Expression of retinoid-related orphan receptor γt (RORγt) on NK22 cells in the peripheral blood and uterine endometrium of women with unexplained recurrent pregnancy loss and unexplained infertility

(リスク因子不明不育症患者と原因不明不妊症患者における末梢血および子宮内膜 NK22 細胞に対する RORγt 発現について)

申請者　　弘前大学大学院医学研究科

成育科学領域　生殖機能病態学教育研究分野

氏　名　　淵之上　康平

指導教授　若林　孝一

Introduction

Natural killer (NK) cells play an important role in innate immune responses (1, 2). NK cells are classified into two groups according to the intensity of CD56 fluorescence on the cell surface. $CD56^{bright}$ cells represent 10% of NK cells in peripheral blood, but mainly consist of uterine NK (uNK) cells, and have a role in cytokine production. $CD56^{dim}$ cells represent the remaining 90% of NK cells in peripheral blood (pNK cells) and their major role is cytotoxicity (3-6).

More than half of women with recurrent pregnancy loss (RPL) are diagnosed as unexplained RPL cases (7). Some of these women may have immune abnormalities, such as abnormal NK cell function or abnormal subpopulations of NK cells. Previously, we reported that a higher percentage of $CD16^{+}/CD56^{dim}$ uNK cells and lower percentage of $CD16^{-}/CD56^{bright}$ uNK cells in the group with miscarriages in subsequent pregnancies than in the group with successful deliveries in subsequent pregnancies in the IVF-ET program (8).

Human NK cells are divided into NK1 , NK2 , NK3 and NKrl subsets on the basis of cytokines produced; NK1 produce IFN-γ and TNF-α; NK2 produce IL-4, IL-5 and IL-13; NK3 produce TGF-β; NKr1 produce IL-10 (9). NK cells may shift from a type 1 to type 2 immune response in a normal pregnancy (10, 11). We have reported an evident type 1 shift of pNK cells in women with recurrent implantation failure (RIF) or RPL patients (12). These results suggested that an increase in the NK1/NK2 ratio might indicate risk for pregnancy failure.

Recently, NK22 cells, a subset of IL-22-producing NK cells were identified (13-15). NK22 cells were reported to exist in the uterine endometrium, and their role is to promote the shed and regeneration of the mucosa during the menstrual cycle (16). Wang et al. reported that the IL-22 receptor was decreased in decidual NK cells of patients

who had miscarriages and that this decrease might prevent the growth and proliferation of a trophoblast (17). Cella et al. suggested that NK22 cells produce IL-22 and other cytokines that prevent inflammation and protect mucosal sites in tonsils or Peyer's patches (15). We previously reported higher production of uterine endometrial NK22 cells in women with RPL (18). NK22 cells are presumed to increase to normalize or regulate cytokine production in women with RPL.

Natural cytotoxicity receptors (NCRs), which include NKp30, NKp44, and NKp46, are expressed on NK cells. NCRs regulate cytotoxicity and cytokine production through signal cascades in NK cells (19). Murine NK22 cells express NKp46 (15, 20, 21) in Peyer's patches, the spleen, and the intestine. NK22 cells of humans express NKp44 (15) in Peyer's patches and tonsils. It has been reported that NK22 cells in the human uterine endometrium express NKp46 (16). Previously, we reported that NK22 cells express NKp46 (18).

NK22 cells express retinoid-related orphan receptor γt (RORγt), a kind of intercellular transcription factor (21, 22). RORγt is a member of the subfamily of RORs (23-26). RORs are considered essential for the genesis of lymph nodes, and RORγt are believed to play a critical role in the regulation of thymopoiesis (27) and lymph node organogenesis (28). RORγt is originally expressed in Th17 cells, which produce IL-22 and IL-17 (29). Indeed, RORγt is considered to be a main regulator of IL-22 and IL-17 (23) in Th17 cells. However, the expression patterns of RORγt in NK22 cells of the peripheral blood and uterine endometrium are unknown.

The aim of this study was to investigate (1) the relationship between NK22 cells and RORγt; (2) which type of NCRs expressed on NK22 cells, NKp44, or NKp46; (3) which cytokines produced by RORγt^{+} NK cells; and (4) the roles of pNK22 cells and uNK22 cells in reproduction.

Materials and methods

Study subjects

Study subjects were recruited from the Department of Obstetrics and Gynecology at Hirosaki University, Hirosaki, Aomori, Japan, between May 1, 2014, and April 30, 2015. All study subjects provided informed consent prior to participating in the study. The study was approved by the Institutional Review Board at Hirosaki University Graduate School of Medicine. In total, 47 women were recruited into this study. Twenty-five of these women who hoped to conceive had no obvious risk factors and were defined as cases of unexplained infertility (uI). Twenty-two of these women had a history of two or more miscarriages without obvious risk factors and were defined as cases of unexplained recurrent pregnancy loss (uRPL). Normal fertile controls were excluded in this study because of ethical reasons and difficulties of performing an endometrial biopsy in healthy fertile women. In contrast, It was reported that NK cells obtained in women with uI showed a similar percentage of $CD56^{bright}$ NK cells in normal women endometrium (30). So, in this study, we tried to clarify the difference of NK cells between women with uRPL and uI for the expression of NK cell surface markers, cytokines production and nuclear receptor. Women with anatomic, endocrine, or autoimmune disorders, including antiphospholipid antibody syndrome and genetic etiologies for uI or uRPL, as well as those with microbial infections were excluded from the study. Patient characteristics are shown in Table 1. There was no significant difference in age and number of prior deliveries between the two groups. The number of pregnancies ($p < 0.01$) and miscarriages ($p < 0.01$) were significantly higher in women with uRPL than in women with uI. The peripheral blood and endometrial samples were obtained during the mid-secretory phase of the normal menstrual cycle according to

basal body temperature. None of the patients had taken oral contraceptives or other medications known to interfere with the menstrual cycle.

Preparation of NK cells from the peripheral blood and uterine endometrium

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using HISTOPAQUE-1077 (Sigma-Aldrich, Inc., St Louis, MO, USA). The uterine endometrial samples were obtained using an endometrial cell sampler (Endosuction; Hakko, Nagano, Japan) and were washed in saline to remove blood. Samples were placed into RPMI-1640 medium (Sigma-Aldrich) 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 lymphocytes were adjusted to 5×10^6 cells/mL.

Expression of natural cytotoxicity receptors and RORγt on NK cells

Multicolor flow cytometric analysis was performed to detect the expression of RORγt in NK cells. Briefly, 10×10^5 PBMCs and uterine endometrial lymphocytes were labeled with monoclonal antibodies (mAbs) for 20 min at room temperature for staining of surface antigens. Anti-CD56-FITC (Becton Dickinson, Franklin Lakes, NJ, USA) and CD334 (NKp44)-phycoerythrin (PE) (Beckman Coulter, Inc., Brea, CA, USA) or CD335 (NKp46)-PE (Beckman Coulter) were used to stain the surface antigens of PBMCs, and anti-CD45-Pacific Blue (Beckman Coulter), anti-CD56-FITC, and NKp44-PE or NKp46-PE were used to stain the surface antigens of uterine endometrial

lymphocytes. The cells were washed in phosphate-buffered saline solution (PBS), and then were fixed with 1% formaldehyde at room temperature. After 10 minutes of fixation, cells were washed in Stain Buffer (Becton Dickinson) and permeabilized by 0.2% saponins for 30 minutes at room temperature. After permeabilization, cells were washed twice in Stain Buffer, and stained for 30 minutes with anti-RORγt-APC (eBioscience, Hatfield, UK). Then, the cells were washed twice with Stain Buffer and resuspended in 250 μL of PBS for subsequent flow cytometric analysis. Finally, intracellular RORγt was measured using multicolor flow cytometry. Appropriate isotype controls were used for each antibody.

