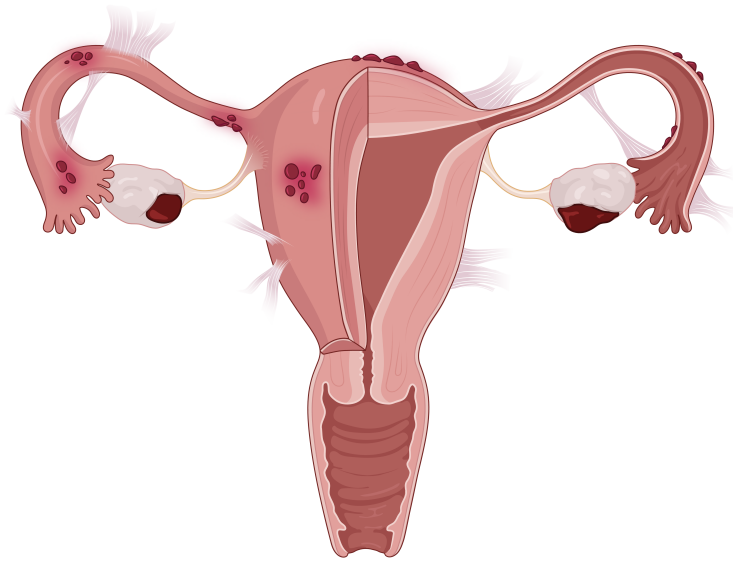




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Assessment of NK Cell Function in the Immune Landscape of Endometriosis

Master's thesis report in Biotechnology

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DEPARTMENT OF LIFE SCIENCES

CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2026
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MASTER'S THESIS REPORT 2026

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Cover image: Schematic illustration of the female reproductive system affected by endometriosis. Created with BioRender.com.

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Abstract

Endometriosis (EN) is a chronic inflammatory disease characterised by the growth of endometrium-like tissue outside the uterus. Altered immune responses, including impaired natural killer (NK) cell cytotoxicity, have been proposed to contribute to disease development. The aim of this study was therefore to assess NK cell function in EN and identify what NK cell receptor interactions contribute to NK cell degranulation and cytotoxic responses towards EN-derived cells. In parallel, protocols for immune cell isolation from EN samples were developed and optimised to support future phenotypic and functional characterisation. Healthy donor (HD) NK cells were co-cultured with EN-derived cultured cells (ENCCs) and their response assessed using a flow cytometry-based combined degranulation and cytotoxicity assay. Addition of antibodies targeting specific NK cell receptors or their ligands enabled the identification of key NK cell receptors involved in EN-induced NK cell activation. Complementary ligand expression on ENCCs was analysed using flow cytometry.

Our studies revealed ENCC-induced NK cell degranulation, demonstrating their ability to activate NK cells. Moreover, receptor-blocking experiments identified DNAM-1, NKG2D, and NKp46 as important contributors to this response. Ligand expression analyses supported the involvement of these activating receptor-ligand interactions, showing high expression of the PVR, Nectin-2, ULBP-2/5/6, and TRAIL-R2. High expression of HLA-ABC, which mainly mediates inhibitory signalling, was also observed on ENCCs. In conclusion, this study demonstrates that ENCCs can be recognised by NK cells through multiple activating receptor pathways and provides further insight into NK cell interactions in EN. The developed protocols for processing EN samples, furthermore, form a foundation for future studies within the EndoMIC project.

Keywords: endometriosis, natural killer cells, co-culture assay, flow cytometry, cytotoxicity, degranulation

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Gabriella Nilsson, Gothenburg, June 2026

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

EN	Endometriosis
ENCCs	Endometriosis-derived culture cells
E:T ratio	Effector-to-target ratio
FSC	Forward scattering
HD	Healthy donor
HLA	Human leukocyte antigen
ILCs	Innate lymphoid cells
KIRs	Killer immunoglobulin-like receptors
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK cells	Natural killer cells
OC	Ovarian cancer
PBMCs	Peripheral blood mononuclear cells
SSC	Side scattering
T _C cells	T cytotoxic cells
T _H cells	T helper cells
trNK cells	Tissue-resident natural killer cells

Contents

List of Acronyms	ix
List of Figures	xiii
List of Tables	xvii
1 Introduction	1
1.1 Purpose and aims	1
1.2 Limitations	2
1.3 Ethical and Societal Aspects	3
2 Theory	5
2.1 The Immune system	5
2.1.1 Three types of cell-mediated immunity	6
2.1.2 NK cell immunology	7
2.1.3 Tissue residency	8
2.2 Endometriosis	9
2.2.1 Prevalence, symptoms, and treatments	9
2.2.2 Postulated origins of endometriosis	10
2.2.3 The microenvironment of endometriosis lesions	11
2.2.4 Endometriosis and the immune system	12
3 Methodology	13
3.1 Culturing of mammalian cells	13
3.2 Flow cytometry	14
3.3 Functional assessment of NK cells	14
4 Methods	15
4.1 Patients and Samples	15
4.1.1 Blood samples	15
4.1.2 Peritoneal lavage samples	16
4.1.3 Endometrioma fluid samples	16
4.1.4 Endometriosis lesion samples	16
4.1.5 Flow cytometry acquisition and compensation	17
4.2 Cell cultures	17
4.3 Functional assays	17
4.3.1 NK cell isolation	18

4.3.2	Degranulation and cytotoxicity assay	18
4.3.3	Identification of ligands on target cells	21
4.4	Statistics	23
4.5	Use of AI tools	23
5	Results	25
5.1	Development of sample processing protocols	25
5.1.1	Blood samples	25
5.1.2	Peritoneal lavage samples	26
5.1.3	Endometrioma fluid samples	26
5.1.4	Tissue samples	27
5.1.5	Flow cytometric assessment of cell composition	29
5.2	Degranulation and cytotoxicity assays	29
5.2.1	Degranulation toward ENCCs	30
5.2.2	Cytotoxicity toward ENCCs	31
5.2.3	Degranulation and cytotoxicity toward K562 cells	33
5.2.4	Degranulation and cytotoxicity toward SK-N-AS cells	34
5.3	Ligand staining	35
6	Discussion	41
6.1	Sample processing	41
6.2	NK Cell Responses and Ligand Expression	42
6.3	Variability in Cytotoxicity Assessment	44
6.4	Characterisation of the Endometriosis-Derived Cultured Cells	44
6.5	NK Cell Source	45
7	Conclusion	47
	Bibliography	49
A	Flow cytometry data	I
A.1	Cell composition assessment	I
A.2	Ligand staining	IV
B	Statistical analyses	V
B.1	ENCC cytotoxicity assessments	V
B.2	K562 degranulation assessment	VI
B.3	K562 cytotoxicity assessment	VII
B.4	SK-N-AS degranulation assessment	IX
B.5	SK-N-AS cytotoxicity assessment	X

List of Figures

2.1	Three types of immune functions, with examples of effector cells, molecules, and mechanisms. Created in Biorender.com.	6
2.2	Activating and inhibitory receptors on NK cells and corresponding endogenous ligands. Created in Biorender.com.	7
2.3	Female anatomy of the lower abdomen and examples of EN lesions. Created in Biorender.com.	9
2.4	Schematic representation of the EN lesion microenvironment. Created in Biorender.com.	11
4.1	Gating strategy for degranulation analysis. (A) Time gate excluding acquisition irregularities. (B) Cell gates separating debris, NK cells, and target cells. (C) Singlet gate excluding doublets. (D) CellTrace gate excluding CellTrace-positive target cells. (E) Live cell gate excluding dead NK cells. (F) CD107a gate identifying CD107a-positive NK cells.	20
4.2	Gating strategy for cytotoxicity analysis. (A) Cell gate as in Figure 4.1B. (B) Singlet gate excluding doublets. (C) CellTrace gate excluding CellTrace-negative NK cells. (D) Dead cell gate identifying dead target cells.	21
5.1	Percentage of CD107 ⁺ HD NK cells following co-culture with ENCCs at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent the mean with standard deviation. Colours of individual data points indicate NK cell donor and symbol shapes indicate the patient-derived ENCCs. Statistical significance was assessed using multiple linear regression and Holm–Bonferroni correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).	30
5.2	Effect size estimates (beta coefficients) for each antibody blocking condition compared to the E:T 1:1 condition. The variance shown is the 95% confidence interval (without corrections for multiple testing).	31
5.3	Percentage of dead ENCCs following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate NK cell donor and symbol shapes indicate ENCC donor.	32

5.4	Effect size estimates for each experimental condition compared to (A) the target only control, and (B) the E:T 1:1 condition. The variance shown is the 95% confidence interval (without corrections for multiple testing).	32
5.5	Percentage of CD107 ⁺ HD NK cells following co-culture with K562 cells at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent means with standard deviation. Colours indicate NK cell donor.	33
5.6	Percentage of dead K562 cells following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate the NK cell donor.	34
5.7	Percentage of CD107 ⁺ HD NK cells following co-culture with SK-N-AS cells at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent means with standard deviation. Colours indicate the NK cell donor.	34
5.8	Percentage of dead SK-N-AS cells following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate the NK cell donor.	35
5.9	Histograms showing staining of target ligands on ENCCs from EndoMIC 8A. Red histograms represent antibody staining of live ENCCs and blue histograms represent negative live ENCC controls (Live/Dead control or Secondary antibody control). (A) HLA-ABC, (B) HLA-E, (C) CD48, (D) MIC-A/B, (E) ULBP-1, (F) ULBP-2/5/6, (G) PVR, (H) Nectin-2, and (I) B7-H6.	36
5.10	Histograms showing staining of target ligands on K562 cells. Green histograms represent antibody staining of live K562 cells and blue histograms represent negative live K562 controls. (A) MIC-A/B, (B) ULBP-1, (C) ULBP-2/5/6, (D) PVR, (E) Nectin-2, and (F) B7-H6.	37
5.11	Plot of CD13-PE-Cy7 and EpCAM-BUV395 staining in ENCCs from EndoMIC 8A (red) and K562 cells (blue).	38
5.12	Histogram showing TRAIL-R2 staining in ENCCs from EndoMIC 8A (red) compared to a negative live ENCC control (blue).	39
A.1	Final CD3 versus CD56 gating plots from representative endometrioma samples processed before and after implementation of the updated staining protocol. (A) Endometrioma sample from EndoMIC 2, processed using the original staining protocol. (B) Endometrioma sample from EndoMIC 8, processed using the updated staining protocol. Corresponding full gating strategies are shown in Figures A.2 and A.3. I	
A.2	Gating strategy for cell composition assessment of endometrioma sample from EndoMIC 2, including gating for (A) cells, (B) single cells, (C) CD15 ⁻ cells, (D) CD45 ⁺ cells, (E) CD3 ⁻ CD56 ⁺ NK cells. II	

A.3	Gating strategy for cell composition assessment of endometrioma sample from EndoMIC 8, including gating for (A) cells, (B) single cells, (C) live cells, (D) CD15 ⁻ cells, (E) CD45 ⁺ cells, (F) CD3 ⁻ CD56 ⁺ NK cells.	III
A.4	Histogram showing TRAIL-R1 staining in ENCCs from EndoMIC 8A (red) compared to a negative live ENCC control (blue). Differences in signal distribution between samples should be interpreted with caution, as compensation analysis identified spillover between fluorescence channels.	IV
B.1	Effect size estimates and 95% confidence intervals for K562 antibody blocking conditions compared to the E:T 1:1 condition (without multiple testing corrections).	VI
B.2	Effect size estimates and 95% confidence interval for K562 experimental conditions compared to (A) target only control, and (B) E:T 1:1 (without multiple testing correction).	VII
B.3	Effect size estimates and 95% confidence intervals for SK-N-AS antibody blocking conditions compared to the E:T 1:1 condition (without multiple testing corrections).	IX
B.4	Effect size estimates and 95% confidence interval for SK-N-AS experimental conditions compared to (A) target only control, and (B) E:T 1:1 (without multiple testing correction).	X

List of Tables

4.1	Flow cytometry staining reagents for patient samples.	17
4.2	Blocking antibodies used for functional assessment.	18
4.3	Overview of reagents added in each condition of the degranulation assay.	19
4.4	Antibody combinations used for target ligand staining.	22
4.5	Staining reagents used for target ligand analysis.	22
5.1	Number of isolated PBMCs from each patient blood sample.	25
5.2	Number of isolated cells from peritoneal lavage samples.	26
5.3	Characteristics of endometrioma fluid samples.	27
5.4	Characteristics of lesion tissue samples.	28
5.5	Holm–Bonferroni-adjusted significance thresholds and corresponding significance levels for blocking antibody conditions compared to the E:T 1:1 condition.	31
5.6	Mean fluorescence intensity (MFI) for ENCC ligand stainings and corresponding negative controls.	37
A.1	Staining reagents used for extended target ligand analysis.	IV
B.1	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards ENCCs, with the target only condition as reference ($\alpha = 0.05$).	V
B.2	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards ENCCs, with the E:T 1:1 condition as reference ($\alpha = 0.05$).	V
B.3	Holm–Bonferroni correction for multiple comparisons of NK cell de- granulation towards K562, with the E:T 1:1 condition as reference ($\alpha = 0.05$).	VI
B.4	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards K562, with the target only condition as reference.	VII
B.5	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards K562, with the E:T 1:1 condition as reference.	VIII
B.6	Holm–Bonferroni correction for multiple comparisons of NK cell de- granulation towards SK-N-AS, with the E:T 1:1 condition as reference.	IX
B.7	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards SK-N-AS, with the target only condition as reference ($\alpha = 0.05$).	X
B.8	Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards SK-N-AS, with the E:T 1:1 condition as reference ($\alpha = 0.05$).	XI

1

Introduction

Endometriosis (EN) is an inflammatory disease estimated to affect approximately 10% of women of reproductive age. The disease is characterised by ectopic growth of endometrium-like tissue outside the uterus and is commonly associated with several pain symptoms as well as infertility. Treatment options are limited and often ineffective or inappropriate for a large proportion of women with EN. There is, therefore, a huge need for alternative treatments.

Dysfunction of the innate and adaptive immune system is evident in women with EN. One such dysfunction observed is reduced cytotoxic activity in natural killer (NK) cells. The cytotoxic activity of NK cells involves degranulation, the secretion of cytotoxic granules that trigger apoptosis of the target cells, such as tumour cells, virus-infected cells, or inflammatory cells. NK cells may therefore be a good target for future treatments of EN. However, to develop such a treatment, more knowledge about NK cells in EN is needed.

1.1 Purpose and aims

This master's thesis is part of the EndoMIC (Endometriosis Molecular and Immunological Characterisation) research project and contributes to the immunological characterisation of EN. Within this project, this thesis focuses on the functional assessment of NK cells in EN.

The initiation of sample collection from EN patients undergoing surgery at Sahlgrenska University Hospital coincided with the start of this thesis work. Consequently, a substantial part of the project involved processing these samples and developing protocols for the isolation of immune cells from the collected EN tissue samples.

To assess NK cell functionality, a combined degranulation and cytotoxicity assay was used to evaluate NK cell degranulation against cells derived from EN lesions and the killing of these target cells. In this *in vitro* assay, blocking antibodies targeting specific NK cell receptors were also used to investigate the contribution of specific NK-target interactions on NK cell functionality toward EN-derived cells.

The following specific aims were defined:

- **Aim 1:** To develop and optimise sample processing protocols for the isolation of immune cells from collected EN samples.
- **Aim 2:** To assess NK cell degranulation and cytotoxicity towards epithelial and stromal cells derived from EN lesions.
- **Aim 3:** To identify NK cell receptors that contribute to NK cell degranulation and cytotoxic responses towards EN-derived cells.

As the EndoMIC research project is still in its early stages, the focus of this thesis is on establishing protocols for immune cell isolation and obtaining preliminary results from *in vitro* assays that can be expanded in future studies. The broader goal is to contribute to a better understanding of the immune landscape in EN, which may ultimately facilitate the development of future immunotherapies.

1.2 Limitations

EN is a complex and heterogeneous disease involving multiple immune and non-immune cell populations. The functional experiments in this study were performed *in vitro* using a co-culture assay with NK cells and target cells derived from EN lesions. Although this approach enabled controlled investigation of NK cell functionality, it represents a simplified model of the *in vivo* environment. EN lesions exist within a complex microenvironment influenced by hormones, inflammatory cytokines, extracellular matrix, and other cell populations. Many of these factors were not replicated under the experimental conditions, which thus represents a limitation of the study. Nevertheless, this simplified system was necessary to enable focused assessment of NK cell responses against EN-derived target cells.

Another limitation is that the target cells isolated from EN lesions were cultured for several passages prior to use in the functional assays. Prolonged *in vitro* culture may alter cellular phenotype, reduce heterogeneity, and affect the expression of ligands for NK cell recognition and cytotoxicity. Thus, the cultured cells may not completely reflect the characteristics of cells in native EN lesions.

The study also lacked healthy endometrial cells as a control target population. Including healthy endometrial tissue would have strengthened the interpretation of whether the observed NK cell responses were specific to EN-derived cells. However, ethical considerations and practical difficulties limited the feasibility of including healthy endometrial tissue in this thesis.

In addition, due to limited NK cell yields from patient samples, the functional assays were performed using NK cells isolated from healthy donor buffy coats rather than from EN patient samples. While the use of EN-derived target cells maintained disease relevance, the responses observed may not fully represent NK cell activity in individuals with EN. However, at this stage, prioritisation of patient-derived immune cells for phenotypic characterisation made this approach necessary.

1.3 Ethical and Societal Aspects

Although EN is a benign disease, it greatly affects approximately 10% of women, with symptoms that often affect these women's physical, mental, and social well-being. There is no cure for EN, and available symptomatic treatments are not suitable or helpful for everyone. Research into the underlying disease process can identify new targets or markers for treatment or diagnosis. By investigating NK cell function in EN, this thesis may contribute to a deeper understanding of disease mechanisms and ultimately support the development of more targeted and effective treatments for EN.

In this project, samples are collected from women undergoing surgery for EN. This raises ethical questions regarding informed consent and the benefit-to-risk ratio. The research has been approved by an ethics review board, and all patients gave informed consent. Further, the surgery would have been performed and the EN lesions removed regardless of whether sample collection was performed. While the sample collection prolongs the operation by a few minutes, this change is considered negligible and does not affect patient health. Thus, collecting samples does not significantly increase surgical risk.

2

Theory

The following chapter provides theoretical background on the immune system, NK cell immunology, and endometriosis, providing context and understanding of the project's main topics.

2.1 The Immune system

The immune system comprises cells and molecules that specialise in protecting against infectious agents and abnormal cells, including virus-infected and transformed cells [1]. It is broadly divided into innate and adaptive immunity. Innate immunity is seen as the first line of defence against infectious agents, with responses occurring within minutes of invasion [2]. It includes phagocytic cells, such as macrophages and neutrophils, antigen-presenting cells, e.g. dendritic cells, and innate lymphoid cells (ILCs), including natural killer (NK) cells.

Adaptive immunity is instead an antigen-specific response by T cells and B cells. T cells coordinate immune responses and kill aberrant cells. B cells produce antibodies that bind specifically to recognised pathogens [1]. This response is slower because it requires clonal expansion and differentiation in response to an antigen. Immune cells are classified based on surface markers called clusters of differentiation (CD) [3]. For example, T cells are categorized into $CD4^+$ T helper (T_H) cells and $CD8^+$ T cytotoxic (T_C) cells. Here, a marker's presence is denoted with $^+$, and its absence would be denoted with $^-$.

Antigen presentation is central to adaptive immunity and is mediated by major histocompatibility complex (MHC) molecules, also called human leukocyte antigens (HLAs) in humans. MHC class I molecules are expressed on most cells and present endogenous peptides to $CD8^+$ T_C cells [4]. These peptides are normally self-derived and tolerated, but in infected or malignant cells, non-self peptides may trigger cytotoxic responses. In contrast, MHC class II molecules are primarily expressed on antigen-presenting cells and present antigens to $CD4^+$ T_H cells, enabling their activation.

A subset of adaptive immune cells persists after infection as memory T and B cells, providing a more robust response upon reinfection. Such memory was considered specific to the adaptive immune system. However, certain innate cells have recently been proposed to also develop a type of immunological memory, called trained immunity [5–8].

2.1.1 Three types of cell-mediated immunity

The immune system can be divided into three categories of cell-mediated effector immunity [9], each targeting distinct threats through distinct effector cells and mechanisms, as illustrated in Figure 2.1. The primary effector cells include both adaptive and innate lymphoid cells.

