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LAKATOS KORNÉL FÜLÖP

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Programvezető: Dr. Poór Gyula, egyetemi tanár

Témavezető: Dr. Fülöp Vilmos, egyetemi tanár

**Novel techniques in gynecological oncological diagnostics and
experimental design.**

PhD thesis

Kornél Fülöp Lakatos

Doctoral School of Molecular Medicine
Semmelweis University



Supervisor: Vilmos Fülöp, MD, D.Sc
Official reviewers: János Szepesi, MD, Ph.D
Anett Czegle Ibolya, MD, Ph.D

Head of the Complex Examination Committee: Szabolcs Várbíró, MD, D.Sc

Members of the Complex Examination Committee: Sándor Nagy, Ph.D
Zoltán Pócs, Ph.D

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List of abbreviations

ANGPT 1/2 – Angiopoietin 1/2
APC – Antigen Presenting Cell
CA 125 – Cancer Antigen 125
CBC – Complete Blood Count
CD – Cluster of Differentiation
Ceacam1 - Carcinoembryonic antigen-related cell adhesion molecule 1
CHM – Complete hydatidiform mole
CTLA-4 - Cytotoxic T-lymphocyte Associated Protein-4
DLL-1 – Delta Like 1 Protein
ECM – Extracellular Matrix
EOC – Epithelial Ovarian Cancer
ETT – Epithelial Trophoblastic Tumor
eVT – Extravillous Trophoblastic Cell
GTD – Gestational Trophoblastic Disease
GTN- Gestational Trophoblastic Neoplasia
HGF- Hepatocyte Growth Factor
HLA – Human Leukocyte Antigen
HMGB1- High Mobility Group Box 1
HSP – Heat Shock Protein
IFN- γ – Gamma Interferon
IL - Interleukin
KIR – Killing Immunoglobulin Like Receptor
LIR-1 – Leukocyte Immunoglobulin Like Receptor -1
Ly49 - C-type lectin-like receptors
MHC – Major Histocompatibility Complex
MMP – Metalloprotease
NCR – Natural Cytotoxicity Killer Receptor
NK- cell – Natural Killer Cell (dNK: decidual NK cell)
NLR – Neutrophil Lymphocyte Ratio
PBMC – Peripheral Blood Mononuclear Cell

PD- L1 – Programmed Death Ligand-1

PD-1 – Programmed Cell Death Protein -1

PGF- Placental Growth Factor

PLAU – Urokinase Plasminogen Activator

PLR – Platelet Lymphocyte Ratio

PSTT- Placental- Site Trophoblastic Tumor

PtdSer - Phosphatidylserine

S1PR5 - Sphingosine-1-phosphate receptor 5

TGF- β – Transforming Growth Factor Beta

Tim-3 - T-cell immunoglobulin and mucin-domain containing-3

TNF- α – Tumor Necrosis Factor Alpha

VEGF – Vascular Endothel Growth Factor

1. Introduction

1.1 Decidual natural killer cells

Natural Killer Cells (NK Cells), alongside T and B cells belong to the family of lymphocytes. NK cells have a pivotal role in detecting and killing virally infected cells, or cancer cells. NK cells form a part of the innate immune system which means that they are capable of reacting fast to many different pathogens forming the immune system's first line of defense (1). NK cells share the same progenitor cell with other lymphocytes but they are unique in many ways. NK cells are patrolling the body through the blood stream and they can enter different types of tissue including the decidua or tumor micro-environment. In contrast to other types of "killing" cells, NK cells can eliminate the target cells (either infected by a virus or showing early signs of malignant degeneration) without prior activation, called priming hence the name natural killer cell (2). The NK cells are also responsible for the production of cytokines, including Interferon Gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) which promote the immune response of other immune cells. They account for 5- 15% percent of peripheral blood lymphocytes and can be found in lymphoid and non- lymphoid tissues like lymph nodes, thymus, tonsils, spleen and the endometrium (3, 4). Their phenotype can vary depending on the tissue they reside. In the peripheral blood there are also two kinds of NK cell subpopulations, the CD 16-, CD 56 bright and the CD 16+, CD 56 diminished cells. The specific decidual type of NK cells play a significant part in coordinating the physiological inflammation at the implantation site and facilitate the angiogenesis (5-7).

