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# Role of Uterine NK Cell Activation Markers NKp46 and NKG2D in Endometriosis-Related Infertility

Alfin Firasy Mufid [1, 2], Fadhil Ahsan [1, 3*], Budi Santoso [1*], Nanda Yuli Rahmawati [2], Fidyah Nanda Alditia [4]

*1- Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*
*2- Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*
*3- Department of Obstetrics and Gynecology, University Hospital Würzburg, University of Würzburg, Würzburg, Germany*
*4- Department of Internal Medicine, Elbe-Elster Klinikum, Finsterwalde, Germany*

## Abstract

**Background:** Endometriosis, affecting 10–40% of infertile women, is associated with endometrial immune dysregulation. Uterine natural killer (uNK) cells play pivotal roles in implantation and endometrial immune regulation. However, the differential expression of the activating receptors NKp46 and NKG2D between $CD56^{bright}$ and $CD56^{dim}$ uNK cell subsets in endometriosis-associated infertility remains poorly characterized. This study investigated these receptor profiles in infertile women with endometriosis.

**Methods:** Endometrial biopsies from 14 women with endometriosis and 21 controls were analyzed by flow cytometry. Disease severity was assessed using the revised American Society for Reproductive Medicine (rASRM) classification. Group comparisons and correlations were evaluated using appropriate statistical tests, with $p<0.05$ considered significant.

**Results:** $NKp46^{+}$ $NKG2D^{-}$ and $NKp46^{-}$ $NKG2D^{-}$ populations were significantly increased in both uNK cell subsets in women with endometriosis compared with controls. In the $CD56^{dim}$ compartment, both populations increased significantly ($p=0.012$ and $p=0.030$, respectively), with comparable increases in $CD56^{bright}$ cells ($p=0.003$ and $p=0.023$). In stage I–II endometriosis, $NKp46^{-}$ $NKG2D^{-}$ populations were significantly increased in both subsets, whereas no significant differences were observed in stage III–IV disease. Additionally, $CD56^{+}$ $NKp46^{+}$ $NKG2D^{-}$ cells showed a significant inverse correlation with the rASRM score.

**Conclusion:** Altered uNK cell phenotypes, particularly those involving NKp46 expression, characterize the eutopic endometrium in endometriosis-associated infertility. Both $CD56^{bright}$ and $CD56^{dim}$ uNK cell subsets showed increased NKp46+ populations, particularly in early-stage endometriosis. These subset-specific changes suggest immune dysregulation that may affect NK-cell cytotoxicity and reproductive function. Larger cohorts with integrated functional assays are needed to clarify the biological and clinical significance of these receptor alterations.

** Corresponding Authors:*
Fadhil Ahsan, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

Budi Santoso, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
*E-mail:*
fadhil.ahsan@fk.unair.ac.id, budi.santoso@fk.unair.ac.id

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## Introduction

Endometriosis is a chronic gynecological condition characterized by the presence of endometrial-like tissue outside the uterine cavity, leading to pelvic pain, dysmenorrhea, and infertility (1). It affects approximately 10% of women of reproductive age and up to 45% of

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women with infertility, making it a significant public health concern (1, 2). Despite extensive research, the exact pathogenesis of endometriosis remains unclear, though it is believed to involve a combination of genetic, hormonal, immunological, and environmental factors (3). One of the critical aspects of endometriosis-related infertility is impaired endometrial receptivity, which is essential for successful embryo implantation (2-4). The endometrium, the inner lining of the uterus, undergoes cyclic changes regulated by ovarian hormones to become receptive to an embryo during a specific window known as the "window of implantation" (5, 6). In women with endometriosis, this process is often disrupted, leading to implantation failure and infertility (2-4, 7).

The immune system, particularly uterine natural killer (uNK) cells, plays a crucial role in establishing and maintaining endometrial receptivity (6, 8, 9). uNK cells are a unique subset of NK cells found in the endometrium, where they contribute to tissue remodeling, angiogenesis, and immune tolerance during early pregnancy (8, 9). These cells are characterized by the expression of CD56, NKp46, and NKG2D, among other markers (9). CD56 is an adhesion molecule that distinguishes two main subsets of NK cells, $CD56^{bright}$ and $CD56^{dim}$ (10). $CD56^{bright}$ uNK cells are primarily involved in cytokine production and tissue remodeling, whereas $CD56^{dim}$ NK cells are more cytotoxic (10, 11). NKp46 is a natural cytotoxicity receptor (NCR) that plays a pivotal role in the activation of NK cells, and NKG2D is an activating receptor that recognizes stress-induced ligands on target cells, mediating cytotoxic responses (11-13). Studies have shown that the number and activity of uNK cells are altered in women with endometriosis, potentially contributing to the impaired endometrial receptivity observed in these patients (2, 4, 14).

Previous studies have demonstrated alterations in NK cell number, receptor expression, and function across different anatomical compartments in women with endometriosis, including the eutopic endometrium, peritoneal fluid, and peripheral blood (14-20). However, these findings are not directly interchangeable because these compartments possess distinct immunological environments and because previous studies have differed in the NK cell populations, receptors, and analytical methods examined. Studies of the eutopic endometrium have primarily evaluated the density or distribution of total $CD56^{+}$ uNK cells and/or NKp46 expression (15, 16, 18), whereas other studies have investigated NK cell receptor expression or NKG2D-related pathways in peripheral blood or peritoneal fluid (17, 19). Although $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets have been examined in peripheral blood in endometriosis, their receptor-specific phenotypic characterization within eutopic endometrial tissue remains largely unexplored.