Immune function	Effector cells	Effector molecules	Effector mechanisms
Type 1 Defence against intracellular pathogens and tumors	T _H 1 ILC1 T _C 1 NK	IFN- γ Granzymes Perforins	Phagocytic macrophage activation Cytotoxicity
Type 2 Defence against large extracellular pathogens and allergens	T _H 2 ILC2	IL-4 IL-5 IL-13 AREG	Alternative macrophage activation IgE antibodies Tissue repair
Type 3 Defence against extracellular microbes	T _H 3 ILC3	IL-17 IL-22	Nutrophil recruitment/Phagocytosis Antimicrobial peptides

Figure 2.1: Three types of immune functions, with examples of effector cells, molecules, and mechanisms. Created in Biorender.com.

Innate lymphoid cells (ILCs) are a recently identified group of innate immune cells comprising five subgroups: NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue inducer (LTi) cells [10]. They are often described as the innate counterparts of T cells, given their similar effector functions and cytokine profiles. For example, NK cells resemble T_C1 cells, a subset of T_C cells characterised by cytotoxic activity and IFN- γ production. Likewise, ILC1s, ILC2s, and ILC3s resemble the T helper cell subsets T_H1, T_H2, and T_H17, respectively, with each pair contributing to one of the three major types of immune responses shown in Figure 2.1.

Type 1 immunity protects against intracellular pathogens and tumours [9,10]. It is mediated in part by NK and T_C1 cells, which trigger apoptosis of aberrant cells by releasing granzymes and perforins. In addition, NK cells, T_C1 cells, ILC1s, and T_H1 cells all produce IFN- γ , which induces classical (M1) macrophage activation, promoting a pro-inflammatory macrophage phenotype specialised in killing intracellular pathogens through phagocytosis.

Type 2 immunity, mediated by ILC2s, T_H2, and T_C, defends against large extracellular pathogens and allergens [9,10]. Key cytokines include IL-4, IL-5, and IL-13, each with multiple functions. For example, IL-5 promotes eosinophil recruitment to defend against multicellular parasites, while IL-4 and IL-13 drive B cell antibody production, including IgE. They also contribute to tissue remodelling and repair. For example, IL-4 and IL-13 induce alternative (M2) macrophage activation, promoting an anti-inflammatory macrophage phenotype that supports wound healing and regulation of inflammation. [10].

Type 3 immunity, driven by ILC3s, T_H17 , and T_C17 cells, targets extracellular microbes [9,10]. Central cytokines include IL-17 and IL-22. IL-17 promotes neutrophil recruitment, enabling efficient clearance of microbes through phagocytosis, while IL-22 induces the production of antimicrobial peptides [9]. LTi cells are sometimes also associated with type 3 immunity. However, their primary role is in fetal development, where they drive the formation of secondary lymphoid structures such as lymph nodes and Peyer's patches [9,10].

2.1.2 NK cell immunology

NK cells comprise approximately 2-15% of blood lymphocytes [11] and are identified by the expression of CD56 and absence of CD3 (a T cell marker). They are commonly divided into $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ subsets, in which the former express high levels of CD56, whereas the latter express lower levels. Approximately 90% of circulating NK cells are $CD56^{\text{dim}}$, whereas the remaining 10% are $CD56^{\text{bright}}$ [11]. However, NK cells are highly heterogeneous and show a diversity of cell receptors and functions from which further NK cell subsets can be defined [12].

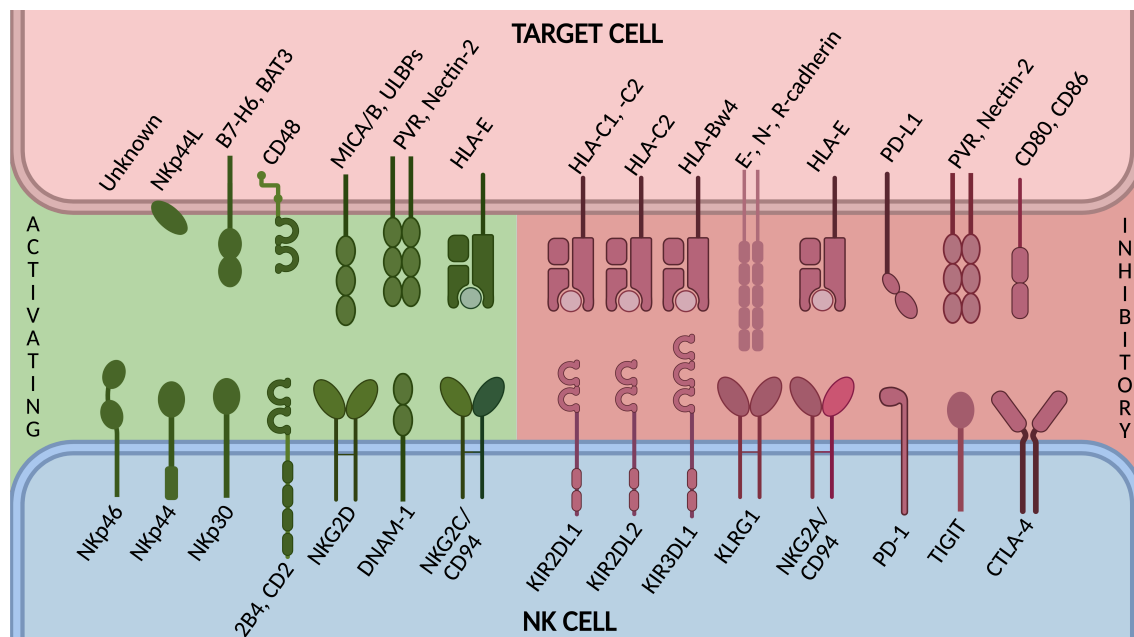


Figure 2.2: Activating and inhibitory receptors on NK cells and corresponding endogenous ligands. Created in Biorender.com.

NK cells are cytotoxic innate lymphoid cells that eliminate stressed cells, including tumour cells, virus-infected cells, or inflammatory cells [13]. Like T_C cells, they induce apoptosis via the release of cytotoxic granules containing perforin and granzymes, but can also act through death receptor pathways (TRAIL/TRAIL-R, FASL/FAS). Unlike T_C cells, which are activated by MHC class I, NK cell activation depends on the balance between activating and inhibitory receptor signals [13]. When the activation signals dominate, NK cells initiate a cytotoxic response. Examples of NK cell receptors and their endogenous ligands are shown in Figure 2.2.

Inhibitory receptors that recognise HLA class I molecules, such as killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A (from now on called NKG2A), are key regulators of NK cell function. According to the “missing-self” hypothesis, NK cells are inhibited by recognition of "self" HLA class I on healthy cells, but become activated when HLA class I expression is reduced or absent [13]. This loss of inhibitory signalling enables NK cells to eliminate tumour or virus-infected cells, which often downregulate HLA class I.

Beyond target recognition, inhibitory receptors also affect NK cell functionality through a process called licensing, or education. NK cells that engage "self" HLA class I via inhibitory receptors become functionally competent, whereas those lacking such interactions remain unlicensed and thus hypofunctional. The strength of inhibitory signalling further tunes responsiveness, such that NK cells receiving strong inhibitory input at steady state are generally more reactive when stimulated [13].

Because KIR and HLA class I genes are inherited independently, mismatches can occur in which inhibitory KIRs lack their corresponding ligands [14]. In such cases, reduced inhibitory signalling during target cell recognition can enhance NK cell activation. This is particularly relevant in allogeneic settings, where donor NK cells may encounter recipient cells lacking ligands for their inhibitory KIRs, leading to increased cytotoxicity, e.g. in transplantation and cancer immunotherapy [13].

2.1.3 Tissue residency

Tissue-resident immune cells reside long-term in specific tissues and do not circulate in the blood or lymph [15]. While they share many functions with circulating immune cells, tissue-resident immune cells also acquire specific properties distinct from those of circulating cells. They are broadly distributed throughout the body and occupy a wide range of peripheral tissues, e.g. skin, brain, liver, lungs, and uterus. They are highly heterogeneous across different tissues due to their phenotypes being shaped by the local tissue environment [15].

Tissue-resident immune cells express tissue residency markers that can be used to differentiate them from circulating immune cells, with common markers for lymphoid tissue-resident immune cells including CD69, CD103, and CD49a [15]. These immune cells are essential for regulating local immune responses and maintaining tissue repair and homeostasis. They keep local immune balance mainly by expressing inhibitory factors and immune checkpoint molecules, such as PD-1, CTLA-4, and TIGIT [15].

Most helper-like ILCs, i.e. ILC1s, ILC2s, and ILC3s, are tissue-resident [16]. In contrast, NK cells are found both resident in tissues and circulating in the blood. Like other tissue-resident cells, tissue-resident NK (trNK) cells have been identified in various tissues and express different markers depending on the tissue in which they reside. For example, trNK cells in the uterus express e.g. CD94/NKG2A, CD49a, and CD103 [15]. These uterine NK (uNK) cells are poorly cytotoxic and instead preferentially produce cytokines [17]. By secreting these cytokines, uNK cells contribute to embryo implantation and are crucial for a successful pregnancy.

2.2 Endometriosis

Endometriosis (EN) is an inflammatory disease characterised by ectopic growth of endometrium-like tissue outside the uterus [18]. The growth of EN lesions is estrogen-dependent, and the condition therefore manifests primarily in women of reproductive age, i.e. between menarche and menopause [19].

The disease is heterogeneous in its presentation and is therefore sometimes divided into three subtypes: superficial peritoneal EN, ovarian endometrioma, and deep infiltrating EN [20]. Lesions are also often accompanied by fibrosis and adhesions [19]. Superficial peritoneal EN lesions are located on the surface of the peritoneum. In contrast, deep infiltrating EN lesions are nodules that have invaded over 5 mm beyond the peritoneum surface, with common locations being the rectovaginal septum, bladder, and bowel [19]. Finally, ovarian endometriomas are ovarian cysts containing a thick, brown fluid, sometimes called chocolate liquid [20]. The female anatomy and exemplifying EN lesions are depicted in Figure 2.3.

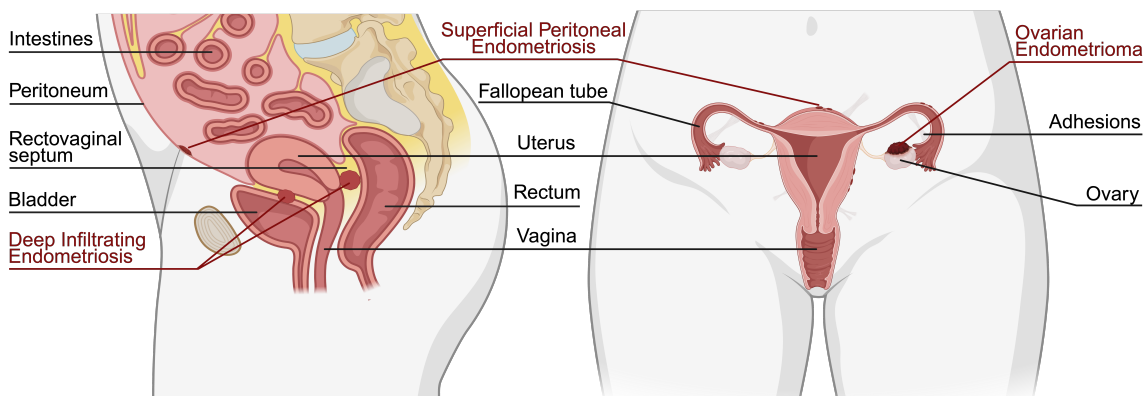


Figure 2.3: Female anatomy of the lower abdomen and examples of EN lesions. Created in Biorender.com.

2.2.1 Prevalence, symptoms, and treatments

EN is estimated to affect approximately 10% of women of reproductive age [21], although the true prevalence is difficult to determine due to diagnostic challenges. In clinical practice, diagnosis is typically based on transvaginal ultrasonography or magnetic resonance imaging (MRI), which quite reliably identify deep infiltrating EN and endometriomas but are less effective for superficial peritoneal EN [18]. The gold standard for diagnosis remains laparoscopy with histological confirmation. However, as an invasive surgical procedure, it is generally reserved for cases in which surgery is also indicated as treatment.

The symptomatic profile of EN varies greatly. Common symptoms include period pain, acyclic pelvic pain, pain during intercourse, and pain during bowel movements or urination [18]. Another common symptom is subfertility, with the condition being associated with a twofold increased risk of infertility [22]. Thus, the disease can

substantially impair physical, mental, and social well-being, reducing quality of life. EN is also associated with an increased risk of ovarian cancer (OC), particularly endometrioid and clear cell subtypes [18]. The lifetime risk is estimated at 2–3%, compared to 1–1.5% in the general population [23,24], corresponding to roughly a twofold increase.

Treatment options for EN are limited and primarily focus on symptom management [18]. Hormonal therapies, including progestins, combined oral contraceptives, and gonadotropin-releasing hormone (GnRH) analogues, are commonly used to suppress estrogen-dependent lesion growth and reduce inflammation [25]. Non-steroidal anti-inflammatory drugs (NSAIDs) may also provide short-term pain relief. Surgical excision of lesions via laparoscopy is also a treatment option. Hysterectomy, the removal of the uterus, with simultaneous lesion excision, is considered a last resort for patients who do not respond to conservative treatment and do not wish to conceive. Symptom and lesion recurrence are quite common after surgery, including hysterectomy, although the estimated recurrence rate varies widely [26–28]. In addition, surgery may result in postoperative scarring and adhesion formation, processes that are also central to the pathophysiology of EN.

Overall, both medical and surgical treatments are ineffective for a large proportion of EN patients and may be associated with side effects or complications [18]. In addition, treatment options are particularly limited for women wishing to conceive, as the commonly used hormonal treatments are contraindicated. There is thus a huge need for alternative treatments of EN.

2.2.2 Postulated origins of endometriosis

Several origins of EN have been proposed, most notably retrograde menstruation, lymphatic or vascular metastasis, and coelomic metaplasia [18]. Retrograde menstruation involves the backward flow of menstrual blood into the peritoneal cavity, transporting endometrial cells. In contrast, lymphatic or vascular metastasis, the transportation of cells through lymphatic channels and veins, can explain the rare occurrence of extrapelvic lesions, such as those in the lungs or brain [19]. Coelomic metaplasia describes the transformation of the coelomic epithelium of the abdomen into endometrial-like tissue, a hypothesis supported by rare cases of EN in men receiving estrogen therapy for prostate cancer [18]. Importantly, these proposed origins are not mutually exclusive and should be viewed as complementary rather than competing.

Retrograde menstruation, first proposed by Sampson in 1927 [29], is the most widely accepted origin in most cases [19]. The theory is supported by epidemiological studies showing associations with increased menstrual exposure, such as obstructed outflow, shorter cycle length, early menarche, and fewer pregnancies [30,31]. Anatomical patterns of lesion distribution further support this theory [32–35]. For example, the left-sided predominance of pelvic EN has been attributed to the presence of the sigmoid colon, which influences peritoneal fluid flow [32]. In addition, genetic studies tracing somatic mutations in EN lesions to eutopic endometrium provide further support for the theory [36].

Retrograde menstruation, however, is very common, occurring in up to 90% of women [37]. Thus, Sampson's theory alone cannot explain why only approximately 10% develop EN. Additional factors are thus required, including the ability of displaced cells to evade immune clearance, adhere to and invade the peritoneal epithelium, and ensure continued growth and survival [38]. Current research focuses on e.g. hereditary or acquired properties of the endometrium and dysfunctional immune clearance [38].

2.2.3 The microenvironment of endometriosis lesions

EN lesions are composed of several cell types, with two major cell types being endometrial epithelial and stromal cells [39,40]. These epithelial and stromal cells are organised in a manner resembling the eutopic endometrium, with, e.g., endometrioid glandular structures composed of epithelial cells. EN lesions are also highly vascularised and innervated [39], and immune cells such as NK cells, T cells, and macrophages are also present (Figure 2.4). Endometrial stem cells are also suspected to play a role in EN, given their ability to regenerate the entire endometrium [40].

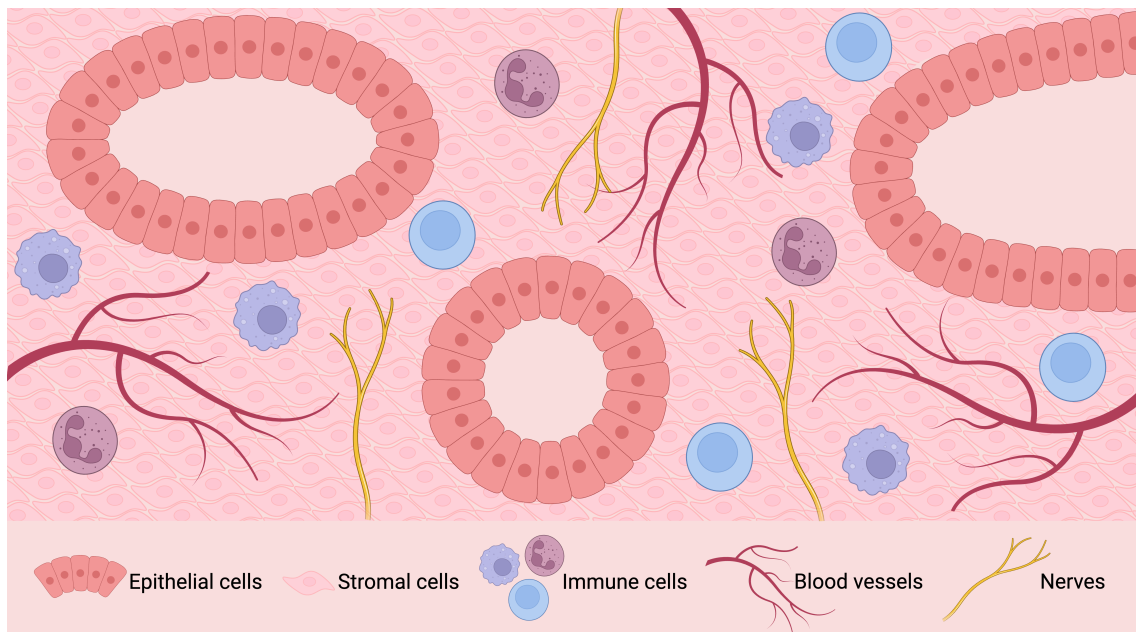


Figure 2.4: Schematic representation of the EN lesion microenvironment. Created in Biorender.com.

Endometrial epithelial and stromal cells in EN lesions actively contribute to lesion establishment and persistence through altered behaviour and signalling. Epithelial cells exhibit changes in gene expression, enhanced migratory abilities, and activation of innate immune pathways [39,40]. Stromal cells in EN lesions, unlike their eutopic counterparts, express enzymes that enable local estrogen synthesis, resulting in elevated estrogen levels that drive inflammation, pain, and lesion growth. They also exhibit altered hormone signalling, including progesterone resistance, which disrupts the mechanisms that usually limit estrogen activity [40]. Further, they secrete cytokines that support proliferation, invasion, and immune modulation.

The prominent vascularisation of EN lesions further supports lesion survival by supplying oxygen and nutrients and facilitating immune cell recruitment [39]. Lesions are also innervated with nerve fibres that are thought to contribute to the pain symptoms of EN. Further, close spatial association between blood vessels and nerves suggests coordinated angiogenic and neurogenic signalling processes [39].

Several genome-wide analyses and meta-analyses have identified multiple genetic loci associated with EN [41,42]. These loci implicate pathways involved in cell adhesion, proliferation, angiogenesis, inflammation, and hormonal regulation [18]. However, these loci explain only a small part of the genetic contribution, with heritability estimated at approximately 50% in population studies [43,44].

2.2.4 Endometriosis and the immune system

The inflammatory microenvironment of EN lesions, and thus the immune system, also evidently plays a vital role in EN. Growing evidence suggests that defective immunological surveillance, encompassing several dysfunctional immune responses, plays a central role in EN [40]. Imbalanced immune cell populations, cellular dysfunctions, and aberrant inflammatory cytokines have been implicated in EN [45]. While both innate and adaptive immune dysfunctions have been reported, reduced NK cell activity has emerged as a central feature of EN, and NK cell-based therapeutic strategies are being explored [46].