The main way how the NK cells perform their killing function is the following. Most of the host cells express the Major Histocompatibility Complex I. (MHC-I) on their cellular surface. Virally infected cells, malignant cells or cells from a different individual (e.g. transplanted tissue) do not express MHC-I receptors identical to the host's so they cannot be identified as 'self'. The MHC-I receptor binds to the CD 8 molecule expressed on the surface of the NK cells and prevents its killing function. Other surface expressed antigens, typically expressed by tumor cells or cells infected by virus, can bind to the NK cell surface receptors enhancing the killing function. The structural classification of these

receptors into families includes the killer immunoglobulin-like receptors (KIR), the natural killer cytotoxicity receptors (NCR) the C-type lectin heterodimers (CD94/NKGs) and the immunoglobulin-like transcripts (8-10). The final outcome will be the result of the delicate balance between the suppressing and activating factors. If the activating factors overcome the suppressing factors, then the NK cell releases its granzyme and perforin containing vesicles. These immunological checkpoints are very important to understand how the innate immune system can protect the body from invasive agents and also how the implantation of an embryo (semi allograft) is possible in the maternal tissue (11).

1.2. Trophoblastic cells

Trophoblastic cells are the first cells to differentiate from the blastocyst during the embryogenesis. They form an outer layer around the growing embryo providing it with nutrients. The trophoblastic cells are originated from the fertilized egg and alongside with the embryonal ectoderm they form the trophoctoderm. As the pregnancy progresses the trophoblastic tissue proliferates and differentiates creating the placenta. This procedure is important to create the optimal environment for receiving the blastocyst. By the end of this procedure a semi allograft (embryo), with different antigen phenotype is able to invade and penetrate the host's (mother) tissue. At the frontier of the maternal- fetal site the syncytiotrophoblast is formed. After the successful penetration both trophoblastic and maternal vascular remodeling are started in the decidua (12). The main types of human trophoblastic tissue are the following: cytotrophoblast, syncytiotrophoblast, intermediate trophoblast or extravillous trophoblast eVT. Based on their localization and function we can talk about invasive eVT that can migrate into the myometrium and form 'giant-cells' and the endovascular trophoblast cells that can invade the lumen of maternal vessels of the decidua.

Immune tolerance at the fetal- maternal interface

The regulatory T- cells (Tregs) produce intracellularly the CTLA-4 (Cytotoxic T-Lymphocyte Associated Protein- 4) after they undergo the maturation process. The

molecule was first described related to Tregs cells, however, it occurs on the surface of CD4⁺ and CD8⁺ T cells as well (13). The main way of its action is the competition with the CD 28 molecule for the B7 receptors. (B-7/1 or CD80 and B- 7/2 or CD 86). CD 28 and B-7 connection is a significant step during the activation of T- cells. By occupying the free B-7 receptors on the surface of the antigen presenting cells (APC) CTLA-4 can compete the activating effect of CD -28 (14). Not only the number of Tregs are elevated in the decidua compared to the periphery but also their CTLA-4 expression. The increased decidual CTLA-4 expression shifts the balance of Th-1/ Th-2 cytokine expression towards Th-1 creating immunosuppression locally. T-cells, B-cells and NK cells present a trans- membrane receptor called PD-1. Upon binding to its ligands PDL-1 and PDL-2 it generates a down regulatory signal on pro- inflammatory T cell activity. PDL-2 is specific to macrophages and dendritic cells, but PDL-1 can be found on many residing immune cells and different types of tissues including syncytiotrophoblast. The PDL-1 expression can be regulated by pro- inflammatory factors and cytokines expressed by leukocytes, including NK cells (IFN- γ , TNF- α). Finally, we have to mention the Tim-3 receptors which can be found on the surface of NK cells, Th1, Th17, NK and NKT-like cells, Tregs, as well as on the surface of antigen-presenting immune cells. Tim-3 has several ligands (PtdSer, HGMB1, Ceacam1) but the Galectin-9 has been in the focus of intensive research due to its relatively specific binding compared to the other ligands. Tim-3 Galectin 9 binding can lead to the apoptosis of Th-1 and Th-17 cells modulating the Th-1, Th-17/ Th-2 balance and causing immune tolerance (15). It has also positive modulatory effect on NK cell cytokine expression including IFN- γ and TNF- α (16).