Expression of intracellular cytokines produced by NK cells

PBMCs and uterine endometrial lymphocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) (25 ng/mL), ionomycin (1 μM), and Brefeldin-A (10 μg/mL) for 4 hours. Lymphocyte stimulation was performed at 37°C in a humidified 5%-CO_2 incubator in RPMI-1640 medium. The cells were then washed in PBS and were stained for 20 min at room temperature for detection of surface antigens. Anti-CD56-FITC or anti-CD56-PE-cy5 (Beckman Coulter), according to the cytokine combination, was used to stain the surface antigens of PBMCs, while anti-CD56-FITC or anti-CD56-PE-cy5, according to the cytokine combination, and anti-CD45-Pacific Blue were used to stain the surface antigens of endometrial lymphocytes. Next, the cells were washed in PBS and were fixed and permeabilized with 250 μL of Cytofix/Cytoperm (Becton Dickinson) for 20 min. Cells were then washed twice in Perm/Wash Buffer (Becton Dickinson) and stained for 30 min with anti-IL-22-PerCP (eBioscience), anti IFN-γ-FITC (Becton Dickinson), anti TNF-α-PE (Beckman Coulter), anti-IL-4-PE (Beckman Coulter), and anti-IL-10-FITC (Beckman Coulter) (Table 2).

Cells were again 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 four-color flow cytometric analysis were performed with a FACSCanto flow cytometer (Becton Dickinson) and a computer interfacing with FACSDiva software (Becton Dickinson) 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 uterine endometrial cells were first gated on anti-CD45-positive events and then on the lymphocyte region by 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). The age distribution and numbers of pregnancies, deliveries, and miscarriages in the two groups were analyzed by *t*-test. The data are presented as the mean with standard deviation. A comparison of RORγt$^+$ NK cells as a subset of NKp44$^+$ NK cells and NKp46$^+$ NK cells was analyzed by the Mann–Whitney U test. The data are presented as the median with interquartile ranges. RORγt$^+$ NK cells in women with uI and uRPL were compared by the Mann–Whitney U-test. The data are presented as the median with interquartile ranges. Correlations between RORγt$^+$ NK cells and cytokine-producing NK cells were analyzed by Pearson's correlation coefficients. Correlations were considered significant if $| r | > 0.4$. For all analyses, a *p*-value of <0.05 was considered to be

statistically significant.

Results

Comparison of the expression levels of RORγt in CD56$^+$/NKp44$^+$ NK cells or CD56$^+$/NKp46$^+$ NK cells between pNK cells and uNK cells in women with uI and uRPL

Representative dot plots of RORγt-expressing NK cells in women with uI and uRPL are shown in Fig. 1, including CD56$^+$/RORγt$^+$, CD56$^+$/NKp46$^+$/RORγt$^+$ and CD56$^+$/NKp44$^+$/RORγt$^+$ cells. The percentage of CD56$^+$/NKp44$^+$/RORγt$^+$ uNK cells was significantly higher than that of CD56$^+$/NKp44$^+$/RORγt$^+$ pNK cells ($p < 0.01$). Similarly, the percentage of CD56$^+$/NKp46$^+$/RORγt$^+$ uNK cells was higher than that of CD56$^+$/NKp46$^+$/RORγt$^+$ pNK cells ($p < 0.01$, Table 3). Then, we evaluated these expression in each group (uRPL and uI). In women with uI, the percentages of CD56$^+$/NKp44$^+$/RORγt$^+$ uNK cells ($p < 0.01$) and CD56$^+$/NKp46$^+$/RORγt$^+$ uNK cells ($p < 0.01$) were higher than those of pNK cells, respectively. Similarly, in women with uRPL, the percentages of CD56$^+$/NKp44$^+$/RORγt$^+$ uNK cells ($p < 0.01$) and CD56$^+$/NKp46$^+$/RORγt$^+$ uNK cells ($p < 0.01$) were significantly higher than those of pNK cells, respectively (Table 4).

Correlations between NKp44$^+$/RORγt$^+$ NK cells or NKp46$^+$/RORγt$^+$ NK cells and IL-22-producing NK cells in women with uI and uRPL

Correlations between NKp46$^+$/RORγt$^+$ NK cells or NKp44$^+$/RORγt$^+$ NK cells and IL-22-producing NK cells are shown in Fig. 2. There were significant positive correlations between the percentage of IL-22-producing pNK cells and NKp46$^+$/RORγt$^+$ pNK cells in women with uI ($r = 0.636$, $p < 0.05$) and those with uRPL ($r = 0.626$, $p < 0.05$) (Fig. 2A(a) and (b)). Moreover, there were significant positive correlations

between the percentage of IL-22-producing uNK cells and NKp46^{+}/RORγt^{+} uNK cells in women with uI (r = 0.616, $p < 0.05$) and those with uRPL(r = 0.634, $p < 0.01$) (Fig. 2B(a) and (b)). On the other hand, there was no correlation between the percentage of IL-22-producing pNK cells and NKp44^{+}/RORγt^{+} pNK cells in women with either uI or uRPL (Fig. 2A(c) and (d)). Similarly, there were no correlations between the percentage of IL-22-producing uNK cells and NKp44^{+}/RORγt^{+} uNK cells in women with either uI or uRPL (Fig. 2B(c) and (d)).

Percentage of CD56^{+}/NKp44^{+}/RORγt^{+} NK cells and CD56^{+}/NKp46^{+}/RORγt^{+} NK cells in women with uI and uRPL

Next, we compared RORγt expression levels between NKp44^{+} NK cells and NKp46^{+} NK cells (Fig. 3). The percentages of NKp46^{+}/RORγt^{+} pNK cells were significantly higher than those of NKp44^{+}/RORγt^{+} pNK cells in women with uI ($p < 0.01$) and in women with uRPL ($p < 0.01$) (Fig. 3A(a) and (b)). Similarly, the percentages of NKp46^{+}/RORγt^{+} uNK cells in women with uI ($p < 0.01$) and in women with uRPL ($p < 0.01$) were significantly higher than percentages of NKp44^{+}/RORγt^{+} uNK cells ($p < 0.01$) (Fig. 3B(a) and (b)).

Expression of RORγt according to the CD56- and NKp46-expressing NK cell subpopulation in women with uI and uRPL

We compared the percentage of cells with RORγt in CD56- and NKp46-expressing NK cell subpopulations between uI and uRPL cases. The percentages of CD56^{bright}/NKp46^{bright}/RORγt^{+} uNK cells and pNK cells in women with uRPL were significantly higher than those in women with uI ($p < 0.05$) (Fig. 4A(a) and B(a)). Moreover, there was a significant positive correlation between the percentage of

$CD56^{bright}/NKp46^{bright}/ROR\gamma t^{+}$ uNK cells and that of $CD56^{bright}/NKp46^{bright}/ROR\gamma t^{+}$ pNK cells ($r = 0.516$, $p < 0.05$).