NK cells from EN patients exhibit altered expression of activating and inhibitory receptors, skewed toward inhibitory signalling [45]. Increased expression of the inhibitory receptors, such as KIR2DL1, has been observed in NK cells from both peritoneal lavage and peripheral blood [47,48]. In addition, elevated expression of NKG2A in peritoneal NK cells, together with increased levels of its ligand HLA-E in EN tissue, suggests enhanced inhibitory signalling that may contribute to reduced NK cell cytotoxicity [49]. Further mechanisms of NK cell inhibition have also been proposed. Elevated levels of soluble ligands for the activating receptor NKG2D (including soluble MIC-A, MIC-B, and ULBP-2) have been detected in the peritoneal fluid of women with EN [45,50]. Despite NKG2D being an activating receptor, these soluble ligands can impair NK cell function, a mechanism also described in tumour immune evasion [13,50].

It is important to note that the findings described above were obtained from NK cells isolated from peripheral blood or peritoneal lavage. Comparatively little is known about NK cells residing within EN lesions themselves. This represents an important knowledge gap, as trNK cells differ substantially from circulating NK cells in terms of phenotype, receptor expression, and functional properties. Further characterisation of lesion-resident NK cells may therefore provide additional insights into the local immune environment of EN lesions. Furthermore, although several NK cell receptors have been implicated in EN, comprehensive characterisation of NK cell receptor profiles remains limited [45]. Targeting these pathways, either by blocking inhibitory receptors or enhancing activating signals, represents a promising strategy for NK cell-based immunotherapy and highlights the importance of identifying relevant receptor targets.

3

Methodology

The following chapter provides the methodological background for cell culture, flow cytometry, and the functional assessment of NK cells. It focuses on the principles underlying these approaches, whereas the specific procedures applied in this project are described in Chapter 4.

3.1 Culturing of mammalian cells

Culturing cells under controlled conditions is essential for in vitro studies. For successful cell culture, both nutritional and physicochemical requirements must be met. The culture medium provides the required nutrients, growth factors, and hormones, and also regulates pH and osmotic pressure [51]. CO₂ incubators are used to control temperature and CO₂ levels, typically maintained at 37°C and 5% CO₂ for mammalian cells [51]. Many mammalian cells are adherent and also require a solid or semisolid support. Others grow directly in the medium as suspension cultures.

A primary cell culture is derived directly from freshly isolated cells and is typically slow-growing, heterogeneous, and retains characteristics of the tissue of origin. Primary cells can be subcultured to establish cell lines that are either finite, with limited proliferative capacity, or continuous, proliferating indefinitely [51].

Subculturing, or passaging, is performed regularly to maintain proliferating cells. Cells should be passaged during the log phase, before reaching confluence. In adherent cultures, confluence is identified microscopically when the surface is fully covered, whereas in suspension cultures it is indicated by clumping and increased turbidity [51]. Medium exhaustion, characterised by nutrient depletion, waste accumulation, and decreased pH due to lactic acid, also signals the need for passaging. For adherent cells, passaging requires detachment from the growth surface, typically using proteolytic enzymes such as trypsin and/or chelating agents such as ethylenediaminetetraacetic acid (EDTA) to break cell attachment [51].

Cryopreservation is used to store cells while maintaining viability. It helps prevent contamination and minimise genetic and functional changes associated with prolonged subculturing [51]. This process relies on controlled freezing rates and cryoprotective agents, such as dimethyl sulfoxide (DMSO), to prevent ice crystal formation. Cryopreserved cells can be stored at -80°C for a few weeks. For long-term storage, cryogenic vials should be kept in liquid nitrogen at -160°C or lower [51].

3.2 Flow cytometry

Flow cytometry is an analytical technique used to measure multiple characteristics of single cells [52]. Cells in suspension are introduced into a sheath fluid stream and aligned into a single file via hydrodynamic focusing. As cells pass through a laser beam, incident light is deflected, a process known as light scattering. There are two types, forward scattering (FSC) and side scattering (SSC), both of which reflect the structural and morphological characteristics of cells [52]. Cells can also be labelled with fluorescent probes, such as antibody conjugates or dyes that bind specific cellular components. These fluorophores are excited by specific wavelengths of light and emit at longer wavelengths, which are detected by the instrument. Because fluorescence intensity is proportional to the number of probes bound to each cell, flow cytometry allows quantitative assessment of cellular features across heterogeneous populations.

Data generated by flow cytometry are interpreted using gating strategies, in which subsets of cells are defined based on combinations of FSC, SSC, and fluorescence parameters [52]. Gating allows exclusion of unwanted events, e.g. debris or dead cells, and thus enables focused analysis of populations of interest. In experiments using multiple fluorophores, spectral overlap can occur as emission spectra are not discrete. This overlap is then corrected through compensation, a mathematical process that subtracts spillover signals between detection channels. Proper gating and compensation are important steps in flow cytometric analysis, as inaccuracies at this stage can substantially affect data interpretation and lead to faulty conclusions.

3.3 Functional assessment of NK cells

Functional assays assess how effector cells respond to stimuli and interact with target cells, giving insight into immune function or dysfunction. NK cell cytotoxicity can be assessed using flow cytometry-based co-culture assays, in which NK cells are incubated with target cells.

NK cell degranulation is often assessed by detecting CD107a, also known as lysosome-associated membrane protein-1 (LAMP-1). This transmembrane protein is one of the most abundant proteins in the lytic granules of NK cells [53,54]. During degranulation, the granule fuses with the NK plasma membrane, transiently exposing CD107a molecules on the NK cell surface, which can be detected using fluorescently labelled antibodies.

A simultaneous assessment of the cytotoxic outcome of the NK cell activity can be obtained by using a viability dye following co-incubation, in order to determine the proportion of non-viable target cells [55]. Target cells can be distinguished from NK cells through prior fluorescent labelling or by phenotypic discrimination. Combining these two readouts yields complementary information on NK cell effector function and deeper knowledge of the immune mechanisms at work.

4

Methods

This chapter describes the methods and procedures applied in this project, including sample collection and processing, cell culture, flow cytometry, and functional assays.

4.1 Patients and Samples

EN samples and matched blood samples were collected from patients diagnosed with EN at Sahlgrenska University Hospital (Gothenburg, Sweden). The samples included blood, peritoneal lavage, endometrioma fluid, and tissue samples from EN lesions. Sample collection was approved by the regional ethics board in Gothenburg (2025-02326-2). All patients were enrolled in accordance with the Declaration of Helsinki and provided written informed consent. EN samples were collected during laparoscopic excision of EN lesions, while blood samples were collected prior to surgery.

Samples were handled under sterile conditions and processed on a laminar airflow (LAF) bench. Unless otherwise stated, centrifugation steps were performed at $350 \times g$ for 7 min at 4°C. Processing protocols were continually evaluated and optimised, with the latest versions described below.

4.1.1 Blood samples

Blood samples were received in EDTA tubes. Two aliquots of whole blood were stored at -80°C. The remaining blood was centrifuged ($1600 \times g$, 10 min, room temperature) to separate plasma. A plasma aliquot was stored at -80°C. The buffy coat layer was then collected, and peripheral blood mononuclear cells (PBMCs) were isolated from it. The buffy coat was diluted in buffered NaCl, and erythrocyte sedimentation was induced using a 2% dextran solution. The upper layer was collected and layered onto Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada) and subjected to density gradient centrifugation ($850 \times g$, 20 min, room temperature, no brake). PBMCs were collected from the interface, washed twice in elutriation buffer (E-buffer), and resuspended in E-buffer.

Cell concentration was determined using a Sysmex XP-100 haematology analyser (Sysmex, Kobe, Japan). The proportion of NK cells, defined as $CD3^-CD56^+$, was assessed by flow cytometry following staining with antibodies against CD3 and CD56 (FITC/PE-Cy5, Beckman Coulter, Brea, CA, USA; cat. no. A07415).

PBMCs were cryopreserved in freezing medium consisting of DMEM (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA). Cells were frozen at -80°C using a controlled-rate freezing container and then transferred to liquid nitrogen for long-term storage.

4.1.2 Peritoneal lavage samples

Peritoneal lavage samples were strained through a 40 μm cell strainer (Corning, Corning, NY, USA), followed by washing with phosphate-buffered saline (PBS). Erythrocytes were lysed with ACK lysis buffer for 5 min, and the cells were then washed with PBS. The cells were then resuspended in PBS, and cell concentration was determined as described above.

Cell composition was analysed by flow cytometry. Cells were stained with LIVE/DEAD Fixable Violet (Invitrogen, ThermoFisher Scientific; cat. no. L34955) following the manufacturer's instructions. Thereafter, the cells were incubated with an FCS solution to reduce non-specific binding and stained with antibodies against CD3, CD56, CD15 (V500, BD Biosciences, San Jose, CA, USA; cat. no. 560779), and CD45 (APC-Cy7, BioLegend, San Diego, CA, USA; cat. no. 323048). The panel is summarised in Table 4.1. NK cells were defined as live $\text{CD15}^{-}\text{CD45}^{+}\text{CD3}^{-}\text{CD56}^{+}$. Cells were cryopreserved for later experiments.

4.1.3 Endometrioma fluid samples

Endometrioma fluid samples from up to two endometriomas per patient were collected and processed separately. Samples were diluted with PBS and strained through a 40 μm cell strainer. The filter was washed with PBS, and the filtrate was centrifuged. Next, the supernatant was transferred to a separate tube, and the pellet was resuspended in PBS. Both fractions were centrifuged again, and the resulting pellets were resuspended in PBS, pooled, and subjected to density gradient centrifugation using Lymphoprep to isolate mononuclear cells. Cell concentration and composition were analysed as described for peritoneal lavage samples. Cells were cryopreserved.

4.1.4 Endometriosis lesion samples

Tissue from up to three lesions per patient was collected and processed separately. Part of each sample was embedded in OCT and snap-frozen for future spatial analysis, while the remaining tissue was used for immune cell isolation as described below.

Tissue samples were minced and enzymatically digested in DMEM (Gibco) containing 1 mg/ml collagenase type II (Sigma-Aldrich; cat. no. C6885) and 100 U/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany; cat. no. 11284932001) for 45 minutes (37°C , 5% CO_2) with regular mixing. The suspension was then strained through a 40 μm cell strainer and washed with PBS. Erythrocytes were lysed with ACK lysis buffer, and the cells were washed and resuspended in PBS. Cell concentration and composition were analysed as described for peritoneal lavage samples. Cells were cryopreserved.

Table 4.1: Flow cytometry staining reagents for patient samples.

Reagent	Clone	Supplier	Cat. no.
CD3/CD56-FITC/PE-Cy5	Unspecified	Beckman Coulter	A07415
CD45-V500	HI30	BD Biosciences	560779
CD15-APC-Cy7	WCD3	BioLegend	323048
LIVE/DEAD Fixable Violet	–	Invitrogen	L34955

4.1.5 Flow cytometry acquisition and compensation

Patient samples were analysed using a CytoFLEX flow cytometer (Beckman Coulter). Spectral overlap was corrected using single-stained compensation controls, consisting of compensation beads for each fluorochrome-conjugated antibody and PBMCs stained with the viability dye (Table 4.1). The resulting compensation matrix was applied to all samples, except PBMCs, before analysis in FlowJo.

4.2 Cell cultures

K562 cells, SK-N-AS cells, and patient-derived cells isolated from EN lesions were cultured. K562 cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco) supplemented with 10% FCS, 1% Penicillin–Streptomycin (PeSt; Gibco), 1% sodium pyruvate (Gibco), and 1% L-glutamine (Gibco). SK-N-AS cells were cultured in RPMI 1640 medium with GlutaMAX (Gibco), supplemented with 10% heat-inactivated FCS and 1% PeSt.

The patient-derived cells consisted of a mixed population of endometrial epithelial and stromal cells, and are hereafter referred to as EN-derived cultured cells (ENCCs). ENCCs were provided by Dr Anna Linder and cultured in DMEM/F-12 medium (Gibco) supplemented with 10% FCS, 1% GlutaMAX (Gibco), 1% PeSt, and 1% Amphotericin B (Gibco). They were cultured in T25 flasks coated with Collagen I (Corning) according to the manufacturer’s instructions.

SK-N-AS cells and ENCCs were adherent and were therefore detached prior to passaging or experimental use. Routine passaging was performed using 0.25% trypsin (Gibco) in PBS for SK-N-AS cells and TrypLE Express (Gibco) for ENCCs. For experimental use, SK-N-AS cells were detached using 0.5 mM EDTA (Invitrogen) in PBS, whereas ENCCs were detached using Accutase (Invitrogen).

4.3 Functional assays

The main functional assay used in this project simultaneously assessed NK cell degranulation and cytotoxicity. Staining of target cell ligands was also performed to complement this assay. Unless otherwise stated, centrifugation steps were performed at $350 \times g$ for 7 min at 4°C for tubes and 3 min for plates.

4.3.1 NK cell isolation

HD NK cells used in functional assays were isolated from buffy coats obtained from Sahlgrenska University Hospital. Buffy coats were diluted in buffered NaCl and mixed with 2% dextran to induce erythrocyte sedimentation. The upper layer was collected and layered onto Lymphoprep supplemented with NaCl. PBMCs were isolated by density gradient centrifugation as described for EndoMIC blood samples, and cell concentration and NK cell percentage were determined.

NK cells were isolated from PBMCs using an NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany; cat. no. 130-092-657) according to the manufacturer's instructions. Briefly, PBMCs were incubated with a biotin-antibody cocktail and magnetic microbeads, and NK cells were isolated by negative selection using a MACS separator and LS columns (Miltenyi Biotec). The unlabelled NK cells were collected in the flow-through, centrifuged, and resuspended in NK cell medium consisting of IMDM supplemented with 10% FCS, 1% PeSt, 1% sodium pyruvate, and 1% L-glutamine. NK cell purity was >90%. Cells used the same day were diluted to 2×10^6 cells/ml. Remaining cells were cryopreserved for later functional assays.

4.3.2 Degranulation and cytotoxicity assay

HD NK cells, either freshly isolated or thawed following cryopreservation, were stimulated overnight with recombinant IL-2 (Proleukin, Clinigen, Burton-on-Trent, UK) at a final concentration of 500 IU/ml. The following day, NK cells were pooled, centrifuged, resuspended in NK cell medium, and cell concentration determined using a Sysmex XP-100. Concentrations were adjusted for each effector-to-target (E:T) ratio.

Target cells were stained with CellTrace Violet (Invitrogen; cat. no. C34557) to distinguish them from NK cells during analysis. K562 cells were stained in suspension, whereas SK-N-AS cells and ENCCs were stained while adherent and subsequently detached using 0.5 mM EDTA or Accutase, respectively. Target cell concentrations were adjusted based on the NK-to-target ratio determined by flow cytometry, by quantifying CellTrace-positive target cells relative to CellTrace-negative NK cells.

Table 4.2: Blocking antibodies used for functional assessment.

Antibody	Clone	Supplier (cat. no.)
aNKp30	F252	Prof. Silvia Pesce, Genoa, Italy
aDNAM-1	F5	Prof. Silvia Pesce
aNKp46	KL247	Prof. Silvia Pesce
aNKp44	KS38	Prof. Silvia Pesce
aCD2	QA196	Prof. Silvia Pesce
aNKG2D	BAT221	Miltenyi Biotec (130-092-686)
aHLA-ABC	A6-136	Prof. Silvia Pesce

Several assay conditions were included. NK cells and target cells were co-cultured at E:T ratios of 5:1 and 1:1. The 1:1 condition was performed both with and without blocking antibodies. Blocking conditions were used to assess the contribution of specific receptor-ligand interactions to NK cell degranulation. Target-only and NK-only conditions were included as controls. Additional NK-only controls with each blocking antibody were used to assess whether the antibodies themselves induced degranulation. Blocking antibodies are listed in Table 4.2, and assay conditions are summarised in Table 4.3.

Table 4.3: Overview of reagents added in each condition of the degranulation assay.

	E:T condition	+ NK receptor blocking	+ Target ligand blocking	Target control	Negative control
20 min pre-incubation					
<i>NK cells</i>	50 µl	50 µl	–	–	50 µl
<i>Target cells</i>	–	–	50 µl	–	–
<i>NK receptor Ab</i>	–	50 µl	–	–	–
<i>Target ligand Ab</i>	–	–	50 µl	–	–
<i>Medium</i>	50 µl	–	–	100 µl	50 µl
4h incubation					
<i>NK cells</i>	–	–	50 µl	–	–
<i>Target cells</i>	50 µl	50 µl	–	50 µl	–
<i>aCD107a</i>	20 µl	20 µl	20 µl	–	20 µl
<i>Medium</i>	30 µl	30 µl	30 µl	50 µl	80 µl

For blocking conditions, NK cells (F252, F5, KL247, KS38, QA196, BAT221) or target cells (A6-136) were pre-incubated with blocking antibodies for 20 min at 37°C and 5% CO₂. The co-cultures were then prepared with a CD107a antibody (BUV395, BD Biosciences; cat. no. 565113) and incubated for 4 h. After incubation, cells were washed with 2 mM EDTA in buffered NaCl and stained with LIVE/DEAD Fixable Near IR (Invitrogen; cat. no. L10119). Degranulation and cytotoxicity were analysed by flow cytometry using a BD LSRFortessa Cell Analyser (BD Biosciences).

The flow cytometry data were analysed and gated in FlowJo. The gating strategy utilised for the degranulation assays (and combined cytotoxicity assessment) is shown in Figures 4.1 and 4.2. The axes for Time, side scatter (SSC), and forward scatter (FSC) were set to linear, whereas the axes for fluorescent labels were set to biexponential. This analysis generated the percentages of live CD107a-positive NK cells and dead target cells.

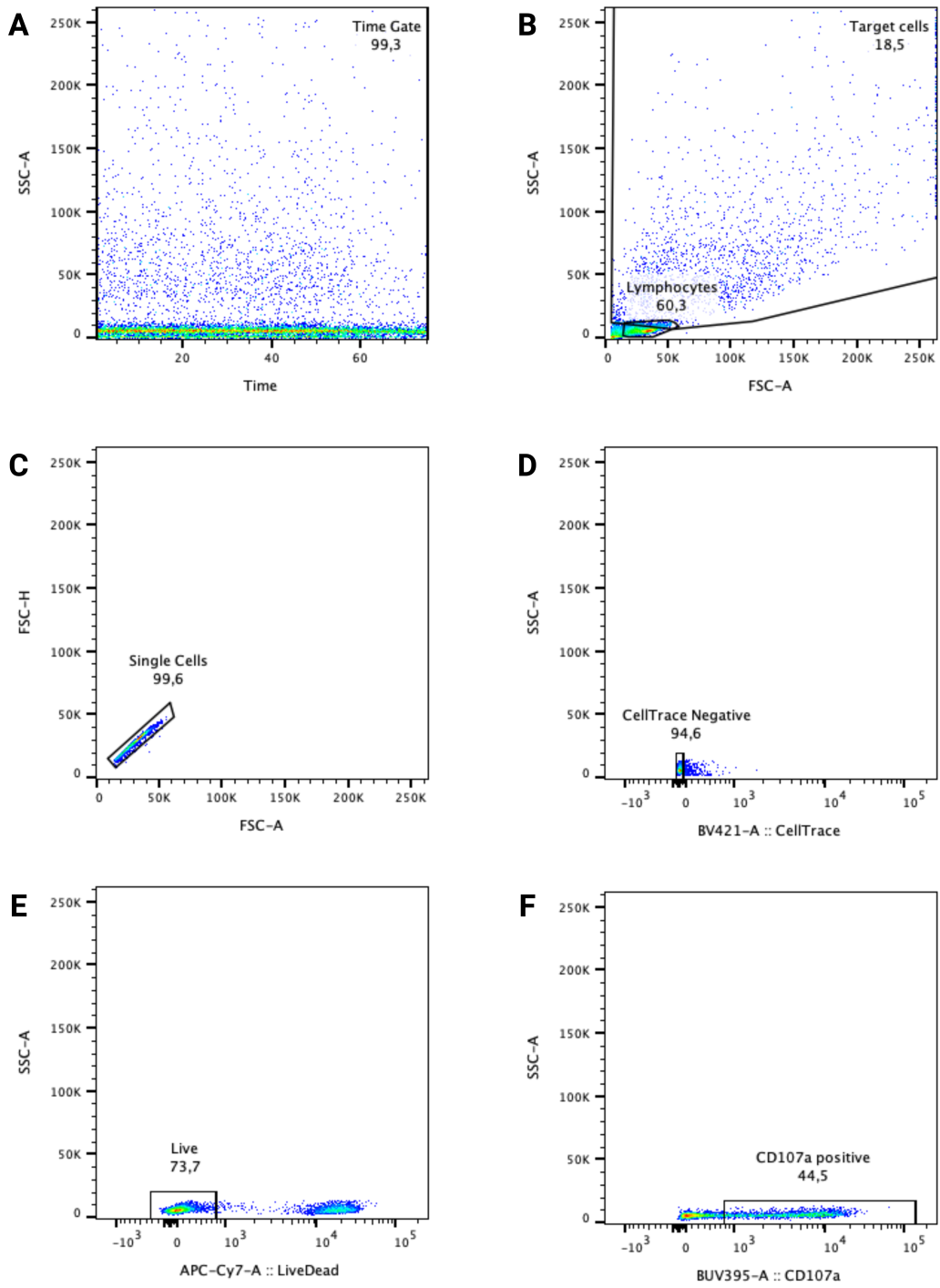


Figure 4.1: Gating strategy for degranulation analysis. **(A)** Time gate excluding acquisition irregularities. **(B)** Cell gates separating debris, NK cells, and target cells. **(C)** Singlet gate excluding doublets. **(D)** CellTrace gate excluding CellTrace-positive target cells. **(E)** Live cell gate excluding dead NK cells. **(F)** CD107a gate identifying CD107a-positive NK cells.

