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Characterization of Natural Killer and Innate Lymphoid Cell Immunity in Ovarian Cancer and Endometriosis

Master thesis report in Biotechnology

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Abstract

Ovarian cancer (OC) is the most lethal gynecological cancer, due to discovery at late stages, vague symptoms and yet no effective screening methods. A common side effect of OC is the buildup of peritoneal fluid or ascites, which contain a large number of immune cells and tumor cells and spheroids. Moreover the carcinoma is heterogeneous and includes various types of tumors with distinctly varying molecular pathways. Endometriosis is a common benign inflammatory disease and is highly associated with certain subtypes of ovarian cancers, including endometrioid and clear cell OC. However, the mechanism behind the transition from endometriosis to OC is not fully understood, but previous research suggests genetic mutations associated with immune escape might play a role.
This project explores the immune landscape in high grade ovarian cancer, with emphasis on phenotypes of natural killer (NK) cells and innate lymphoid cells (ILCs) in ascites and matched peripheral blood mononuclear cells (PBMCs). Using spectral flow cytometry with clustering and dimensionality reduction, distinct immune cell populations were identified. Two populations were found that were significantly enriched in ascites, including one clear population of NK cells with clear tissue resident properties recognized by high expression of markers CD49a, CD103 and CD69 as well as of NK cell receptors, including NKG2A which aligns with previous results in the research group.
In parallel, six NK cell receptor-related SNPs were analyzed using public GWAS datasets for endometriosis and endometrioid and clear cell OC. The results showed slight significance for SNPs in genes associated with the NK cell receptors NKG2A, NKp30, and HLA-B-21. These results may have relevance in the susceptibility for ovarian cancer and impaired NK cell regulation in endometriosis and ovarian cancer highly associated with endometriosis.
These findings further deepened the understanding of variances in immunity in ovarian cancer and endometriosis and could in the future potentially identify immunotherapeutic targets in tissue resident populations in ascites fluid.

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Contents

1

Introduction

1.1 Project Introduction

Ovarian cancer (OC) is the deadliest gynecological cancer, which puts a high demand on novel treatment options. Moreover, ovarian cancer is often discovered in later stages where metastasis has already occurred, which makes it hard to treat. A known risk factor for getting ovarian cancer is endometriosis (EN), which is a hormone driven, benign inflammatory disease where uteri lining proliferate outside the uterus. However more needs to be discovered about the transition from endometriosis to ovarian cancer and the immune responses. This master thesis is performed in Karin Sundfeldt group, whose research focuses on gynecological cancers and endometriosis. The group consists of a range of expertise, including engineers, gynecoligal surgeons, immunologists and bioinformaticians. The supervisor of this thesis; Elin Bernson's research broadly focuses on Natural Killer (NK) cells and innate lymphioid cells (ILCs) and their role in OC and EN. A symptom of ovarian cancer is fluid buildup in the peritoneum and abdomen, so called ascites fluid. Ascites fluid contains a large quantity of immune cells, including NK-cells and ILCs. It is therefore of great interest to study the ascites and characterize the phenotypes of these immune cells.

1.1.1 Sample collection

Samples from high grade serous ovarian cancer (HGSOC) were collected from 2020-2024 as a part of the so called OCANK study. These samples included ascites and blood samples of patients with suspected HGSOC that had not been treated with chemotherapy. The samples were taken during debulking surgery (tumor removing surgery) or paracentesis (emptying of excessive abdominal fluid) performed before the surgery.

For this master thesis project, 18 ascites and matched PBMC samples from HGSOC patients, collected from OCANK cohort are analyzed. Human peripheral mononuclear cells (PBMCs) are blood circulating cells identified as any cell with a round nucleus[1]. This classification includes immune cells as lymphocytes, monocytes, NK cells and dendritic cells. To compare the immune profile of the HGSOC samples, healthy donor cells were used as controls.

1.2 Aims

So on, the aims for this master thesis project are the following:

- Characterize the NK and ILC immune landscape in ascites and matched PBMCs from HGSOC and compare the results with PBMCs from healthy donors. The analysis is carried out with spectral flow cytometry and data is further analyzed in the flow cytometry software Flowjo.
- Analyze public GWAS summary statistics to investigate whether genetic polymorphisms associated with NK cell receptor genes are associated with endometriosis, clear cell OC and endometrioid OC.

2

Theoretical Background

This section provides theoretical background for the reader to understand the role of immune responses and mechanisms behind the diseases studied that are the foundation of the results. .

2.1 Immune system

The immune system is the body's natural defense against harmful pathogens. There are two distinctions in the immune system; the innate (general) and adaptive (specific) immunity. The innate and adaptive immune system cooperate and the immune cells have different roles. Adaptive immunity include T cells, B cells and antibodies. T cells and B cells originate from stem cells in the bone marrow, where T cells coordinate immune response and attack infected cells whereas B cells are responsible for producing antibodies, which bind specifically to the pathogens. T cells are categorized into T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) [2]. Classifying immune cells as $^+$ or $^-$ according to CD (Cluster of differentiation) is a method to identify phenotypes of different immune cells [3], [2]. For instance $CD8^+$ T cells express the surface marker CD8, whereas $CD8^-$ do not.

A crucial part of the immune system is antigen presentation, where small fragments of specific pathogenic cells are presented at the cell surface by antigen-presenting cells. These antigen-presenting cells include dendritic cells, macrophages, and B cells [2]. These presenting molecules are known as major histocompatibility complex (MHC) molecules and are divided into two classes: MHC class I and MHC class II. MHC class I present antigens of intracellular pathogens to $CD8^+$ T cells, which induces apoptosis of the infected cells, whereas MHC class II present antigens of extracellular pathogens to $CD4^+$ T cells, which induces activation of other immune cells.

The innate immune system is the body's first-line defense against pathogens. Unlike the adaptive immune system it is not tailored to attack a specific pathogen [4]. In the innate immune system, a range of cells and guarding mechanisms work together. Key features of the innate immune systems include physical barriers (skin, mucous membranes, saliva), innate immune cells (neutrophils, macrophages, natural killer cells, innate lym-

phoid cells), inflammatory responses (chemical signals as cytokines induce inflammation which calls immune cells to the site of infection) and the complement system (proteins that increase immune response by marking pathogens that will be destroyed). The innate immune system is effective in stopping infections early on, but for specificity and recurring pathogens the adaptive immune system is crucial and the two need to work together in order to achieve homeostasis.

Moreover, another classification of immune responses is type 1 and type 2 immunity. Type 1 immunity is driven by T-helper 1, Th1 ,cells which produce cytokines such as IL-2 and IFN-γ and lymphotoxin-α, inducing phagocytic macrophage activation. These responses are typically active against intracellular pathogens such as viruses and some bacteria [5]. Type 2 response is initiated by T-helper 2, Th2, cells that produce predominantly interleukins, including IL-4, IL-5, IL-9, and IL-13. Additionally, type 2 immunity promote antibody production and is primarily effective against extracellular pathogens, such as parasites. It also plays a role in tissue repair and immune recovery. [5].

Immunotherapy represents a promising advancement in modern medicine, aiming to enhance the body's natural immune responses to treat diseases such as cancer. By engineering or boosting the activity of a patient's own immune cells, immunotherapy facilitate highly personalized and targeted treatments [6]. This path holds particular promise in oncology, where immune cells can be directed to recognize and eliminate tumor cells, paving the way for more effective and personalized therapies. In 2018 the Nobel Prize in Medicine was awarded to Tasuko Honjo and James Allison for their discoveries of immune checkpoint inhibition molecules [7]. These molecules include the death molecule (PD-1) and the cytotoxic T-lymphocyte antigen-4 (CTLA-4), which both suppress T-cell activity: allowing cancer cells to evade the immune system. These discoveries led to development of immune checkpoint inhibitors - therapies where these receptors are blocked, and therein recover T-cell activity and promote tumor elimination. These therapies have significantly advanced the survival outcome for certain cancers, but are unfortunately not effective against all tumor types.

2.1.1 NK and ILC immunology

Innate lymphoid cells (ILCs) are types of cells in the innate immune system. Their role is to produce cytokines and modulate immune responses. Unlike T lymphocytes, ILCs do not express various antigen-specific receptors; instead, their activity is modulated by quickly reacting to signals or cytokines in tissues [8]. Classically, ILCs are divided into three groups; group 1, 2 and 3. Each group is assigned to the ILCs according to factors such as their function, development, and the cytokines they produce. Within the three groups, there are subsets of cells, where a novel approach is to divide these into five major subsets [8], including

- ILC1, helper cells that produce cytokines. Stimulated by tumor cells, microbes and

viral infections. Promote type 1 immune response (i.e activates macrophages)

- ILC2, helper cells that produce cytokines. Mediate type 2 immune responses through alternative macrophage activation. React to allergens and extracellular parasites.
- NK cells that are classified as group 1 ILC.
- ILC3 are cytotoxic cells that are involved in the response against extracellular bacteria and fungi. Also active in intestinal homeostasis.
- Lymphoid tissue inducer cells (LTIs) are responsible for secondary lymphoid structures. They are stimulated by mesenchymal organizer cells.

These subsets can be identified based on presentation of surface markers, expression transcription factors [9]. These characterizations according to surface markers are as follows:

- **ILC1**: $CD127^+$, $CD161^+$, $KLRG1^+$, $CRTH2^-$, $cKit^-$
- **ILC2**: $CD127^+$, $CD161^+$, $CRTH2^+$, $cKit^{+/-}$, $KLRG1^+$
- **ILC3**: $CD127^+$, $CD161^+$, $cKit^+$, $NKp44^{+/-}$, $NKp46^+$, $CRTH2^-$

As mentioned, a class 1 ILC is the NK cell. The function of NK cells is similar to that of their adaptive immune cell counterparts, cytotoxic $CD8^+$ T-cells, but without antigen specificity [10]. Since the discovery in 1975, NK cells have since captivated great interest in immunology due to their ability to rapidly eradicate virally infected and tumor cells without prior sensitization - a major advantage compared to adaptive immune cells. The activity of NK cells is closely regulated through the balance activating and inhibiting receptors that interact with ligands on potential target cells [11].

NK cells develop from hematopoietic stem cells in the bone marrow, which differentiate into common lymphoid progenitors and gradually mature into NK cells [12]. Mature NK cells are generally divided into two subsets according to their surface markers: $CD56^{dim}$ and $CD56^{bright}$ [12]. $CD56^{bright}$ NK cells are mainly present in lymphoid tissues and are responsible for production of cytokines, while $CD56^{dim}$ cells reside in peripheral blood and display cytotoxic activity. $CD56^{bright}$ NK cells produce a variety of cytokines, including interferon-gamma ($IFN\gamma$), tumor necrosis factor α ($TNF\alpha$), interleukins, and chemokines.