Therefore, the purpose of the present study was to characterize both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets and assess NKp46 and NKG2D expression within each subset in eutopic endometrial tissue from infertile women with and without endometriosis. To our knowledge, this is among the first studies to provide simultaneous subset-specific and receptor-specific phenotyping of eutopic endometrial NK cells using flow cytometry.

## Methods

***Study design:*** This observational study included 35 women of reproductive age with primary or secondary infertility who underwent diagnostic laparoscopy. Fourteen women had laparoscopically confirmed endometriosis, including 6 women with rASRM stage I–II disease and 8 women with rASRM stage III–IV disease. Twenty-one infertile women without laparoscopic evidence of endometriosis served as controls. Ethical approval was granted by the Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia (No. 129/EC/KEPK/FKUA/2020) on June 8, 2020, and all participants provided written informed consent before enrollment. Participants were recruited consecutively during the recruitment period and were included if they met the predefined inclusion and exclusion criteria. As this was an exploratory observational study and all eligible participants available during the recruitment period were included, no formal a priori power calculation was performed. Medical histories and physical examinations were conducted to confirm eligibility according to the criteria. Confidentiality and data anonymity were maintained throughout the study, with all identifying information replaced by unique codes. The study was conducted at the Kendangsari Hospital and Institute of Tropical Disease, Airlangga University, Surabaya, between September 2020 and February 2021, following the principles of the Declaration of Helsinki.

Women of reproductive age (18-45 years) diagnosed with primary or secondary infertility were

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included in the study. The endometriosis group consisted of women diagnosed with endometriosis based on laparoscopic examination. The control group included infertile women undergoing laparoscopy for other benign gynecological conditions, such as ovarian cysts or tubal factor infertility, without any evidence of endometriosis. Exclusion criteria were pregnancy, smoking, endocrine disorders (*e.g.*, thyroid disease, diabetes mellitus), gastrointestinal diseases, mixed gynecological pathologies, and unexplained infertility.

The extent of endometriosis was assessed using the revised American Society for Reproductive Medicine (rASRM) classification, which allowed for standardized grading of the disease severity (21). Endometrial tissue samples were collected during the secretory phase of the menstrual cycle (days 21-24) using a Pipelle sampler. This phase was chosen to ensure consistency in the menstrual cycle phase among participants, which is critical for accurately assessing endometrial receptivity. The collected samples were immediately placed in a transport medium and processed for flow cytometry analysis.

***Sample processing and flow cytometry analysis:*** Endometrial tissue samples were processed to obtain a single cell suspension. Briefly, endometrial samples were collected and minced into small fragments. The tissue fragments were enzymatically digested using a collagenase and DNase I solution at 37°*C* for 30-60 *min* with gentle agitation. Post digestion, the cell suspension was mechanically dissociated by pipetting and filtered through a 70 $\mu m$ cell strainer to remove debris. The filtered suspension was centrifuged at 300 *g* for 10 *min*, and the cell pellet was resuspended in PBS. Red blood cells were lysed using an ammonium chloride-based red blood cell lysis buffer. Cell viability, assessed with trypan blue in a hemocytometer, was consistently above 90%.

***Flow cytometry analysis:*** The cells were stained with fluorochrome-conjugated antibodies against CD3, CD56, NKp46, and NKG2D. Specifically, the following antibodies were used: PerCP anti-human CD3 antibody (Clone: OKT3, Cat. No. 317335), APC anti-human CD56 (NCAM) antibody (Clone: HCD56, Cat. No. 318309), PE anti-human CD335 (NKp46) antibody (Clone: 9E2, Cat. No. 331908), and FITC anti-human CD314 (NKG2D) antibody (Clone: 1D11, Cat. No. 320806) (BioLegend, USA). Flow cytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences, USA), and data were analyzed with FlowJo software. Compensation controls and isotype controls ensured accurate data analysis. Data were acquired using CellQuest software (BD Biosciences, USA) and analyzed with FlowJo software. The analysis followed a sequential gating strategy, and NK cells were identified as $CD3^-$ $CD56^+$ events after sequential lymphocyte and singlet gating. Within this population, $CD56^{bright}$ and $CD56^{dim}$ subsets were distinguished based on CD56 fluorescence intensity. Expression of NKp46 and NKG2D was evaluated within each CD56 subset using a four-quadrant analysis. Because the study included multiple samples and several subgroup-specific analyses, a representative gating strategy is provided to illustrate the analytical workflow, while the statistical graphs display the quantitative results from all analyzed participants.

***Statistical analysis:*** Data normality was assessed using the Shapiro-Wilk test. Based on the distribution, either a parametric (independent t-test) or a nonparametric (Mann-Whitney U test) test was applied. Correlation analysis was conducted with Spearman's rank correlation coefficient (ρ) for two continuous variables. Statistical significance was set at $p<0.05$.

## Results

The demographic characteristics of the study participants are summarized in table 1. Of the 35 subjects, the median age of patients in the endometriosis group was 29 years (range 24-37), while the control group had a median age of 30 years (range 25-41). The majority of patients in both groups had primary infertility, with 11 in the endometriosis group and 18 in the control group. Secondary infertility was present in 3 patients in each group. Notably, dysmenorrhea was more frequently reported in the control group (14 patients) compared to the endometriosis group (4 patients).