There were no significant differences between the percentages of women with uI and uRPL of $CD56^{bright}/NKp46^{dim}/ROR\gamma t^{+}$ (Fig. 4A(b) and B(b)), $CD56^{dim}/NKp46^{bright}/ROR\gamma t^{+}$ (Fig. 4A(c) and B(c)), or $CD56^{dim}/NKp46^{dim}/ROR\gamma t^{+}$ (Fig. 4A(d) and B(d)) in uNK and pNK cells.

Correlations between the percentages of RORγt-positive cells and cytokine-producing cells among $CD56^{bright}$ cells in women with uI and uRPL

The correlations between the percentages of RORγt-positive cells and cytokine-producing cells among $CD56^{bright}$ cells are shown in Table 5 and Fig. 5. There was a significant negative correlation between the percentage of $CD56^{bright}/ROR\gamma t^{+}$ uNK cells and $CD56^{bright}/IFN\text{-}\gamma^{+}$ uNK cells in uRPL cases ($r = -0.635$, $p < 0.05$) (Fig. 5A). Similarly, there was a significant negative correlation between the percentage of $CD56^{bright}/ROR\gamma t^{+}$ uNK cells and $CD56^{bright}/TNF\text{-}\alpha^{+}$ uNK cells in uRPL cases ($r = -0.644$, $p < 0.05$) (Fig. 5B).

There were no correlations between $CD56^{bright}/ROR\gamma t^{+}$ NK cells and $CD56^{bright}$/IL-4 or $CD56^{bright}$/IL-10 cells in either pNK cells or uNK cells of uI or uRPL cases (Table 5).

Discussion

Although NK22 cells are NK cells, they have a role independent from that of other NK cells (14). In general, NK cells show cytotoxicity against pathogens or tumor cells; however, the cytotoxicity of NK22 cells is weak and their main role is believed to be the production of IL-22 to protect mucosa (20-22, 31). NK22 cells exist not only in the intestine (21, 22), spleen (21), tonsil (15), and skin (20, 22), but also in the uterine

endometrium and aborted or terminated decidua (16-18).

With regard to the definition of NK22 cells, it has been proposed that NK22 are those cells that produce IL-22 because of stimulation by PMA and ionomycin. However, stimulation by PMA and ionomycin is pharmacological and not physiological, and new markers to identify intact NK22 cells are needed. The results of the present study have shown that RORγt can be a candidate marker to define NK22 cells, since RORγt is detectable without any stimulation. RORγt , on the other hand, is proposed to be a proper marker of Th17 cells (14), as RORγt is expressed on this T cell subset.

The expression of RORγt in decidual Th17 cells has been shown to be higher than that in the peripheral blood, as the percentage of Th17 cells in decidua is higher than that in the peripheral blood (32). However, there were no such reports on RORγt-positive NK cells in the peripheral blood and uterine endometrium. The present study showed for the first time that RORγt$^+$ NK cells are present in the uterine endometrium and peripheral blood and that NK22 cells represent a subset of RORγt$^+$ NK cells. We have shown the presence of NK22 cells in the uterine endometrium and peripheral blood of women with RPL [17]. In the present study, the percentage of RORγt$^+$ NK cells in uNK cells was higher than that in pNK cells. This suggests that the percentage of NK22 cells in uNK cells might be higher than that in pNK cells.

We previously shown that the expression of NKp46 was decreased in pNK and uNK cells in women with RPL (33), and that cytokine production by NK cells was associated with NKp46 expression (34-37). Although NKp46 was shown to be expressed in uterine endometrial NK22 cells, it was not clear whether NKp44 was also expressed in uNK cells. As shown in Fig. 2, NKp44$^+$ NK cells were detected among uNK cells, but there was no correlation between CD56$^+$/NKp44$^+$/RORγt$^+$ NK cells and CD56$^+$/IL-22$^+$ NK cells in either pNK cells and uNK cells. On the contrary, there were significant

correlations between $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in both pNK and uNK cells. Moreover, the percentage of $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells was significantly higher than that of $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells in both pNK cells and uNK cells (Fig. 3). These results strongly suggest that most NK22 cells in the peripheral blood and uterine endometrium are consistent with $NKp46^+$ NK cells. Among pNK and uNK cells, $CD56^{bright}/NKp46^{bright}$ cells are the cytokine producers (35). As shown in Fig. 4, the percentage of $ROR\gamma t^+$ NK cells among $CD56^{bright}/NKp46^{bright}$ uNK cells from women with uRPL was significantly higher than that from women with uI. Based on these results and our previous findings that NK22 cells of women with uRPL were elevated (18), we infer that NK22 cells are a subset of $ROR\gamma t^+$ NK cells. With regard to RORγt function, Awasthi et al. have demonstrated that RORγt is involved in IL-22 production from Th17 cells (38). Although we did not determine the quantity of IL-22 production in this study, it is likely that the increased $CD56^{bright}/NKp46^{bright}/ROR\gamma t^+$ NK cells in women with uRPL would result in excessive IL-22 production. Of interest, there was a significant positive correlation between the percentage of $CD56^{bright}/NKp46^{bright}/ROR\gamma t^+$ uNK cells and that of $CD56^{bright}/NKp46^{bright}/ROR\gamma t^+$ pNK cells. Whether changes in pNK synchronize with those of uNK has been a matter of debate (39). Our previous study (37) showed that pNK cells reflected changes in uNK cells by the expression of NKp46.

NK cells are activated in response to cytokines such as IL-2 and IL-23, while NK cells elicit an immune response by secreting IFN-γ or TNF-α. It was reported that the population of NK1 type cells in decidua of spontaneous miscarriage was higher than that in decidua of early pregnancy (9). We have shown that type 1 cytokines such as INF-γ or TNF-α, which are produced by NK cells, are increased in women with RPL(12), and that IL-22 production was elevated in the uNK cells of women with RPL.

In addition, there were negative correlations between IL-22 and IFN-γ or TNF-α (18). As shown in Fig. 5, there was a negative correlation between the percentages of $CD56^{bright}$/RORγt^{+} cells and $CD56^{bright}$/IFN-γ^{+} or $CD56^{bright}$/TNF-α^{+} uNK cells in women with uRPL, suggesting that RORγt could modulate the production of IL-22. The expression of RORγt might increase the production of IL-22 by NK22 cells in women with uRPL. Therefore, it is suggested that an increase in IL-22 by NK cells decreases type 1 cytokine production to adjust the cytokines balance from type 1 to type 2 for the maintenance of pregnancy. Wang et al. reported that the IL-22 receptor, IL-22R1, was decreased in the decidual NK cells of women with RPL (17). Taken together, these results suggest that in women without RPL, RORγt expression and IL-22 production are controlled to maintain a type 2-dominant intra-uterine environment.

In conclusion, in this study, we demonstrated that RORγt is expressed in NK cells in the uterine endometrium and that $CD56^{+}$/NKp46^{+}/RORγt^{+} cells were positively correlated with $CD56^{+}$/IL-22^{+} cells in both PB and EM of women with either uI or uRPL. In addition, we showed that the percentage of $CD56^{bright}$/$NKp46^{bright}$/RORγt^{+} cells in PB and EM from uRPL cases was significantly higher than that from uI cases, while the percentage of $CD56^{bright}$/RORγt^{+} cells in EM were negatively correlated with that of $CD56^{bright}$/IFN-γ^{+} and $CD56^{bright}$/TNF-α^{+} cells from uRPL cases, suggesting that RORγt could be a useful tool to investigate the role of NK22 cells in conjunction with uRPL.