1.3 Human uterine NK cells

The human uterine natural killer cells (uNK cells or decidual NK cells) are of a different phenotype than the peripheral NK cell subset. The uNK cells as well as the peripheral NK cells are CD3⁻ and CD 15⁻, CD 14⁻. Most of the uNK cells are CD 16⁻ and CD56 bright which phenotype is related to diminished cytotoxicity. In contrast, most of the peripheral NK cells are CD 16⁺ and CD56 diminished. Another difference is that the uterine NK cells' expression of KIRs (killer cell immunoglobulin- like receptors) takes place at a

higher level compared to the peripheral phenotype and they are also expressing VEGF, placental growth factor (PGF), ANG2 and TGFB1 (6). The proportion of uNK cells among other leukocytes in early pregnancy is extremely high, accounting 70% of all white blood cells (3, 8). This high percentage persists until the 20th week and then it starts to decline until it reaches 10- 35% around the end of pregnancy (17) (according to our unpublished data- flow cytometry). The high number of uNK cells in the decidua during implantation and throughout early pregnancy and their angiogenic cytokine expression suggest the importance of uNK cells in decidual angiogenesis. It is also important to comment that decidual NK cells can be divided in two main groups according to their origins. Some of them are already in the endometrium before the conception and implantation like they were „awaiting” for pregnancy (endometrial NK cells or eNK cells). Endometrial NK cells can be characterized by the following phenotype: CD 56+, CD 3, CD 16, CD 94+, CD 9+, CD 57, HLA-DR+, CD 69+, CD158b+, NKB1+n and L-selectin. After activation by cytokines, most importantly IL-15, they become mature uterine NK cells (3). The other subset of dNK cells are recruited from peripheral blood during the formation of the placenta (6).

1.4 Syncytiotrophoblast and NK cell interaction

By week 20 of human pregnancy the placenta provides a 15 m² surface for direct contact between the embryo and the mother. Although there is no mixture between the maternal and fetal blood, the syncytium covered chorion is submerged into maternal blood. The maternal NK cells, among other types of circulating and uterine residing leukocytes, have direct connection to the embryonic syncytium. The trophoblastic cells do not express conventional MHC-I antigens but they do express moderately polymorphic HLA-C and monomorphic HLA-G and HLA-E molecules. Trophoblastic tissue is unique in a way that it decreases its HLA-A and B expression after IL-10 exposition. The decreased number of these two surface antigens may make the trophoblastic cells less vulnerable against cytotoxic cells including NK cells (1). In contrast to other types of trophoblastic cells (e.g. cytotrophoblastic cells, extravillous trophoblastic cells) the syncytiotrophoblast does not express HLA-G in significant amounts but its PDL-1 expression is augmented.

PDL-1 is one of the well-known immune checkpoint inhibitors. It binds specifically to the PD-1 receptor which was originally discovered on the surface of T- cells. NK cell subsets are similarly able to express PD-1 receptors and, alongside with the cytotoxic T-cells, are the main contributors of the first line anti -tumor immunity (18).

1.5 NK cells and cytotrophoblast

Cytotrophoblast form the population of trophoblastic cells which resides below the confluent syncytium. Due to their higher mitotic activity, they provide a continuous replacement for the syncytium. Cytotrophoblast are different from the syncytiotrophoblast in their surface antigens and cytokine expression. Their PDL-1 expression is very diminished compared to the syncytium but on the other hand they express HLA-G. After its first isolation arose the question whether the HLA-G was an evolutionary remnant, or it has a crucial function in the immune response evasion. It seems that expressing monomorphic MHC-I receptor (HLA-G) is a weaker killing trigger for cytotoxic cells than expressing an MHC-I receptor with a clearly ‘not- self’ pattern or not expressing it at all (1). It is also important to notify that HLA-G is not expressed by all the trophoblastic cell subsets but the cytotrophoblast and extravillous trophoblast. It is also important to mention the Galectin-9 and Tim-3 expression. There are several types of Galectins involved in human trophoblast maturation, differentiation and placenta formation (19, 20). Galectin-1 is important in the forming of syncytium by the fusion of the cytotrophoblast. Galectin -9 has a soluble form and it can be also found on the outer surface of the cellular membrane. It is a relatively specific ligand to the Tim-3 receptor among others such as Ceacam1, HMGB1 and PtdSer. Tim-3 is a co-inhibitory receptor expressed by T-regulator cells, Cytotoxic T cells, NK cells and other antigen presenting cells belonging to the innate immune system (such as macrophages and dendritic cells) (21). The Tim-3 and Galectin-9 connection is necessary for the NK cells to produce IFN- γ . In human peripheral blood, it is on NK cells that the highest level of Tim-3 expression takes place. Not only is Tim-3 expressed on all CD56dimCD16+ NK cells, but it is also possible to induce its expression on CD56brightCD16– NK cells following stimulation by IL-12, IL-15, and IL-18. It has been indicated by in vitro data that Tim-3 plays a role