Nk cell cytotoxicity is provoked when the cell engages with MHC class 1 molecules through its inhibitory receptors, while simultaneously recognizing stress induced ligands through their activating receptors [13]. NK cell activity is mediated by the balance between activating and inhibitory signals generated through receptor-ligand interactions [13]. When the activating signals outweigh inhibitory ones, the NK cell releases cytotoxic granules

containing perforin and granzymes, inducing apoptosis in the target cell [13]. This shift occur from a reduction in inhibitory signals or an increase in activating signals, shifting the balance and promoting NK cell mediated cytotoxicity.

Cytotoxic NK cells identify target cells by detecting the expression levels of HLA class I molecules, a type of MHC class I molecule [14]. This is described as the "missing self theory," suggesting NK cells recognize and eradicate cells without adequate HLA class I expression [15]. Inhibitory receptors on the NK cell surface - such as CD94/NKG2A (referred to as NKG2A), and killer-cell immunoglobulin-like receptors (KIRs) interact with these HLA molecules to mediate cytotoxic activity [15], [14]. Both HLA class I molecules and KIRs are highly polymorphic allowing for a diversity of immune responses. HLA class I molecules are divided into classical (HLA-A, -B and -C) and non-classical (HLA-E, -F, -G and -H) subtypes [16]. Classical HLA molecules are primarily recognized by KIRs. Specifically, HLA-C are divided into two variants, C1 and C2, which bind to KIR2DL2/L3 and KIR2DL1 respectively [14].HLA-B and HLA-C interact with KIR3DL1. The non-classical HLA-E molecule is recognized by NKG2A receptor. Another inhibitory receptor is LILRB1, which display broad specificity for HLA class I molecules and is also expressed on many lymphoid cells [13].

The role of inhibitory receptors is complex, as they do not only supress NK cell function. NK cells undergo a process called "education," whereby previous interactions with MHC class I molecules via their inhibitory receptors enhance their responsiveness toward aberrant cells lacking MHC class I molecules [17]. Thus, inhibitory interactions can enhance immune responses, with KIRs and NKG2A play essential roles in the education process.

Not all inhibitory receptors are HLA class I specific. Under pathological conditions, other inhibitory receptors, - such as PD-1, TIM-3, and TIGIT - are frequently highly expressed. These receptors inhibit immune responses, contributing to immune evasion by abnormal cells [13].

In contrast, activating receptors trigger cytotoxicity when NK cells encounter a target cell. A major group of activating receptors are natural cytotoxicity receptors (NCRs), including NKp46, NKp44, and NKp30. NCRs can also be expressed by ILC3s (notably NKp44 and NKp46) and by T-cell subsets expressing NKp30 [13]. Another crucial activating receptor is NKG2D, which is expressed on both cytotoxic T cells and NK cells. NKG2D interacts with MICA/B and ULBPs, which are frequently upregulated in virally infected and tumor cells, promoting their elimination. NKG2C - the activating counterpart of NKG2A - also recognizes HLA-E, although with lower binding affinity [13]. Additionally, several activating co-receptors — such as DNAM-1, NKp80, and NTB-A further enhance NK cell function. Particularly, DNAM-1 binding has shown increased efficacy in NK cell-mediated elimination of certain tumor cells, making it a potential therapeutic target [13].

The highly polymorphic nature of NK cell receptors and ligands results in allelic variations among individuals, influencing immune responses and diseases outcomes. Prior research

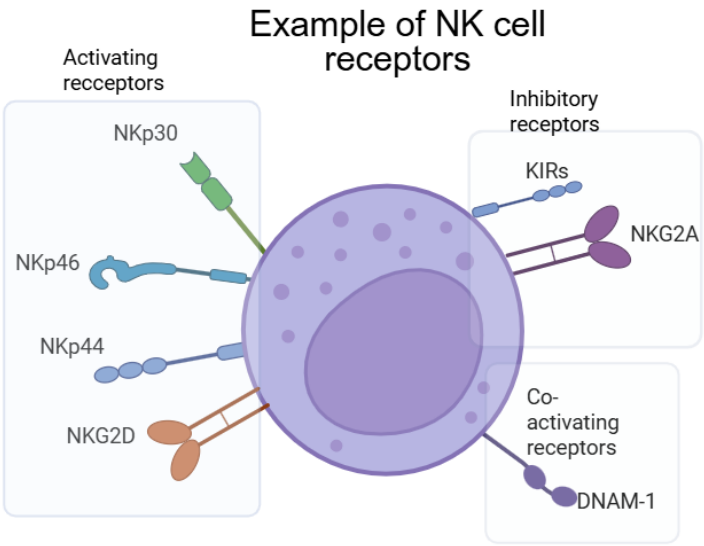

Figure 2.1: Examples of Nk cell receptors. Figure created in Biorender.com

by the Berg-Thorén group has identified six single nucleotide polymorphisms (SNPs) that are associated with NK cell activity. These include:

- rs1049174 (KLRK1 gene encoding NKG2D, G/C)
- rs763361 (DNAM-1 variant, T/C)
- rs986475 (NKp30 gene variant, A/G)
- rs1052248 (NKp30 gene variant, T/A)
- rs1983526 (NKG2A gene variant)
- rs1050458 (HLA-B-21 gene variant) [18].

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Relevance of these SNPs can be seen below.

- HLA-B-21 (rs1050458 A/G): This SNP causes two distinct variants of the leader peptide of HLA-B, yielding M or T protein. The genotype M/M and M/T (A allele) are termed M/x and T/T (G allele) are termed T variant [18]. HLA-B as a class I molecules presents leader peptides contributing to the folding and function of HLA-E. Patents with the M/x genotype display a reliance on NKG2A regulation and NK education, while the T variant rely more on KIR NK education-and signalling. In acute myeloid leukemia (AML) patients that recieved IL-2 based immunotherpy, the M/x patients displayed more cytotoxicity in NKG2A$^+$ NK cells, and displayed improved survival outcomes [19]

- DNAM-1 Also known as Gly307Ser(rs763361 T/C), the T variant have been associated with elevated risk for diabetes and autoimmune diseases [20] [21]. This propose a role in DNAM-1 polymorphisms in immune regulation.
- NKG2D (rs1049174 G/C) The SNP in the KLRK1 gene encoding the NKG2D receptor has been associated with high and low NK cell activity. The C allele was associated with low NK activity, and individuals with this allele have an elevated cancer risk [22].
- NKG2A (rs1983526 G allele) located in the KLRC1 gene, in close proxinity to KLRK1. This SNP has been associated with better responses in AML patients treated with HDC/IL-2 immunotherapy. Patients carrying the G allele exhibited better response, proposing a cancer halting role [23].
- NKp30 (rs986475 A/G): Non-G alleles of this SNP were associated with increased expression of activating NKp30 receptors in AML patients, suggesting enhanced NK cell cytotoxic activity [24].
- The rs1052248 T allele encoding NKp30 has been reported to have a protective effect in susceptibility for developing autoimmune disease as rheumatoid arthritis and ankylosing spondylitis, suggesting a potential role in immunomodulation [25].

While polymorphisms alter the activity of NK cells and other lymphocytes, environmental factors can also alter the activity of immune cells.

2.1.1.1 Tissue Resident ILCs and NK cells

Immune cells have different phenotypes depending on the tissue they are reciding in. This is referred to as tissue residency, or tissue resident immune cells (TRICs). TRICS differ from their blood- circulating immune cell counterparts, and can be identified with phenotypic surface markers [26].

Tissue resicency is acquired at different time-points for different immune cells, whereas some inherit it during embryogenesis and some if being stimulated in an immune response. How long the TRICS persist in the tissue depends, as some proliferate through self renewal and some depend on interaction with other circulating immune cells [26]. TRICs possess great heterogeneity, and their main functions include local immune response regulation, maintaining tissue homeostasis, pathogen defense, and regulating tissue repair.

ILC cells are commonly tissue resident in mucosal tissues, and their activity plays a crucial role in the environment homeostasis of the host tissue [26]. Tissue resident NK cells acquire their phenotypic properties during different time points. These include local progenitor cells in the tissue in embryonic and adult stages, progenitor cells in bone marrow in adulthood or the NK cells transform into tissue resident phenotypes upon entering the specific tissue [26]. TrNK cells have been discovered in various tissues as

in the uterus, intestine, skin, adipose tissue and liver. Depending on the tissue the NK cells are situated in, they express different surface markers. These tissue resident markers are frequently also found on trT cells, including CD103, CD49a and CD69 [12]. Specific Markers expressed on trNK cells in the uterus are for example CD94, NKG2A, CD49a and CD103 [26].

Recent research has emphasized the importance of trNK cells in the microenvironment of OC tumors. Here Bernson et al found that trNK cells were identified in both tumor tissue and ascites of high-grade serous Ovarian Cancer [27]. The results were that these trNK cells displayed up-regulated expression of NKGA receptors compared to their counterparts circulating peripheral blood. Moreover functional assays showed that these trNK cells responded strongly to ovarian tumor cells, despite the fact that these trNK exhibit an inhibitory phenotype. Interestingly the most abundant trNK cells in ascites were the $CD56^{bright}$ phenotype, ultimately questioning the previous belief that the cytokine producing $CD56^{bright}$ cells are not cytotoxic. Thus, these findings put an emphasis on the importance of improving phenotypic and functional characterization of trNK cells in Ovarian Cancer and other diseases [27]. In addition identifying immunological targets in trNK cells could be a way for novel immunotherapeutic treatments against diseases with a high abundance of TRICs.

2.2 Ovarian cancer

Ovarian cancer (OC) is a lethal gynecological disease affecting approximately 1% of women. In fact, it is the most deadly gynecological cancer with a five-year relative survival rate of only 51.6% [28]. Due to vague symptoms and lack of effective screening methods, the disease is commonly discovered at a late stage (typically stage III-IV), which complicates treatment [29]. Additionally, severe cases of OC frequently relapse with shorter disease-free periods, potentially culminating in resistance against chemotherapy; resulting in fatal outcomes for many patients.

Moreover, OC is pathologically diverse, and the success of treatment varies among patients [28]. Standard treatment typically includes tumor removal surgery combined with chemotherapy [29]. Therefore, given the severe side effects of chemotherapy, the risk of surgery, and the high rates of recurring disease, novel treatment strategies are of interest for OC.

OC can be classified into different subtypes according to the originating cell type. Epithelial OC that originates from the surface layer of the ovary is the most common type, accounting for 90% of the OC cases [30]. Epithelial OC is further classified into five major subtypes—high-grade serous (HGSOC), endometrioid (EOC), clear cell (OCCC), mucinous, and low-grade serous, listed according to frequency in descending order [31].

