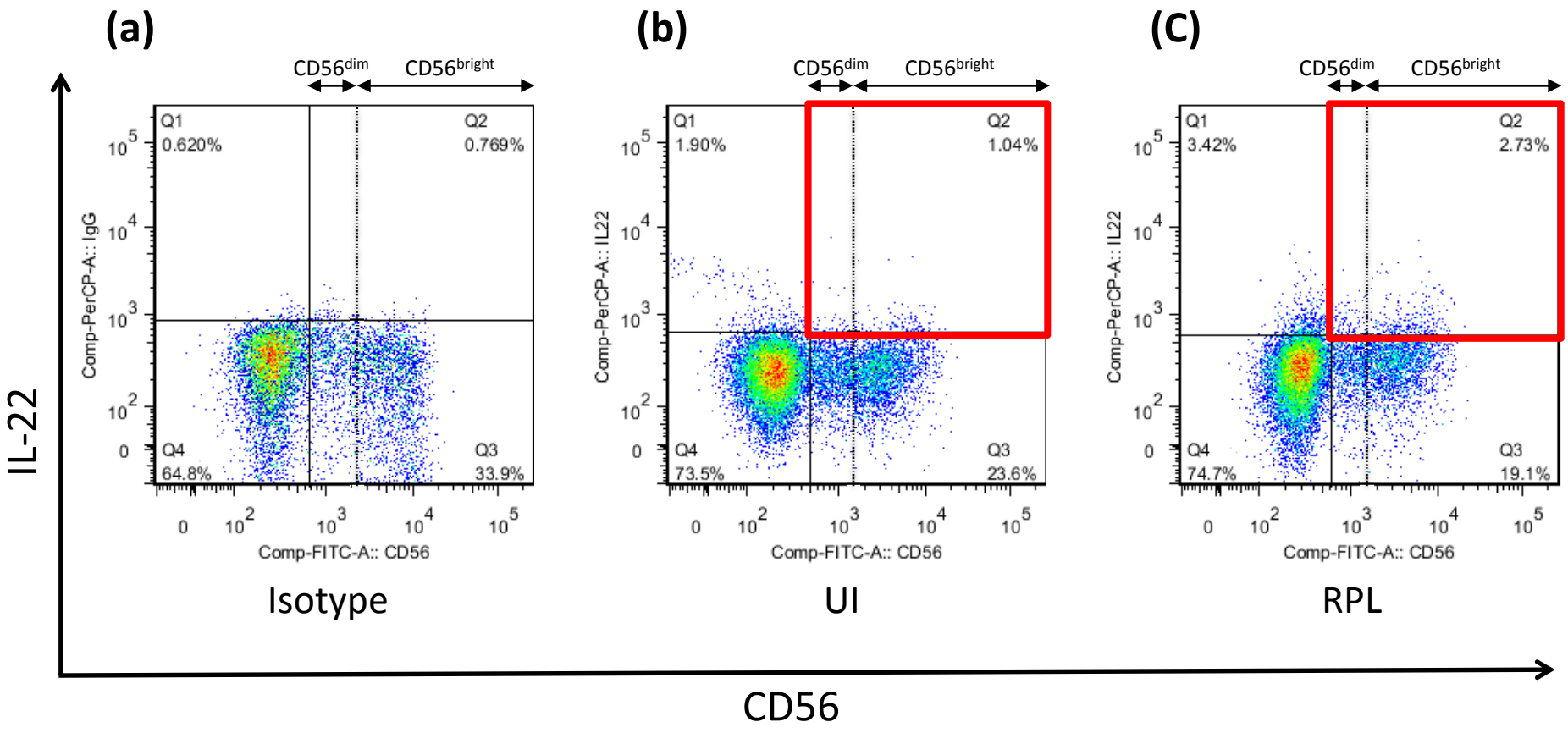

Figure 1: Representative dot plots of cytokines producing NK cells in total uterine endometrial NK cells.
(d) Representative dot plot of IFN-γ producing NK cells
(e) Representative dot plot of TNF-α producing NK cells