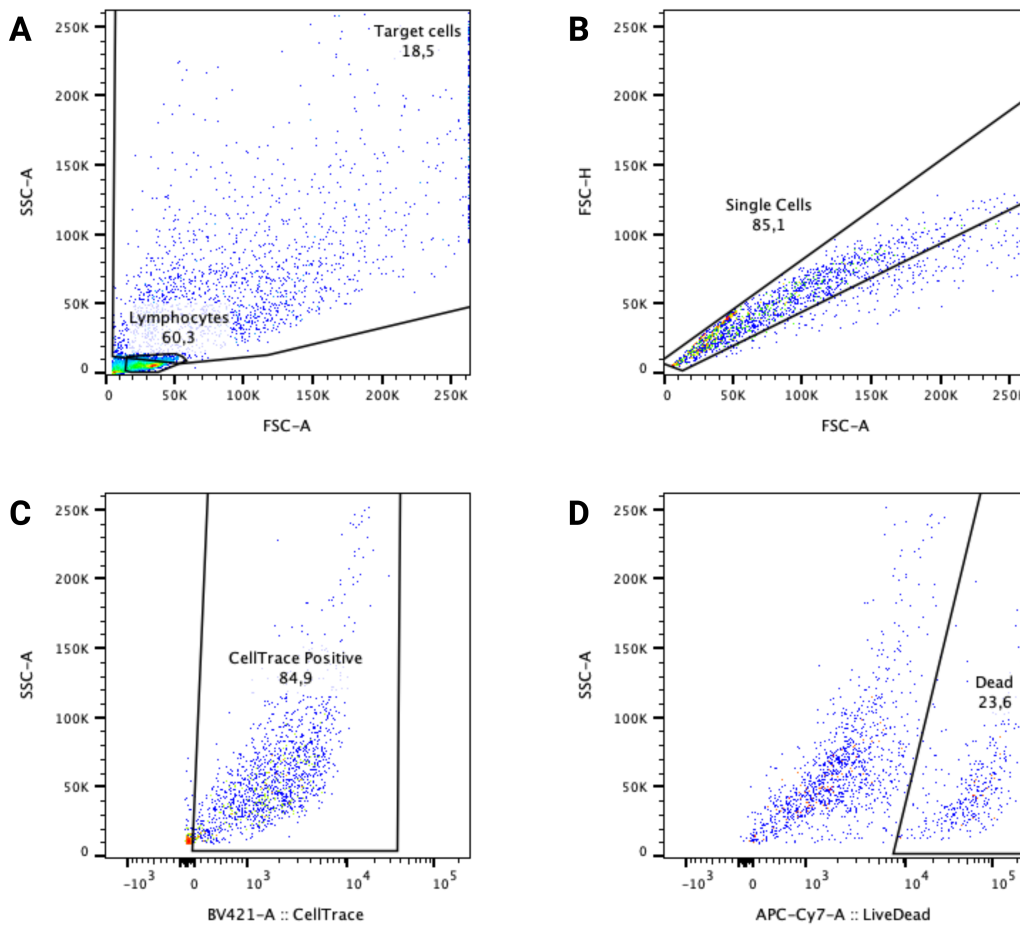


Figure 4.2: Gating strategy for cytotoxicity analysis. **(A)** Cell gate as in Figure 4.1B. **(B)** Singlet gate excluding doublets. **(C)** CellTrace gate excluding CellTrace-negative NK cells. **(D)** Dead cell gate identifying dead target cells.

4.3.3 Identification of ligands on target cells

Surface expression of NK cell receptor ligands on ENCCs and K562 cells was assessed by flow cytometry to correlate NK cell receptor-blocking results with ligand levels. Briefly, cells were harvested and washed in E-buffer. Cells were then resuspended in E-buffer and distributed into wells for staining of different ligands. All wells were stained with LIVE/DEAD Near IR. Additional ligand-specific antibodies were added to individual wells as shown in Table 4.4. Antibodies used are listed in Table 4.5.

Cells were first labelled with LIVE/DEAD Fixable Near IR according to the manufacturer's instructions. Thereafter, primary antibodies were added to the indicated wells, incubated for 30 min, and washed with E-buffer. For samples requiring secondary antibody detection, cells were then blocked with goat serum diluted in E-buffer for 10 min to reduce non-specific binding. After blocking, cells were incubated with a fluorophore-conjugated goat anti-mouse secondary antibody (Table 4.5) for 30 min, washed again, and resuspended in E-buffer for flow cytometry acquisition.

Table 4.4: Antibody combinations used for target ligand staining.

Well	Ligands
1	Live/Dead + HLA-ABC + HLA-E + CD48
2	Live/Dead + MIC-A/B + ULBP-1 + ULBP-2/5/6
3	Live/Dead + B7-H6 (+ Secondary antibody)
4	Live/Dead + PVR (+ Secondary antibody)
5	Live/Dead + Nectin-2 (+ Secondary antibody)
6	Live/Dead + Secondary antibody control
7	Live/Dead control

Table 4.5: Staining reagents used for target ligand analysis.

Antibody	Fluorophore	Clone	Supplier (cat. no.)
HLA-ABC	FITC	G46-2.6	BD Biosciences (555552)
HLA-E	PE-Cy7	3D12	BioLegend (342608)
CD48	BV421	TÜ145	BD Biosciences (582718)
MIC-A/B	viobright FITC	6D4	Miltenyi Biotec (130-106-100)
ULBP-1	PerCP	170818	R&D Systems, Minneapolis, MN, USA (FAB1380C)
ULBP-2/5/6	APC	165903	R&D Systems (FAB1298A)
B7-H6	–	875001	R&D Systems (MAB7144)
PVR	–	5A10	Prof. Silvia Pesce
Nectin-2	–	L14	Prof. Silvia Pesce
Secondary	AlexaFluor 488	Polyclonal	Invitrogen (A-11001)

In the FlowJo analysis, a time gate was first applied to exclude acquisition irregularities and unstable flow. Cells were then gated based on forward scatter (FSC) and side scatter (SSC) to exclude debris, followed by singlet gating to remove doublets. Dead cells were excluded using LIVE/DEAD Fixable Near IR. Ligand expression was then assessed within the relevant live singlet target cell populations. Median fluorescence intensity (MFI) was used as a measure of relative ligand expression level.

The antibody panel was later updated and expanded. Antibodies for EpCAM (BUV395; BD Biosciences, cat. no. 745704) and CD13 (PE-Cy7; BD Biosciences, 338439) were added in order to identify epithelial and stromal cells, respectively, within the ENCC population. An extra well with antibodies for TRAIL-R1 (PE; BD Biosciences, 564180) and TRAIL-R2 (APC; BioLegend, 307407) were also added. The antibodies used for the expanded panel are listed in Appendix A.2.

4.4 Statistics

Statistical analysis was performed using GraphPad Prism (version 10.6.1). Multiple linear regression analyses were performed to assess the effects of experimental conditions on NK cell degranulation and cytotoxicity. NK cell donor, target cell donor, and experimental condition were included as independent variables in the models. For degranulation analyses, blocking conditions were compared with the unblocked E:T 1:1 condition. For cytotoxicity analyses, separate models were constructed using either the target only condition or the E:T 1:1 condition as the reference condition. Multiple comparisons were controlled for using the Holm–Bonferroni method.

4.5 Use of AI tools

AI tools, including Grammarly and ChatGPT, were used as support during the thesis process, primarily for language refinement and for identifying grammatical mistakes and spelling errors. While independent literature searches were performed using databases and search engines such as PubMed and Google Scholar, ChatGPT was also occasionally used to assist in identifying potentially relevant literature. All AI-generated suggestions were critically evaluated and revised before inclusion.

5

Results

In this chapter, the results obtained during the project are presented, including sample processing optimisation, NK cell degranulation and cytotoxicity assays, and target ligand stainings.

5.1 Development of sample processing protocols

As previously mentioned, the final methods for processing patient samples are described in the Methods chapter. The protocols, however, have been optimised during this master's thesis. The most important updates made are described below.

5.1.1 Blood samples

The blood processing protocol was revised twice. For the first five patients (EndoMIC 1–5), 1 mL of whole blood was collected separately and centrifuged at $1000 \times g$ for 10 minutes at 4°C for plasma isolation, while the remaining whole blood was used for PBMC isolation. As shown in Table 5.1, this protocol (v1) resulted in PBMC yields of 4–7 million cells, excluding the low yield observed for EndoMIC 2.

Table 5.1: Number of isolated PBMCs from each patient blood sample.

Patient ID	Protocol	Isolated cells
EndoMIC 1	v1	6×10^6
EndoMIC 2	v1	0.4×10^6
EndoMIC 3	v1	4×10^6
EndoMIC 4	v1	7×10^6
EndoMIC 5	v1	5×10^6
EndoMIC 6	v2	0.3×10^6
EndoMIC 7	v2	1.9×10^6
EndoMIC 8	v2	0.3×10^6
EndoMIC 9	v3	2×10^6
EndoMIC 10	v3	9×10^6

The protocol was then revised to enable plasma isolation from the whole blood sample. In the second version of the protocol (v2), used for EndoMIC 6–8, the entire blood collection tube was centrifuged at $350 \times g$ for 7 minutes. The plasma fraction was subsequently collected, while the remaining cellular fractions were used for PBMC isolation. As shown in Table 5.1, PBMC yields were generally lower with v2 than with v1, prompting further optimisation of the protocol.

The relative centrifugal force of $350 \times g$ in v2 was initially selected to preserve cell viability. However, the buffy coat layer was difficult to distinguish following centrifugation, suggesting that a substantial fraction of PBMCs might have remained suspended in the plasma fraction and were removed during plasma collection. Following consultation with personnel at Sahlgrenska University Hospital with expertise in blood component separation, a third protocol version (v3) was implemented. Samples were centrifuged at $1600 \times g$ for 10 minutes, resulting in a clearly distinguishable buffy coat layer that could be collected separately for PBMC isolation. PBMC yields obtained using v3 were comparable to those achieved with v1 (Table 5.1), while enabling plasma isolation from the same sample.

5.1.2 Peritoneal lavage samples

In Table 5.2, the number of cells isolated from the peritoneal lavage of each patient is shown. No major modifications to the protocol were required for peritoneal lavage samples. While a wide range of cell yields was observed across samples, no consistent relationship with protocol modifications could thus be identified.

Table 5.2: Number of isolated cells from peritoneal lavage samples.

Patient ID	Isolated cells
EndoMIC 1	3×10^6
EndoMIC 2	2.4×10^6
EndoMIC 3	–
EndoMIC 4	4×10^6
EndoMIC 5	1×10^6
EndoMIC 6	13×10^6
EndoMIC 7	1×10^6
EndoMIC 8	0.16×10^6
EndoMIC 9	1×10^6
EndoMIC 10	0.2×10^6

5.1.3 Endometrioma fluid samples

Several processing protocols were evaluated for isolation of cells from endometrioma fluid samples. The main protocol consisted of filtration and density gradient centrifugation to isolate mononuclear cells.

For the EndoMIC 4 sample, filtration followed by repeated PBS washes was evaluated to increase total cell yield. Although this approach yielded substantially higher cell counts, the proportion of lymphocytes was low. To further enrich immune cells, CD15 depletion was performed on 100 million of the isolated cells. However, only 0.2 million cells remained following depletion.

For EndoMIC 6 onward, cell recovery was optimised through additional centrifugation steps. Following the observation that the supernatant from the first wash after filtration remained visibly brown, it was collected and re-centrifuged to recover residual cells. In addition, the cell pellet was often poorly compacted, likely due to the high viscosity of the samples. An additional wash step of the pellet was therefore also introduced to improve cell recovery. As shown in Table ??, overall cell yields appeared to increase following implementation of these modifications.

Table 5.3: Characteristics of endometrioma fluid samples.

Patient ID	Endometrioma	Isolated cells
EndoMIC 1	I II	Pooled: 11×10^6
EndoMIC 2	I	1.4×10^6
EndoMIC 3	—	—
EndoMIC 4	I	800×10^6
EndoMIC 5	—	—
EndoMIC 6	I	21.6×10^6
EndoMIC 7	—	—
EndoMIC 8	I	132×10^6
	II	23×10^6
EndoMIC 9	I	250×10^6
EndoMIC 10	—	—

5.1.4 Tissue samples

Protocols for single cell isolation from tissue samples were also developed and optimised. In addition, snap freezing of part of the tissue samples was introduced for EndoMIC 7 onward. To isolate single cells, several adjustments to the tissue dissociation protocol were evaluated, primarily regarding the enzymatic digestion conditions. Initial tissue dissociation experiments (EndoMIC 2, 4, and 5) were performed using a digestion medium containing both collagenase type II and type IV at 1 mg/mL each. While these conditions enabled tissue dissociation, further optimisation was performed to improve cell recovery.

Table 5.4: Characteristics of lesion tissue samples.

Patient ID	Lesion	Snap frozen	Isolated cells
EndoMIC 1	–	–	–
EndoMIC 2	1	No	0.3×10^6
	2	No	0.1×10^6
	3	No	0.4×10^6
EndoMIC 3	–	–	–
EndoMIC 4	1	No	1×10^6
	2	No	1×10^6
	3	No	0.4×10^6
EndoMIC 5	1	No	$-.^* \times 10^6$
	2	No	0.4×10^6
	3	No	0.3×10^6
EndoMIC 6	1	No	$-.^* \times 10^6$
	2	No	0.2×10^6
	3	No	0.3×10^6
EndoMIC 7	1	Yes	–
	2	Yes	–
	3	Yes	3.6×10^6
EndoMIC 8	1	Yes	0.36×10^6
	2	Yes	0.68×10^6
	3	Yes	1.2×10^6
EndoMIC 9	1	Yes	3.3×10^6
	2	Yes	4.2×10^6
	3	Yes	1.2×10^6
EndoMIC 10	1	Yes	2.0×10^6
	2	Yes	0.3×10^6
	3	Yes	4.6×10^6

* Cell counts could not be determined using the default Sysmex analysis. From EndoMIC 7 onward, manual adjustment of the integration limits allowed for cell counts in such cases.

For EndoMIC 6, several modifications were introduced. The incubation time was extended from 45 to 90 minutes, the digestion volume was increased from 1 to 2 ml, and collagenase type II was used alone in place of the previous combination of types II and IV. While these modifications visually improved tissue digestion, they did not substantially increase cell yields (Table 5.4). An observation made for these EndoMIC 6 tissue samples, and also true for earlier patients' tissue samples, was that the samples frequently developed a gel-like consistency after digestion. As this can be caused by release of extracellular DNA from damaged cells, this indicated a need for DNase supplementation in the digestion medium.

The protocol was thus further refined for EndoMIC 7. The incubation time was reduced to 45 minutes to minimise potential loss of surface marker expression associated with prolonged enzymatic treatment, while the increased digestion volume was maintained. In addition, DNase I was added to the digestion medium to reduce sample viscosity. Although only one tissue sample from EndoMIC 7 was processed for single-cell isolation, cell yield appeared to improve. This protocol was therefore implemented for subsequent patient samples, in which cell yields have generally also been higher (Table 5.4).

In parallel with these adjustments, thorough mechanical mincing prior to enzymatic digestion was also found to be critical for efficient tissue dissociation. Considerable variability between samples was observed, likely reflecting differences in both tissue composition and sample mass used for dissociation. In particular, processing samples rich in scar tissue appeared to yield lower cell counts.

5.1.5 Flow cytometric assessment of cell composition

The staining strategy used to assess cell composition in peritoneal lavage, endometrioma fluid, and tissue samples was adjusted throughout sample collection. During flow cytometry analysis, a substantial proportion of cells from endometrioma fluid and tissue samples appeared to be simultaneously positive for multiple markers. This was suspected to result from non-specific antibody binding. An additional incubation step with 5% FCS in bNaCl prior to antibody staining was therefore introduced to reduce non-specific binding and improve staining quality. Despite the protocol modification, no major improvement in population separation was observed (see Appendix A.1). Several cell populations remained poorly resolved. For example, cells appeared in intermediate regions of the CD3 versus CD56 plots rather than forming distinct clusters (see Figure A.1). However, increased familiarity with the datasets and staining patterns over the course of the study enabled more consistent identification of these populations in subsequent analyses.

In addition to the observed staining challenges, low viability was observed when thawing one of the cryopreserved endometrioma fluid samples from EndoMIC 4. Thus, to enable monitoring of viability and exclusion of non-viable cells during cell composition analysis, a live/dead viability marker was introduced.

5.2 Degranulation and cytotoxicity assays

To investigate the mechanisms underlying NK cell-mediated responses against patient-derived ENCCs, a combined degranulation and cytotoxicity assay was performed using healthy donor NK cells co-cultured with ENCCs. Co-cultures with K562 and SK-N-AS cells were also performed as controls. Blocking antibodies targeting activating NK cell receptors and the inhibitory HLA-ABC target ligands were used to assess the contribution of specific receptor–ligand interactions to NK cell activation and target cell killing. NK cell degranulation was evaluated by CD107a surface expression, while cytotoxicity was assessed in parallel using a live/dead marker to quantify target cell death.

5.2.1 Degranulation toward ENCCs

Experiments were performed using NK cells derived from three healthy donors and ENCCs derived from two EN patients. As shown in Figure 5.1, large variance was introduced by the different NK donors. To account for donor-dependent variability while assessing the effect of individual blocking conditions, statistical analysis was performed using multiple linear regression including NK cell donor, ENCC donor, and experimental condition as separate variables:

$$\text{Degranulation} \sim \text{Intercept} + \text{NK cell donor} + \text{Patient ID} + \text{Condition}$$

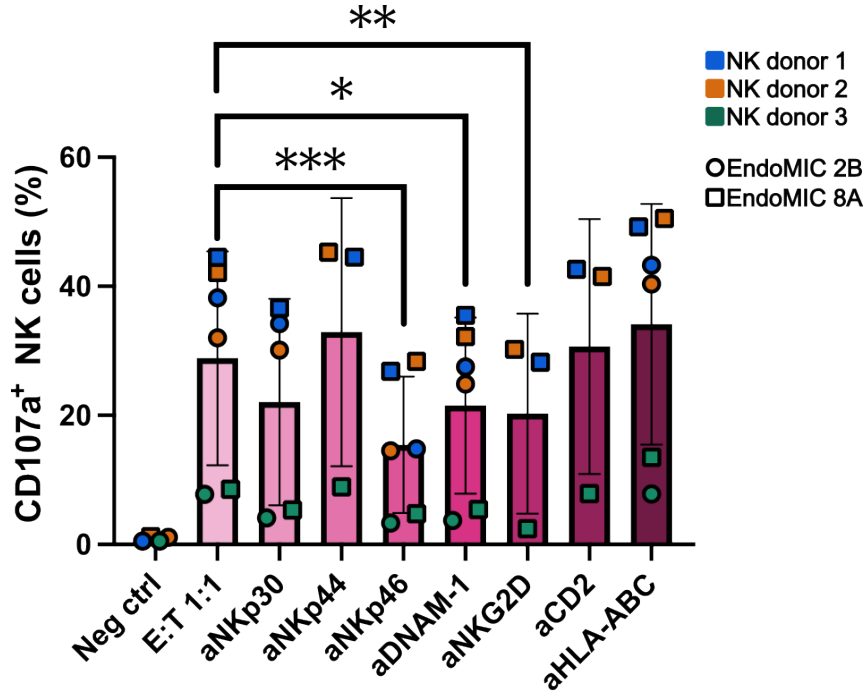


Figure 5.1: Percentage of CD107⁺ HD NK cells following co-culture with ENCCs at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent the mean with standard deviation. Colours of individual data points indicate NK cell donor and symbol shapes indicate the patient-derived ENCCs. Statistical significance was assessed using multiple linear regression and Holm–Bonferroni correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Effect size estimates (beta coefficients) for the different blocking antibody conditions were calculated relative to the E:T 1:1 condition. The estimates and 95% confidence intervals (without correction for multiple testing) are shown in Figure 5.2. Multiple testing correction was then performed using the Holm–Bonferroni method. The adjusted significance levels and the resulting significances are shown in Table 5.5. Significant comparisons are also shown in 5.1. Blocking the activating receptors NKp46, NKG2D, and DNAM-1 significantly reduced degranulation toward ENCCs. The increase in degranulation following HLA-ABC blockade, with a p-value of 0.0359, was not significant after multiple testing correction (Holm–Bonferroni adjusted threshold of 0.0125).