Although both are classified as serous carcinoma, high-grade and low-grade variants display distinct morphologies and molecular pathways [31]. Low grade serous carcinomas

are frequently associated with early mutations in KRAS or BRAF, and are also found in benign and borderline areas of the neoplasm. High-grade serous carcinoma (HGSOC), is the most common subtype of OC, and is believed to primarily originate from the fallopian tube epithelium [31]. HGSOC is associated with mutations in TP53, occurring in up to 97% of cases. Moreover, HGSOC is frequently linked with BRCA1-and BRCA2-mutations, both germline and somatic within the neoplasm [31]. Women with germline BRCA mutations face a significant risk of developing HGSOC with a lifetime risk of 40-60%, compared to approximately 1% for those without such mutations [29].

Endometrioid and Clear Cell OC are the second and third most common epithelial subtypes, respectively. Both are typically diagnosed at earlier stages (I-II), leading to a better survival prognosis than HGSOC [31]. Ovarian Clear Cell Carcinoma (OCCC), is frequently recognized by cells with clear cytoplasm and distinct cell membranes. Despite frequent early diagnosis of OCCC, advanced cases have a poor prognosis due to low response to chemotherapy [31]. Endometrioid OC (EOC), often co-exist with synchronous endometrial carcinoma [32]. A known risk factor for both EOC and OCCC is endometriosis, with studies estimating that of 25-80% of patients with these tumors also have endometriosis [32]. The variation in reported percentages is due to inconsistent findings across studies, resulting in the wide percentage range.

The spread of OC cells typically causes accumulation of peritoneal fluid or ascites [33]. Malignant ascites contain both OC carcinoma cells and spheroids, along with immune cells, including tissue-resident NK cells and ILCs. An emerging immunotherapeutic strategy could therein be to implement immune checkpoint blockade to enhance the cytotoxic activity of these tissue resident immune populations. However, previous attempts with implementation of checkpoint inhibitors targeting PD-1 and CTLA-4 in OC have shown limited success, emphasizing the need to find new targets [34].

2.3 Endometriosis

Endometriosis (EN) is a common inflammatory disease affecting approximately 10% of women of reproductive age. However, it is challenging to diagnose; a definitive diagnosis requires laparoscopy, as visualization with ultrasound is not always sufficient. These diagnostic limitations make it difficult to know the true prevalence of EN [35]. EN does not currently have a cure. Treatment is therefore directed at symptom relief, usually involving hormonal therapy and, in more severe cases, surgical removal of endometrial lesions.

EN occurs when endometrium-like tissue grows outside the uterus, forming endometrial lesions. These are commonly found in the fallopian tubes, ovaries, and peritoneal cavity [35]. Much like the uterine endometrial lining, these ectopic lesions respond to hormonal signals, causing thickening, shedding and bleeding during the menstrual cycle. However, because there is no outlet, this causes inflammation and pain. Symptoms include painful

and heavy menstruation, pain during intercourse, gastrointestinal issues and infertility [36]. Interestingly, symptom severity is not necessarily correlated with lesion size or number, as some patients are completely asymptomatic despite having large or many lesions [36]. EN is a major cause of infertility: an estimated 25-50% of infertility cases are associated with EN, and about 30-50% EN patients experience infertility [37]. Asymptomatic EN patients often go undiagnosed until they investigate infertility [36].

EN can be classified into subtypes where the most common types are deep infiltrating, ovarian and peritoneal EN. Ovarian EN causes ovarian cysts referred to as endometriomas, or "chocolate cysts", filled with old blood [38]. Endometriomas can cause local inflammation that can trigger formation of scar and fibrous tissue. In severe cases, this fibrous tissue can form adhesions that can cause pelvic organs - such as the bladder - to adhere to surrounding pelvic tissue, causing dysfunction and pain [36].

The disease pathogenesis of EN is complex and not fully understood. The most widely recognized theory involves retrograde menstruation, referring to the reverse flow of menstrual blood through the fallopian tube, causing endometrial fragments to implant on ectopic tissues. An extension of this theory suggests that progenitor epithelial cells and mesenchymal stem cell-like cells in the peritoneal cavity differentiate into ectopic implants. Another proposed theory include coelomic metaplasia, in which peritoneal or ovarian epithelial cells transform into endometrial tissue [38]. Although retrograde menstruation occurs in approximately 90% of women, EN only affects 10%, suggesting other contributing factors such as genetic susceptibility and immune dysfunction play major roles [39].

Inflammation and imbalances in immunity are central in EN pathogenesis. Impaired immune responses for NK cells, T cells and macrophages have been seen in the peritoneum, endometrium and blood in EN patients. These altered responses ultimately result in elevated levels of pro-inflammatory cytokines such as IL-6 and TNFα. Moreover, EN is a hormone-driven disease characterized by excessive cell proliferation, angiogenesis, and inflammation linked to altered progesterone and estrogen signaling. Persistent activation of estradiol and reduced progesterone responsiveness create the characteristic hormonal profile seen in EN, with estrogen dominance and progesterone resistance [39].

Furthermore, evidence suggests EN patients have immune cell impairments that contribute to disease progression [40]. For example, NK cells in both peripheral blood and peritoneal fluid of EN patients have reduced cytotoxic activity, potentially impairing clearance of ectopic endometrial tissue. This impairment might be caused by altered NK cell phenotypes, changes in surface markers, and alternative cytokine production. Additionally, the inflammatory environment of the peritoneum can influence the NK cytotoxicity, as cytokines in the peritoneum may suppress NK cell function [40].

EN, particularly ovarian EN, is a recognized risk factor in developing Ovarian Cancer (OC), where benign endometriomas undergo malignant transition [41]. While somatic mutations, such as those in ARID1A, are common in OCCC and EOC, their exact role

in the transition is not fully understood. Absent expression of ARID1A protein has been shown to be highly associated with early stage OCCC [42].

However, paired-sample data from the Sundfeldt group challenge the idea that somatic cancer associated mutations are the main drivers of the transformation from EN to OC. In this study, 11 patients that had undergone surgery removing endometriomas, who later developed OC were examined. Tumor and lesion tissue from both disease stages were genetically profiled [43]. Mutations associated with cancer such as ARID1A were present in EN lesions years before the malignant transition into OC, and were not directly related to the malignant transition [43]. In fact, the data showed that persistent growth of endometriomas may halt tumor progression. However, it was found that genetic aberrations altering immune response were significant early events in the malignant transition from EN to OC as well as genes associated with immune escape. Evidently, the most common shared mutations were found in genes encoding HLA class I and class II molecules, challenging the theory that EN associated OC occur due to somatic cancer associated mutations. Thus, these findings suggest genetic aberrations in genes affecting immune escape play a role in the progression from ovarian EN to OC [43].

2.4 Purpose

- To characterize the immune landscape within ascites and matched PBMCs of HGSOC patients, with emphasis on identifying distinct populations of NK cells and ILCs in the tissue resident cells. The future goal with this is to identify immunotherapeutic targets against OC in NK cells and ILCs.
- Establish whether publicly available GWAS data from EN patients and EOC and OCCC portray different prevelance of the six SNP alleles known to alter cytotoxicity or response to immunotherapy compared to healthy controls.

2.5 Limitations / Demarcations

- Will not analyze ascites and PBMC samples from other types of OC other than HGSOC.
- Will only look at the six SNPs that the six SNPs that are proposed to be associated with altered cytotoxicity or alternative response towards immunotherapy.

3

Methods

3.1 Study design

3.1.1 Antibody panel design

The group has previously designed an antibody panel to facilitate the simultaneous detection of multiple targets. Antibodies and fluorophore were carefully selected in order to identify specific cell populations while minimizing spectral overlap. The antibodies stain the cells either by binding intracellular (IC) or extracellular (EC), and require different procedures for staining the cells.

For this analysis, the cell types of interest are mainly ILCs and NK cells including their tr subsets. For a more detailed view of the panel design and what surface marker each antibody binds to, see the Appendix.

The panels designed for this experiment are the following:

- a NK/ILC panel containing 31 markers. The markers include both functional markers and markers to identify populations of NK and ILC and tissue residency markers.

- an Immune panel of 13 markers. These included markers for other immune cell populations.

To exclude cell populations that are not of interest to analyze a so called dump channel is implemented. Multiple markers (commonly lineage markers) that are not of interest conjugated to the same fluorochrome, facilitating a more simple exclusion of these markers for the analysis[44].

Due to time limitation, only the NK/ILC panel will be analyzed for this master thesis, as these results are of the greatest interest.

3.1.2 Flow cytometry

Flow cytometry is a characterization technique used to analyze physical, chemical, biological and morphological characteristics of single cells in suspension. The method is widely applied in biological sciences for immunophenotyping and enables visualization of diverse phenotypes solution. Prior to analysis cells are typically stained with viability dyes, DNA stains or fluorophore-conjugated antibodies. These antibodies bind to specific targets such as proteins, surface markers or DNA, enabling cell characterization[45].

The main components of the flow cytometer are the fluidics system, optical system and electronics. The fluidics system transports the sample through the system. The optical system includes lasers (excitation light sources), lenses, and filters that focus and direct excitation light. Emitted and scattered light is captured by detectors and converted into photocurrent. The electronics system then converts the photocurrent into a digital signal for downstream analysis. Cells or particles are suspended in solution and drawn into the system through a thin nozzle, where the cells are surrounded by sheath fluid (a physiological buffer for the cells) and organized into a single cell stream. The stream passes through the interrogation point (or laser intercept), where laser light passes through each single cell[46].

When the laser passes the cell, the light will scatter providing morphological information. The information gathered depends on the angle that the light is collected- the forward scatter (FSC) correlates with cell size and side scatter (SSC) reflects granularity. Simultaneously the laser excites the fluorophores conjugated to the antibody, causing them to emit fluorescence, which is collected by the detectors and computed[46].

An objection in multicolor flow cytometry is spectral overlap, where emission spectra of fluorochromes emitting close in wavelength interfere. To correct for this, a process called compensation applies mathematical adjustments to subtract overlapping signals[45].

However a limitation of traditional flow cytometry is the limited to the number of available detectors and lasers, which restrains parameters that can be measured simultaneously[45]. This issue can be avoided using flow cytometry which captures the whole spectrum of each fluorochrome, facilitating management of nearby-emitting fluorphores.