The investigation into NK cell populations within the context of endometriosis provides valuable

**Table 1.** Demographic characteristics of study participants

| Demographic characteristics | Endometriosis (n=14) | Control (n=21) |
|---|---|---|
| Median age (years, range) | 29 (24-37) | 30 (25-41) |
| Primary infertility (n) | 11 | 18 |
| Secondary infertility (n) | 3 | 3 |
| Dysmenorrhea (n) | 4 | 14 |

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insights into the disease's immune landscape. Flow cytometry was used to quantify $CD56^{+}$ expression, an NK cell marker, and to distinguish two $CD3^{-}$ lymphocytic cell subsets, namely, $CD56^{bright}$ and $CD56^{dim}$. $CD56^{dim}$ and $CD56^{bright}$ cells were significantly different between endometriosis and control cases with p=0.049 and p=0.028, respectively (Figure 1A and 1B). Comparing CD56 expression among endometriosis stages I–II, III–IV, and controls, both $CD56^{dim}$ and $CD56^{bright}$ cell frequencies were significantly higher in early endometriosis (stage I/II) compared to controls (p=0.029 and p=0.032, respectively), but no significant differences were found for stage III–IV (Figure 1C and 1D). Advanced endometriosis (stage III–IV) did not significantly differ from controls in the percentage of $CD56^{dim}$ or $CD56^{bright}$ cells.

Scatter plots show the percentages of $CD56^{dim}$ and $CD56^{bright}$ cells in the endometriosis and control groups (A–B) and in the early (stage I–II), advanced (stage III–IV), and control groups (C–D). Medians and interquartile ranges are displayed. The p-values are indicated, and each dot represents one subject.

Next, NKp46 and NKG2D expression patterns were evaluated within the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets (Figure 2). In the $CD56^{dim}$ compartment, there were no significant differences between the endometriosis and control groups in $NKp46^{+}$ $NKG2D^{+}$ (p=0.152) (Figure 2A) and $NKp46^{-}$ $NKG2D^{+}$ (p=0.0816) (Figure 2C). However, the populations of $CD56^{dim}$ $NKp46^{+}$ $NKG2D^{-}$ and $NKp46^{-}$ $NKG2D^{-}$ were significantly higher in the endometriosis group compared to controls (p=0.0115, p=0.0300, respectively) (Figures 2B and 2D), indicating an altered NK cell receptor phenotype. Additionally, within the $CD56^{bright}$ compartment, both the $NKp46^{+}$ $NKG2D^{-}$ and $NKp46^{-}$ $NKG2D^{-}$ populations were also significantly increased in the endometriosis group relative to controls (p=0.0027, p=0.0224, respectively) (Figures 2F and 2H). No significant differences were observed for the remaining expression patterns, namely $NKp46^{+}$ $NKG2D^{+}$ (p=0.1100, Figure 2E) and $NKp46^{-}$ $NKG2D^{+}$ (p=0.4645, Figure 2G).

The scatter plots depict the percentages of $CD56^{dim}$ (A–D) and $CD56^{bright}$ (E–H) cells expressing four distinct receptor phenotypes, $NKp46^{+}$ $NKG2D^{+}$, $NKp46^{+}$ $NKG2D^{-}$, $NKp46^{-}$ $NKG2D^{+}$, and $NKp46^{-}$ $NKG2D^{-}$, in endometriosis patients and controls. Medians and interquartile ranges are shown. The p-values are indicated for each comparison, and each dot represents one subject.

Since several NK cell receptor phenotypes varied between the endometriosis and control groups,