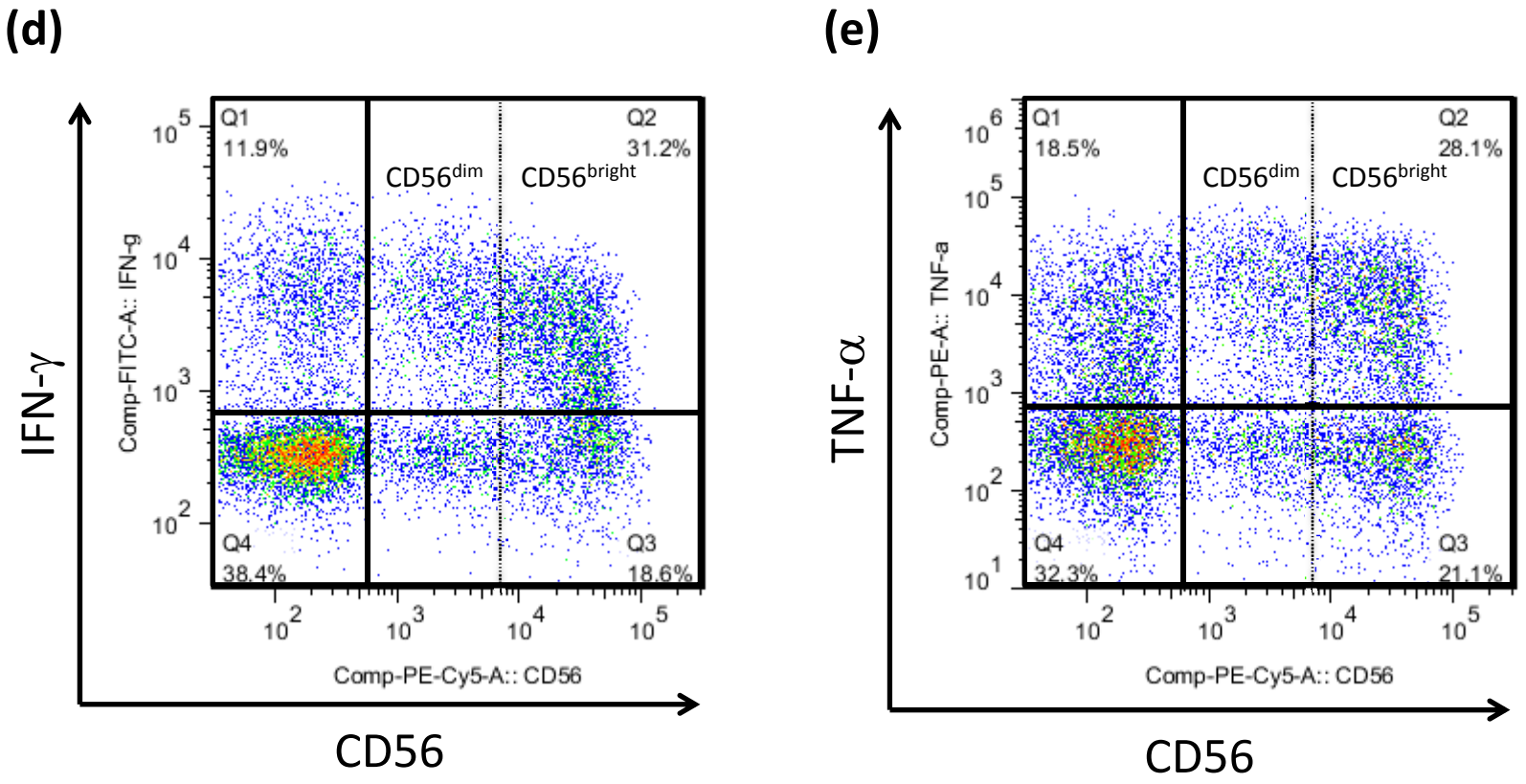

Figure 2: Correlation of the percentage of IL-22 producing NK cells in total uterine endometrial NK cells stimulated with IL-23 with those with PMA/ionomycin in women with UI