3.1.3 Spectral flow cytometry

Spectral flow cytometry is an advanced technique that enhances traditional flow cytometry by capturing the entire emission spectrum from each fluorescently labeled cell, contrary to only detecting light at a fixed wavelength bands in conventional flow cytometry. Instead of each fluorophore being assigned to one detector only, a series of detectors is used in spectral flow cytometry to capture the full spectral signature across a broader wavelength. This offers greater sensitivity and improved separation of fluorophores with overlapping emission spectra and thereby enable the simultaneous use of more parameters

than previous techniques[47].

A major advantage of this technology is its unmixing algorithm where the software can disentangle complex spectral signals into contributions from individual fluorochromes. This replaces the compensation which is more limited and prohibits the use of fluorochromes with comparable emsission profiles[47].

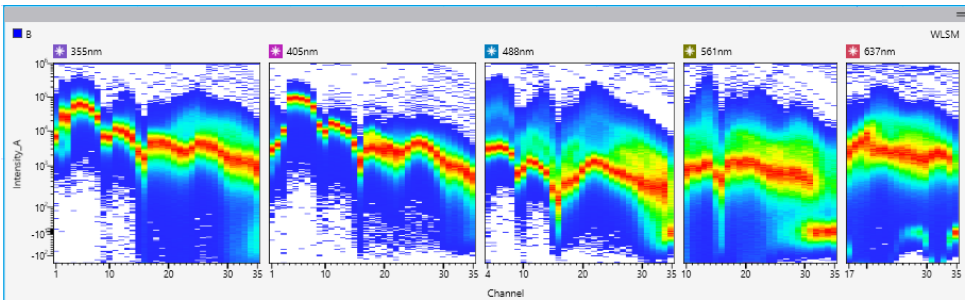


Figure 3.1: Example picture of spectral signanature from an experiment including all fluorochromes used

In addition spectral systems can also detect and subtract cellular autofluorescence, - the inherent emission by certain amino acids - and treats it as a separate parameter. This further enhances the signal resolution and improves the accuracy of identified cell populations[47]. These advantages makes spectral flow cytometry especially well suited for high dimensional immune profiling, rare cell population and conducting experiments involving large antibody panels[47].

3.1.4 Analysis of spectral flow cytometry results

The output for flow cytometry data is collected into fcs (flow cytometry standard) files. These are analyzed in software FlowJo, a program specialized for analysis of flow cytometry data. In FlowJo gates identifying certain populations according to parameters size, fluorescence. These gates can be performed in a hierarchical manner, identifying cell populations within the gates.

In addition to conventional gating, dimensionality reduction techniques such as t-distributed stochastic neighboring embedding (t-SNE) and uniform Manifold Approximation and Projection (UMAP) which enables high-dimensional visualization of data. Here, identification and comparison of cellular phenotypes is made possible. Algorithms reduce the dimensionality of the complex datasets and enables visualization of cell populations in a two-dimensional cluster graphs. Differences between these strategies include that t-SNE separates close clusters but can sometimes disrupt the overall structures, while UMAP preserves local and global structures better[48].

4

Materials & Methods

Here is a section for materials and methods for a more in depth understanding of techniques used in this project.

4.1 Laboratory practices

PBMCs and ascitic mononuclear cells (aMNCs, mononuclear cells in ascites) were stained with flourochrome conjugated antibodies and further analyzed with spectral flow cytometry and FlowJo for the laboratory procedures.

4.1.1 Staining procedure

4.1.1.1 Antibody titration staining procedure

Peripheral blood mononuclear cells (PBMCs) and NK cells were isolated and resuspended to a concentration of $4 * 10^6$ cells/mL in IMDM medium, one batch containing interleukin (IL-2) or without. The cells were then incubated overnight in 37^o. The following day cryopreserved ascites was thawed until only a small bit of ice remained, and cold medium was added dropwise followed by dilution in an additional 8 ml of cold medium. Cells were then centrifuged and washed with bNaCl and concentration adjusted to $4 * 10^6$ cells/mL.

Antibody titrations were performed on 96-well U-bottom plates, where 2-fold serial dilutions were prepared in stain buffer (extracellular) or Perm/Wash buffer (intracellular). 50μL of cell suspension was added per well, along with 150μL of bNaCl. Following centrifugation, cells were stained with 100 μL viability dye and incubated in the fridge, followed by a wash step.

Cells were stained with the EC antibody mix (in Brilliant Stain Buffer) during fridge incubation, followed by additional washing steps. Then, 100μL of BD Cytofix/Cytoperm was added to each well and incubated for fixation and permeabilization. Cells were then washed twice with Perm/Wash buffer and resuspended in NaCl containing 2mM EDTA.

For IC staining, the fixation step with BD Cytofix/Cytoperm was followed by two washes

with Perm/Wash buffer, and 50μL of the IC antibody mix was added to the respective wells. Unstained controls were treated with Perm/Wash buffer. Samples were incubated in the fridge, followed by two washes with BD Perm/Wash buffer. The final step was the same as for the extracellular plate, with resuspension in NaCl containing 2mM EDTA. Both plates were then incubated overnight at 4°C.

4.1.2 Sample collection

The ascites samples collected as part of the OCANK study. Here, the samples were acquired during either tumor removal surgery or paracentesis (removal of peritoneal fluid). HGSOC was suspected for every patients, but a conclusive diagnosis of the tumor type was set during the surgery. Collection of these samples are performed with ethical approval.

4.1.3 Sample staining procedure

The 18 HGSOC samples and the corresponding PBMCs were cryoconserved prior to experiments, and six samples were stained at the time. First, the samples were thawed in water bath followed by drop-wise addition of chilled cell medium (RPMI + 10% FCS and 1 % L glutamine). The cells were centrifuged, washed in Buffered NaCl and counted followed by concentration adjustment to 10 million cells/mL. 100 μL of suspension was then added to each well in the plate. Ab mixes for extra-and intracellular staining mixed with Brilliant stain Buffer and Perm/Wash Buffer respectively. Cell viability was checked with Live/Dead dyes (Fixable Olive for NK/ILC plate and Far Red for Immune panel, both diluted to 1:10000), followed by 20 minutes of fridge incubation. After initial washes in buffered NaCl, Extracellular Antibody cocktails were added to respective wells and Perm/Wash for unstained controls, followed by fridge incubation. The cells were then fixed and permeabilized using in BD Cytofix/Cytoperm solution. Intracellular staining was performed after this step with the intracellular Antibody mix, where Perm/Wash was added to unstained controls. After the final wash, cells were resuspended in NaCl + 2mM EDTA and incubated in the fridge overnight. A more detailed workflow is found in the protocol used for the procedure.

4.2 Spectral flow cytometry and analysis

Antibody titrations were performed to determine the antibody concentration providing the best signal. The plates were analyzed on Sony ID7000 spectral flow cytometer. The output data from this step was processed in FlowJo, and median fluorescence intensity values were exported to a table for further calculation.

To assess the best signal-to-noise ratio for each antibody, the stain index (SI) was calcu-

lated for each dilution[49], defined as:

$$SI = \frac{MFI_{\text{pos}} - MFI_{\text{neg}}}{2 \times SD_{\text{neg}}} \tag{4.1}$$

Here MFI_{pos} and MFI_{neg} represent respective median fluorescence intensity of positive and negative populations, and SD_{neg} refers to the standard deviation of the negative population. The optimal antibody concentration is therefore defined as the dilution with the highest stain index yield, representing the maximal separation between negative and positive populations[49].

4.2.1 Spectral flow cytometry

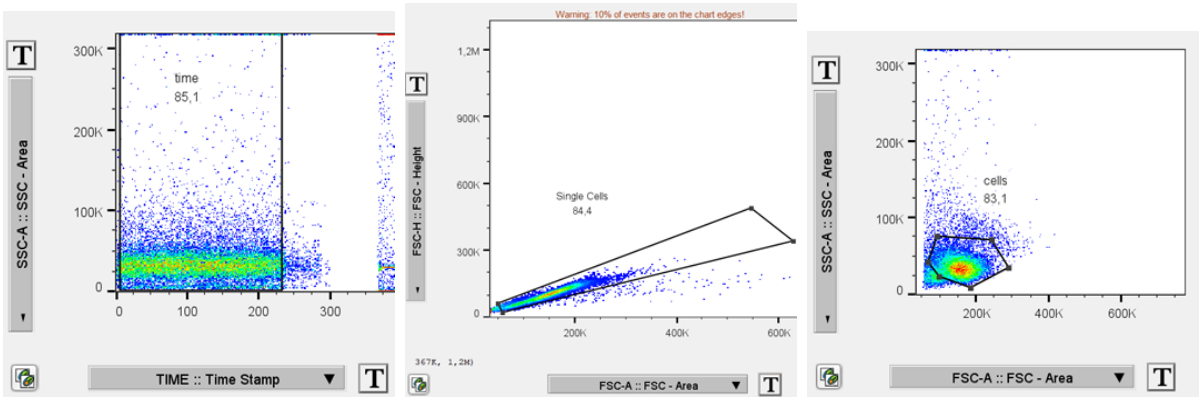
Spectral flow cytometry analysis was performed on a Sony ID7000 spectral flow cytometer. The instrument was calibrated according to standard QC procedures. Pre-recorded spectral profiles of fluorochromes used in the panel were loaded from the fluorochrome library from previous experiments of single stain controls. Instrumental parameters (e.g FSC/SSC gains and PMT voltages) were set according to each panel. Sample acquisition was performed in standardized mode with a 5-minute acquisition time per sample gate. Auto fluorescence was set individually in each sample group and spectral unmixing was performed after data acquisition.

4.3 FlowJo Analysis

4.3.1 Gating strategy

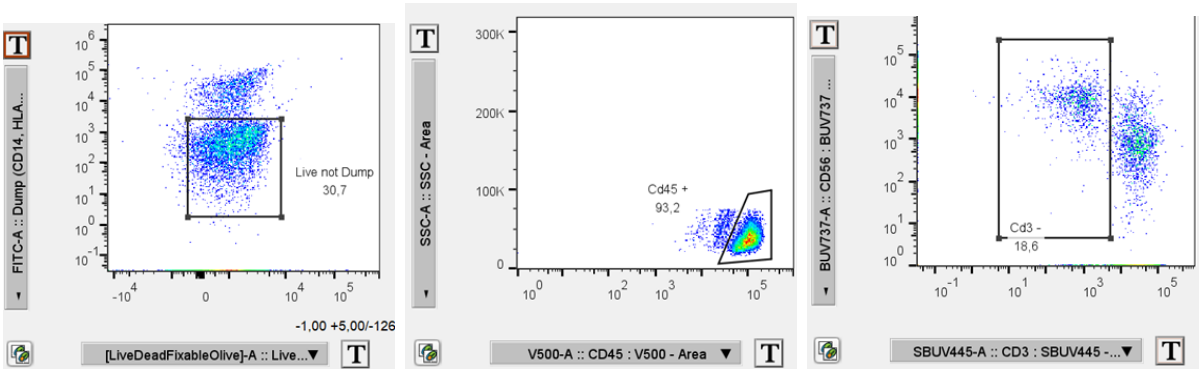
Immune cell populations of interest were gated in FlowJo. The gates in this project were set to isolate ILCs and NK cells. These gates were the following:

Example gating strategies for NK/ILC panel



(a) Time gate **(b)** Single cells gate **(c)** Cells gate

Figure 4.1: Gates to isolate precise cell populations. a) Is a time gate that ensure data quality by avoiding irregularities in the acquisition as clogs. b) Single cell gates avoiding doublets, allowing for analysis of single cells. c) Cell gate differentiating debris from actual cells.