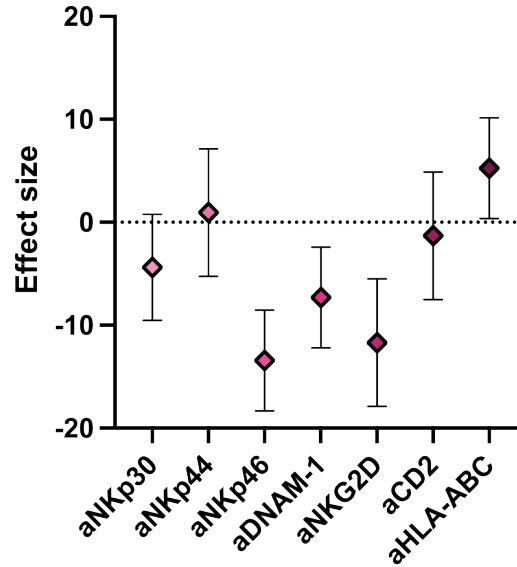


Figure 5.2: Effect size estimates (beta coefficients) for each antibody blocking condition compared to the E:T 1:1 condition. The variance shown is the 95% confidence interval (without corrections for multiple testing).

Table 5.5: Holm–Bonferroni-adjusted significance thresholds and corresponding significance levels for blocking antibody conditions compared to the E:T 1:1 condition.

Condition	P-value	Holm–Bonferroni thresholds			Significance
		$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.001$	
aNKp46	< 0.0001	$\frac{0.05}{7} = 0.00714$	0.00143	0.000143	***
aNKG2D	0.0006	$\frac{0.05}{6} = 0.00833$	0.00167	0.000167	**
aDNAM-1	0.0048	$\frac{0.05}{5} = 0.01000$	0.00200	0.000200	*
aHLA-ABC	0.0359	$\frac{0.05}{4} = 0.01250$	0.00250	0.000250	ns
aNKp30	0.0932	$\frac{0.05}{3} = 0.01667$	0.00333	0.000333	ns
aCD2	0.6674	$\frac{0.05}{2} = 0.02500$	0.00500	0.000500	ns
aNKp44	0.7568	$\frac{0.05}{1} = 0.05000$	0.01000	0.001000	ns

5.2.2 Cytotoxicity toward ENCCs

In the simultaneous cytotoxicity assessments, the differences between conditions were not as pronounced. In Figure 5.3, a large variance introduced through the ENCC donor can be seen. In particular, a higher percentage of dead ENCCs is seen for EndoMIC 2B compared to EndoMIC 8A. This is especially evident in the target only condition. Viability assessed by trypan blue exclusion prior to co-culture did not reveal a corresponding difference in viability between the cell cultures. Because some conditions (E:T 5:1, aNKp44, aNKG2D, and aCD2) were assessed only using EndoMIC 8A cells, the corresponding mean percentages of dead ENCCs may be lower than those of conditions that also included data from EndoMIC 2B.

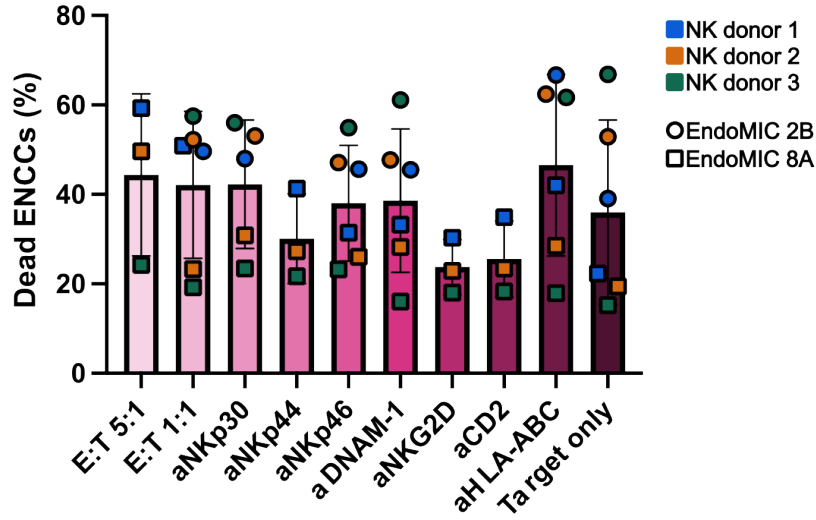


Figure 5.3: Percentage of dead ENCCs following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate NK cell donor and symbol shapes indicate ENCC donor.

Due to donor-dependent variances, multiple linear regression models were also used for the statistical analysis of the cytotoxicity results for ENCCs:

$$\text{Cytotoxicity} \sim \text{Intercept} + \text{NK cell donor} + \text{Patient ID} + \text{Condition}$$

Two different models were used, one where the experimental conditions were compared to target only (Figure 5.4A), and one where comparisons were made to the E:T 1:1 condition (Figure 5.4B). After Holm-Bonferroni correction, the only statistically significant effect was the E:T 5:1 condition compared to the target only condition (see Appendix B.1).

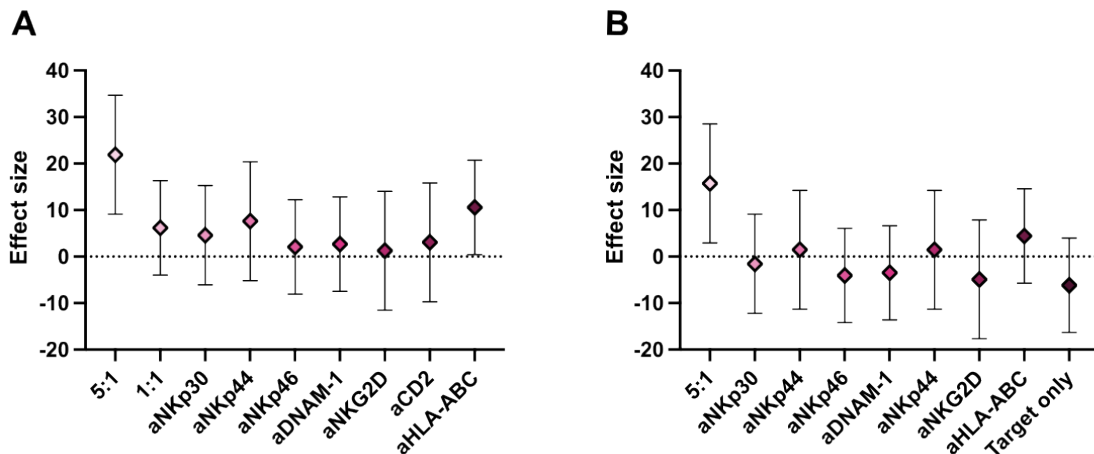


Figure 5.4: Effect size estimates for each experimental condition compared to (A) the target only control, and (B) the E:T 1:1 condition. The variance shown is the 95% confidence interval (without corrections for multiple testing).

5.2.3 Degranulation and cytotoxicity toward K562 cells

To validate NK cell functionality and provide reference target cells for comparison, degranulation and cytotoxicity were assessed using K562 cells. The degranulation results are shown in Figure 5.5. Overall, NK cells exhibited high degranulation responses against K562 cells. The lower response of NK donor 3 is still evident, and multiple linear regression was thus used:

$$\text{Degranulation} \sim \text{Intercept} + \text{NK cell donor} + \text{Condition}$$

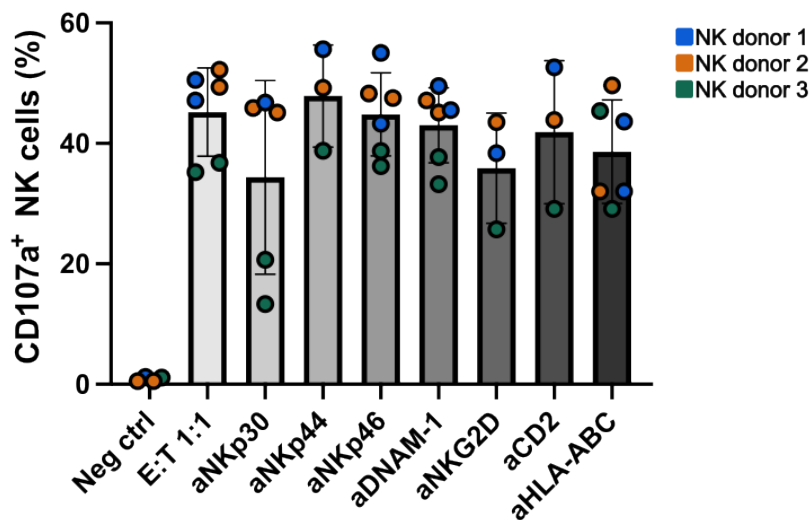


Figure 5.5: Percentage of CD107⁺ HD NK cells following co-culture with K562 cells at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent means with standard deviation. Colours indicate NK cell donor.

Compared with the E:T 1:1 condition, no receptor blockade resulted in a significant change in NK cell degranulation. The blockade of NKp30 had the largest effect. However, the resulting reduction in degranulation was not significant after Holm-Bonferroni correction (see Appendix B.2).

In Figure 5.6, the results from the simultaneous cytotoxicity assessment are shown. None of the blocking conditions remained significant following Holm-Bonferroni correction. However, significant differences were observed between the target-only, E:T 1:1, and E:T 5:1 conditions (Appendix B.3). Compared to the ENCC assay, the cytotoxicity assessment showed lower variability across experiments.

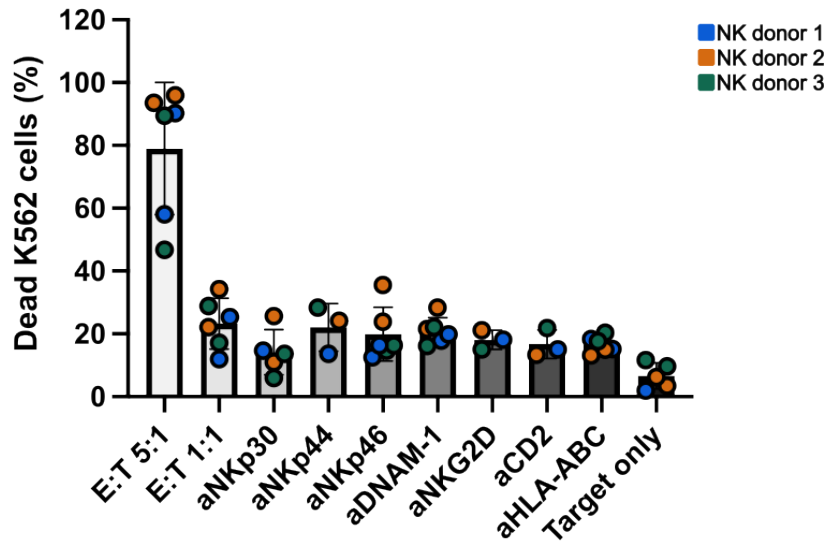


Figure 5.6: Percentage of dead K562 cells following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate the NK cell donor.

5.2.4 Degranulation and cytotoxicity toward SK-N-AS cells

The adherent SK-N-AS cells were also used as a control/reference target cell for the degranulation and cytotoxicity assessments. As an adherent cell line, they provide a target cell model more comparable to ENCCs. SK-N-AS cells have also been used previously in the laboratory, providing a well-characterised reference model with known experimental outcomes, including reduced NK cell responses following NKp46 blockade.

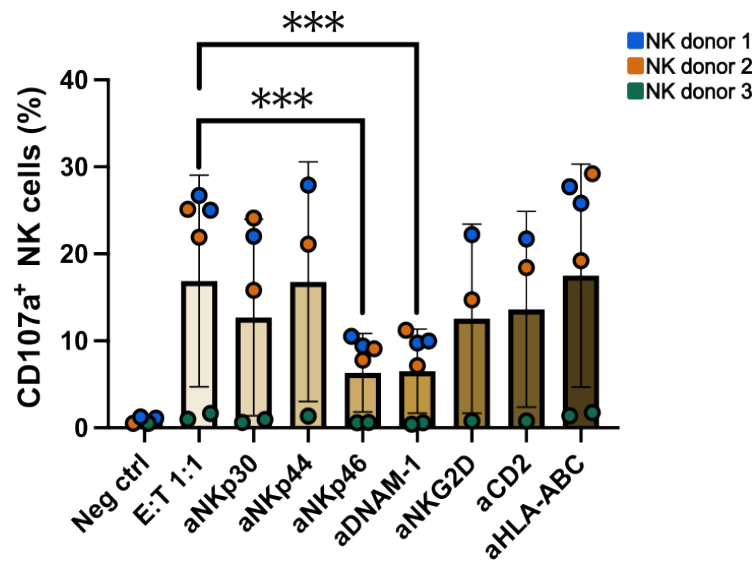


Figure 5.7: Percentage of CD107⁺ HD NK cells following co-culture with SK-N-AS cells at an E:T ratio of 1:1, with blocking antibodies as indicated. Bars represent means with standard deviation. Colours indicate the NK cell donor.

The degranulation results are shown in Figure 5.7. Compared with the E:T 1:1 condition, receptor blockade of DNAM-1 and Nkp46 resulted in a significant change in NK cell degranulation (see Appendix B.4). The lower degranulation ability of the NK cells from donor 3 is also clearly apparent in Figure 5.7.

As shown in Figure 5.8, the cytotoxicity assessment of SK-N-AS cells exhibited considerable variability between experiments, similar to that observed for the ENCCs. Consequently, no conditions were significant following Holm–Bonferroni correction for multiple comparisons (see Appendix B.5).

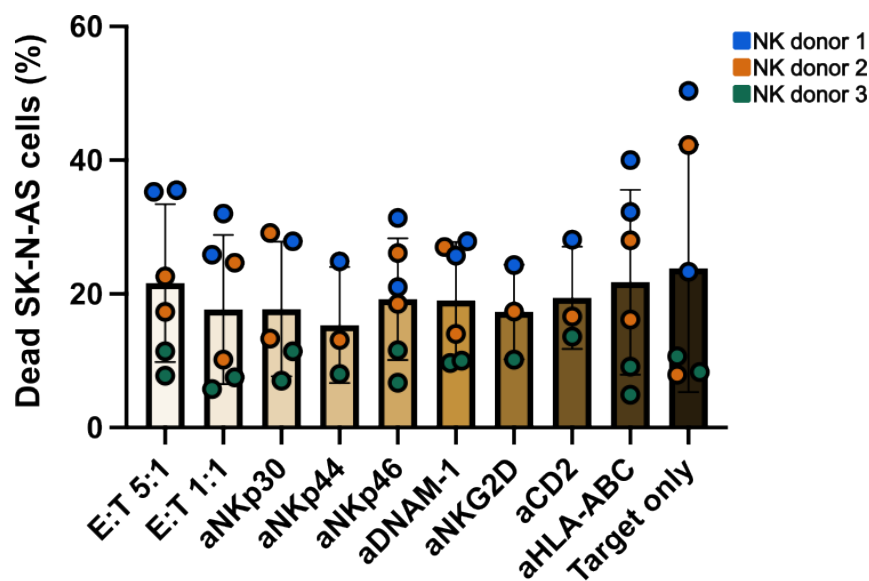


Figure 5.8: Percentage of dead SK-N-AS cells following co-culture with healthy donor NK cells at different E:T ratios and blocking conditions. Bars represent means with standard deviation. Colours indicate the NK cell donor.

5.3 Ligand staining

To complement the degranulation and cytotoxicity assays, ligand expression on ENCCs was assessed by flow cytometry. Histograms showing staining of live ENCCs for the nine investigated ligands, together with their respective controls (secondary antibody control or Live/Dead control), are presented in Figure 5.9. Mean fluorescence intensity (MFI) values for each ligand staining and corresponding control are summarised in Table 5.6.

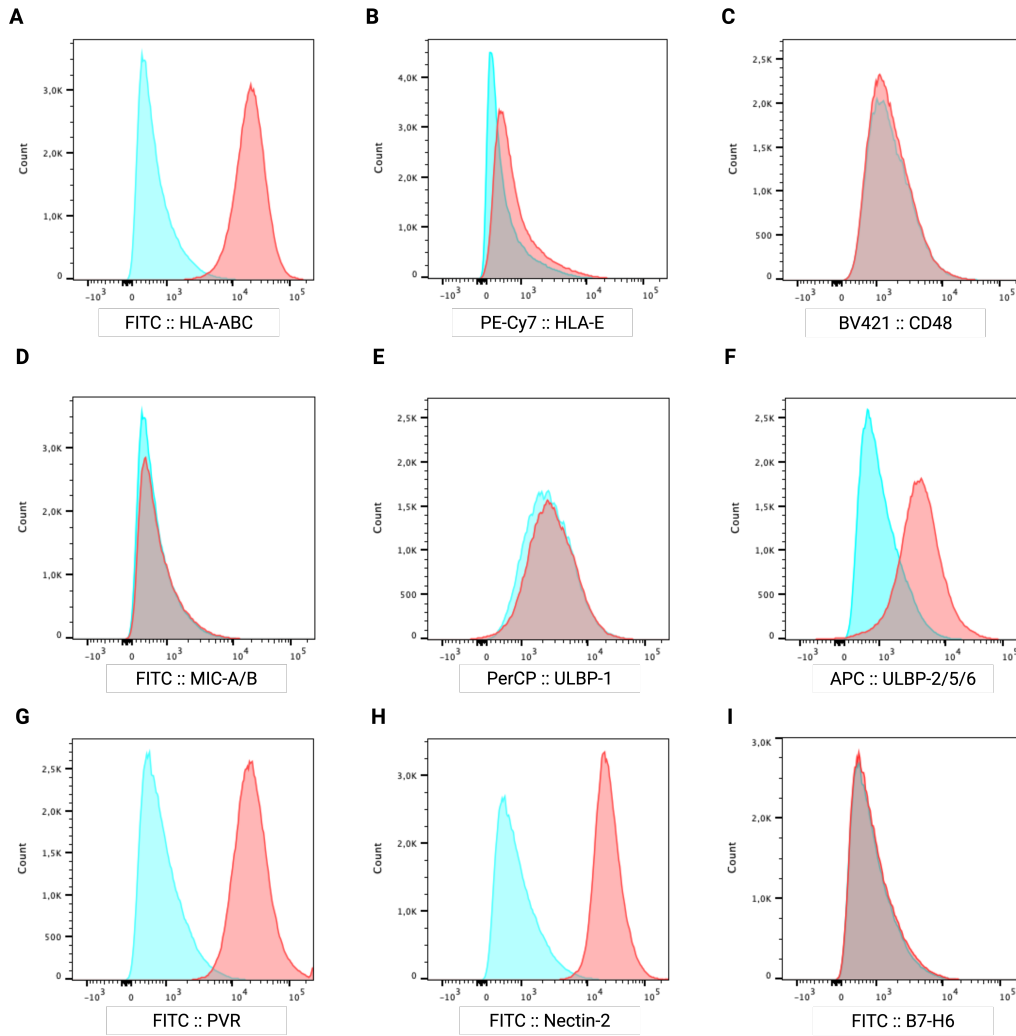


Figure 5.9: Histograms showing staining of target ligands on ENCCs from EndoMIC 8A. Red histograms represent antibody staining of live ENCCs and blue histograms represent negative live ENCC controls (Live/Dead control or Secondary antibody control). (A) HLA-ABC, (B) HLA-E, (C) CD48, (D) MIC-A/B, (E) ULBP-1, (F) ULBP-2/5/6, (G) PVR, (H) Nectin-2, and (I) B7-H6.