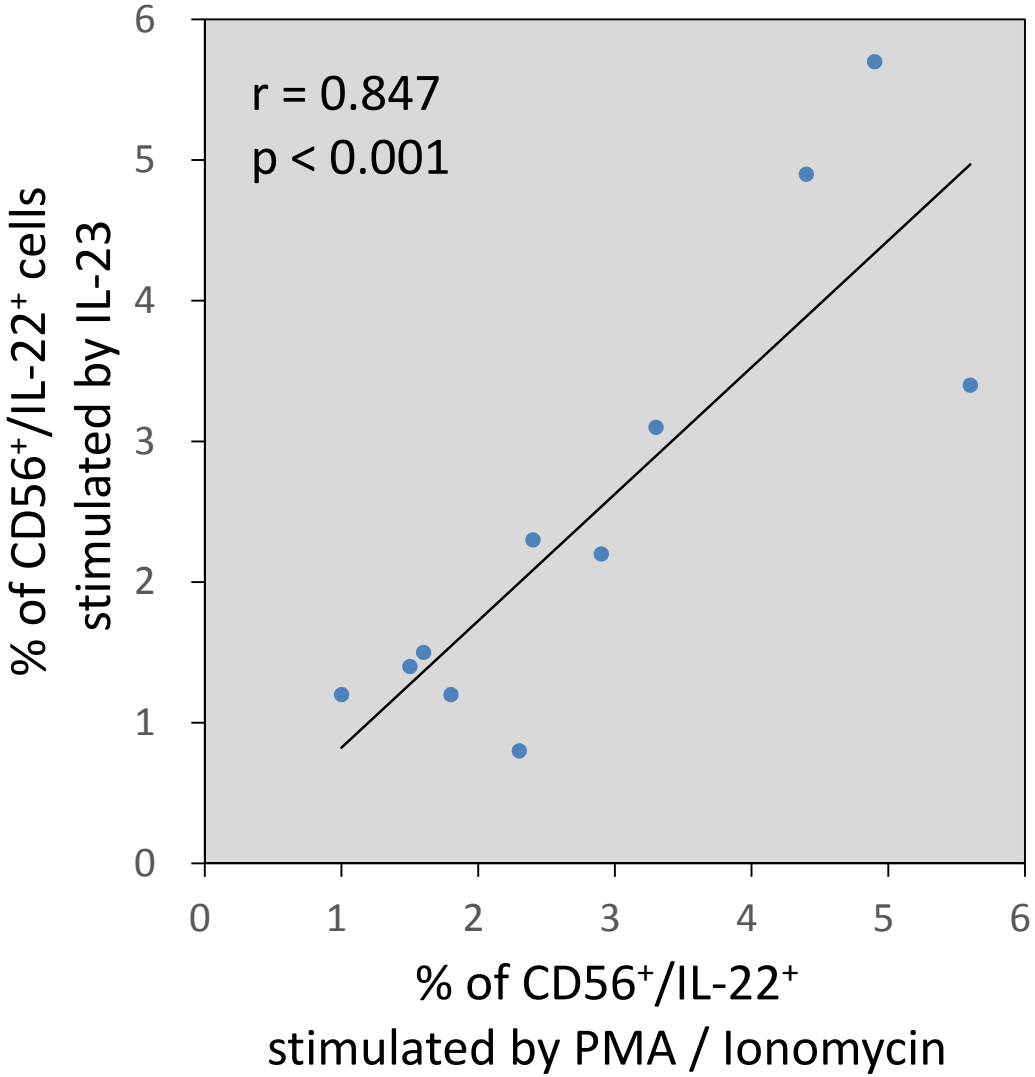

Figure 3: The percentage of $CD56^{+}/IL\text{-}22^{+}$ NK cells (NK22 cells) in the peripheral blood stimulated with PMA/ionomycin