(a) Live not dump gate **(b)** Leukocyte gate **(c)** NK & ILC gate

Figure 4.2: Gates to find a) Live cells that are not in the dump channel, ensure analyis of Live cells. b)Leukocyte gate to find $CD45^+$ cells, i.e leukocytes. c)The NK & ILC gate to select $CD3^-$ cells, with the emphasis of finding NK cells and ILC cells for further investigation.

The axis for TIME, SSC and FSC were set to linear, and set to biexponential for live dead, dump, CD45, CD3, and CD56.

4.3.2 Dimensionality reduction and clustering tools

To find clusters of similar cells within the dimensionality reduction graphs, the FlowJo the clustering algorithm PhenoGraph was implemented. PhenoGraph uses nearest neighbor

algorithms, implementing phenotypically similar clusters of cells [50]. Unlike other plug-ins, PhenoGraph generates the number of clusters itself, rather than setting the number of clusters beforehand.

4.4 Cytotoxic SNP-analysis

Publicly available genome-wide association studies (GWAS) summary statistics for endometrioid and clear cell OC as well as endometriosis (EN) were obtained from several GWAS platforms. These included the MRC IEU OpenGWAS platform [51], the NHGRI-EBI Catalog of human genome-wide association studies[52], and FinnGen[53] (FinnGen only EN data, with data on EN subtypes). The initiative Finngen is described as, "The FinnGen study is a large-scale genomics initiative that has analyzed over 500,000 Finnish biobank samples and correlated genetic variation with health data to understand disease mechanisms and predispositions. The project is a collaboration between research organizations and biobanks within Finland and international industry partners"[53]. Two releases from the FinnGen study was studied, including release R9 (from 2022) and R12 (2025) and more participants where recruited in the newer release, ultimately having an effect on the results. Moreover, not all six SNPs of interest were present in the different datasets.

The GWAS summary statistics were commonly provided in VCF (.vcf.gz) or tab-delimited (.tsv.gz) format. Processing of the data was conducted in Linux environment using Windows Subsystem for Linux (WSL) along with tools as bcftools (for handling VCF files) and zgrep (searching in compressed files). To inspect whether a target SNP was present in the dR, zgrep was used along with a list of rsIDs (SNP identifiers). Matches and their corresponding rows were extracted and saved for further analysis (see code in abstract).

The GWAS summary statistics contained information such as: chromosome and position, alleles (reference/alternative), alternative allele frequency (n reference population and effect population), effect size (beta or ES), Standard error (SE), statistical significance (p-value or -log10 p-value).

The key parameter of greatest interest is p-value, representing the probability that the observed association between a SNP allele and the trait of interest (EN and OC in this case) is due to random variation in the data, assuming the null hypothesis is true. For instance, if the SNP rs1050458(A/G) has a p-value of 0.05, this implies a 5% probability that the observed association occurred by chance. Traditionally in statistics, a p-value < 0.05 is viewed as a significance threshold [54]. However, as GWAS involves millions of SNPs and so on statistical tests, this threshold is insufficient. Due to linkage disequilibrium - the tendency for nearby genetic variants to be inherited together - the number of statistically independent tests is estimated to one million. In order to correct for this and avoid family-wise error rate, Bonferroni correction is applied[55]. This involves dividing

the classical significance threshold by the number of statistically independent tests , N (SNPs inherited together is viewed as one test), resulting in the adjusted significance level:

$$\frac{0.05}{1{,}000{,}000} = 5 \times 10^{-8} \tag{4.2}$$

Therefore, the widely accepted genome-wide significance threshold is $p < 5 * 10^{-8}$, which reduces the risk of acquiring false positives[56]. While SNPs with p-values larger than the $p < 5 * 10^{-8}$ do not meet the the criteria of genome wide significance, this does not necessarily mean that they are not biologically relevant. Such SNPs may still be of interest if they are located near biologically relevant genes, notably if they display consistent results across independent datasets or are consistent with know biological pathways.

For this SNP analysis, the cutoff set to identify SNPs of interest was set at the classical significance threshold of $p < 0.05$ as the aim of this analysis is not to identify genome wide significance, but rather to investigate if these SNP alleles occur at a higher rate in EN and OC possibly associated with EN. Thus, there is a risk of acquiring false positive results, but allows to capture potential associations. This approach can help determine whether the genetic variations linked to impaired immune function are more common in EN cases and in ovarian cancers suspected to be associated with EN (specifically endometrioid and clear cell subtypes). However, these findings need further validation to confirm their biological significance.

In the table 4.1 below, it is stated whether the SNP is correlated with impaired cytotoxicity or reduced response to immunotherapy. Categorized as impaired and not impaired.

rsID	**Receptor**	**Impaired**	**Not impaired**
rs1050458	HLA-B	G	A
rs1049174	NKG2D	C	G
rs763361	DNAM-1	T	C
rs1983526	NKG2A	C	G
rs986475	NKp30	G	A
rs1052248	NKp30	A	T

Table 4.1: Cytotoxic and non-cytotoxic alleles of SNP associated with NK cell function

5

Results

5.1 Antibody titration

After applying gates on the cells and calculating the stain index of each antibody, final concentrations for each antibody were obtained. Multiple experiments were performed, and after trial and error with the settings of the Sony ID7000 and laboratory procedures, the final concentrations for each antibody was calculated, seen in the table below.

Table 5.1: Extracellular Antibody Titrations and Volumes

Marker	**Titration**	**Volume per sample (µL)**
CD3	1:20	2.5
CD19	1:20	2.5
HLA-DR	1:80	0.625
CD14	1:80	0.625
CD45	1:20	2.5
CXCR3	1:20	2.5
NKG2C	1:20	2.5
Tim-3	1:20	2.5
NKG2D	1:40	1.25
LILRB1 (CD85j)	1:20	2.5
TRAIL	1:20	2.5
CD16	1:160	0.3125
TIGIT	1:20	2.5
CRTH2	1:20	2.5
NKG2A	1:40	1.25
CD56	1:40	1.25
NKp46	1:40	1.25
CD69	1:40	1.25
CTLA-4	1:50	1
KIR2DL1	1:50	1
KIR3DL1	1:50	1
CD158b/j	1:50	1
CD127	1:50	1
KLRG1	1:50	1
NKp30	1:100	0.5
NKp44	1:160	0.3125
LAG-3	1:40	1.25
PD-L1	1:20	2.5
CD161	1:50	1
CD49a	1:50	1
CD103	1:50	1
PD-1	1:80	0.625
cKit	1:20	2.5
Total Volume		**49.5**
Brilliant Stain Buffer to add		**0.5**

Intracellular Antibody	Titration	Volome per sample (µL)
Perforin	1:50	1
Granzyme B	1:20	2.5
CD15	1:80	0.625
Tot VOL		**13.5**
PermWash to add		**36.5**

Table 5.2: Intracellular antibody panel (excluding fluorochromes).

5.2 Results from NK/ILC panel

Firstly, the CD3$^-$ gated cell populations were concatenated into a new file. Prior to concatenation keywords were set to distinguish in between aMNCs, PBMCs and HD PBMCs. Also cell counts were normalized against the sample with the smallest cell population prior to the concatenation - in order to make the results more interpretable.

A UMAP was generated from the concatenated file, reducing the dimensionality of the data to two dimensions. This enables for visual exploration of complex data with many parameters. From the set keywords, the UMAP was plotted for every sample type respectively. Thereafter, PhenoGraph was generated for the UMAP. PhenoGraph is a clustering algorithm that groups cells according to their phenotype (according to expression of surface markers), and especially leverages in finding rare cell populations that are difficult to distinguish with conventional gating. All markers were included in the cluster generation.

The UMAP with all the samples and generated clusters is shown in figure 5.1.

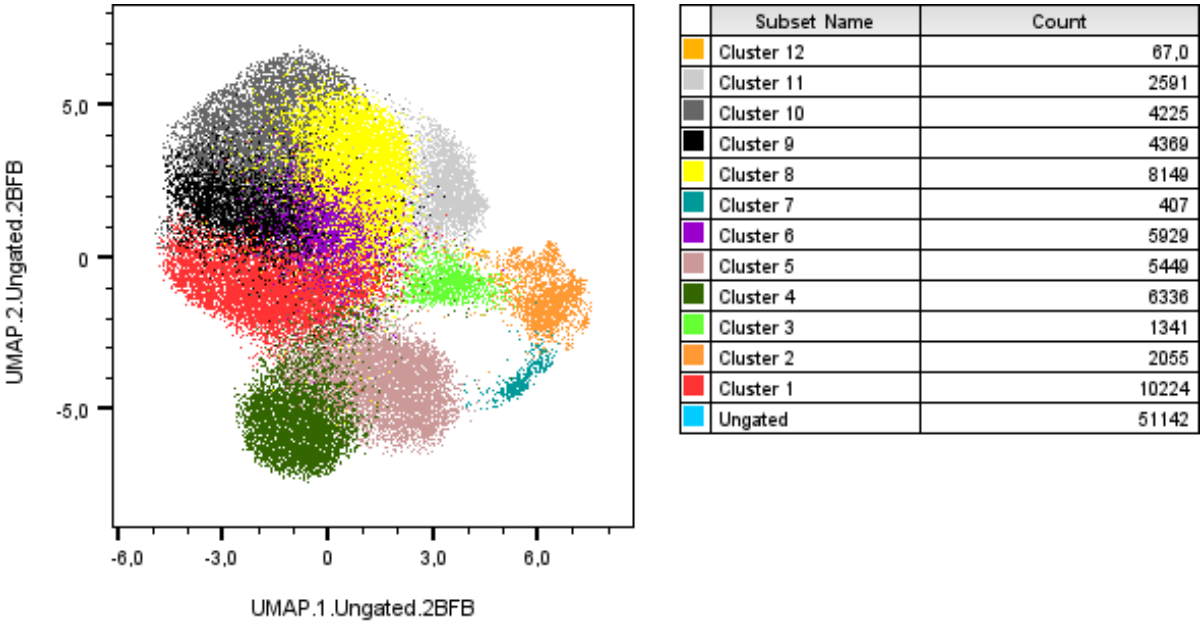


Figure 5.1: The different clusters generated by PhenoGraph applied on the UMAP

As seen in figure 5.1, there are distinct clusters in the graph, indicating the clustering algorithm was successful.