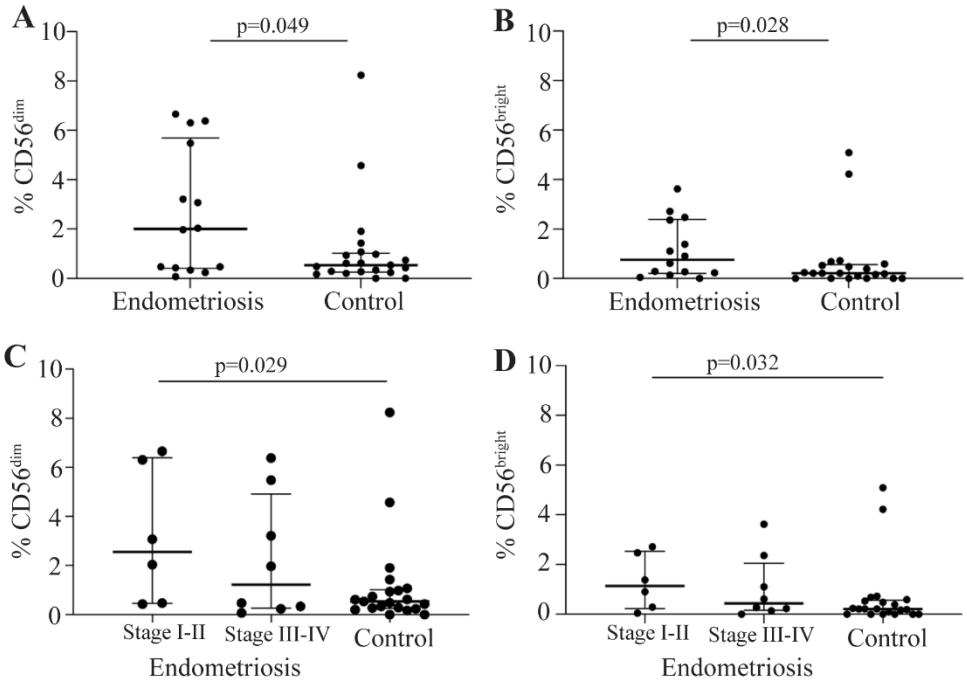



**Figure 1.** $CD56^{bright}$ and $CD56^{dim}$ uterine NK cell populations in endometriosis and control groups

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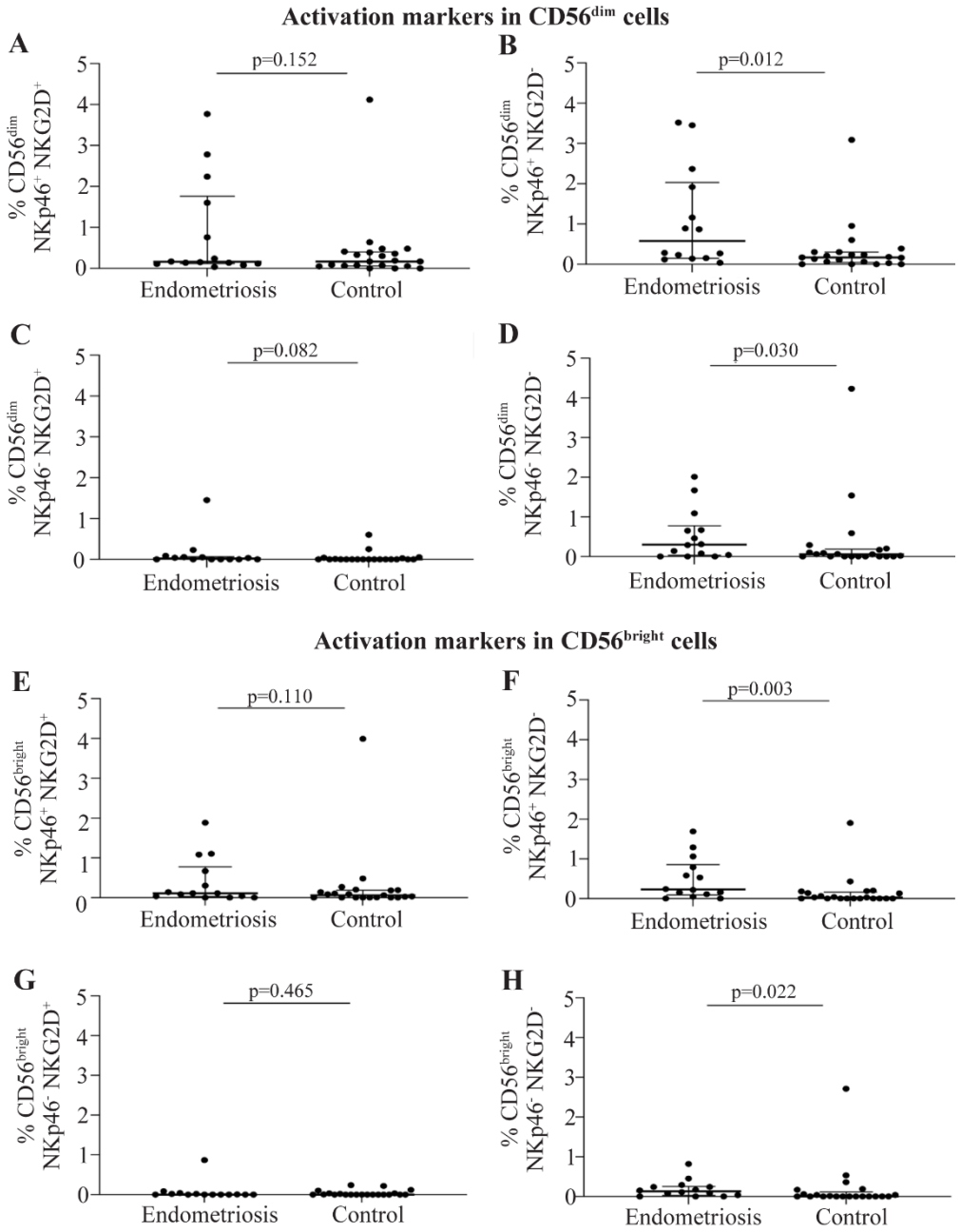



**Figure 2.** NKp46 and NKG2D receptor phenotypes within CD56$^{dim}$ and CD56$^{bright}$ uterine NK cell subsets in endometriosis and control groups

these populations were examined based on endometriosis stage (Figure 3). For CD56$^{dim}$ cells, the frequencies of NKp46$^{+}$ NKG2D$^{-}$ and NKp46$^{-}$ NKG2D$^{-}$ populations were significantly higher in patients with stage I–II endometriosis compared to controls (p=0.005 and p=0.032, respectively; Figures 3B and 3D), whereas no significant difference was observed between stage III–IV and controls. The remaining subsets, NKp46$^{+}$ NKG2D$^{+}$ (p=0.169, Figure 3A) and NKp46$^{-}$ NKG2D$^{-}$ (p=0.282, Figure 3C), showed no significant differences. For CD56$^{bright}$ cells, the frequencies of NKp46$^{+}$ NKG2D$^{-}$ and NKp46$^{-}$ NKG2D$^{-}$ populations were significantly higher in patients with stage I–II endometriosis compared to controls (p=0.001 and p=0.046, respectively; Figures 3F and 3H), whereas no significant difference was observed between stage III–IV and controls. The remaining subsets, NKp46$^{+}$ NKG2D$^{+}$ (p=0.286) and NKp46$^{-}$ NKG2D$^{+}$ (p=0.989), showed no sig-