as a co-receptor on NK cells, promoting the production of IFN- γ . Unlike its role in cell death induction among Tim-3-expressing Th1 cells, galectin-9 is indispensable to Tim-3-mediated IFN- γ production in NK cells. Anti-Tim-3 antibody blockade or reduced Tim-3 expression is associated with decreased production of IFN- γ (21).

1.6 Natural killer cells and invasive extravillous trophoblast (eVT)

Among the cytotrophoblast and syncytiotrophoblast, the extravillous trophoblast is the 3rd highlighted trophoblast subset in human placenta. Whether the extravillous invasive trophoblast derives from the cytotrophoblast pool or it has its own progenitor cell pool is still not clear (22). It has several roles during the forming of the structure of the placenta and vascularization (10). One of them is to anchor the chorionic villi into the maternal decidua by invading it. This invasion has been in the focus of many immunological studies as a unique example of physiological suppressed semi-allogeneic versus host reaction (1). eVT also participates in the neovascularization. The main kinds of eVT are the interstitial extravillous trophoblast and the intravascular extravillous trophoblast. The interstitial eVT are penetrating the maternal decidua forming columns that connect the fetal side of the placenta with the maternal side. During the invasion they cause extracellular protein matrix (ECM) remodeling and they also interact with the spiral arterioles of the maternal decidua causing apoptosis of some vessels but also dilatation of the remaining ones leading to a greater volume of maternal blood flow (10). The interstitial eVT migrate into the myometrium, then become the 'giant cells' meanwhile the endovascular eVT can be found inside the maternal vessels. First let us take a look on the mechanisms of the interstitial eVT invasion. One of the proteases that promotes the eVT migration is the urokinase plasminogen activator (PLAU). Another protease described is the metalloprotease 2 (MMP2) which is activated through the p38/MAPK pathway. This pathway can be activated by the Galectin -9 binding to Tim-3 receptor. This allows the autocrine 'self- activation' of the protease function as eVT can produce Galectin-9 (23). As the eVT migrates in the decidua it goes under the effect of different immune cells and cytokines of the host. Some factors, such as the hepatocyte growth factor and EGF stimulates the eVT meanwhile the IFN- γ and transforming growth factor-

β can limit the invasion (24, 25). IFN- γ is also produced by the uNK cells and it also have immunosuppressive effects as we will discuss it later. It is also known that the physiological hypoxic microenvironment at early pregnancy can promote the eVT invasion (10, 26). eVT cells express a spectrum of special MHC-I molecules the HLA-G, HLA-E and HLA-C. In contrast to other cells, are not involved in the expression of the classic HLA-A and HLA-B antigens. Most of the uNK cell receptors recognize the HLA-G antigen as a ligand preventing its killing function. Cytokines produced by uNK cells, including IFN- γ and TNF- α , granulocyte macrophage colony stimulating factor (GCSF), as well as IL-10, IL-8 may regulate trophoblast invasion (27, 28).

1.7 NK cells and vascular remodeling

uNK cell produce not only vasoactive factors and angiokines but enzymes and apoptotic factors which have an important role in vascular structuring and remodeling. Metalloproteases (MMP) participate in the degradation of the extracellular matrix (ECM), which facilitates the growth and the migration of endothelial cells. ANGPT1/2 is important in the coordination of vascular mural cell apoptosis. VEGF a well-known angiogenic factor is pivotal at many steps of vascular genesis and also remodeling. It contributes to the formation of tip cells and the proliferation of endothelial cells. Tip cells are specialized endothelial cells. During vascularization the tip cell which are spear headed and have a low proliferative profile make the way in the ECM for the newly formed vessels. They are followed by the stalk cells (another specialized endothelial cell population) that elongates the vessel. The PGF and DLL1, produced by NK cells, contribute to vessel enlargement. Activation receptors, for example, Ly49 and NCR1 in mice or S1PR5 in humans, are responsible for the homing, activation and maturation of angiogenic uNK cells (6). With regard to endothelial cells, PGF represents an angiogenic, chemotactic and survival factor. It was isolated first in human placenta (hence the name Placental Growth Factor) but normally it is moderately expressed by normal embryonic tissues and adult tissues. It is important during decidual vascularization and is mainly produced by decidual NK cells (29).