When the UMAPs generated from each sample population were put on top on each other, it generated the graph seen in figure 5.2.

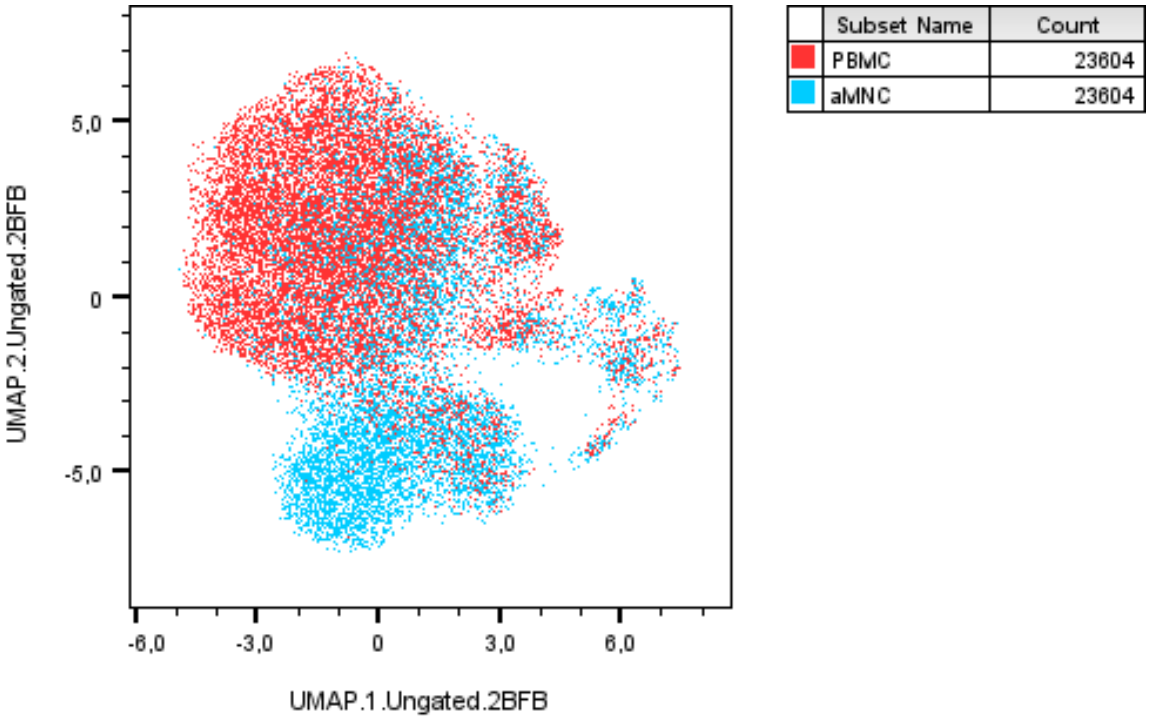
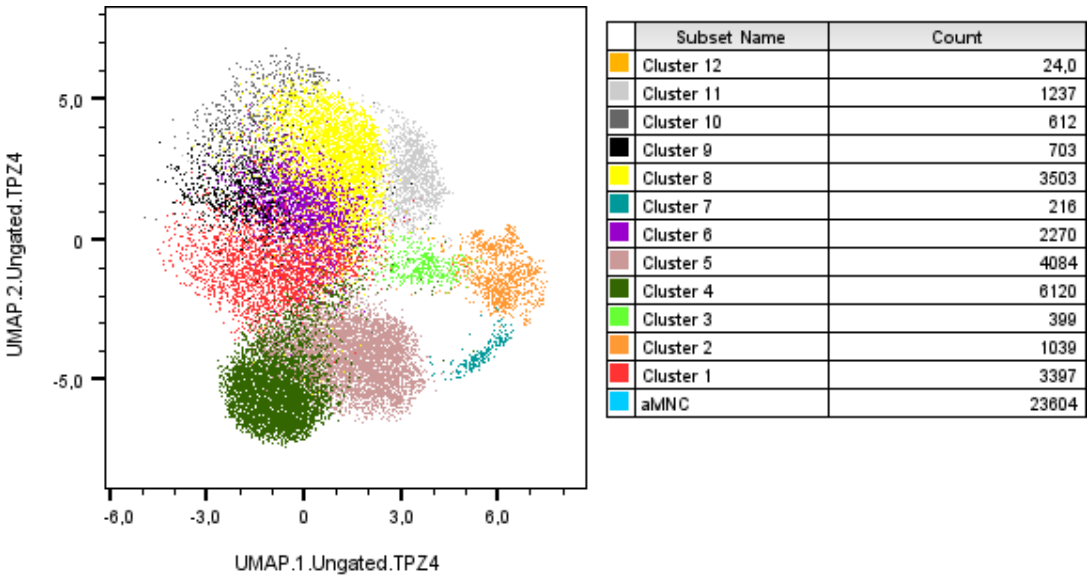


Figure 5.2: The UMAP derived from aMNCs and PBMCs ovarlayed on one another

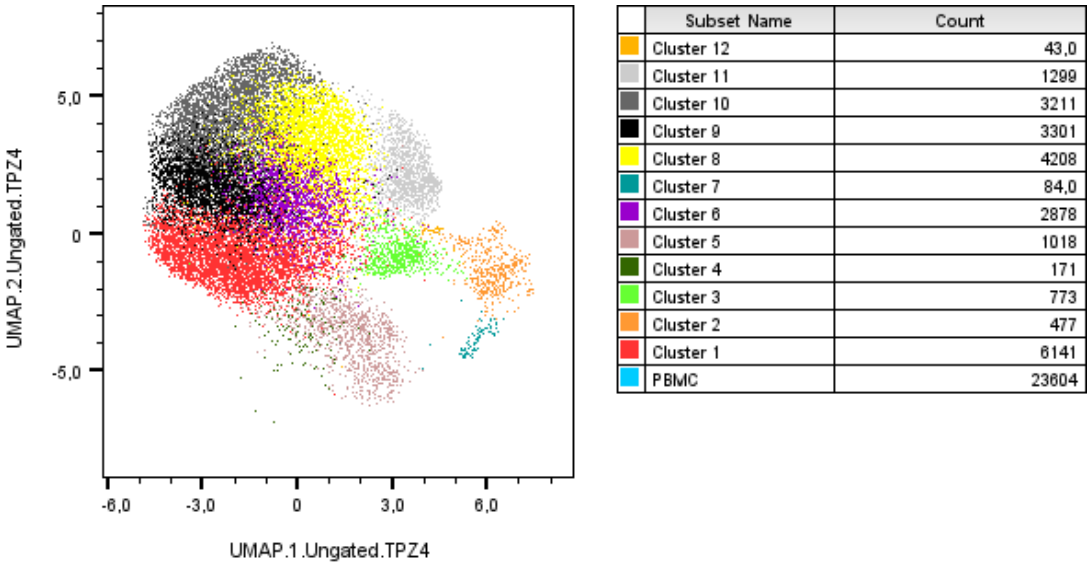
It is observed that there is a distinct shared population between figure 5.1 and 5.2, the beige and dark green clusters in the bottom of figure 5.1 and the clear population of aMNCs seen in blue in the bottom of figure 5.2. This indicates that darkgreen cluster four and beige cluster five are distinctly abundant in ascites.

These results are further verified in figure 5.3, where the clusters were applied on PBMCs and aMNCs respectively, forming the graphs seen in 5.3



Subset Name	Count
Cluster 12	24,0
Cluster 11	1237
Cluster 10	612
Cluster 9	703
Cluster 8	3503
Cluster 7	216
Cluster 6	2270
Cluster 5	4084
Cluster 4	6120
Cluster 3	399
Cluster 2	1039
Cluster 1	3397
aMNC	23604

(a) aMNCs



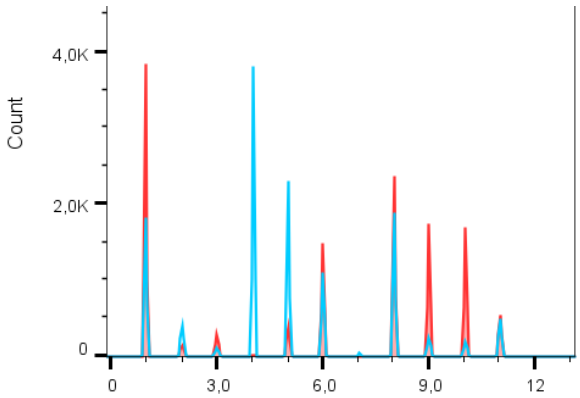
Subset Name	Count
Cluster 12	43,0
Cluster 11	1299
Cluster 10	3211
Cluster 9	3301
Cluster 8	4208
Cluster 7	84,0
Cluster 6	2878
Cluster 5	1018
Cluster 4	171
Cluster 3	773
Cluster 2	477
Cluster 1	6141
PBMC	23604

(b) PBMCs

Figure 5.3: UMAP with applied clusters for aMNCs and PBMCs

In figure 5.3, once again, a clear cluster four and five is seen for the aMNC population, but not for the PBMCs.

The relative expression of each cluster in each cell type is seen in figure 5.4.



	Subset Name	Count
	aMNC	23604
	PBMC	23604

Figure 5.4: Histogram of abundance of each cluster per cell type

The peaks in figure 5.4 suggest that aMNCs have a high abundance of cells mainly in cluster four and cluster five.