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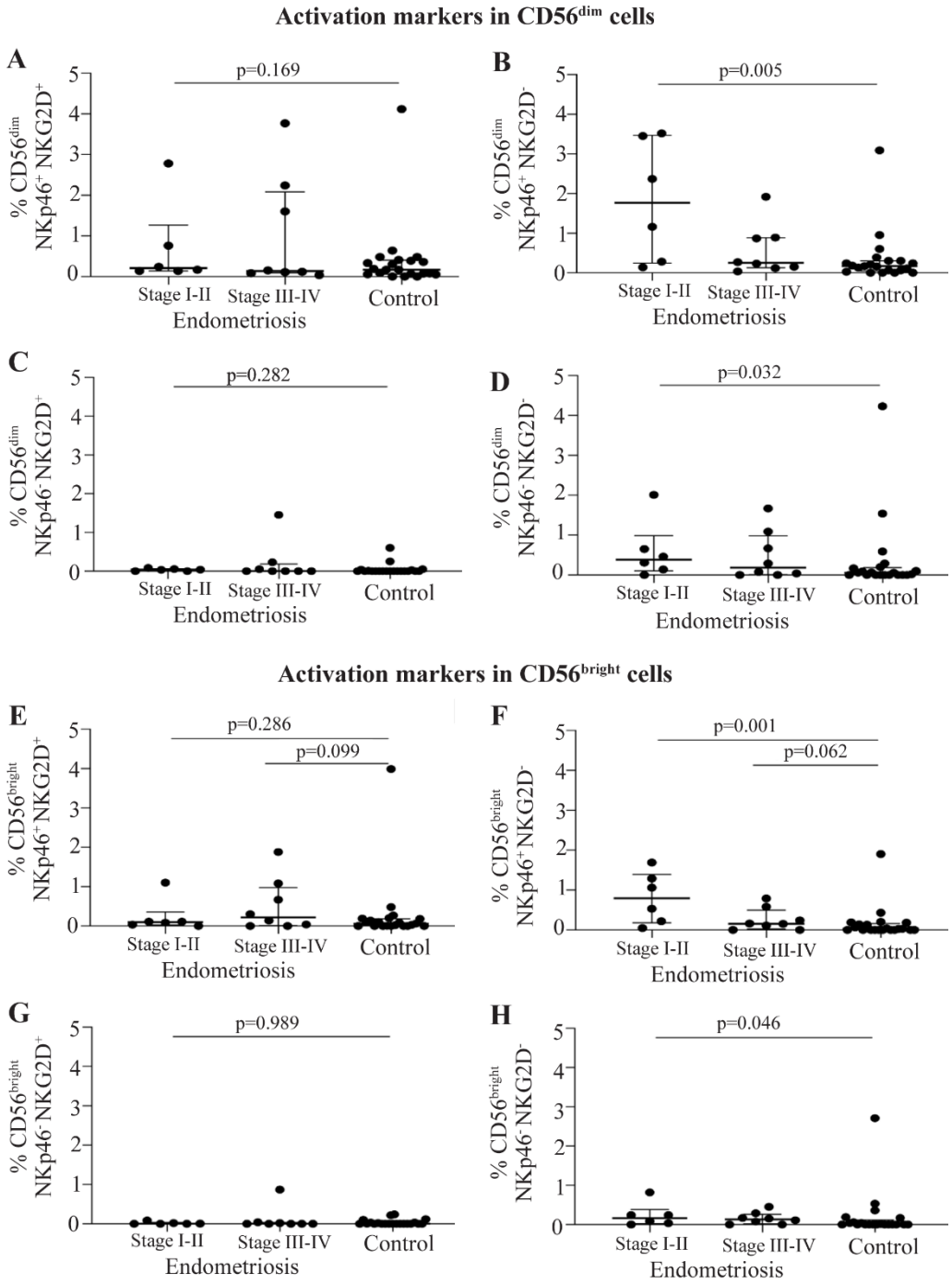



**Figure 3.** NKp46 and NKG2D receptor phenotypes within CD56$^{dim}$ and CD56$^{bright}$ uterine NK cell subsets across endometriosis stages

nificant differences (Figures 3E and 3G).

The scatter plots illustrate the percentages of CD56$^{dim}$ (A–D) and CD56$^{bright}$ (E–H) cells exhibiting different activation phenotypes (NKp46$^{+}$ NKG2D$^{+}$, NKp46$^{+}$ NKG2D$^{-}$, NKp46$^{-}$ NKG2D$^{+}$, and NKp46$^{-}$ NKG2D$^{-}$) in early (I–II), advanced (III–IV) endometriosis, and control groups. Medians and interquartile ranges are indicated. The p-values are shown for groups, and each dot represents one subject. The relationship between NK cell activation and the total rASRM score, along with endometriosis and adhesion subscores, was further investigated. Among all activation marker combinations shown in figure 4A-4H, a signifi-