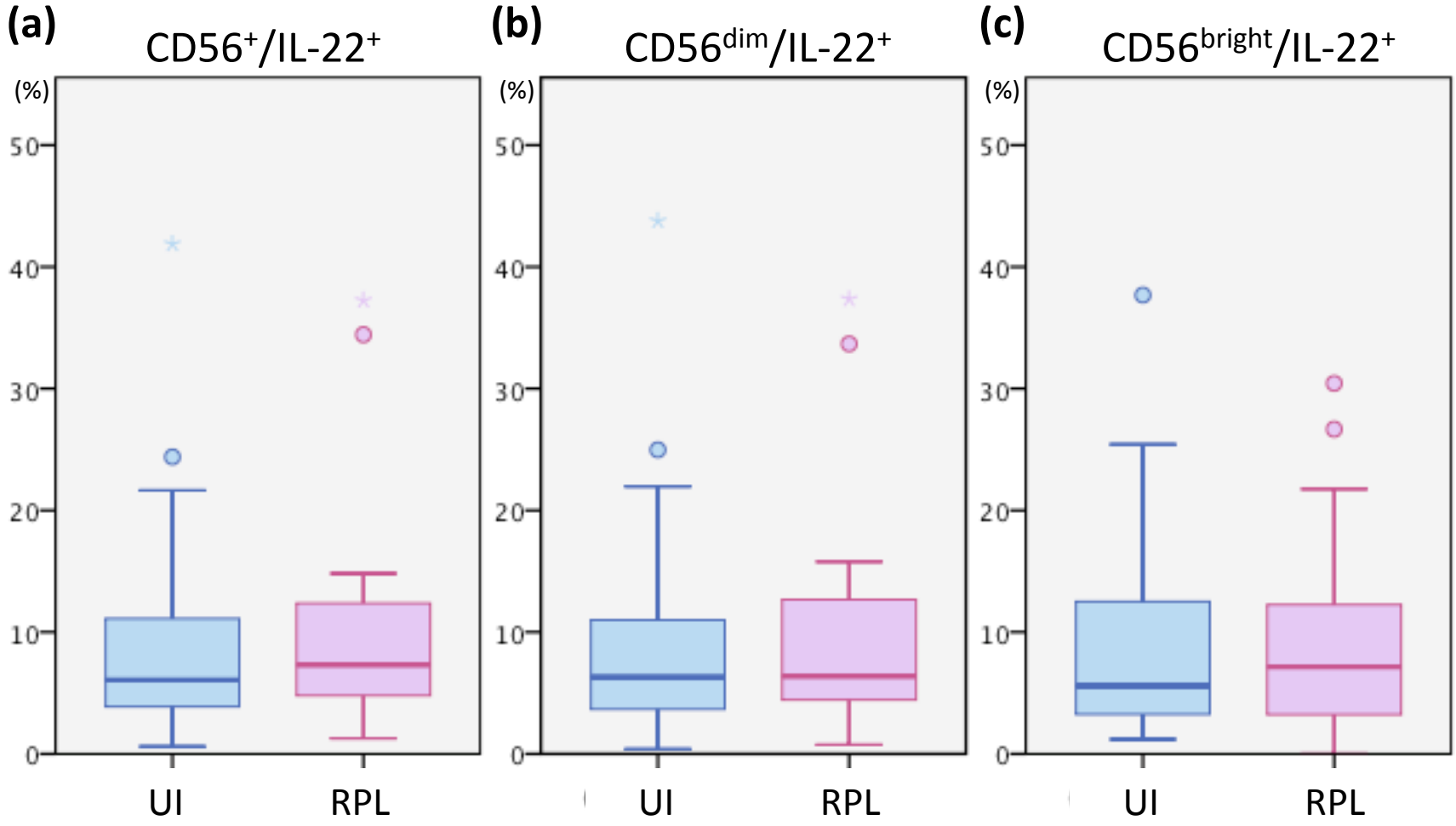

Figure 4: The percentage of $CD56^{+}/IL\text{-}22^{+}$ NK cells (NK22 cells) in the endometrium stimulated with PMA/ionomycin