Through the feature cluster explorer, the separate expression of each marker in each cluster could be visualized in a heat map, seen in figure 5.5

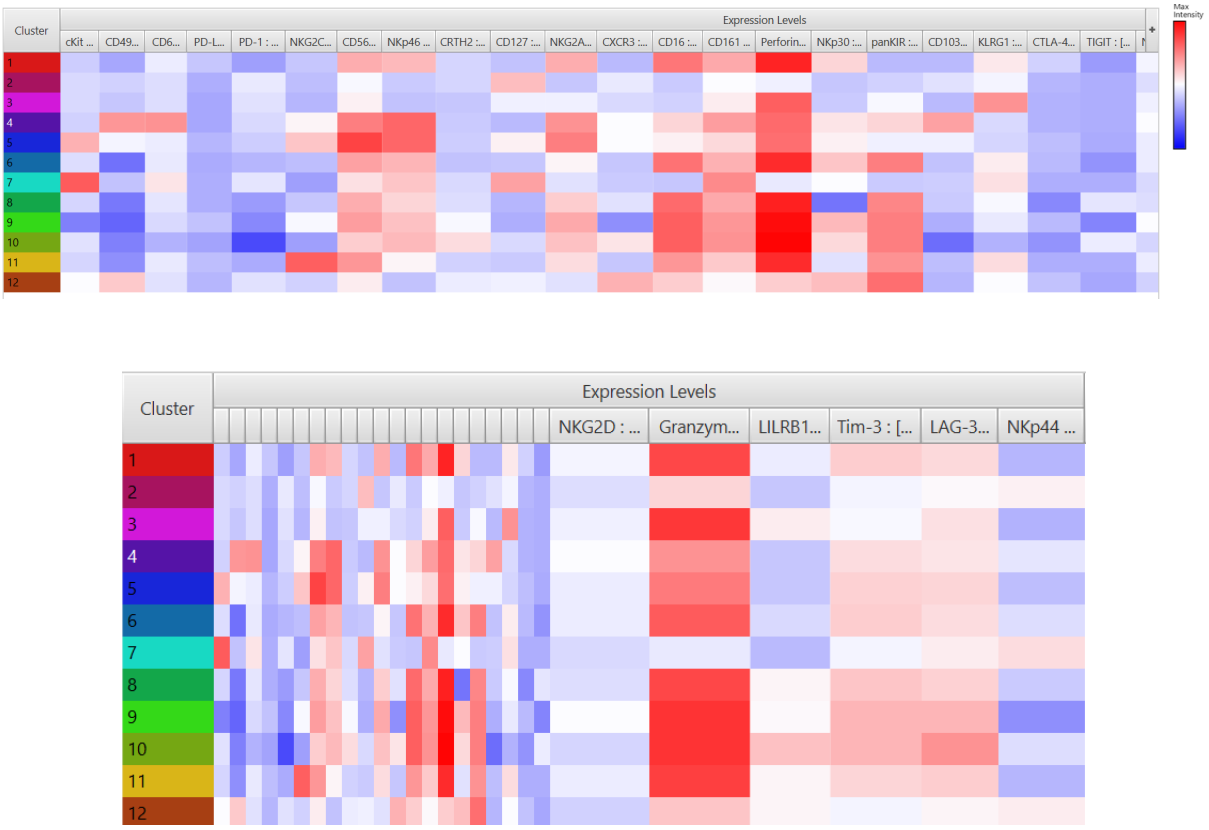


Figure 5.5: Heatmap of the surface marker expression for each cluster

It is seen that cluster four has a higher relative expression of NKp46, CD69, CD49a, CD56,

and NKG2A, and CD103 and no expression of CD127. These expression levels indicates that cluster four are NK-cells and that they are tissue resident. This is indicated by the fact that they express both CD49a and CD103 and are not ILCs as they lack CD127 expression. However, it does have a high expression of the ILC marker CD161, but the expression is red for a majority of clusters, indicating possible error.

Moreover, cluster five also had a very high abundance for aMNCs. As previously stated, ILCs can be identified according to the expression of surface markers:

- **ILC1**: $CD127^{+}$, $CD161^{+}$, $KLRG1^{+}$, $CRTH2^{-}$, $cKit^{-}$
- **ILC2**: $CD127^{+}$, $CD161^{+}$, $CRTH2^{+}$, $cKit^{+/-}$, $KLRG1^{+}$
- **ILC3**: $CD127^{+}$, $CD161^{+}$, $cKit^{+}$, $NKp44^{+/-}$, $NKp46^{+}$, $CRTH2^{-}$

Cluster five has a high expression for cKit, NKG2C, CD56, NKp46 and NKG2A, indicating some form of NK cell, but the high expression of cKit could possibly point towards an ILC population.

Referring to these expression profiles, potential candidates according to the results from figure5.5, a potential candidate for an ILC population is cluster seven. It has relative high expression of cKit, CD127, CD161, NKp44, NKp46 and are $CRTH2^{-}$. This indicate an ILC3 population. However, the expression of KLRG1 would contradict this but overall ILC3 is a match for this population.

5.2.1 Limitations

Due to experimental errors during the first day of performing the experiment, the first 6 samples were not included in the analysis. Moreover, because of to unmixing and errors in removing the autofluorescence, the separation of live dead cells was not adequate. Due to these factors, the results might not provide a realistic view on the true immune landscape as the live dead gate could not distinguish the dead cell population, ultimately affecting the other gates. Moreover, the fluorochrome conjugated to NKp30 had degraded, which affects the result for a large panel. These limitations also have an effect on the FlowJo analysis, altering the outcomes of PhenoGraph and UMAP as these are based on algorithms implemented on the data input. Hence, these results should be interpreted with caution.

5.3 SNP analysis

The SNP study based on GWAS data, publicly available datasets of EN was used. Of these, not all consisted of all of the 6 SNPs of interest for this report. Below is specified in how many datasets each SNP occurred.

- **rs1050456, HLA-B-21** occurred in 11 datasets
- **rs10522448, NKp30** occurred in all 40 datasets
- **rs1983526, NKG2A** occurred in all 40 datasets
- **rs1049174, NKG2D** occurred in 39 datasets
- **rs986475, NKp30** occurred in 38 datasets
- **rs763361, DNAM-1** occured in all 40 datasets

5.3.1 Significant Results

Table 5.3: SNPs with nominal significance ($p < 0.05$) in Endometriosis datasets

Dataset	rsID	Receptor	p-value	Case	Controls
ukb-b-10903	rs1050458	HLA-B-21	0.01898	3809	459124
R9 Finngen N14_ENDOMET_INFERT	rs1983526	NKG2A	0.0411889	3206	219462
R9 Finngen N14_ENDOMER_NOS	rs1052248	NKp30	0.0285076	2982	219462
R9 Finngen N14_ENDOMER_NOS	rs986475	NKp30	0.0393378	2982	219462
R9 Finngen N14_ENDO_INTESTINE	rs1049174	NKG2D	0.0321721	436	219462
R9 Finngen N14_ENDO_INTESTINE	rs1983526	NKG2A	0.0105653	436	219462
R12 Finngen N14_ENDOMER_NOS	rs986475	NKp30	0.0138692	4314	293373

Note that R9 is an earlier version of the data and that more patients with the trait was recruited in R12 whereas some SNPs alleles now showed to be non-significant according to our cutoff.

SNPs with nominal significance ($p < 0.05$) in Clear cell and Endometrioid OC datasets:

Dataset	rsID	Receptor	p-value	Case	Controls
ieu-a-1124	rs1050458	HLA-B-21	0.0133	1366	40941

6

Discussion

6.1 Characterization of NK/ILC panel

The results from the spectral flow cytometry provide insights into the immune landscape within ascites in high-grade ovarian cancer (HGSOC). Spectral flow cytometry and subsequent analysis in FlowJo implementing UMAP dimensionality reduction and the clustering algorithm PhenoGraph revealed distinct immune cell populations enriched in ascites. In particular, cluster four and five were distinctly more abundant in ascites, compared to matched samples of PBMC, suggesting tissue resident phenotypes.

Cluster four appeared as the most abundant cluster in aMNCs as visualized in figure 5.4. Analyzing the heatmap in figure 5.5 showed this cell population expressed CD49a, NKp46, CD103, CD69, and NKG2A, while lacking CD127 expression, consistent with tissue resident NK (trNK) cells. These results align with previous work from Bernson et al., which established that NKG2A^{+} trNK as highly abundant in ascites, playing a potential role in ovarian Cancer (OC) surveillance [27]. Notably, trNK cells in ascites have demonstrated clear cytotoxic capability against tumors, despite a majority being the phenotype CD56^{bright} typically viewed as non-cytotoxic.

Furthermore, cluster five was likewise enriched in the aMNC population. This cluster displayed a varying expression of characteristic markers, including cKit, NKG2C, CD56, NKp46, and NKG2A. Although the high expression of cKit suggests that this could be an ILC population, the expression profile rather indicates an NK cell subset. Interestingly, this population does not express tissue residency markers despite the high abundance in ascites.

Cluster seven, though rare, emerged as a possible ILC population, expressing CD127, cKit, CD161, NKp44, and NKp46, with absent CRTH2 expression. This profile aligns quite well with ILC3, although it expresses KLRG1 which is not typical for ILC3s. With only 407 cells as seen in 5.1, this population is rare, but could still be functionally relevant. Seen in figure 5.3, the expression is higher in aMNCs than for PBMCs, indicating tissue residency.

Overall, the NK/ILC panel yielded promising results for immune cell characterization in ascites fluid, although the results were influenced by several technical limitations.

Degradation of NKp30 likely resulted in a shift in the emission spectra. As the panel consists of many antibodies conjugated to fluorochromes with similar emission spectra, one single shift could ultimately affect the entire panel. This led to unmixing issues, limiting the function of algorithms to pinpoint the spectral signals of each marker and could have reduced the marker resolution affecting the gating. Moreover there were difficulties in gating the live cells as the antibodies in the dump channel interfered with the viability dye. This diminished the separation between the live and dead cell populations, which complicated the process of gating live cells.

Analytically, these limitations impacted beyond just marker interpretation. As clustering algorithms like PhenoGraph and dimensionality reduction algorithms such as UMAP consider all dimensions, one faulty marker can disrupt the clustering. Hence, this could explain the difficulties in interpreting the expression profiles in figure 5.5.

To conclude, these findings indicate that the NK/ILC panel shows great promise for discovering the immune landscape in tissues and fluids. With the limitation that working with panels this big is a sensitive process, and therefore small mistakes can greatly affect the outcome. Moreover, this analysis showed that the implementation of algorithms to identify clusters is a powerful tool when detecting rare cell populations. However, the limitations mentioned presumably affected the marker intensities, reducing the performance of the clustering algorithms.

For future experiments the panel will have to be tweaked further and the experiments will have to be repeated according to the new experimental settings. In addition, more samples originating from other subtypes of OC will be analyzed, as well as ascites from other forms of cancer.

6.2 SNP-analysis

Unfortunately, the results from the SNP analysis were not conclusive, as most p-values were high and significance of SNPs were inconsistent across datasets. Moreover, only the Finngen data provided allele frequency information for both the control and study populations, making it difficult to determine which SNPs occurred at an abnormal rate and contributed to the significant findings.