Acknowledgement

This study was supported by JSPS KAKENHI Grant number 24592456. The authors thank Tsushima Y. for her help in performing the FACScanto.

Disclosure

No authors associate with any conflict of interest.

References

1. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol. 2001;13(4):458-64.

2. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. Curr Opin Immunol. 2006;18(4):391-8.

3. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22(11):633-40.

4. 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. Dev Immunol. 1991;1(3):169-90.

5. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol. 2002;2(9):656-63.

6. Caligiuri MA. Human natural killer cells. Blood. 2008;112(3):461-9.

7. Saito S, Nakashima A, Shima T. Future directions of studies for recurrent miscarriage associated with immune etiologies. J Reprod Immunol. 2011;90(1):91-5.

8. 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(6):413-22.

9. Higuma-Myojo S, Sasaki Y, Miyazaki S, Sakai M, Siozaki A, Miwa N, et al.

Cytokine profile of natural killer cells in early human pregnancy. Am J Reprod Immunol. 2005;54(1):21-9.

10. 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. Eur J Immunol. 2005;35(10):3054-63.

11. Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, et al. Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle. Fertil Steril. 2002;77(5):1032-7.

12. 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. Fertil Steril. 2008;89(1):157-65.

13. Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S, Ribeiro VS, Mandelboim O, Renauld JC, et al. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. J Immunol. 2009;183(10):6579-87.

14. Cooper MA, Colonna M, Yokoyama WM. Hidden talents of natural killers:

NK cells in innate and adaptive immunity. EMBO Rep. 2009;10(10):1103-10.

15. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 2009;457(7230):722-5.

16. 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(7):3913-8.

17. 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. Int J Clin Exp Pathol. 2013;6(9):1781-90.

18. Kamoi M, Fukui A, Kwak-Kim J, Fuchinoue K, Funamizu A, Chiba H, et al. NK22 Cells in the Uterine Mid-Secretory Endometrium and Peripheral Blood of Women with Recurrent Pregnancy Loss and Unexplained Infertility. Am J Reprod Immunol. 2015;73(6):557-67.

19. Biassoni R, Cantoni C, Marras D, Giron-Michel J, Falco M, Moretta L, et al. Human natural killer cell receptors: insights into their molecular function and structure. J Cell Mol Med. 2003;7(4):376-87.

20. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M,

Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity. 2008;29(6):958-70.

21. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol. 2009;10(1):83-91.

22. Luci C, Reynders A, Ivanov, II, Cognet C, Chiche L, Chasson L, et al. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. Nat Immunol. 2009;10(1):75-82.

23. Solt LA, Burris TP. Action of RORs and their ligands in (patho)physiology. Trends Endocrinol Metab. 2012;23(12):619-27.

24. Jetten AM, Joo JH. Retinoid-related Orphan Receptors (RORs): Roles in Cellular Differentiation and Development. Adv Dev Biol. 2006;16:313-55.

25. Burris TP, Busby SA, Griffin PR. Targeting orphan nuclear receptors for treatment of metabolic diseases and autoimmunity. Chem Biol. 2012;19(1):51-9.

26. Mullican SE, Dispirito JR, Lazar MA. The orphan nuclear receptors at their 25-year reunion. J Mol Endocrinol. 2013;51(3):T115-40.

27. Kurebayashi S, Ueda E, Sakaue M, Patel DD, Medvedev A, Zhang F, et al. Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid

organogenesis and controls apoptosis during thymopoiesis. Proc Natl Acad Sci U S A. 2000;97(18):10132-7.

28. Eberl G, Littman DR. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. Immunol Rev. 2003;195:81-90.

29. Graeber KE, Olsen NJ. Th17 cell cytokine secretion profile in host defense and autoimmunity. Inflamm Res. 2012;61(2):87-96.

30. Matteo MG, Greco P, Rosenberg P, Mestice A, Baldini D, Falagario T, et al. Normal percentage of CD56bright natural killer cells in young patients with a history of repeated unexplained implantation failure after in vitro fertilization cycles. Fertil Steril. 2007;88(4):990-3.

31. Veiga-Fernandes H, Kioussis D, Coles M. Natural killer receptors: the burden of a name. J Exp Med. 2010;207(2):269-72.

32. Nakashima A, Ito M, Yoneda S, Shiozaki A, Hidaka T, Saito S. Circulating and decidual Th17 cell levels in healthy pregnancy. Am J Reprod Immunol. 2010;63(2):104-9.

33. Fukui A, Funamizu A, Yokota M, Yamada K, Nakamua R, Fukuhara R, et al. Uterine and circulating natural killer cells and their roles in women with recurrent pregnancy loss, implantation failure and preeclampsia. J Reprod Immunol.

2011;90(1):105-10.

34. Fukui A, Ntrivalas E, Fukuhara R, Fujii S, Mizunuma H, Gilman-Sachs A, et al. 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(6):371-80.

35. Yokota M, Fukui A, Funamizu A, Nakamura R, Kamoi M, Fuchinoue K, et al. 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(3):202-11.

36. Funamizu A, Fukui A, Kamoi M, Fuchinoue K, Yokota M, Fukuhara R, et al. 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(4):359-67.

37. Fukui A. Role of NK cells in women with recurrent pregnancy loss and implantation failure. Acta Obst Gynaec Jpn. 2011;63(12):2167-84.

38. Awasthi A, Kuchroo VK. Th17 cells: from precursors to players in inflammation and infection. Int Immunol. 2009;21(5):489-98.

39. Moffett A, Regan L, Braude P. Natural killer cells, miscarriage, and infertility.

BMJ. 2004;329(7477):1283-5.

Figure Legends

Fig. 1 Representative dot plots of RORγt-expressing NK cells. A gate was set on the lymphocyte region by characteristic forward and scatter parameters. Lymphocytes were classified into $CD56^+$ and $CD56^-$ cells, and then $CD56^+$ cells were divided into $NKp44^+$ and $NKp44^-$ or $NKp46^+$ and $NKp46^-$ cells. The ordinate in each plot indicates RORγt-expressing cells. **A** Peripheral blood NK (pNK) cells: (a) Isotype control; (b) $CD56^+/ROR\gamma^+$ NK cells of unexplained infertility (uI); (c) $CD56^+/ROR\gamma^+$ NK cells of unexplained recurrent pregnancy loss (uRPL). **B** uterine endometrial NK (uNK) cells: (a) Isotype control; (b) $CD56^+/ROR\gamma^+$ NK cells of uI; (c) $CD56^+/ROR\gamma^+$ NK cells of uRPL. RED square in **A** and **B** shows $CD56^+/ROR\gamma t^+$ NK cells. **C** pNK cells: (a) Isotype control; (b) $CD56^+/NKp46^+/ROR\gamma^+$ NK cells of uI; (c) $CD56^+/NKp46^+/ROR\gamma^+$ NK cells of uRPL; (d) $CD56^+/NKp44^+/ROR\gamma^+$ NK cells of uI; (e) $CD56^+/NKp44^+/ROR\gamma^+$ NK cells of uRPL. **D** uNK cells: (a) Isotype control; (b) $CD56^+/NKp46^+/ROR\gamma^+$ NK cells of uI; (c) $CD56^+/NKp46^+/ROR\gamma^+$ NK cells of uRPL; (d) $CD56^+/NKp44^+/ROR\gamma^+$ NK cells of uI; (e) $CD56^+/NKp44^+/ROR\gamma^+$ NK cells of uRPL. RED square in **C** and **D** shows $CD56^+/NKp46^+/ROR\gamma t^+$ or $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells.