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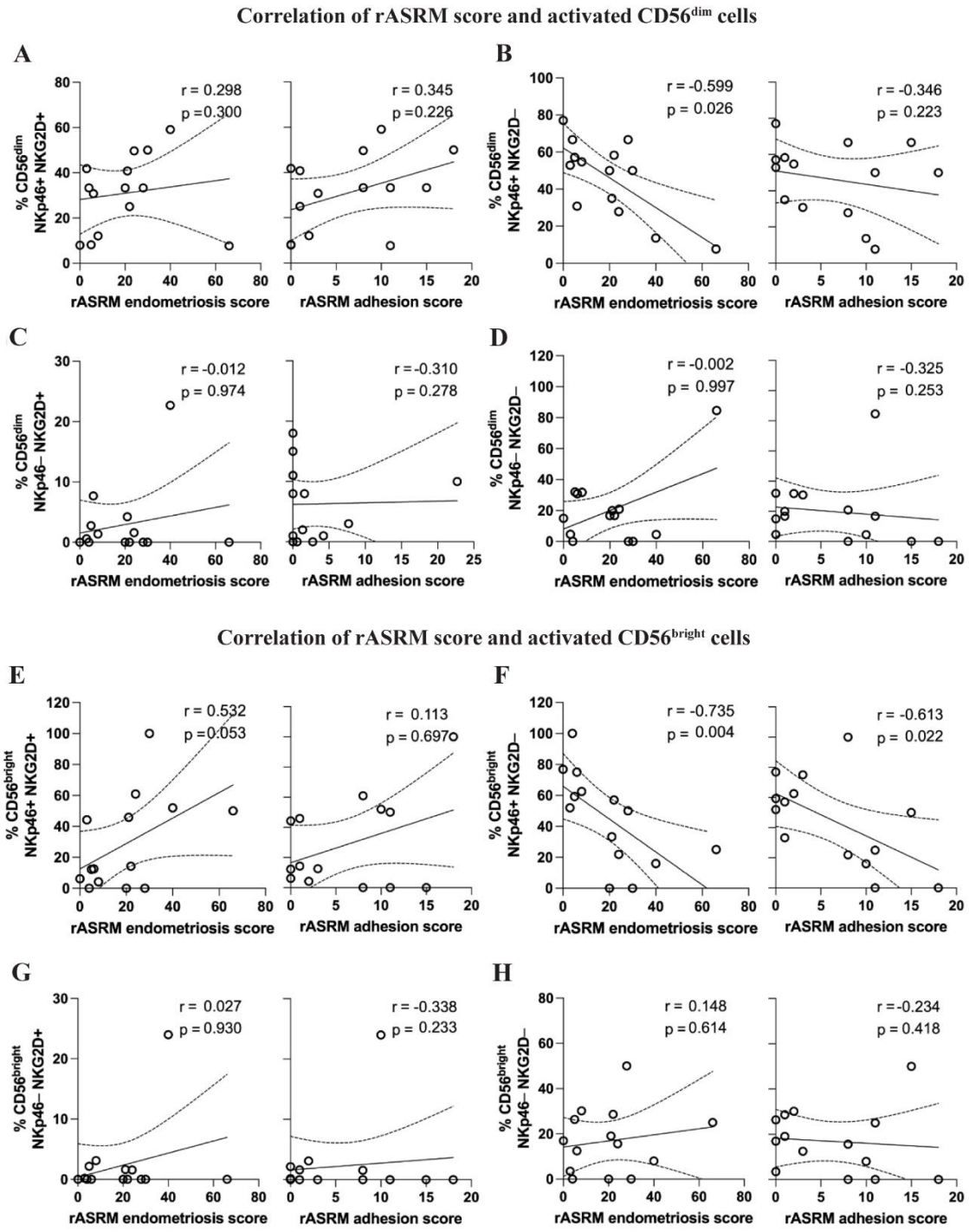



**Figure 4.** Spearman **co**rrelation between rASRM **s**cores and NK cell receptor phenotypes among endometriosis cases

cant inverse correlation was found between the rASRM endometriosis score and the percentage of $CD56^{dim}$ $NKp46^{+}$ $NKG2D^{-}$ cells (r=-0.599, p= 0.026), while no significant correlation was observed between this cell population and the rASRM adhesion score (Figure 4B). Additionally, there were no significant correlations between other activation states of $CD56^{dim}$ cells and rASRM scores. For $CD56^{bright}$ cells, $NKp46^{+}$ $NKG2D^{-}$ cells (Figure 4F) showed significant inverse correlations with both the rASRM endometriosis and adhesion scores (r=-0.735, p=0.004; r=

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-0.613, p=0.022, respectively). Furthermore, although CD56$^{bright}$ NKp46$^{+}$ NKG2D$^{+}$ cells showed a trend toward a positive correlation with the rASRM endometriosis score, this was not statistically significant (r=0.532, p=0.053) (Figure 4E). No significant correlations were found between the other two CD56$^{bright}$ cell populations (NKp46$^{-}$ NKG2D$^{+}$ and NKp46$^{-}$ NKG2D$^{-}$) and rASRM scores (Figures 4G and 4H).

Scatter plots show the correlations between rASRM endometriosis and adhesion scores and the percentages of CD56$^{dim}$ (A–D) and CD56$^{bright}$ (E–H) cells with distinct activation phenotypes (NKp46$^{+}$ NKG2D$^{+}$, NKp46$^{+}$ NKG2D$^{-}$, NKp46$^{-}$ NKG2D$^{+}$, and NKp46$^{-}$ NKG2D$^{-}$). Spearman's correlation coefficients and p-values are displayed and each dot represents one endometriosis case.

## Discussion

This study offers novel insights into immune dysregulation in endometriosis by focusing on a subset-specific analysis of NK cell receptor phenotypes in the eutopic endometrium. Our study uniquely provides a detailed analysis of the differential expression of two activation markers, NKp46 and NKG2D, in NK cell subsets (CD56$^{bright}$ and CD56$^{dim}$) in infertile women. The present study thus provides a more detailed characterization of local endometrial NK cell alterations than analyses of total CD56$^{+}$ NK cells alone. The significant findings of heightened NKp46 expression in early-stage endometriosis (stage I-II), particularly in both CD56$^{dim}$ and CD56$^{bright}$ NK cells, underscore the importance of early immune changes in disease progression. This novel focus on NKp46-mediated activation provides a new perspective on the immune mechanisms that may contribute to the pathophysiology of endometriosis and its associated infertility.