1.8 NK cells and the endovascular extravillous trophoblast

In human pregnancy the spiral arterioles of the maternal decidua undergo a structural reorganization after the implantation occurs. As a result of it, the arterioles that were highly resistant and provided lower blood flow change to be less resistant but of a bigger lumen letting more maternal blood to flow through (30). This is important for the proper oxygen and nutrient supply of the embryo. Endovascularly and interstitially located extravillous trophoblast cells have an important function in the induction of vascular smooth muscle cell (VSMCs) and endothelial cell (ECs) loss as well as in the remodeling of the ECM. Endovascular eVTs migrate into the lumen of the arterioles and form trophoblast bungs. With regard to the remodeling, the interaction with the ECs seems to play a crucial role, while, due to their location, interstitial eVT are the first to enter into interaction with VSMCs. It remains as yet undecided whether endovascular eVT originate in trophoblasts migrating down the spiral artery or, alternatively, they result from the invasion of the decidual interstitial eVT. The most likely answer is a combination of the two mechanisms described above (31, 32). uNK cells and macrophages can be found in high numbers around the decidual part of the spiral arterioles at the beginning of pregnancy. It is very likely that the structural and morphologic changes, such as vacuolisation, fibrinoid degeneration or muscular hypertrophy and dilatation prior the arrival of eVT is regulated by these immune cells (33).

1.9 The Galectin-9 – HLA-G circle

Galectin-9 is 36kD molecular weight B- galactoside lectin protein. It was first isolated in 1997 from mouse embryonic kidney. The different types of galectins play a major part in the process of human placenta formation (34). Some of the galectins have immune modulating and anti- tumor suppressive effects. Galectin-1 causes the apoptosis of activated T- cells and the activation of Tregs and dendritic cells (35). Galectin-3 can suppress anti- apoptotic effects. Galectin- 7 can modulate lymphocyte and monocyte apoptosis. Galectin-9 positively stimulates the differentiation of Treg cells and the apoptosis of Th1 and Tc cells as well as the suppression of Th 17 differentiation (34, 36,

37). In human, Galectin-9 is expressed from early pregnancy and data support that its levels remain high until term (20). Others suggest that there is a difference between the serum levels of Galectin-9 as it gradually grows during the pregnancy being higher in the second and third trimester (38). It can be detected on cytotrophoblast, eVT and the syncytium intra and extracellularly (20). Tim-3 receptor expression is upregulated on uNK cells in the decidua. The specific Galectin-9 and Tim-3 receptor connection takes part in inducing immune suppression against the trophoblastic cells (39). The Tim-3 – Galectin-9 binding enhances cytokine production in leukocytes (21). Data support that Tim-3 Galectin-9 binding enhances IFN- γ production in uNK cells (16). Cellular effects of IFN-gamma include antigen processing and presentation, up-regulation of pathogen recognition, antiviral response, cell proliferation inhibition and effects on apoptosis, activation of microbicidal effector functions, immunomodulation, and migration of leukocytes (40). Besides modulating leukocyte function, IFN- γ upregulates HLA-G expression on trophoblastic cells (41). It has been shown in vitro that IFN- γ , produced by leukocytes, enhances the HLA-G production on trophoblastic cell culture line JEG-3 (42). HLA-G has cytotoxicity suppression on various immune cells, including uNK cells, cytotoxic T cells (Tc cells), dendritic cells (DC) through HLA-G and IL-2, IL-4, KIR2DL4 binding (43). It is easy to see the chain of reactions between the Galectin-9 production of trophoblastic cell populations and its immune modulatory effects, but it needs more scientific evidence to evaluate its importance in the physiological invasion of the trophoblastic cells. The existence and importance of each of the separate step is well supported by experimental data, but functional studies in vitro and especially in vivo still await.