Fig. 2 Correlations between the percentage of $CD56^+/NKp46^+/ROR\gamma t^+$ or $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and the percentage of $CD56^+/IL\text{-}22^+$ NK cells. The ordinate indicates the percentage of $CD56^+/IL\text{-}22^+$ NK cells. The abscissa indicates the percentage of $CD56^+/NKp46^+/ROR\gamma t^+$ or $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells. **A** pNK cells: (a) Correlation between $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in unexplained infertility (uI); (b) Correlation between $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in unexplained recurrent

pregnancy loss (uRPL); (c) Correlation between $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uI; (d) Correlation between $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uRPL. **B** uNK cells: (a) Correlation between $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uI; (b) Correlation between $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uRPL; (c) Correlation between $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uI; (d) Correlation between $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells in uRPL. Correlation between the percentage of $CD56^+/NKp46^+/ROR\gamma t^+$ or $CD56^+/NKp44^+/ROR\gamma t^+$ NK cells and $CD56^+/IL\text{-}22^+$ NK cells was analyzed by Pearson's linear correlation.

Fig.3 The percentage of $ROR\gamma t^+$ NK cells in $NKp44^+$ NK cells or $NKp46^+$ NK cells. **A** Comparison between the percentages of $CD56^+/NKp44^+/ROR\gamma t^+$ pNK cells and $CD56^+/NKp46^+/ROR\gamma t^+$ pNK cells: (a) unexplained infertility (uI); (b) recurrent pregnancy loss (uRPL). **B** Comparison between the percentages of $CD56^+/NKp44^+/ROR\gamma t^+$ uNK cells and $CD56^+/NKp46^+/ROR\gamma t^+$ uNK cells: (a) uI; (b) uRPL. BLUE box shows the percentage of $CD56^+/NKp44^+/ROR\gamma t^+$ cells. Box and whisker plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. GREEN box shows the percentage of $CD56^+/NKp46^+/ROR\gamma t^+$ cells. Differences between each group were analyzed by Mann-Whitney *U*-test. * $p < 0.01$.

Fig. 4 The percentage of $ROR\gamma t^+$ NK cells in each subpopulation by CD56 and NKp46.

A Comparison between the percentages of unexplained infertility (uI) and unexplained recurrent pregnancy loss (uRPL) of pNK cells: (a) the percentages of $CD56^{bright}$/$NKp46^{bright}$/RORγt cells; (b) the percentages of $CD56^{bright}$/$NKp46^{dim}$/RORγt cells; (c) the percentages of $CD56^{dim}$/$NKp46^{bright}$/RORγt cells; (d) the percentages of $CD56^{dim}$/$NKp46^{dim}$/RORγt cells. **B** Comparison between the percentages of uI and uRPL of uNK cells: (a) the percentages of $CD56^{bright}$/$NKp46^{bright}$/RORγt cells; (b) the percentages of $CD56^{bright}$/$NKp46^{dim}$/RORγt cells; (c) the percentages of $CD56^{dim}$/$NKp46^{bright}$/RORγt cells; (d) the percentages of $CD56^{dim}$/$NKp46^{dim}$/RORγt cells. Box and whisker plots: bar (horizontal line) = median; box = 25th and 75th percentiles; whiskers = extend to the extreme values. LIGHT BLUE box shows uI and RED box shows uRPL. Differences between each group were analyzed by Mann-Whitney *U*-test. * $p < 0.05$.

Fig. 5 Corralations between the percentages of $CD56^{bright}$/$ROR\gamma t^{+}$ uNK cells and cytokine-producing $CD56^{bright}$ uNK cells in women with unexplained recurrent pregnancy loss. The ordinate indicates the percentage of cytokine-producing $CD56^{bright}$ uNK cells. The abscissa indicates the percentage of $CD56^{bright}$/$ROR\gamma t^{+}$ uNK cells.: **A** Correlation between $CD56^{bright}$/$ROR\gamma t^{+}$ uNK cells and IFN-γ-producing $CD56^{bright}$ uNK cells. **B** Correlation between $CD56^{bright}$/$ROR\gamma t^{+}$ uNK cells and TNF-α-producing $CD56^{bright}$ uNK cells. Corralations between the percentages of $CD56^{bright}$/$ROR\gamma t^{+}$ uNK cells and cytokine-producing $CD56^{bright}$ uNK cells in women with unexplained recurrent pregnancy loss. The ordinate indicates the percentage of cytokine-producing $CD56^{bright}$ uNK cells were analyzed by Pearson's liner correlation.

Table 1 Age and obstetrical histories of women with unexplained infertility and unexplained recurrent pregnancy loss

	uI (n=25)	uRPL (n=22)	*p* -value
Age (years)	35.5 ±3.8	36.7±5.4	N.S.
No. of pregnancies	0.6 ± 0.7	2.9 ± 1.3	<0.01
No. of deliveries	0.2 ± 0.4	0.3 ± 0.6	N.S.
No. of abortions	0.4 ± 0.6	2.3 ± 0.9	<0.01

All data were shown mean ± standard division
uI, unexplained infertility; uRPL, unexplained recurrent pregnancy loss

Table 2 The combination of monoclonal antibodies using for flow cytometric analysis in peripheral blood and uterine endometrium

Peripheral blood				
NKp44/RORγt	CD56-FITC	NKp44-PE	RORγt-APC	
NKp46/RORγt	CD56-FITC	NKp46-PE	RORγt-APC	
IL-22	CD56-FITC	IL-22-PerCP		
IFN-γ/TNF-α	CD56-PE-cy5	INF-γ-FITC	TNF-α-PE	
IL-4/IL-10	CD56-PE-cy5	IL4-PE	IL10-FITC	
Uterine endometrium				
NKp44/RORγt	CD56-FITC	NKp44-PE	RORγt-APC	CD45-PacificBlue
NKp46/RORγt	CD56-FITC	NKp46-PE	RORγt-APC	CD45-PacificBlue
IL-22	CD56-FITC	IL-22-PerCP		CD45-PacificBlue
IFN-γ/TNF-α	CD56-PE-cy5	INF-γ-FITC	TNF-α-PE	CD45-PacificBlue
IL-4/IL-10	CD56-PE-cy5	IL4-PE	IL10-FITC	CD45-PacificBlue

Table 3 Comparison the percentage of $CD56^+/NKp44^+/ROR\gamma t^+$ or $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells between pNK cells and uNK cells

	pNK cells		uNK cells		
	median(%)	Interquartile range	median(%)	Interquartile range	*p* -value
uI					
$CD56^+/NKp44^+/ROR\gamma t^+$	0.17	0.08-0.58	1.25	0.97-1.41	<0.01
$CD56^+/NKp46^+/ROR\gamma t^+$	2.4	1.19-3.67	4.6	3.87-6.52	<0.01
uRPL					
$CD56^+/NKp44^+/ROR\gamma t^+$	0.32	0.24-0.70	1.68	0.45-3.11	<0.01
$CD56^+/NKp46^+/ROR\gamma t^+$	1.87	1.46-2.50	5.57	3.42-8.86	<0.01