Our findings support the broader concept that endometrial NK cell populations and receptor phenotypes are altered in endometriosis, although the direction and nature of these changes vary between studies. Giuliani et al. reported alterations in uNK cell populations and NKp46-associated phenotypes in eutopic endometrial tissue from women with infertility and endometriosis (15). In contrast, studies of peritoneal fluid have reported alterations in natural cytotoxicity receptors, including NKp30 and NKp46 (16), whereas other studies have described changes in NKG2D and NKG2D-ligand pathways (17, 19). Circulating CD56$^{+}$ NKG2D$^{+}$ NK cell populations have also been associated with endometriosis and postoperative fertility outcomes (19), and reduced peripheral NK cell cytotoxicity has been reported in women with a history of endometriosis (20). These findings are complementary but cannot be directly equated with the present results because they concern different anatomical compartments and, in some cases, different methodological approaches. The present study extends this literature by simultaneously characterizing CD56$^{bright}$ and CD56$^{dim}$ NK cell subsets and evaluating NKp46 and NKG2D expression within each subset directly in eutopic endometrial tissue. This approach permits identification of subset-specific receptor phenotypes that may be obscured when total CD56$^{+}$ NK cells were analyzed as a single population.

The current findings complement and differ from previous immunohistochemical studies. These studies mainly assessed the density, location, or overall tissue staining of CD56$^{+}$ uNK cells and NKp46 (15, 18). In contrast, flow cytometry allows for detailed single-cell analysis of CD56$^{bright}$ and CD56$^{dim}$ NK cell subsets and the expression of NKp46 and NKG2D. Differences across studies may be due to biological heterogeneity or variations in sampling, disease stage, menstrual cycle, tissue processing, patient traits, marker choices, and analysis methods. Notably, the higher frequency of NKp46$^{+}$ NKG2D$^{-}$ populations in our cohort should not be viewed as contradictory to earlier studies demonstrating reduced NKp46 staining or altered NKp46/CD56 ratios, as these measures do not reflect the same biological phenomena. Tissue immunohistochemistry and subset-specific flow cytometry offer complementary insights into uNK cell biology.

NK cells are the main CD56-expressing lymphocytes and are broadly classified into two subsets based on CD56 density, CD56$^{dim}$ and CD56$^{bright}$, which exhibit distinct phenotypic and functional properties (11, 12, 22). In peripheral blood, approximately 90% of human NK cells are CD56$^{dim}$, while 10% are CD56$^{bright}$ (12, 23); this distribution pattern observed in peripheral blood should not be extrapolated to the endometrium, where uNK cells are tissue-resident populations that fluctuate with endometrial physiology and differ from circulating NK cells (22, 24). NK cell populations increase during the menstrual cycle, peaking during decidualization in early pregnancy (22, 24). The proliferation of CD56$^{bright}$ NK cells is associated with critical functions in implantation, angiogene-

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sis, and pregnancy maintenance (11, 24-26). $CD56^{bright}$ NK cells are primarily involved in producing pro-inflammatory cytokines, while $CD56^{dim}$ NK cells are more cytotoxic (11, 26), which define their significant role in immune regulation. The $CD56^{bright}$ findings are particularly relevant to the endometrial environment because $CD56^{bright}$ uNK cells are abundant in the endometrium and participate in cytokine production, tissue remodeling, vascular regulation, and immune modulation. In the present study, the $CD56^{bright}$ $NKp46^{+}$ $NKG2D^{-}$ population was increased in early-stage endometriosis and showed inverse associations with both rASRM endometriosis and adhesion scores. These findings raise the possibility that altered receptor phenotypes within the $CD56^{bright}$ compartment may be associated with early local immune dysregulation. However, the present study did not directly measure cytokine production, angiogenic activity, tissue remodeling, or endometrial receptivity. Therefore, the observed $CD56^{bright}$ phenotype should be considered a candidate marker of local immune dysregulation rather than direct evidence of impaired $CD56^{bright}$ NK cell function.

Leukocyte populations in the endometrium play pivotal roles in cellular regeneration and secretion processes during the menstrual cycle (27). Disruptions in these leukocyte populations can lead to uterine dysfunctions, including infertility (27). Endometriosis contributes to infertility through chronic inflammatory processes that impair oocyte function and cause defects in the endometrium, hindering implantation and decidualization (2, 28). Inflammatory conditions lead to decreased progesterone receptor expression, resulting in progesterone resistance and increased estrogen levels due to aromatase P450 expression in the endometrium of women with endometriosis (29).

Endometriosis is associated with a chronic inflammatory state in the pelvic cavity (1, 2). This inflammation results in an altered cytokine milieu, characterized by elevated levels of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β (30, 31). These cytokines can influence the activation and function of uNK cells by promoting their proliferation and enhancing their cytotoxic activity (20). The study indicates a significant increase in $NKp46^{+}$ uNK cells in women with endometriosis, particularly in early stages (stage I-II). NKp46 is a natural cytotoxicity receptor that plays a pivotal role in activating NK cells and mediating their cytotoxic response (12, 13). The upregulation of NKp46 in endometriosis suggests that these cells are in a heightened state of activation, potentially contributing to the inflammatory environment observed in endometriosis. Increased NKp46 expression may be influenced by hormonal, genetic, and inflammatory factors. Estrogen, which is often elevated in endometriosis, can enhance NK cell cytotoxic activity, while progesterone typically has an immunosuppressive effect (32, 33). Genetic predispositions, such as polymorphisms in NK cell receptors, may also contribute to increased NKp46 expression (18). Inflammatory cytokines in the endometriotic environment can further drive NK cell activation and NKp46 expression (34). These factors collectively suggest that heightened NKp46 expression in endometriosis may contribute to both enhanced immune surveillance and increased tissue damage through cytotoxic mechanisms.