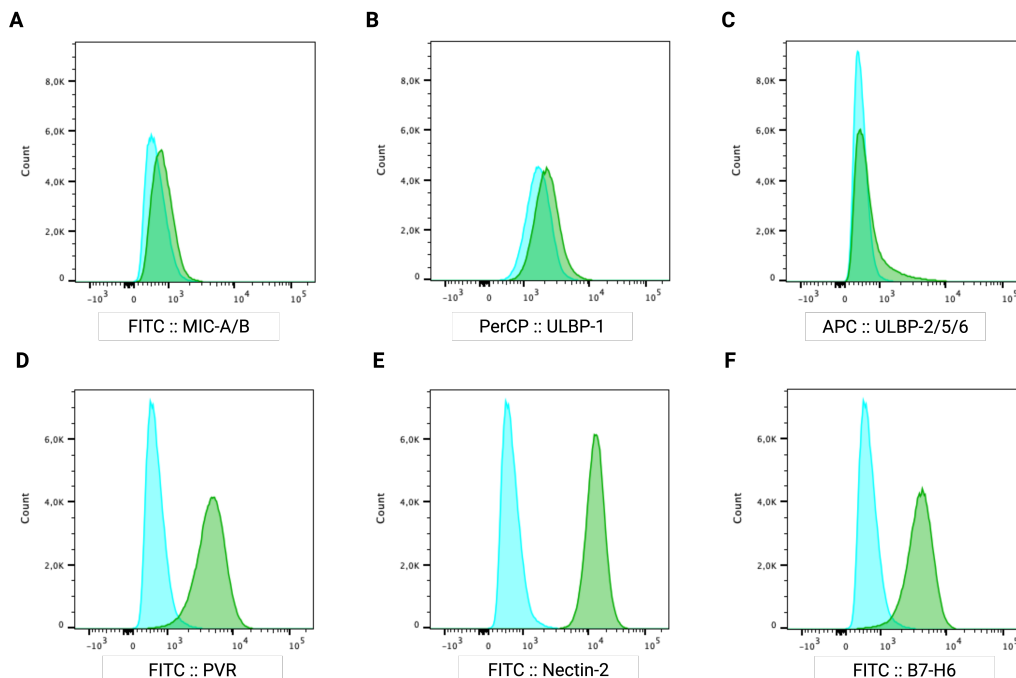
As shown in Figure 5.9A, HLA-ABC, which are ligands for inhibitory KIRs, were highly expressed on ENCCs from EndoMIC 8A. Strong expression of PVR and Nectin-2, ligands for the activating receptor DNAM-1, was also observed (Figure 5.9G-H). MFI values for these were over 20-fold higher than for their controls (Table 5.6). ULBP-2/5/6, ligands for the activating receptor NKG2D, also exhibited increased expression, with a more than four-fold higher MFI compared to the control.

In contrast, no clear increase in staining intensity relative to the controls was observed for HLA-E, CD48, MIC-A/B, ULBP-1, or B7-H6 (Table 5.6), and no distinct shift was apparent in the corresponding histograms (Figure 5.9B-E and I). These ligands correspond to the NK-cell receptors NKG2A (HLA-E), 2B4 and CD2 (CD48), NKG2D (MIC-A/B and ULBP-1), and NKp30 (B7-H6).

Table 5.6: Mean fluorescence intensity (MFI) for ENCC ligand stainings and corresponding negative controls.

Ligand	Control MFI	Ligand MFI
HLA-ABC	802	23171
HLA-E	722	1240
CD48	2181	2158
MIC-A/B	802	955
ULBP-1	3564	3901
ULBP-2/5/6	1295	5390
PVR	1097	27601
Nectin-2	1097	25915
B7-H6	1097	1166

Ligand staining was also performed on K562 cells, which were included as a control cell line. However, due to a technical issue during flow cytometric acquisition, only a very limited number of events were recorded for the first sample containing K562 cells stained for HLA-ABC, HLA-E, and CD48. These stainings were therefore excluded from further analysis. Histograms for the remaining six ligand stainings and their corresponding controls are shown in Figure 5.10.

**Figure 5.10:** Histograms showing staining of target ligands on K562 cells. Green histograms represent antibody staining of live K562 cells and blue histograms represent negative live K562 controls. (A) MIC-A/B, (B) ULBP-1, (C) ULBP-2/5/6, (D) PVR, (E) Nectin-2, and (F) B7-H6.

For the K562 cells, strong expression of PVR and Nectin-2 was observed (Figure 5.10D-E), with clear separation between stained and control populations. B7-H6 expression was also clearly detected (Figure 5.10F), in contrast to the ENCCs from EndoMIC 8A. MIC-A/B, ULBP-1, and ULBP-2/5/6 exhibited only limited increases in fluorescence intensity relative to their corresponding controls (Figure 5.10A-C).

An extended flow cytometry panel was further evaluated to enable characterisation of ENCC subpopulations and investigate target ligand expression on specific subpopulations. Thus, CD13 and EpCAM were included in the panel to distinguish stromal and epithelial cell populations, respectively. However, expansion of the panel resulted in substantial spectral spillover between multiple fluorophores, and the applied compensation matrix was insufficient to fully correct for these effects.

Despite these technical limitations, evaluation of the CD13 and EpCAM stainings provided preliminary information regarding the composition of the ENCCs (at the point of the experiment). As shown in Figure 5.11, a distinct CD13-positive ENCC population is observed, whereas no clearly defined EpCAM-positive population could be identified. This staining pattern suggests that the ENCCs from EndoMIC 8A used in the experiment were predominantly stromal cells.

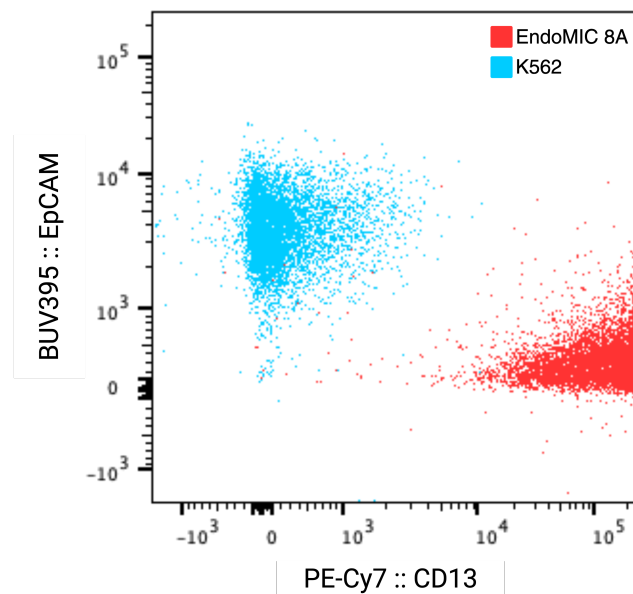


Figure 5.11: Plot of CD13-PE-Cy7 and EpCAM-BUV395 staining in ENCCs from EndoMIC 8A (red) and K562 cells (blue).

The extended flow cytometry panel also included markers for TRAIL-R1 and TRAIL-R2. While TRAIL-R1-PE staining could not be reliably interpreted due to spillover-related effects (see Appendix A.2), the TRAIL-R2-APC signal was less affected and considered interpretable. As shown in Figure 5.12, ENCCs exhibited increased TRAIL-R2 staining relative to the negative live-cell control, indicating expression of TRAIL-R2 within the cultured ENCC population.

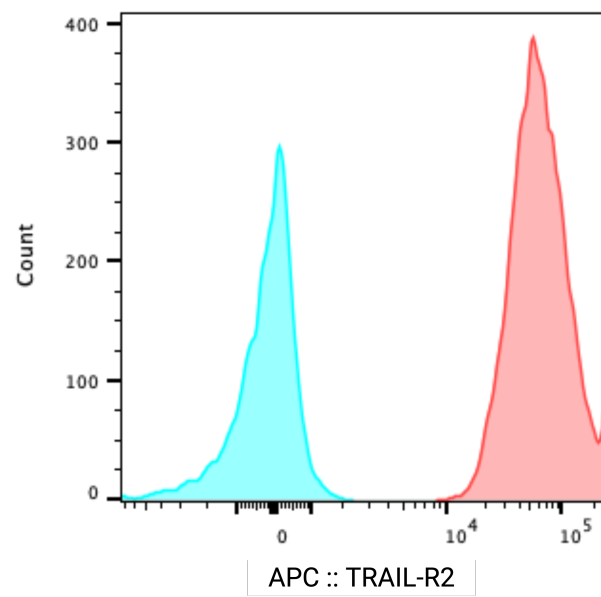


Figure 5.12: Histogram showing TRAIL-R2 staining in ENCCs from EndoMIC 8A (red) compared to a negative live ENCC control (blue).

6

Discussion

In this chapter, the findings on NK cell recognition of ENCCs are discussed, with particular focus on the receptor-ligand interactions identified through functional assays and ligand staining experiments. In addition, protocols for isolating immune cells from EN samples are discussed. Although these cells were not included in the functional experiments presented in this thesis, establishing reliable isolation procedures represents an important step towards future studies of these immune cells. Finally, the characterisation of the ENCC cultures, methodological limitations, and future research directions are discussed.

6.1 Sample processing

The revisions to the blood processing protocol were primarily driven by the need to enable plasma isolation from the entire blood sample for cfDNA analysis by collaborating researchers within the EndoMIC study, while maintaining sufficient PBMC recovery. Initial modifications resulted in reduced PBMC yields, likely due to suboptimal separation of the plasma and cellular fractions during centrifugation. Subsequent adjustment of the centrifugation conditions improved buffy coat separation and restored PBMC yields to levels comparable to those achieved with the original protocol. As the formation and recovery of the buffy coat are influenced by centrifugation conditions [56], these observations highlight the importance of balancing efficient separation of plasma and cellular fractions with optimal recovery of viable PBMCs for downstream applications.

For endometrioma fluid processing, the high viscosity of the samples, along with the presence of dead cells and cellular debris, complicated sample processing and immune cell recovery. Similar observations have previously been reported by Xu et al. [57]. Protocols for immune cell isolation from endometrioma fluid are also largely absent from the literature, limiting direct methodological comparisons. A major challenge was balancing total cell recovery with enrichment of immune cell populations. While omitting density gradient centrifugation increased cell yield, it reduced mononuclear cell enrichment, and CD15 depletion provided only limited additional benefit. In contrast, an additional wash and re-centrifugation step improved cell recovery while maintaining mononuclear cell enrichment, likely by reducing cell loss associated with sample viscosity. The final protocol therefore represented a compromise between maximising cell yield and enriching for immune cells for downstream analyses.

Tissue processing also presented several methodological challenges. While several modifications were evaluated, the addition of DNase I to the dissociation medium appeared to provide the greatest improvement in cell recovery and was supported by published protocols for dissociation of endometrial tissue [58]. However, considerable variability in cell recovery remained, which may partly reflect differences in fibrosis between samples. Fibrotic tissue, which is commonly associated with endometriosis, is characterised by extensive extracellular matrix deposition, particularly collagen [59]. This may impede both enzymatic and mechanical dissociation. Further optimisation may therefore be achieved through more standardised tissue processing procedures. Automated dissociation systems, such as the gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec), may be of interest and have previously been used for dissociation of endometrial and endometriosis tissue [60]. However, as that protocol was developed for single cell RNA sequencing and employed different digestion conditions, its suitability for immune cell isolation and subsequent phenotypic and functional characterisation remains to be evaluated.

Finally, assessment of cell composition using flow cytometry proved more challenging than anticipated. Several cell populations remained poorly resolved despite introduction of an FCS incubation step intended to reduce non-specific antibody binding. Consequently, interpretation of the cell composition data required careful gating and remained highly uncertain. Importantly, this analysis was intended primarily as a preliminary assessment of the isolated cell populations. More extensive phenotypic characterisation of immune cells from the EN samples using spectral flow cytometry is planned in future studies. The current protocol was considered sufficient for this initial assessment, but the increased complexity of future analyses may reveal a need to further optimise the isolation and staining procedures.

6.2 NK Cell Responses and Ligand Expression

Previous studies have reported impaired NK cell function in endometriosis and have proposed that reduced immune surveillance may contribute to lesion persistence [45–49]. Although NK cells reportedly recognise and kill EN-derived stromal cells *in vitro* [61], relatively little is known about the receptor-ligand interactions governing these responses. In this study, we therefore combined receptor blocking experiments with ligand expression profiling to investigate the mechanisms underlying NK cell recognition of patient-derived ENCCs. To our knowledge, receptor-blocking experiments of this kind have not previously been performed using patient-derived ENCCs. Our results demonstrated that ENCCs induce NK cell degranulation and identified DNAM-1, NKG2D, and NKp46 as important contributors to this response.

Both DNAM-1 and NKG2D are well-established contributors to NK cell activation and target cell recognition, including in tumour immunity [50,62]. The consequences of these receptor-ligand interactions in EN, however, may be more complex. The DNAM-1 ligands PVR and Nectin-2 can also engage inhibitory receptors such as TIGIT [62], potentially shifting the balance between activating and inhibitory signalling of these ligands depending on the receptor repertoire of the responding

NK cells. Similarly, elevated levels of soluble NKG2D ligands have previously been detected in the peritoneal fluid of women with endometriosis and have been proposed to impair NK cell function [50]. The contribution of these receptor–ligand interactions in EN may therefore be influenced by both activating and inhibitory mechanisms, warranting further investigation.

The involvement of NKp46 was particularly interesting given the limited understanding of the ligand(s) mediating NKp46 signalling. Such NKp46-dependence has previously been observed in SK-N-AS cells within the research group (Islamagic et al., unpublished) and was also replicated in the present study. Ongoing research in Professor Fredrik Bergh Thorén’s group (University of Gothenburg) has suggested a potential role for TRAIL and its receptors in NKp46-mediated signalling (Blomén et al., submitted). In this context, the high expression of TRAIL-R2 observed on ENCCs is intriguing. Although this interaction was not directly investigated, the findings support further investigation of the NKp46-TRAIL-R pathway in EN.

In contrast, NKp30 appeared to play a limited role in NK cell recognition of ENCCs, with no detectable expression of its ligand, B7-H6, and a limited effect on degranulation following NKp30 blockade. This differed from K562 cells, where high B7-H6 expression was accompanied by a marked reduction in degranulation following NKp30 blockade, consistent with the established role of NKp30 in K562 recognition [63]. Given the concordance between ligand expression and receptor-blocking results in both ENCCs and K562 cells, this strengthens the interpretation that NKp30-mediated signalling is not a major contributor to NK cell recognition of ENCCs under the conditions examined.

In addition to activating ligands, ENCCs also expressed high levels of HLA-ABC. Although HLA-ABC blockade did not significantly increase degranulation following correction for multiple testing, a trend towards increased NK cell activation was observed. These findings suggest that HLA-ABC expression may limit NK cell responses towards ENCCs. This is also consistent with previous studies proposing that enhanced inhibitory signalling contributes to impaired NK cell function in EN, including reports of altered expression of inhibitory NK cell receptors and their ligands [47–49]. In addition, the relatively modest increase observed following HLA-ABC blockade may, in part, reflect KIR-HLA mismatches between NK cell donors and ENCCs. As the extent of HLA-mediated inhibition depends on interactions between HLA class I and inhibitory KIRs, donor-specific differences in KIR-HLA compatibility may have influenced the magnitude of the inhibitory signal [14].

Taken together, these findings suggest that ENCCs express sufficient activating ligands to be recognised by healthy donor NK cells and do not appear intrinsically resistant to NK cell activation. This implies that impaired immune surveillance in EN also involves alterations in NK cell phenotype or the lesion microenvironment. The limited number of patient-derived cultures analysed should, however, be considered when interpreting these findings, and future studies including a larger number of patient-derived ENCCs and appropriate non-EN control cells will be important to determine the extent to which these observations are generalizable across EN lesions and whether they are specific to ENCCs.

6.3 Variability in Cytotoxicity Assessment

Although degranulation provides a useful measure of NK cell activation, it is not a direct measurement of target cell death. Cytotoxicity was therefore assessed as a complementary measure of NK cell function towards ENCCs. However, substantial variability was observed in cytotoxicity experiments involving ENCCs, reducing data robustness and limiting interpretability. The observed variability is likely attributable to the cells' adherent nature. Fewer ENCCs than expected were recovered during flow cytometry analysis, suggesting that viable cells may have remained attached to the culture plate and thus been excluded from analysis. Such selective loss of viable cells could lead to an overestimation of cytotoxicity and contribute to variability between experiments. Consistent with this interpretation, less variability was observed when K562 cells were used as targets. Because K562 cells grow in suspension, these findings suggest that recovery of adherent target cells contributed to the variability observed for ENCCs. Higher baseline levels of target cell death were also observed with ENCCs from EndoMIC 2B than EndoMIC 8A, including in target only controls. Although EndoMIC 2B cells were derived from a late passage, viability estimates prior to co-culture did not indicate marked differences between the cultures, suggesting that other technical factors may have contributed to this observation.

Future studies may thus benefit from alternative approaches for quantifying NK cell-mediated killing of the adherent ENCCs. Live cell imaging platforms, such as the Incucyte system (Sartorius), allow direct monitoring of target cell viability in culture and may therefore circumvent limitations associated with target cell recovery [64]. In addition, such continuous live cell imaging enables real-time assessment of cytotoxicity and provides a more robust characterisation of NK cell-mediated killing dynamics than conventional endpoint assays [64].

6.4 Characterisation of the Endometriosis-Derived Cultured Cells

Phenotypic characterisation of the ENCC cultures indicated a predominance of CD13⁺ stromal cells, while no clearly distinguishable EpCAM⁺ epithelial population was identified. One possible explanation is selective expansion of stromal cells during in vitro culture, as prolonged maintenance of endometrial epithelial cells is technically challenging and often requires specialised culture conditions [65]. This interpretation is also consistent with observations from the EndoMIC study, in which epithelial cells appear to survive fewer passages than stromal cells. Consequently, the receptor-ligand interactions and NK cell responses described in the present study are likely primarily driven by interactions with stromal ENCCs. This should be considered when interpreting the findings, as epithelial and stromal cells may differ in their susceptibility to NK cell recognition and in their expression of NK cell receptor ligands. Nevertheless, stromal cells represent a key cellular component of EN lesions and contribute to inflammation, extracellular matrix remodelling, angiogenesis, and lesion growth [40]. The findings therefore still remain biologically relevant.

Optimisation of culture conditions to improve preservation of epithelial populations is currently ongoing within the EndoMIC study and may facilitate future comparisons between epithelial and stromal ENCCs. Analysis of earlier-passage cultures may also allow epithelial populations to be included before their representation declines during prolonged culture. Combining these cultures with an Incucyte-based NK cell killing assay may provide a useful approach for investigating potential differences in NK cell susceptibility between epithelial and stromal cell populations, as these populations can be distinguished by morphology during live cell imaging.

6.5 NK Cell Source

The use of HD NK cells enabled a standardised assessment of ENCC susceptibility to NK cell-mediated responses. However, the findings may not fully reflect the immune environment present in patients with EN, where impaired NK cell cytotoxicity has been reported in both peripheral blood and peritoneal fluid [45,46]. The observation that HD NK cells readily degranulated in response to ENCCs suggests that the ENCCs are not intrinsically resistant to NK cell recognition. Instead, alterations in NK cell phenotype and function may contribute to the impaired immune surveillance observed in EN. There is already evidence supporting this notion, including reports of increased expression of inhibitory KIRs on NK cells isolated from the peritoneal cavity [47,48]. Similarly, the high expression of PVR and Nectin-2 observed on ENCCs may have different functional consequences *in vivo*, where elevated expression of the inhibitory receptor TIGIT could shift the balance away from DNAM-1-mediated activation.

Tissue-resident NK (trNK) cells differ phenotypically and functionally from circulating NK cells, displaying distinct receptor repertoires, cytokine profiles, and cytotoxic capacities. NK cells residing within EN lesions may therefore possess functional characteristics that are not fully captured by experiments performed using HD NK cells from peripheral blood. Planned phenotypic characterisation of EN-derived immune cells using spectral flow cytometry may help address some of these questions by providing detailed information regarding NK cell phenotype and receptor expression. For example, such analyses could determine whether the activating receptors identified as important in this study, as well as inhibitory receptors such as TIGIT, are expressed by NK cells within the lesion microenvironment, thereby providing insight into the balance between activating and inhibitory signalling. Such analyses may thus facilitate interpretation of future functional studies and help determine whether receptor pathways identified in the present study are likely to be active or inhibited within the EN microenvironment.