Comparison the percentage of $CD56^+/NKp44^+/ROR\gamma t^+$ or $CD56^+/NKp46^+/ROR\gamma t^+$ NK cells between pNK cells and uNK cells 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.

uI, unexplained infertility; uRPL, unexplained recurrent pregnancy loss

pNK cells, peripheral blood NK cells; uNK cells, uterine endometrial NK cells

Table 4 Correlations between the percentages of RORγt positive $CD56^{bright}$ cells and cytokines producing $CD56^{bright}$ cells

	uI		uRPL	
Cytokine producing $CD56^{bright}/ROR\gamma t^{+}$ NK cells	r	*p*	r	*p*
pNK cells				
Interferon-γ	0.359	0.383	0.226	0.53
Tumor necrosis factor-α	0.288	0.489	0.253	0.479
IL-4	0.276	0.549	0.621	0.479
IL-10	0.639	0.122	0.601	0.188
uNK cells				
Interferon-γ	-0.65	0.235	-0.635	<0.05
Tumor necrosis factor-α	-0.641	0.243	-0.644	<0.05
IL-4	0.071	0.911	0.438	0.326
IL-10	0.41	0.493	0.277	0.546

Correlations between the percentages of RORγt positive $CD56^{bright}$ cells and cytokines producing $CD56^{bright}$ cells were analyzed by Pearson's linear correlation. Correlations were considered significant if $| r | > 0.4$ and p-value < 0.05.

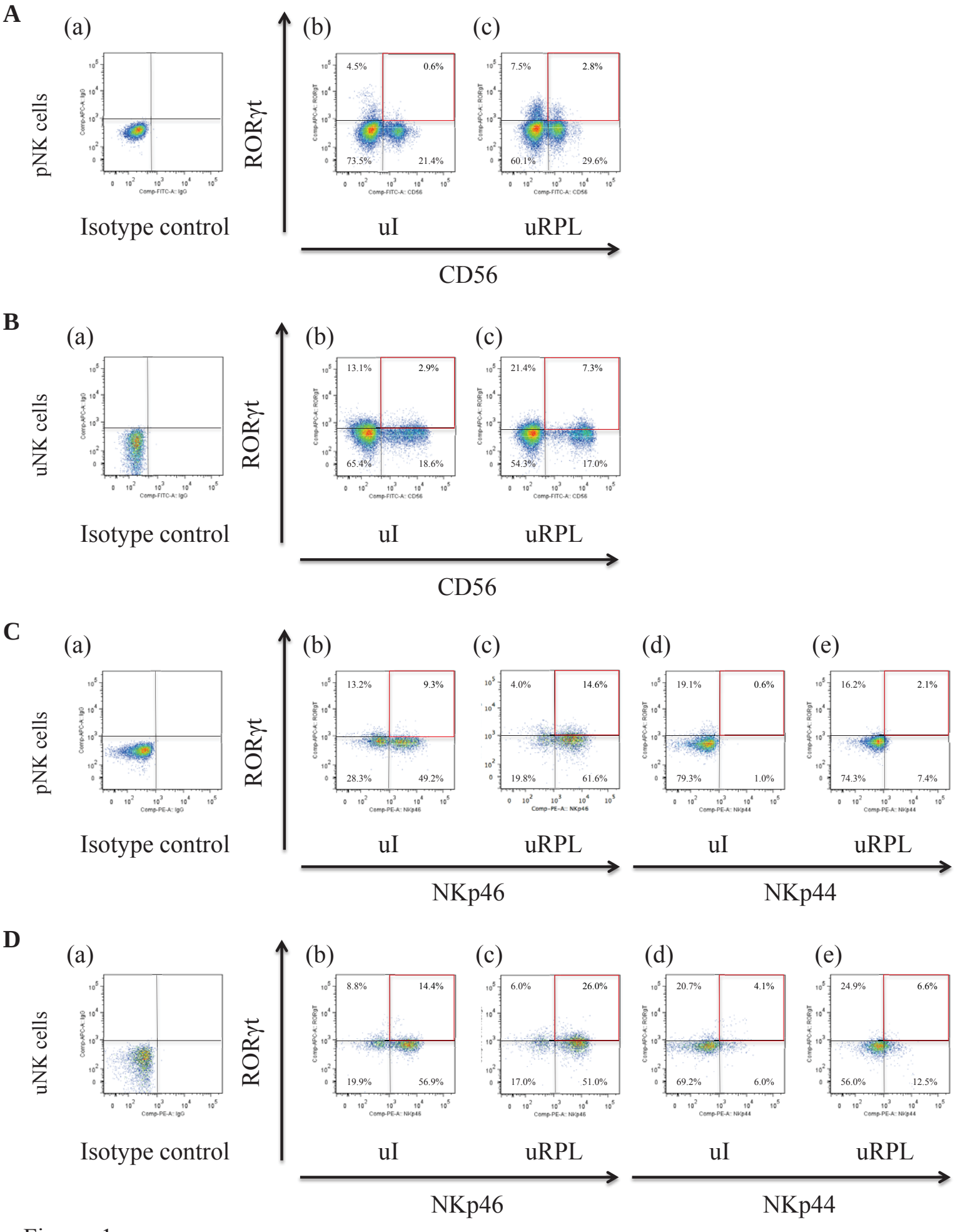
A
(a)
(b)
(c)
pNK cells
RORγt
4.5%
0.6%
73.5%
21.4%
7.5%
2.8%
60.1%
29.6%
Isotype control
uI
uRPL
CD56
B
(a)
(b)
(c)
uNK cells
RORγt
13.1%
2.9%
65.4%
18.6%
21.4%
7.3%
54.3%
17.0%
Isotype control
uI
uRPL
CD56
C
(a)
(b)
(c)
(d)
(e)
pNK cells
RORγt
13.2%
9.3%
28.3%
49.2%
4.0%
14.6%
19.8%
61.6%
19.1%
0.6%
79.3%
1.0%
16.2%
2.1%
74.3%
7.4%
Isotype control
uI
uRPL
uI
uRPL
NKp46
NKp44
D
(a)
(b)
(c)
(d)
(e)
uNK cells
RORγt
8.8%
14.4%
19.9%
56.9%
6.0%
26.0%
17.0%
51.0%
20.7%
4.1%
69.2%
6.0%
24.9%
6.6%
56.0%
12.5%
Isotype control
uI
uRPL
uI
uRPL
NKp46
NKp44