Our results align with previous studies showing increased NK cell activity in endometriosis. Giuliani et al. reported increased NK cell activation in the endometrial stroma of women with endometriosis and unexplained infertility (15). Similarly, Parkin and Fazleabas observed heightened NK cell activity in endometriosis, potentially impairing endometrial receptivity and embryo implantation (8). The significant increase in NKp46 expression on both $CD56^{bright}$ and $CD56^{dim}$ NK cells in women with endometriosis indicates a heightened activation state, contributing to the chronic inflammatory environment observed in endometriosis. In contrast, the reports by Funamizu et al. and Xu showed that NKp46 expression in peritoneal NK cells is lower in endometriosis cases than in controls, highlighting differences in regulation between the ectopic and eutopic endometrial compartments (16, 17). It is speculated that eutopic endometrium in endometriosis cases is more cytotoxic than in peritoneal environment, leading to reduced endometrial receptivity while maintaining the growth of ectopic implant.

Our study did not find significant differences in NKG2D expression between the endometriosis and control groups, which is in agreement with a previous report (17). NKG2D is an essential activating receptor that recognizes stressed cells and initiates immune responses (35). The lack of significant change in our study suggests that NKp46-mediated activation might not play a dominant role in the context of eutopic endometrium in endometriosis. Interestingly, previous reports showed that soluble NKG2D levels and circulating

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$CD56^+$ $NKG2D^+$ cells are significantly higher in patients with endometriosis compared to controls, suggesting a systemic role for this molecule in the disease (36, 37). The mixed results in systemic and local NKG2D expression warrants further investigation. Understanding the regulatory mechanisms underlying NK cell activation in endometriosis could provide deeper insights into the disease's pathophysiology.

Our analysis also explored the correlation between NK cell activation and the severity of endometriosis. Significant increases in $NKp46^+$ NK cells were observed in early-stage endometriosis (stage I-II) compared to controls, but not in advanced stages (stage III-IV). This suggests that NK cell activation may be more critical in the early stages of endometriosis, potentially contributing to the initial establishment and progression of the disease. A previous study by Giuliani et al. revealed that elevated populations of $CD56^+$ $NKp46^+$ cells may be at greater risk for reproductive disorders resulting from an inflammatory environment during implantation (15). The altered activation status of NK cells, particularly the increased expression of NKp46, highlights potential therapeutic targets (38). Modulating NK cell activity could help restore immune balance in the endometrium and improve fertility outcomes in women with endometriosis (15). Future studies should focus on the functional consequences of altered NK cell activation and explore potential interventions that could mitigate these effects.

Our findings also provide a nuanced perspective on NK cell involvement in endometriosis, particularly highlighting the differential roles of NK cell subsets. Previous studies have reported heterogeneous alterations in NK cell number, receptor expression, and function in endometriosis, rather than a uniform increase in NK cell activity across all anatomical compartments (14-20). In the present study, the $CD56^{dim}$ $NKp46^+$ $NKG2D^-$ population showed an inverse correlation with the rASRM endometriosis score, suggesting that this phenotype was more prominent in early-stage disease and decreased with increasing disease severity. Additionally, the significant inverse correlations observed in $CD56^{bright}$ $NKp46^+$ $NKG2D^-$ cells with both endometriosis and adhesion scores indicate that these cells may have a more direct role in modulating disease severity than previously thought. The absence of significant group-level differences in NKG2D-positive populations in the present eutopic endometrial samples, despite previous reports of alterations in circulating $NKG2D^+$ NK cells or soluble NKG2D-related ligands (19), further underscores the compartment-specific nature of NK cell dysregulation in endometriosis. Systemic or soluble alterations in the NKG2D axis may therefore not necessarily be reflected by surface NKG2D expression in eutopic endometrial NK cell subsets.

The limitations of the current study should be underlined. First, the sample size, while adequate for initial observations, limits the generalizability of the findings. Larger, multicenter studies are needed to validate these results and explore the clinical implications of NK cell alterations in endometriosis. Second, the cross-sectional design precludes the assessment of temporal changes in NK cell activity, which could provide valuable insights into the progression of endometriosis. Third, the study did not directly assess the functional consequences of NK cell phenotypes, including cytotoxicity, degranulation, cytokine production, or tissue remodeling. Finally, the use of tissue dissociation for flow-cytometric analysis provides detailed single-cell phenotyping but does not preserve the spatial localization of NK cells within the endometrium. Larger multicenter studies integrating multiparameter flow cytometry, functional assays, and spatial tissue analysis are needed to validate and extend these findings.

## Conclusion

This study demonstrates subset-specific alterations in NK cell receptor phenotypes in the eutopic endometrium of infertile women with endometriosis. The $CD56^{bright}$ and $CD56^{dim}$ compartments showed distinct alterations in $NKp46^+$ $NKG2D^-$ populations, particularly in early-stage disease, whereas NKG2D-positive populations did not show significant overall group-level differences. A notable strength of this study is the simultaneous characterization of both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets together with NKp46 and NKG2D expression within eutopic endometrial tissue. This subset-specific approach provides a more refined assessment of local NK cell phenotypes than analysis of total $CD56^+$ NK cells alone. Collectively, these findings support the presence of subset-specific dysregulation of endometrial NK cells in endometriosis-associated infertility. Further research is needed to elucidate the mechanisms underlying these changes and their impact on fertility outcomes.

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## Conflict of Interest

The authors declare that they have no conflicts of interest.

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