Direct functional assessment of trNK cells isolated from EN lesions remains technically challenging due to limited cell yields and the relatively large numbers required for functional assays. However, the planned phenotypic characterisation of lesion-derived immune cells may help bridge part of this knowledge gap. In addition, the snap-frozen lesion samples collected will enable future spatial analyses aimed at elucidating the localisation and phenotype of NK cell populations within EN lesions.

7

Conclusion

In conclusion, this study demonstrates that patient-derived ENCCs can activate NK cells and identifies DNAM-1, NKG2D, and NKp46 as important contributors to this response. To the best of our knowledge, this is the first study to investigate the receptor pathways underlying NK cell recognition of EN-derived cells using these receptor-blocking experiments. The receptor-blocking and ligand expression analyses showed concordance between receptor dependence and ligand availability, supporting a role for these pathways in NK cell recognition of ENCCs. The findings further suggest that ENCCs are not intrinsically resistant to NK cell recognition by functionally competent NK cells. Together with previous reports of impaired NK cell function in EN, these observations support the notion that alterations in NK cell phenotype and the lesion microenvironment may contribute to deficient immune surveillance in the disease. Further characterisation of EN-derived immune cells, particularly trNK cell populations, will therefore be important for understanding how these interactions are regulated *in vivo*.

The protocols developed in this study for isolating immune cells from EN samples enable such further characterisation. Although further refinement is still warranted, these protocols provide an important foundation for future studies aimed at phenotypic and functional characterisation of immune cells within EN lesions. Together, the findings and methodological advances presented in this thesis contribute to a deeper understanding of NK cell interactions in EN and provide a framework for future investigations of immune regulation in the disease.

Bibliography

1. Wang R, Lan C, Benlagha K, Camara NOS, Miller H, Kubo M, et al. The interaction of innate immune and adaptive immune system. *MedComm*. 2024; 5(10):e714. doi: 10.1002/MC02.714.
2. Murphy K, Weaver C. *Janeway's immunobiology*. 9th ed. New York, NY: Garland Science, Taylor & Francis Group; 2017. p. 6–13.
3. Bernard A, Boumsell L. The clusters of differentiation (CD) defined by the First International Workshop on human leucocyte differentiation antigens. *Hum Immunol*. 1984;11(1):1–10. doi: 10.1016/0198-8859(84)90051-X.
4. Rock KL, Reits E, Neefjes J. Present Yourself! By MHC Class I and MHC Class II Molecules. *Trends Immunol*. 2016;37(11):724–737. doi: 10.1016/J.IT.2016.08.010.
5. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajani-refah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014;345(6204). doi: 10.1126/SCIENCE.1251086.
6. Logie C, Stunnenberg HG. Epigenetic memory: A macrophage perspective. *Semin Immunol*. 2016;28(4):359–367. doi: 10.1016/J.SMIM.2016.06.003.
7. Romee R, Schneider SE, Leong JW, Chase JM, Keppel CR, Sullivan RP, et al. Cytokine activation induces human memory-like NK cells. *Blood*. 2012; 120(24):4751–4760. doi: 10.1182/BLOOD-2012-04-419283.
8. Cerwenka A, Lanier LL. Natural killer cell memory in infection, inflammation and cancer. *Nature Reviews Immunology*. 2016;16(2):112–123. doi: 10.1038/NRI.2015.9.
9. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol*. 2015; 135(3):626–635. doi: 10.1016/J.JACI.2014.11.001.
10. Vivier E, Artis D, Colonna M, Diefenbach A, Santo JPD, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. *Cell*. 2018;174(5):1054–1066. doi: 10.1016/J.CELL.2018.07.017.
11. Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, et al. Practical NK cell phenotyping and variability in healthy adults. *Immunologic Research*. 2015;62(3):341–356. doi: 10.1007/S12026-015-8664-Y.
12. Fu B, Tian Z, Wei H. Subsets of human natural killer cells and their regulatory effects. *Immunology*. 2014;141(4):483. doi: 10.1111/IMM.12224.

13. Wolf NK, Kissiov DU, Raulet DH. Roles of natural killer cells in immunity to cancer, and applications to immunotherapy. *Nature Reviews Immunology*. 2022; 23(2):90–105. doi: 10.1038/s41577-022-00732-1.
14. Kulkarni S, Martin MP, Carrington M. The Ying and Yang of HLA and KIR in Human Disease. *Seminars in immunology*. 2008;20(6):343. doi: 10.1016/J.SMIM.2008.06.003.
15. Li J, Xiao C, Li C, He J. Tissue-resident immune cells: from defining characteristics to roles in diseases. *Signal Transduct Target Ther*. 2025;10(1):1–37. doi: 10.1038/S41392-024-02050-5.
16. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*. 2015; 350(6263):981–985. doi: 10.1126/SCIENCE.AAC9593.
17. Gaynor LM, Colucci F. Uterine natural killer cells: Functional distinctions and influence on pregnancy in humans and mice. *Frontiers in Immunology*. 2017; 8:257756. doi: 10.3389/FIMMU.2017.00467.
18. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med*. 2020; 382(13):1244–1256. doi: 10.1056/NEJMRA1810764.
19. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Viganò P. Endometriosis. *Nature Reviews Disease Primers*. 2018;4(1):1–25. doi: 10.1038/S41572-018-0008-5.
20. Imperiale L, Nisolle M, Noël JC, Fastrez M. Three Types of Endometriosis: Pathogenesis, Diagnosis and Treatment. State of the Art. *J Clin Med*. 2023; 12(3):994. doi: 10.3390/JCM12030994.
21. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*. 2018;51:1–15. doi: 10.1016/J.BPOBGYN.2018.06.001.
22. Prescott J, Farland LV, Tobias DK, Gaskins AJ, Spiegelman D, Chavarro JE, et al. A prospective cohort study of endometriosis and subsequent risk of infertility. *Hum Reprod*. 2016;31(7):1475–1482. doi: 10.1093/HUMREP/DEW085.
23. Pearce CL, Templeman C, Rossing MA, Lee A, Near AM, Webb PM, et al. Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case-control studies. *Lancet Oncol*. 2012; 13(4):385–394. doi: 10.1016/S1470-2045(11)70404-1.
24. Hermens M, Altena AM van, Nieboer TE, Schoot BC, Vliet HA van, Siebers AG, et al. Incidence of endometrioid and clear-cell ovarian cancer in histological proven endometriosis: the ENOCA population-based cohort study. *Am J Obstet Gynecol*. 2020;223(1):107.e1–107.e11. doi: 10.1016/J.AJOG.2020.01.041.
25. Kalaitzopoulos DR, Samartzis N, Kolovos GN, Mareti E, Samartzis EP, Eberhard M, et al. Treatment of endometriosis: a review with comparison of 8 guidelines. *BMC Women's Health*. 2021;21(1):1–9. doi: 10.1186/S12905-021-01545-5.
26. Vignali M, Bianchi S, Candiani M, Spadaccini G, Oggioni G, Busacca M. Surgical treatment of deep endometriosis and risk of recurrence. *J Minim Invasive Gynecol*. 2005;12(6):508–513. doi: 10.1016/J.JMIG.2005.06.016.

27. Veth VB, Keukens A, Reijs A, Bongers MY, Mijatovic V, Coppus SF, et al. Recurrence after surgery for endometrioma: a systematic review and meta-analyses. *Fertil Steril*. 2024;122(6):1079–1093. doi: 10.1016/J.FERTNSTERT.2024.07.033.
28. Rizk B, Fischer AS, Lotfy HA, Turki R, Zahed HA, Malik R, et al. Recurrence of endometriosis after hysterectomy. *Facts Views Vis ObGyn*. 2014;6(4):219.
29. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol*. 1927;14(4):422–469. doi: 10.1016/S0002-9378(15)30003-X.
30. Barbieri RL. Reproductive history and endometriosis among premenopausal women. *Obstetrics and Gynecology*. 2004;104(5):965–974. doi: 10.1097/01.AOG.0000142714.54857.F8.
31. Olive DL, Henderson DY. Endometriosis and mullerian anomalies. *Obstetrics and Gynecology*. 1987;69(3):412–415.
32. Al-Fozan H, Tulandi T. Left lateral predisposition of endometriosis and endometrioma. *Obstetrics & Gynecology*. 2003;101(1):164–166. doi: 10.1016/S0029-7844(02)02446-8.
33. Vercellini P, Chapron C, Fedele L, Frontino G, Zaina B, Crosignani PG. Evidence for asymmetric distribution of sciatic nerve endometriosis. *Obstetrics and Gynecology*. 2003;102(2):383–387. doi: 10.1016/S0029-7844(03)00532-5.
34. Vercellini P, Chapron C, Fedele L, Gattei U, Daguati R, Crosignani PG. REVIEW: Evidence for asymmetric distribution of lower intestinal tract endometriosis. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2004;111(11):1213–1217. doi: 10.1111/J.1471-0528.2004.00453.X.
35. Vercellini P, Abbiati A, Viganò P, Somigliana ED, Daguati R, Meroni F, et al. Asymmetry in distribution of diaphragmatic endometriotic lesions: evidence in favour of the menstrual reflux theory. *Human Reproduction*. 2007;22(9):2359–2367. doi: 10.1093/HUMREP/DEM224.
36. Suda K, Nakaoka H, Yoshihara K, Ishiguro T, Tamura R, Mori Y, et al. Clonal Expansion and Diversification of Cancer-Associated Mutations in Endometriosis and Normal Endometrium. *Cell Reports*. 2018;24(7):1777–1789. doi: 10.1016/J.CELREP.2018.07.037.
37. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstetrics and gynecology*. 1984;64(2):151–154.
38. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertility and Sterility*. 2012;98(3):511–519. doi: 10.1016/J.FERTNSTERT.2012.06.029.
39. Greaves E, Critchley HO, Horne AW, Saunders PT. Relevant human tissue resources and laboratory models for use in endometriosis research. *Acta Obstetrica et Gynecologica Scandinavica*. 2017;96(6):644–658. doi: 10.1111/AOGS.13119.
40. Gołabek-Grenda A, Olejnik A. In vitro modeling of endometriosis and endometriotic microenvironment – Challenges and recent advances. *Cellular Signalling*. 2022;97:110375. doi: 10.1016/J.CELLSIG.2022.110375.
41. Rahmioglu N, Nyholt DR, Morris AP, Missmer SA, Montgomery GW, Zondervan KT. Genetic variants underlying risk of endometriosis: insights from

- meta-analysis of eight genome-wide association and replication datasets. *Human Reproduction Update*. 2014;20(5):702–716. doi: 10.1093/HUMUPD/DMU015.
42. Sapkota Y, Steinhorsdottir V, Morris AP, Fassbender A, Rahmioglu N, Vivo ID, et al. Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. *Nature Communications*. 2017;8(1):15539. doi: 10.1038/NCOMMS15539.
 43. Saha R, Pettersson HJ, Svedberg P, Olovsson M, Bergqvist A, Marions L, et al. Heritability of endometriosis. *Fertility and Sterility*. 2015;104(4):947–952. doi: 10.1016/J.FERTNSTERT.2015.06.035.
 44. Treloar SA, O'Connor DT, O'Connor VM, Martin NG. Genetic influences on endometriosis in an Australian twin sample. *Fertility and Sterility*. 1999; 71(4):701–710. doi: 10.1016/S0015-0282(98)00540-8.
 45. Symons LK, Miller JE, Kay VR, Marks RM, Liblik K, Koti M, et al. The Immunopathophysiology of Endometriosis. *Trends Mol Med*. 2018;24(9):748–762 . doi: 10.1016/J.MOLMED.2018.07.004.
 46. Evert JHV, Paap R, Nap A, Molen R van der. The Promises of Natural Killer Cell Therapy in Endometriosis. *International Journal of Molecular Sciences*. 2022;23(10):5539. doi: 10.3390/IJMS23105539.
 47. Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. *Fertility and Sterility*. 2002;77(2):297–302. doi: 10.1016/S0015-0282(01)02964-8.
 48. Matsuoka S, Maeda N, Izumiya C, Yamashita C, Nishimori Y, Fukaya T. Expression of inhibitory-motif killer immunoglobulin-like receptor, KIR2DL1, is increased in natural killer cells from women with pelvic endometriosis. *American Journal of Reproductive Immunology*. 2005;53(5):249–254. doi: 10.1111/J.1600-0897.2005.00271.X.
 49. Galandrini R, Porpora MG, Stoppacciaro A, Micucci F, Capuano C, Tassi I, et al. Increased frequency of human leukocyte antigen–E inhibitory receptor CD94/NKG2A–expressing peritoneal natural killer cells in patients with endometriosis. *Fertility and Sterility*. 2008;89(5):1490–1496. doi: 10.1016/J.FERTNSTERT.2007.05.018.
 50. González-Foruria I, Santulli P, Chouzenoux S, Carmona F, Batteux F, Chapron C. Soluble Ligands for the NKG2D Receptor Are Released during Endometriosis and Correlate with Disease Severity. *PLOS ONE*. 2015;10(3):e0119961. doi: 10.1371/JOURNAL.PONE.0119961.
 51. Mani S, Singh M, Kumar A. *Animal Cell Culture: Principles and Practice*: Springer International Publishing; 2023.
 52. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. *Critical Reviews in Biotechnology*. 37(2):163–176. doi: 10.3109/07388551.2015.1128876.
 53. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods*. 2004;294(1-2):15–22. doi: 10.1016/J.JIM.2004.08.008.

54. Lorenzo-Herrero S, Sordo-Bahamonde C, Gonzalez S, López-Soto A. CD107a degranulation assay to evaluate immune cell antitumor activity. *Methods in Molecular Biology*. 2019;1884:119–130. doi: 10.1007/978-1-4939-8885-3_7.
55. Kandarian F, Sunga GM, Arango-Saenz D, Rossetti M. A Flow Cytometry-Based Cytotoxicity Assay for the Assessment of Human NK Cell Activity. *Journal of Visualized Experiments : JoVE*. 2017;2017(126):56191. doi: 10.3791/56191.
56. Harrison TE, Bowler J, Cheng CI, Reeves KD. Optimizing Platelet-Rich Plasma: Spin Time and Sample Source. *Bioengineering*. 2023;10(11):1270. doi: 10.3390/BIOENGINEERING10111270.
57. Xu X, Chen Y, Yu Q, Wang J, Xu P, Zhu L, et al. The presence of living endometrial cells in ovarian endometriotic cyst fluid may contribute to the recurrence of endometriosis after surgical excision of endometriomas. *Journal of Ovarian Research*. 2022;15(1):89. doi: 10.1186/S13048-022-01018-9.
58. Vento-Tormo R, Hoo R. Endometrium dissociation with collagenase v1. 2019; doi: 10.17504/PROTOCOLS.IO.76THREN.
59. Mu X, Bellayr IH, Walters TJ, Li Y. Mediators leading to fibrosis - how to measure and control them in tissue engineering. *Operative techniques in orthopaedics*. 2010;20(2):110. doi: 10.1053/J.OT0.2009.10.003.
60. Tan Y, Flynn WF, Sivajothi S, Luo D, Bozal SB, Davé M, et al. Single-cell analysis of endometriosis reveals a coordinated transcriptional programme driving immunotolerance and angiogenesis across eutopic and ectopic tissues. *Nature Cell Biology*. 2022;24(8):1306–1318. doi: 10.1038/S41556-022-00961-5.
61. Yao J, Zhang L jiao, Zhou Z, Hua M fang. NK-92 cells activated by IL-2 inhibit the progression of endometriosis in vitro. *Journal of Obstetrics and Gynaecology*. 2024;44(1):2372682. doi: 10.1080/01443615.2024.2372682.
62. Paolini R, Molfetta R. Dysregulation of DNAM-1-Mediated NK Cell Anti-Cancer Responses in the Tumor Microenvironment. *Cancers*. 2023;15(18):4616. doi: 10.3390/CANCERS15184616.
63. Zhuang X, Veltri DP, Long EO. Genome-Wide CRISPR Screen Reveals Cancer Cell Resistance to NK Cells Induced by NK-Derived IFN- γ . *Frontiers in Immunology*. 2019;10:495684. doi: 10.3389/FIMMU.2019.02879/TEXT.
64. Croom-Perez TJ, Robles-Carillo LD, Oyer JL, Dieffenthaler TA, Hasan MF, Copik AJ. Kinetic, imaging based assay to measure NK cell cytotoxicity against adherent cells. *Methods in Cell Biology*. 2023;178:63–91. doi: 10.1016/BS.MCB.2022.07.012.
65. Riaz MA, Kary FL, Jensen A, Zeppernick F, Meinhold-Heerlein I, Konrad L. Long-Term Maintenance of Viable Human Endometrial Epithelial Cells to Analyze Estrogen and Progestin Effects. *Cells*. 2024;13(10):811. doi: 10.3390/CELLS13100811/S1.

A

Flow cytometry data

A.1 Cell composition assessment

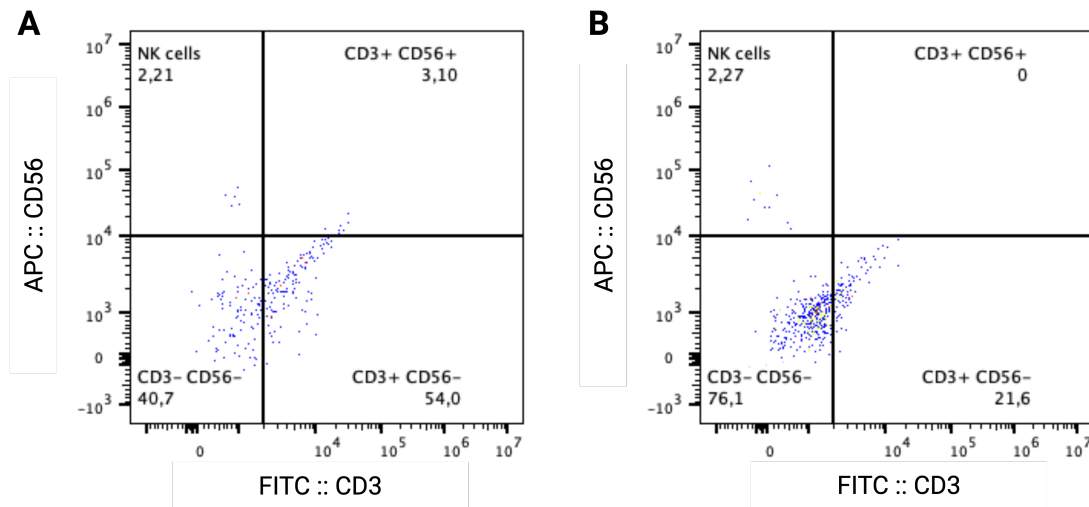


Figure A.1: Final CD3 versus CD56 gating plots from representative endometrioma samples processed before and after implementation of the updated staining protocol. (A) Endometrioma sample from EndoMIC 2, processed using the original staining protocol. (B) Endometrioma sample from EndoMIC 8, processed using the updated staining protocol. Corresponding full gating strategies are shown in Figures A.2 and A.3.