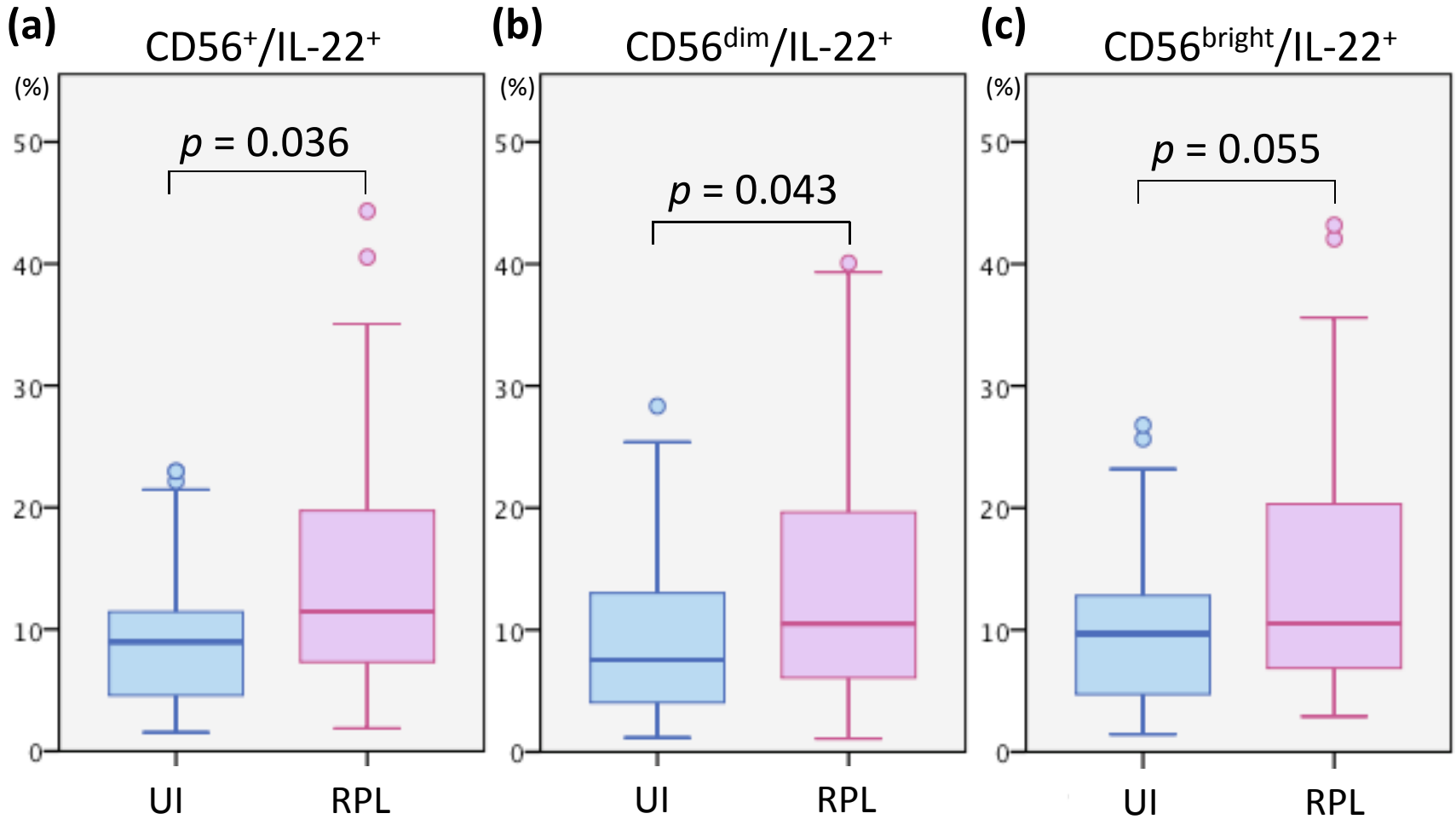

Figure 5: Correlation between the percentage of $CD56^{bright}/IL\text{-}22^{+}$ cells and IFN-γ or TNF-α producing $CD56^{bright}$ NK cells stimulated with PMA/ionomycin in women with RPL