Figure. 1

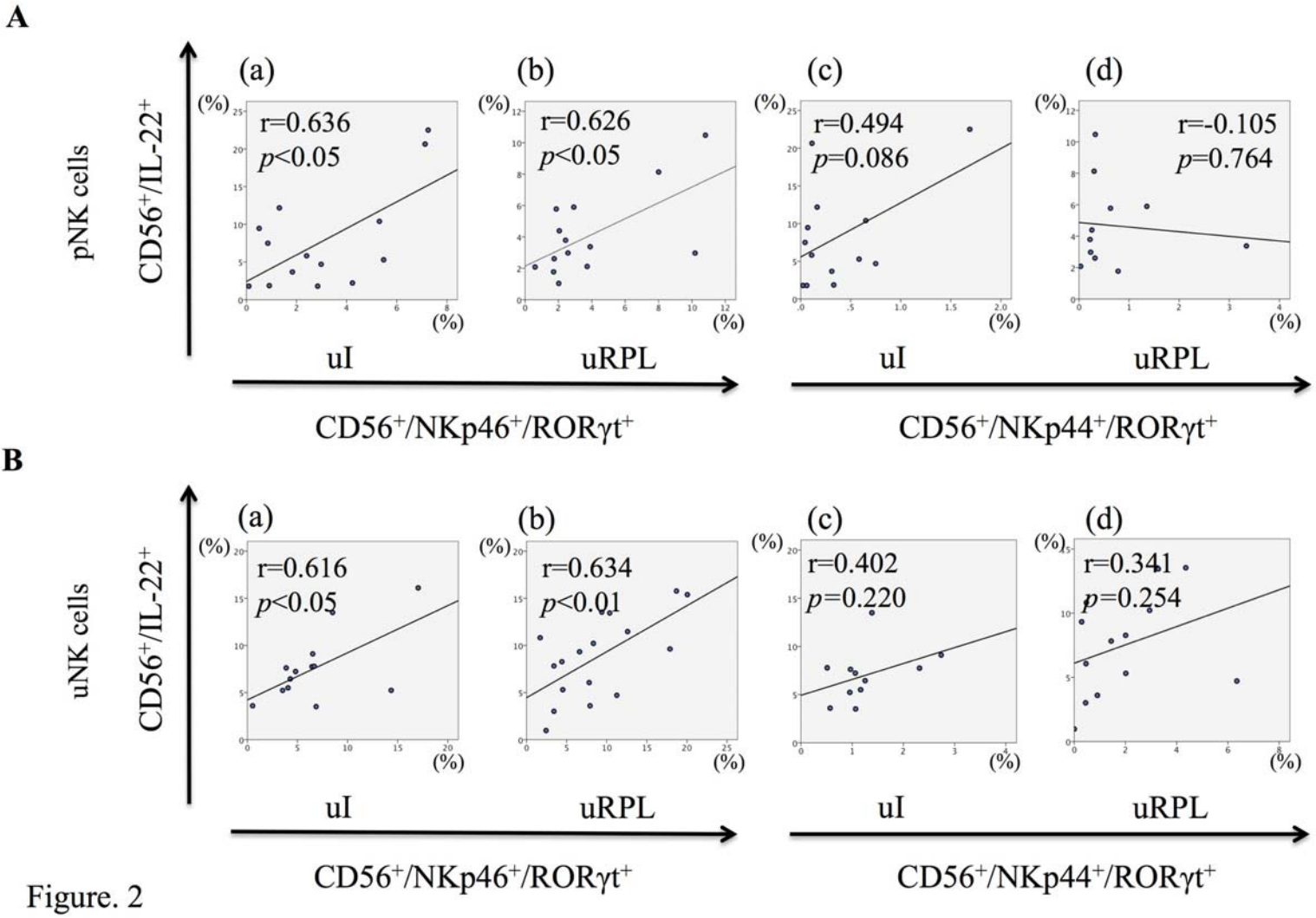
A
pNK cells
CD56+/IL-22+
(a)
r=0.636
p<0.05
(b)
r=0.626
p<0.05
(c)
r=0.494
p=0.086
(d)
r=-0.105
p=0.764
(%)
uI
uRPL
CD56+/NKp46+/RORγt+
CD56+/NKp44+/RORγt+
B
uNK cells
CD56+/IL-22+
(a)
r=0.616
p<0.05
(b)
r=0.634
p<0.01
(c)
r=0.402
p=0.220
(d)
r=0.341
p=0.254
(%)
uI
uRPL
CD56+/NKp46+/RORγt+
CD56+/NKp44+/RORγt+

Figure. 2

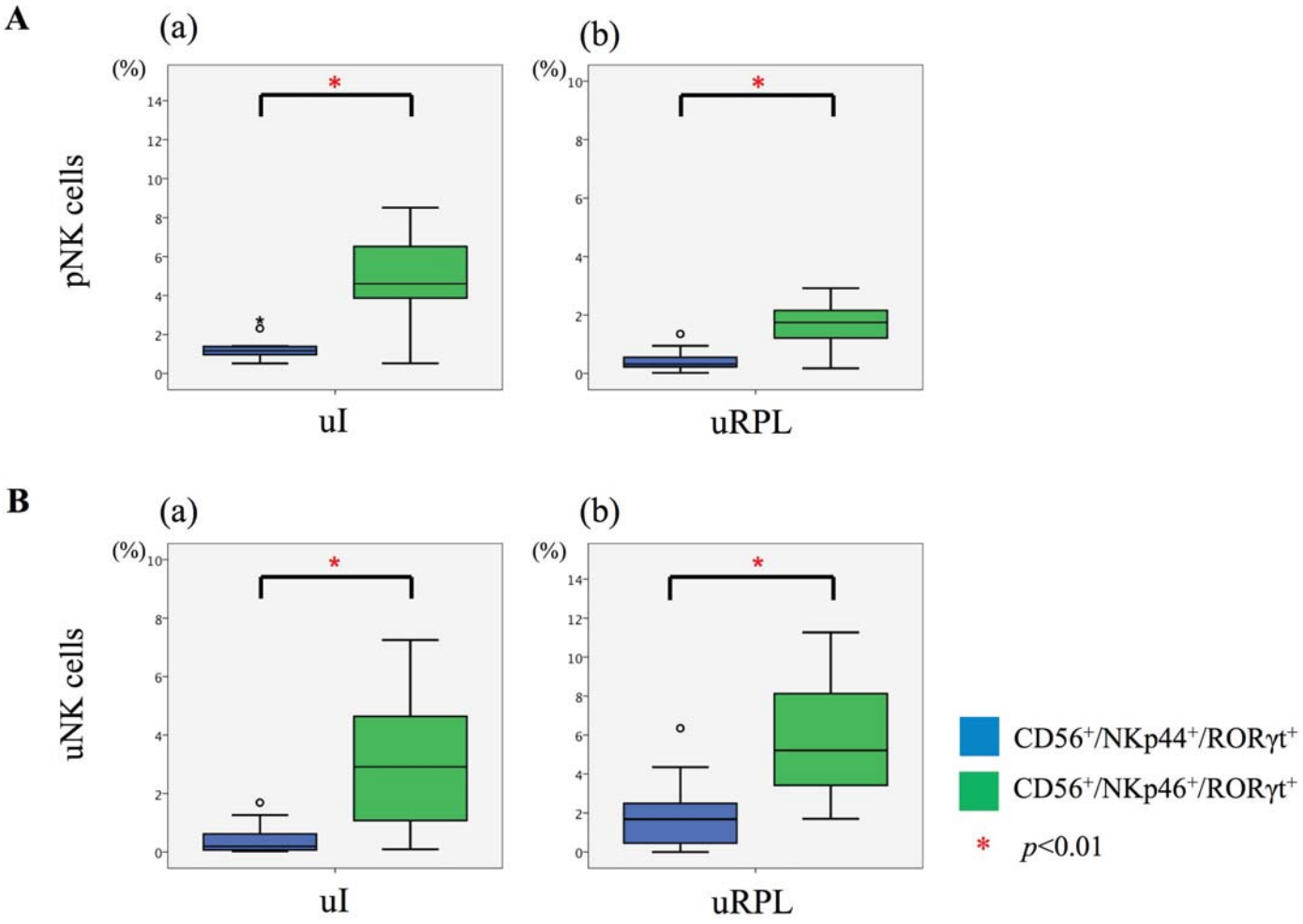
A
(a)
(b)
(%)
pNK cells
uI
uRPL
B
uNK cells
CD56+/NKp44+/RORγt+
CD56+/NKp46+/RORγt+
* p<0.01