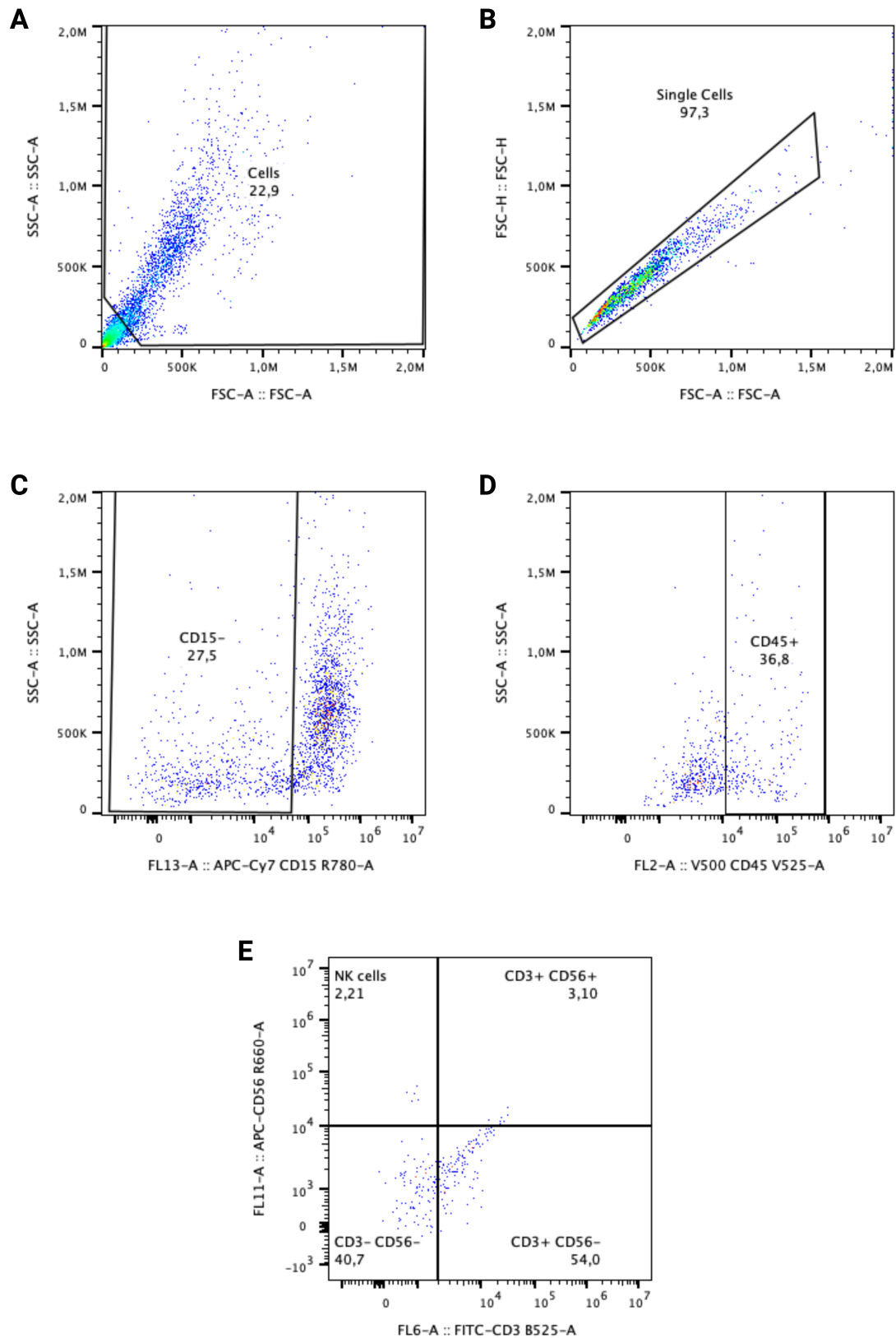


Figure A.2: Gating strategy for cell composition assessment of endometrioma sample from EndoMIC 2, including gating for (A) cells, (B) single cells, (C) CD15⁻ cells, (D) CD45⁺ cells, (E) CD3⁻CD56⁺ NK cells.

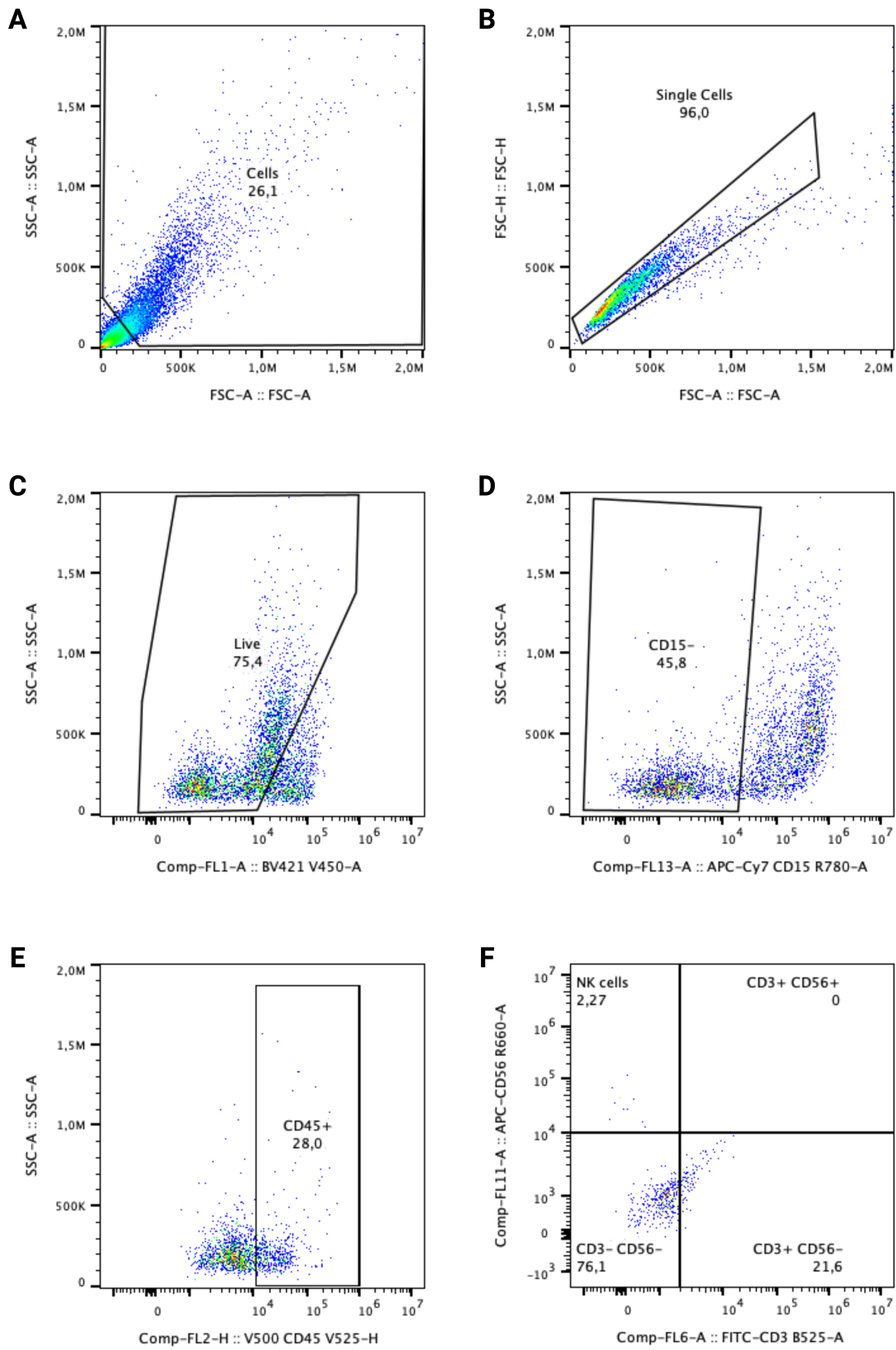


Figure A.3: Gating strategy for cell composition assessment of endometrioma sample from EndoMIC 8, including gating for (A) cells, (B) single cells, (C) live cells, (D) CD15⁻ cells, (E) CD45⁺ cells, (F) CD3⁻CD56⁺ NK cells.

A.2 Ligand staining

Table A.1: Staining reagents used for extended target ligand analysis.

Antibody	Fluorophore	Clone	Supplier (cat. no.)
CD13	PE-Cy7	L138	BD Biosciences (338439)
EpCAM	BUV395	KS1/4	BD Biosciences (745704)
HLA-ABC	FITC	G46-2.6	BD Biosciences (555552)
HLA-E	APC	3D12	BioLegend (342606)
CD48	PE	TÜ145	BD Biosciences (552855)
MIC-A/B	viobright FITC	6D4	Miltenyi Biotec (130-106-100)
ULBP-1	PerCP	170818	R&D Systems, Minneapolis, MN, USA (FAB1380C)
ULBP-2/5/6	APC	165903	R&D Systems (FAB1298A)
TRAIL-R1	PE	S35-934	BD Biosciences (564180)
TRAIL-R2	APC	DJR2-4 (7-8)	BioLegend (307407)
B7-H6	–	875001	R&D Systems (MAB7144)
PVR	–	5A10	Prof. Silvia Pesce
Nectin-2	–	L14	Prof. Silvia Pesce
Secondary	AlexaFluor 488	Polyclonal	Invitrogen (A-11001)

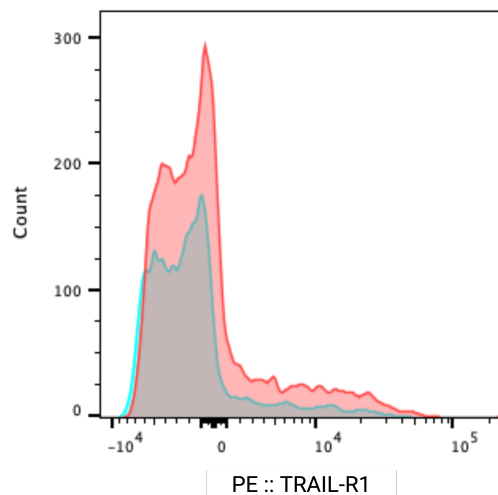


Figure A.4: Histogram showing TRAIL-R1 staining in ENCCs from EndoMIC 8A (red) compared to a negative live ENCC control (blue). Differences in signal distribution between samples should be interpreted with caution, as compensation analysis identified spillover between fluorescence channels.

B

Statistical analyses

B.1 ENCC cytotoxicity assessments

Table B.1: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards ENCCs, with the target only condition as reference ($\alpha = 0.05$).

Condition	P-value	Holm–Bonferroni threshold	Significance
E:T 5:1	0.0014	0.00556	*
aHLA-ABC	0.0414	0.00625	ns
E:T 1:1	0.2252	0.00714	ns
aNKp44	0.2337	0.00833	ns
aNKp30	0.3880	0.01000	ns
aDNAM-1	0.5967	0.01250	ns
aCD2	0.6300	0.01667	ns
aNKp46	0.6790	0.02500	ns
aNKG2D	0.8429	0.05000	ns

Table B.2: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards ENCCs, with the E:T 1:1 condition as reference ($\alpha = 0.05$).

Condition	P-value	Holm–Bonferroni threshold	Significance
E:T 5:1	0.0174	0.00556	ns
Target only	0.2252	0.00625	ns
aHLA-ABC	0.3825	0.00714	ns
aNKp46	0.4191	0.00833	ns
aNKG2D	0.4402	0.01000	ns
aDNAM-1	0.4880	0.01250	ns
aCD2	0.6240	0.01667	ns
aNKp30	0.7671	0.02500	ns
aNKp44	0.8183	0.05000	ns

B.2 K562 degranulation assessment

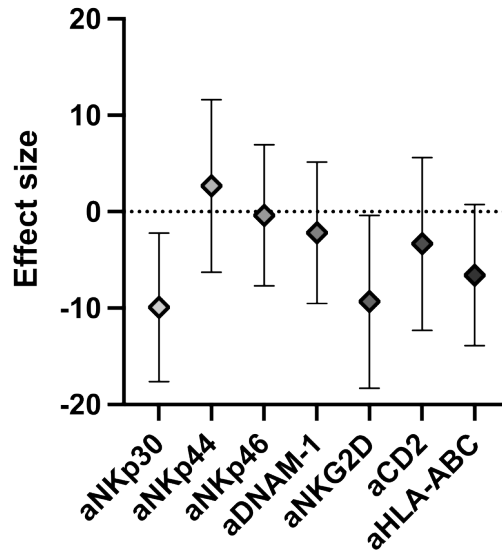


Figure B.1: Effect size estimates and 95% confidence intervals for K562 antibody blocking conditions compared to the E:T 1:1 condition (without multiple testing corrections).

Table B.3: Holm–Bonferroni correction for multiple comparisons of NK cell degranulation towards K562, with the E:T 1:1 condition as reference ($\alpha = 0.05$).

Condition	P-value	Holm–Bonferroni threshold	Significance
aNKp30	0.0135	0.00714	ns
aNKG2D	0.0418	0.00833	ns
aHLA-ABC	0.0760	0.01000	ns
aCD2	0.4525	0.01250	ns
aDNAM-1	0.5460	0.01667	ns
aNKp44	0.5471	0.02500	ns
aNKp46	0.9190	0.05000	ns

B.3 K562 cytotoxicity assessment

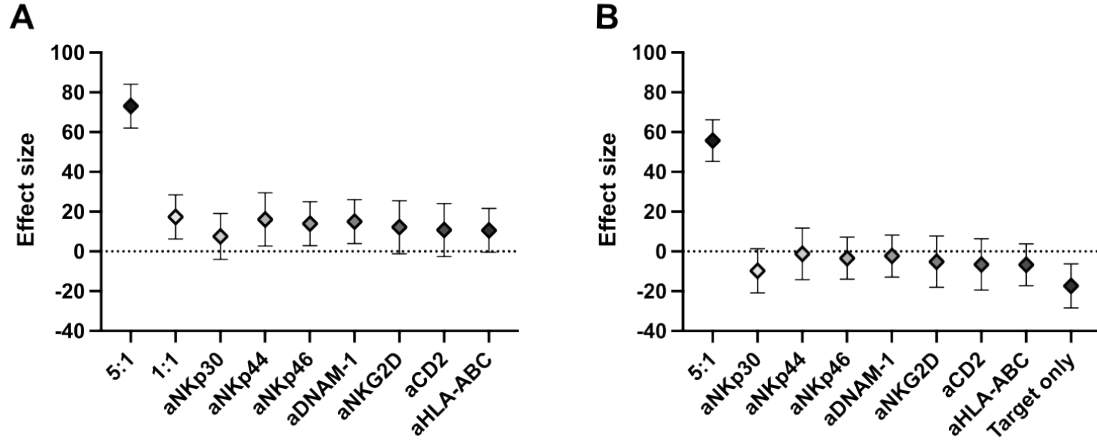


Figure B.2: Effect size estimates and 95% confidence interval for K562 experimental conditions compared to (A) target only control, and (B) E:T 1:1 (without multiple testing correction).

Table B.4: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards K562, with the target only condition as reference.

Condition	P-value	Holm–Bonferroni thresholds			Significance
		$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.001$	
E:T 5:1	< 0.0001	0.00556	0.00111	0.000111	***
E:T 1:1	0.0031	0.00625	0.00125	0.000125	*
aDNAM-1	0.0092	0.00714	0.00143	0.000143	ns
aNKp46	0.0150	0.00833	0.00167	0.000167	ns
aNKp44	0.0194	0.01000	0.00200	0.000200	ns
aHLA-ABC	0.0595	0.01250	0.00250	0.000250	ns
aNKG2D	0.0729	0.01667	0.00333	0.000333	ns
aCD2	0.1097	0.02500	0.00500	0.000500	ns
aNKp30	0.1926	0.05000	0.01000	0.001000	ns

Table B.5: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards K562, with the E:T 1:1 condition as reference.

Condition	P-value	Holm–Bonferroni thresholds			Significance
		$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.001$	
E:T 5:1	< 0.0001	0.00556	0.00111	0.000111	***
Target only	0.0031	0.00625	0.00125	0.000125	*
aNKp30	0.0825	0.00714	0.00143	0.000143	ns
aHLA-ABC	0.2066	0.00833	0.00167	0.000167	ns
aCD2	0.3128	0.01000	0.00200	0.000200	ns
aNKG2D	0.4238	0.01250	0.00250	0.000250	ns
aNKp46	0.5213	0.01667	0.00333	0.000333	ns
aDNAM-1	0.6608	0.02500	0.00500	0.000500	ns
aNKp44	0.8495	0.05000	0.01000	0.001000	ns

B.4 SK-N-AS degranulation assessment

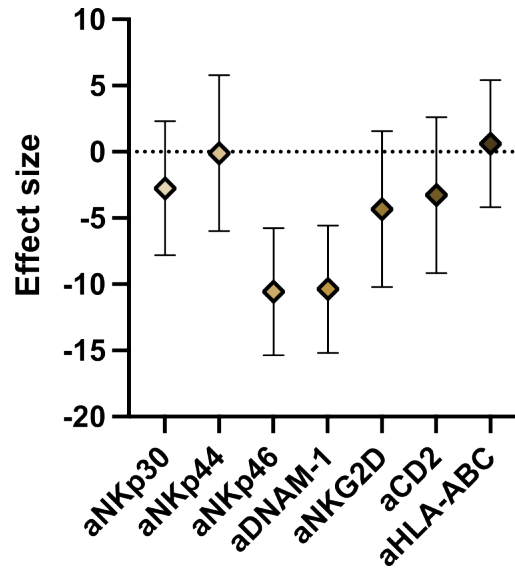


Figure B.3: Effect size estimates and 95% confidence intervals for SK-N-AS antibody blocking conditions compared to the E:T 1:1 condition (without multiple testing corrections).

Table B.6: Holm–Bonferroni correction for multiple comparisons of NK cell degranulation towards SK-N-AS, with the E:T 1:1 condition as reference.

Condition	P-value	Holm–Bonferroni thresholds			Significance
		$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.001$	
aNKp46	0.0001	0.00714	0.00143	0.000143	***
aDNAM-1	0.0001	0.00833	0.00167	0.000167	***
aNKG2D	0.1432	0.01000	0.00200	0.000200	ns
aCD2	0.2653	0.01250	0.00250	0.000250	ns
aNKp30	0.2746	0.01667	0.00333	0.000333	ns
aHLA-ABC	0.7958	0.02500	0.00500	0.000500	ns
aNKp44	0.9711	0.05000	0.01000	0.001000	ns

B.5 SK-N-AS cytotoxicity assessment

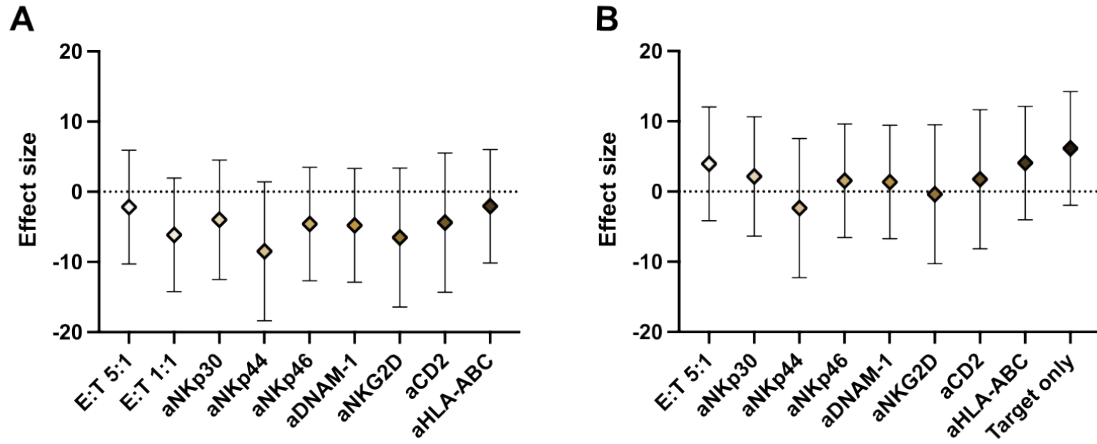


Figure B.4: Effect size estimates and 95% confidence interval for SK-N-AS experimental conditions compared to (A) target only control, and (B) E:T 1:1 (without multiple testing correction).

Table B.7: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards SK-N-AS, with the target only condition as reference ($\alpha = 0.05$)

Condition	P-value	Holm–Bonferroni threshold	Significance
aNKp44	0.0911	0.00556	ns
E:T 1:1	0.1331	0.00625	ns
aNKG2D	0.1909	0.00714	ns
aDNAM-1	0.2399	0.00833	ns
aNKp46	0.2572	0.01000	ns
aNKp30	0.3497	0.01250	ns
aCD2	0.3759	0.01667	ns
E:T 5:1	0.5893	0.02500	ns
aHLA-ABC	0.6085	0.05000	ns

Table B.8: Holm–Bonferroni correction for multiple comparisons of cytotoxicity towards SK-N-AS, with the E:T 1:1 condition as reference ($\alpha = 0.05$)

Condition	P-value	Holm–Bonferroni threshold	Significance
Target only	0.1331	0.00556	ns
aHLA-ABC	0.3151	0.00625	ns
E:T 5:1	0.3283	0.00714	ns
aNKp30	0.6108	0.00833	ns
aNKp44	0.6337	0.01000	ns
aNKp46	0.7028	0.01250	ns
aCD2	0.7230	0.01667	ns
aDNAM-1	0.7353	0.02500	ns
aNKG2D	0.9377	0.05000	ns

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