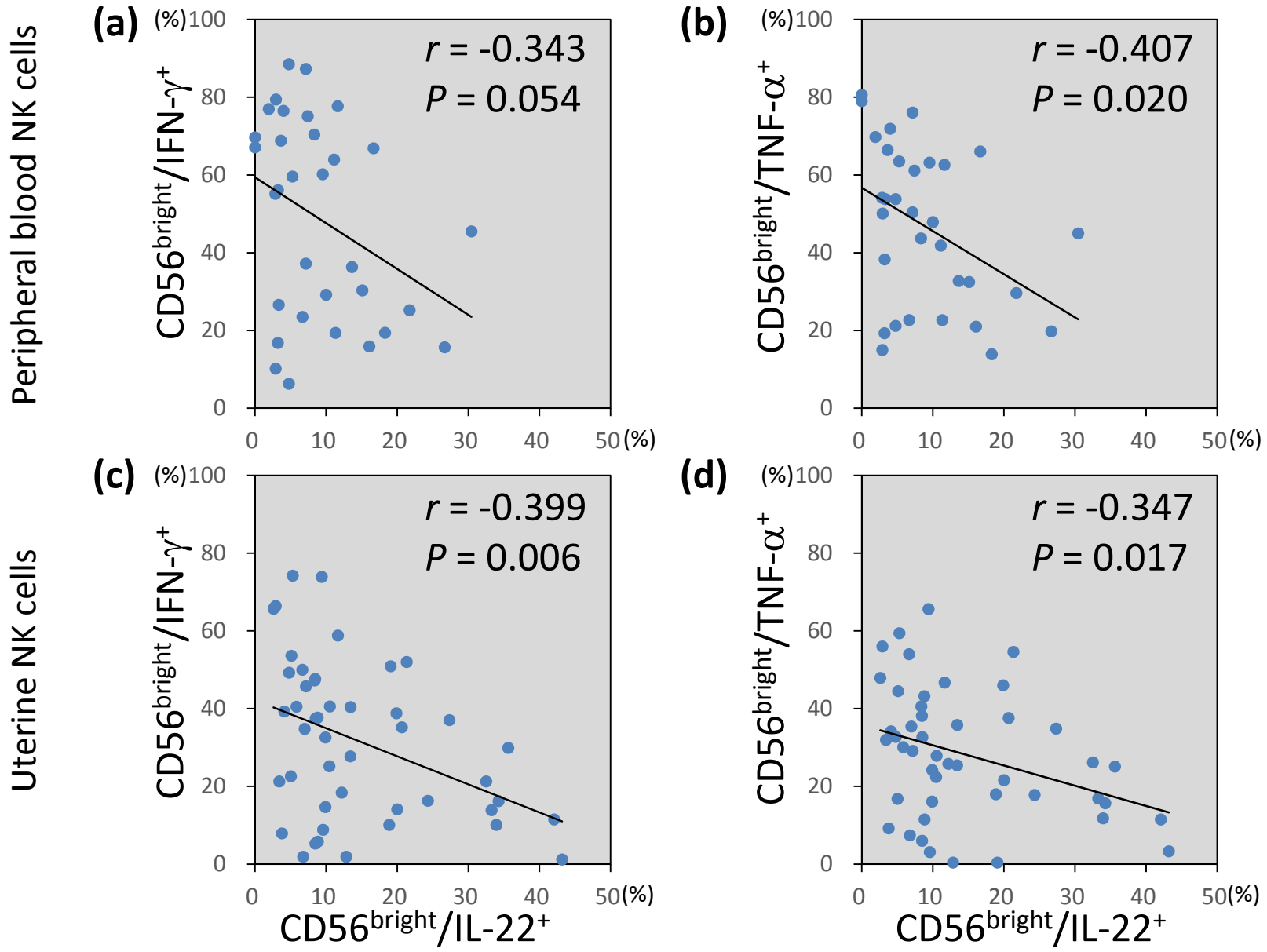

Figure 6: Correlation between the percentage of IL-22 producing NK cells and NKp46 expressing NK cells in peripheral blood