Moreover, not all datasets contained the six SNPs of interest. For example, Finngen did not include the HLA-B-21 SNP, which would have been particularly relevant given its significance in OCCC, as well as the genetic aberrations shared in HLA genes between OC and EN suggested by Linder et. al. [43]. The Finngen dataset also provided the most detailed information about the endometriosis, including subtypes such as ovarian endometriosis, which is known to have a higher likelihood of progressing into OC compared to other forms of the disease.

Despite these limitations, some SNPs especially in NKG2A, NKp30, and HLA-B-21

showed an abundance of low p-values in multiple datasets, suggesting a potential link between altered NK receptor function and susceptibility to disease progression in EN and OC associated with EN. Similarly, rs1050458 (HLA-B-21), known to affect NK cell education, was significantly associated with clear cell ovarian cancer, a subtype frequently linked to endometriosis. These genetic alterations could impact NK cell activity and immune function in the context of tumor elimination in patients with a history of endometriosis.

Though the p-values are not low enough to conclusively determine a causal effect for the traits studied, the patterns observed suggest potential effects. Further validation with larger cohorts, detailed genomic analysis could further validate these results.

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A

Appendix 1

A.1 Antibodies

A.1.1 Antibody list and marker

Immune Marker Classification

1. NK Cell Markers

Table A.1: NK and Immune Markers with Tags / Functions

Marker	**Tags / Indicates**
LAG-3	NK, T, Tregs, B
NKG2A	NK, $CD8^+$ T (some TrNK)
TIGIT	NK, T
Perforin	NK, cytotoxic T cells
CD158b/j	Inhibitory NK cell receptors (KIR family)
KIR3DL1	Inhibitory NK cell receptor
KIR2DL1	Inhibitory NK cell receptor
CTLA-4	NK, T
NKp30	NK
TRAIL	NK
KLRG1	Senescent or terminally differentiated NK and T cells
NKp44	Activating NK cell receptor
CD69	NK, ILCs, resident memory T, $\gamma\delta$T (exhausted NK cells when expressed in context)
NKG2C	Activating NK cell receptor
Granzyme B	Cytotoxic granule enzyme in NK and $CD8^+$ T cells

Continued on next page

Marker	Tags / Indicates
CD85j/LILRB1	NK, myeloid cells, macrophages, DCs, B, T
NKG2D	NK, $\gamma\delta$T, CD8 T
CD279 (PD-1)	NK, T, B, macrophages, subset of DC
CD161	NK, peripheral T (including $\gamma\delta$T and $\alpha\beta$T), NKT, ILC
CD103	Tissue-resident aNK
CXCR3 (CD183)	Decidual-like NK, effector T cells, Th1 $CD4^+$ T cells
CD16	NK, T
NKp46	NK, T (low)
CD56	NK, ILC3
CD45	Leukocytes: NK, NKT, ILC
CD127	ILCs, memory T cells
CD294 (CRTH2)	Basophils, eosinophils, TH2, ILC2
cKit (CD117)	ILC2 (low), ILC3
CD14	Monocytes/macrophages
CD15	Granulocytes, monocytes, macrophages
HLA-DR	APCs (DCs, monocytes, B, T)
CD19	B cells
CD3	Pan-T cell marker

2. ILC / Tissue-Resident Markers

Marker	Tags / Indicates
CD127	ILCs, memory T cells
CD294 (CRTH2)	Basophils, eosinophils, TH2, ILC2
cKit (CD117)	ILC2 (low), ILC3
CD49a	Tissue-resident aNK, uNK, endometrial NK (eNK)
CD69	ILCs also resident memory T, gdT, (exhausted NK cells when expressed in combination with CXCR6)
CD161	Also on ILCs (pro-inflammatory marker)
CD103	Also tissue-resident aNK
CD56	Also ILC3
NKG2A	Tissue resident in uterus environment
CD45	Also ILC lineage

3. Dump Channel Markers

Marker	Tags / Indicates
CD14	Monocytes, macrophages
HLA-DR	APCs (DCs, monocytes, B, T)
CD19	B cells
CD15	Granulocytes, monocytes, macrophages

A.1.2 Technical information of antibodies

Marker	Fluorochrome	Detection Ex/Em	EC/IC
LAG-3	Spark Plus UV 395	UV laser 355nm. 355/385	EC
CD127	BV650	Violet laser 405nm. 405/645	EC
CD294 (CRTH2)	BV605	Violet laser 405nm. 405/603	EC
NKG2A	BV711	Violet laser 405nm. 405/711	EC
TIGIT	PerCP-Fire 806	Blue laser 488nm. 488/800	EC
Perforin	Pe-Dazzle 594	Blue, green or yellow-green. 566/610	IC
cKit (CD117)	APC	Red laser 633-640nm. 650/660	EC
CD158b/j (KIR2DL2/L3/S2)	PE-Vio770	Violet laser 405nm. 405 / 711	EC
KIR3DL1	PE-Vio770	Violet laser 405nm. 405/711	EC
KIR2DL1	PE-Vio770	Violet laser 405nm. 405/711	EC
CTLA-4	PE-Fire744	Blue, Green/ yellow-green. 565/ 744 (	EC
NKp30	PE-Fire 810	Blue, yellow-green. 488/561/810	EC
CD49a	APC-Fire750	Red laser 633-640nm. 650/ 788	EC
TRAIL	PE	Blue, green, yellow-green. 565/578	EC
KLRG1	SparkNIR-685	Red laser 640nm (?). 665/685	EC
NKp44	Spark-Red 718	Red laser 640nm. 695/ 715	EC
CD69	BUV496	UV laser 355 nm. 348/496	EC
CD274 (PD-L1)	BUV563	UV laser 355 nm. 348/ 563	EC
Tim-3	Real Yellow 703	Blue, green, yellow-green. 565/703	EC
NKG2C	BUV661	UV laser 355 nm. 348/661	EC

Marker	Fluorochrome	Detection	EC/IC
Granzyme B	Real Blue 705	Blue laser 488nm. 498/ 781	IC
CD85j/LILRB1	Real Blue 744	Blue laser 488nm(?). 488/744	EC
NKG2D	Real Blue 780	Blue laser 488nm. 488/780	EC
CD279 (PD-1)	BUV615	UV laser 355 nm. 348/ 616	EC
CD161	PE-Cy5	Blue laser 488nm. 488/ 667	EC
CXCR3 (CD183)	BV750	Violet laser 405nm. 405/750	EC
CD103	Starbright UV 795	UV laser 355nm. 338/792nm	EC
CD3	Starbright UV 445	UV laser 355 nm. 345/439	EC
CD14	FITC	Blue laser 488nm. 498/525	EC
CD16	BV786	Violet laser 405nm. 405/786	EC
HLA-DR	FITC	Blue laser 488nm. 498/525	EC
NKp46	BV421	Violet laser 405nm. 405/421	EC
CD56	BUV737	UV laser 355 nm. 498/732	EC
CD45	V500	Violet laser 405nm. 405/500	EC
CD19	FITC	Blue laser 488nm. 498/525	EC
CD15	FITC	Blue laser 488nm. 498/525	IC

A.1.3 Clone, Catalogue number and recommended dilution of Antibody

Marker	Source (Cat. No.)	Clone	Conjugation	Dilution
LAG-3	Sony Biotechnology (2446770)	11C3C65	Spark PLUS UV 395	1:40
CD127	Sony Biotechnology (2356630)	?	Brilliant Violet 650	1:50
CRTH2	Sony Biotechnology (2350610)	BM16	Brilliant Violet 605	1:20
NKG2A	Sony Biotechnology (2475780)	S19004C	Brilliant Violet 711	1:40
TIGIT	Sony Biotechnology (2463745)	A15153G	PerCP-Fire 806	1:20
Perforin	Sony Biotechnology (2140660)	dG9	Pe-Dazzle 594	1:50
cKit	Sony Biotechnology (2166030)	104D2	APC	1:20
CTLA-4	Sony Biotechnology (2448230)	BNI3	PE-Fire744	1:50
NKp30	Sony Biotechnology (2226195)	P30-15	PE-Fire 810	1:100
CD49a	Sony Biotechnology (2241585)	TS2/7	APC-Fire750	1:50
TRAIL	Sony Biotechnology (2141030)	RIK-2	PE	1:20
KLRG1	Sony Biotechnology (2438700)	SA231A2	Spark Red 685	1:50
CD69	BD (750214)	FN50	BUV496	1:40
PD-L1	BD (741423)	MIH1	BUV563	1:20
TIM-3	BD (571470)	7D3	Real Yellow 703	1:20
NKG2C	BD (749842)	134591	BUV661	1:20

Granzyme B	BD (570274)	GB11	Real Blue 705	1:20
LILRB1	BD (757063)	GHI/75	Real Blue 744	1:20
NKG2D	BD (569322)	1D11	Real Blue 780	1:40
PD-1	BD (612991)	EH12.1	BUV615	1:80
CXCR3	BD (746895)	1C6/CXCR3	BV750	1:20
KIR2DL2/L3/S2	Milteny (130-116-835)	REA1006/DX27	PE-Vio770	1:50
KIR3DL1	Milteny (130-116-826)	REA1005/DX9	PE-Vio770	1:50
KIR2DL1	Milteny (130-120-447)	REA284/143211	PE-Vio770	1:50
NKp44	Biolegend (285099)	P44-8	Spark-Red 718	1:160
CD161	Bio-Rad (MCA1266SBB580)	PK136	StarBright Blue 580	1:50
CD103	Bio-Rad (MCA1416SBUV795)	LF61	Starbright UV 795	1:50
CD3	Bio-Rad (MCA463SBUV445)	UCHT1	Starbright UV 445	1:20
CD56	BD (612766)	NCAM16	BUV737	1:40
NKp46	Biolegend (331913)	9E2	BV421	1:40
CD45	BD (560777)	HI30	V500	1:20
CD16	BD (563690)	3G8	BV786	1:160
CD14	BD (345784)	MΦP9	FITC	1:80
CD15	BD (555401)	HI98	FITC	1:80
HLA-DR	BD (347400)	L243	FITC	1:80
CD19	BD (345788)	SJ25C1	FITC	1:20
TCR	BD (569510)	11F2	BV650	1:50
TCR V	Milteny (130-119-365)	REAL277	–	1:50
TCR V2	Milteny (130-131-916)	REA771	PE-Vio615	1:50
CD4	BD (560345)	RPA-T4	V450	1:20
CD8	BD (568284)	HIT8a	BB700	1:40
CD14	BD (345786)	MΦP9	Per-CP	1:5
CD15	Biolegend (301919)	HI98	Alexa Fluor 700	1:20
CD19	BD (560177)	SJ25C1	APC-H7	1:20

A.2 Code for WSL

zgrep -F -f snplist.txt dataset.vcf.gz | grep -w -f snplist.txt > matches.txt

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