Figure. 3

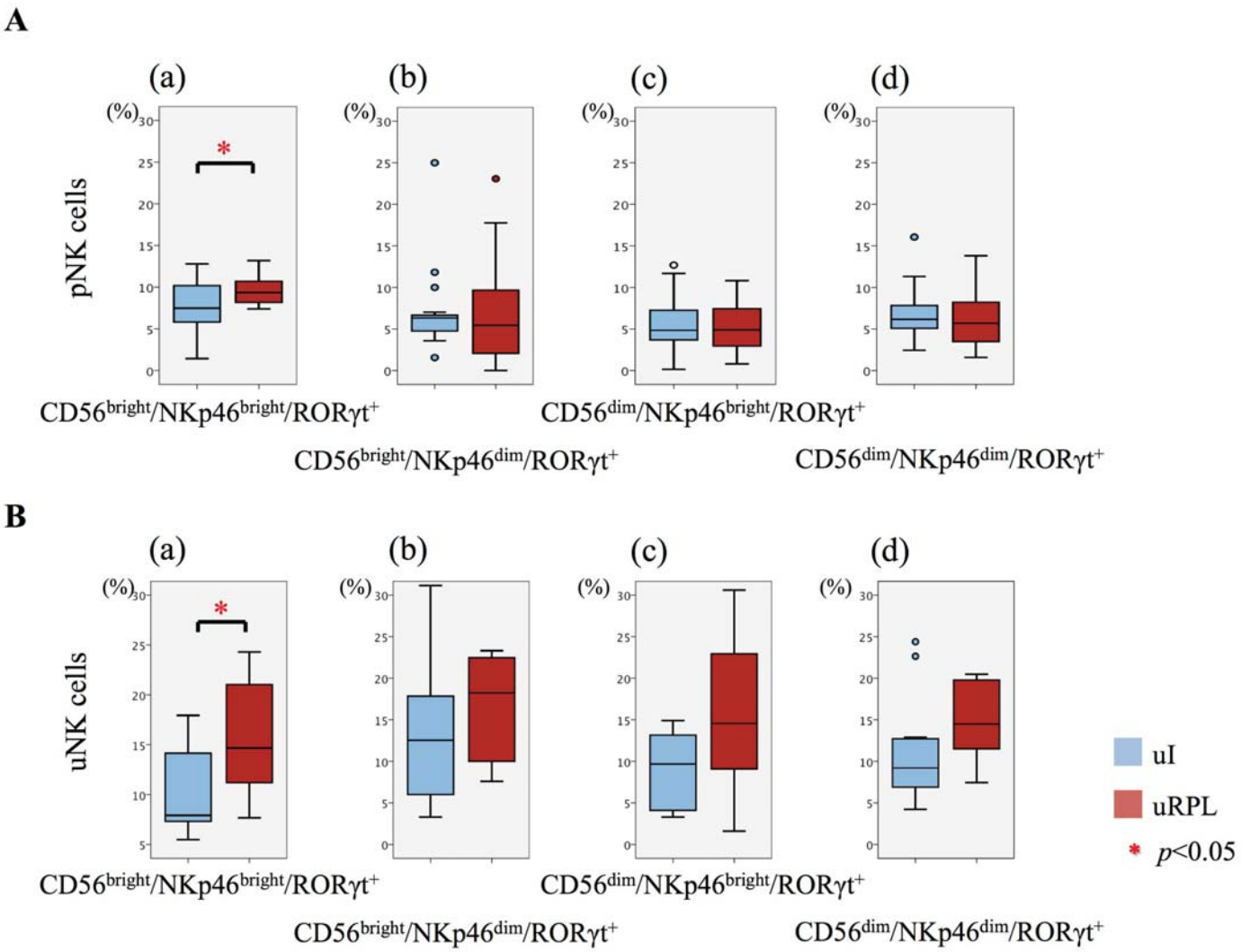
A
(a)
(b)
(c)
(d)
(%)
pNK cells
*
CD56bright/NKp46bright/RORγt+
CD56dim/NKp46bright/RORγt+
CD56bright/NKp46dim/RORγt+
CD56dim/NKp46dim/RORγt+
B
uNK cells
uI
uRPL
* p<0.05

Figure. 4

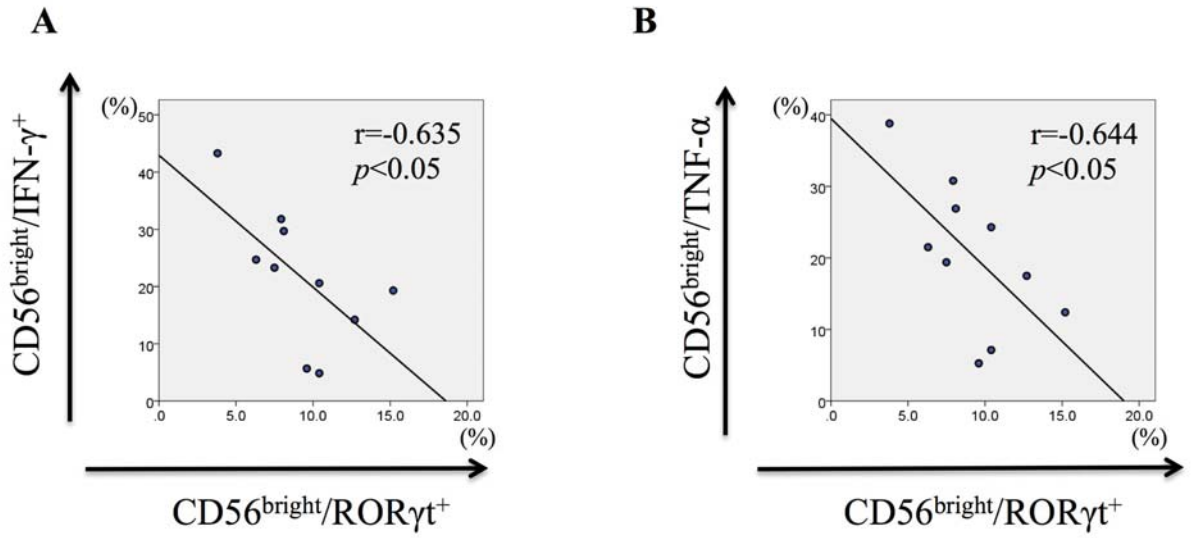
A
CD56bright/IFN-γ+
(%)
50
40
30
20
10
0
.0
5.0
10.0
15.0
20.0
(%)
r=-0.635
p<0.05
CD56bright/RORγt+
B
CD56bright/TNF-α
(%)
40
30
20
10
0
.0
5.0
10.0
15.0
20.0
(%)
r=-0.644
p<0.05
CD56bright/RORγt+

Figure. 5