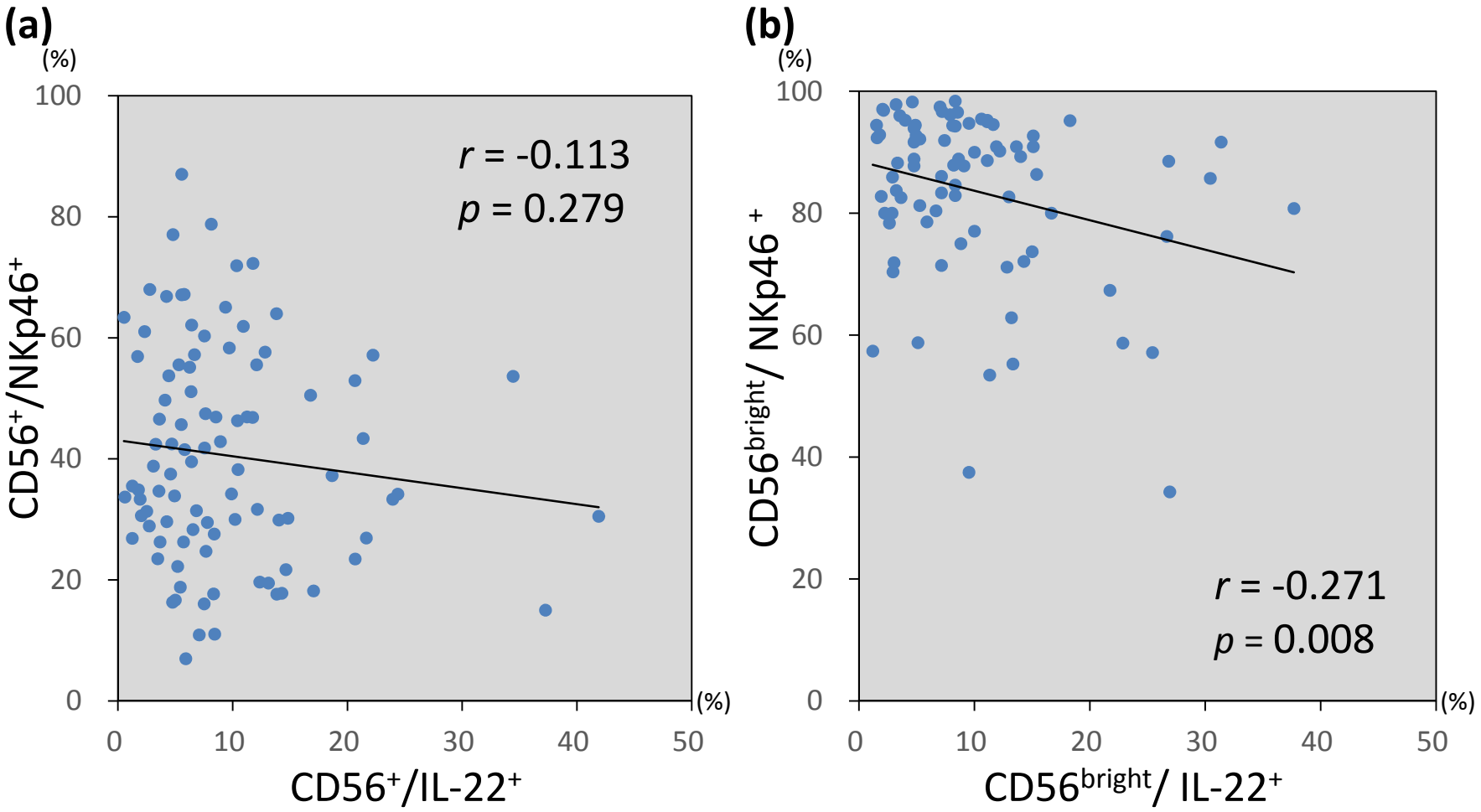

Table 2 The Combination of Monoclonal Antibodies Using for Flow Cytometric Analysis in Peripheral Blood and Endometrium

Peripheral blood				
IL-22	CD56-FITC	IL-22 PerCP		
IFN-γ/TNF-α	CD56-PerCP-Cy5.5	IFN-γ FITC	TNF-α PE	
IL-4/IL-10	CD56-PerCP-Cy5.5	IL-4-PE	IL-10 FITC	
TGF-β1	CD56-FITC	TGF-b1-APC		
NKp46	CD56-FITC	NKp46-PE		

Endometrium				
IL-22	CD45-PacificBlue	CD56-FITC	IL-22 PerCP	
IFN-γ/TNF-α	CD45-PacificBlue	CD56-PerCP-Cy5.5	IFN-γ FITC	TNF-α PE
IL-4/IL-10	CD45-PacificBlue	CD56-PerCP-Cy5.5	IL-4-PE	IL-10 FITC
TGF-β1	CD45-PacificBlue	CD56-FITC	TGF-b1-APC	
NKp46	CD45-PacificBlue	CD56-FITC	NKp46-PE	

Table 1 Age and Obstetrical Histories of Women with Recurrent Pregnancy Losses and Unexplain

	RPL (n = 43)	UI (n = 38)	*P* -value
Age (years)	34 (30.0-38.0)	35 (31.8-39.0)	N.S.
No. of pregnancies	4 (3-5)	1 (0-1)	< 0.001
No. of deliveries	0 (0-1)	0 (0-0)	< 0.05
No. of miscarriages	3 (2-4)	0 (0-1)	< 0.001
BMI	20.8 (19.5-23.2)	20.0 (18.4-22.4)	N.S.

All data were shown median (interquartile range).

RPL, recurrent pregnancy loss

UI, unexplained infertility

BMI, body mass index