1.10 The measurement of CA 125 binding capacity of different leukocyte subtypes in epithelial ovarian cancer

Researchers immunized mice with an epithelial ovarian cancer cell line (EOC) to find a reliable biomarker for this type of ovarian tumor. Finally, a monoclonal antibody was created which reacted with sera from EOC patients (44, 45). The new antibody was

labelled as “OC 125” and the antigen was called CA 125. Later it became clear that the CA 125 antigen was an epitope of a bigger glycoprotein from the mucin family (MUC 16) (46). Serum levels of CA 125 proved to have great clinical value in the past decades. The higher concentrations in peripheral blood serum indicates poorer prognosis, it can be also used for follow up and as indicator of recurrence (47). Furthermore, it seems that MUC 16 has an active role in tumor propagation by being attached (actively and passively) to the leukocyte cell surface activating immunosuppressive mechanisms and also creating a mechanical barrier between the leukocyte and its target (48-50). The majority of the experiments supporting this theory are based on conventional flow cytometry and immune fluorescent labelling. The exact measurement of surface bound antibodies is limited as a result of auto-fluorescence.

These limitations were discussed in a series of studies from the PNP Research Corporation, Thorlabs, Inc., and Obstetrics and Gynecology Epidemiology Center, Brigham and Women’s Hospital, in which the authors outlined a novel technology that revolutionizes the methods of evaluating the degree of MUC16-binding to PBMCs. In the following, I would like to refer to our former paper published in 2022 (51). “This technology involves: 1) plasmonic gold nanoparticles (PNPs) conjugated to anti-CA125 antibodies as optical nanoprobe; 2) high contrast darkfield microscopy to detect PNPs bound to PBMCs and 3) a computational algorithm for automated counting of the bound nanoparticles. PNPs have a unique property called plasmonic resonance, which causes them to strongly scatter specific wavelengths of light to enable single nanoparticle detection. Data were presented from women with EOC showing that their PBMCs (collected before therapy) had greater levels of MUC16 bound to their cell surfaces compared to PBMCs from healthy women (52). In subsequent work, we improved the technique by adding fluorescence capacities to the microscope, enabling analyses on subsets of PBMCs (53, 54). EOC cancer patients exhibited higher levels of MUC16 bound to T cells, NK cells and B cells compared to healthy controls (54).”

Our research involved clinical samples from one patient provided by the Massachusetts General Hospital and Obstetrics and Gynecology Epidemiology Center, Brigham and Women’s Hospital. The experiments were performed at the Kevin Elias Gynecologic

Oncology Laboratory at the Brigham and Women's Hospital, Massachusetts and at Thorlabs, Virginia. The patient samples were from a 21-month-long course of treatment. The CA 125 binding of different leukocyte subtypes was correlated with levels of serum CA 125, complete blood count and medical treatment data which, in our opinion, indicate that this novel technology will afford a fresh insight into the clinical course of EOC.

2. Objectives

2.1 A novel methodology for the isolation of Decidual Natural Killer cells from fresh, term placentas.

dNK cells were in the focus of many immunological studies of the past decades. Various cell lines can be purchased on the market and there are several protocols for peripheral NK cell enrichment and isolation. However, the isolation of dNK cells from the fetal-maternal interface is still an exquisite way of NK cell culturing. For future immunological studies of the fetal-maternal interface, our goal was to develop a better protocol for dNK isolation from fresh, term placenta.

2.2 Alteration of the phenotype and cytotoxicity of dNK cells after the exposure to different trophoblastic cell lines.

Many of the processes of placental formation are recapitulated in placental neoplasms. Gestational trophoblastic disease (GTD) covers a wide variety of trophoblastic pathologies including gestational trophoblastic neoplasia (GTN). They are extremely rare but can appear during or after pregnancy. GTN includes invasive hydatidiform mole, placental -site trophoblastic tumor (PSTT), epithelioid trophoblastic tumor (ETT) and choriocarcinoma(55). PSTT is originated from interstitial trophoblasts, while hydatidiform moles and choriocarcinoma arise from villous trophoblasts (56). The ways

in which GTN tumors avoid the maternal immune system probably show similarities to healthy trophoblastic cells. The immune suppressive effect of trophoblastic cells on peripheral NK cells *in vitro* have been demonstrated, but how malignant and benign trophoblasts interact specifically with dNK cells is not known (42, 57). In this study, we isolated dNK cells from fresh, end term placenta and co-cultured them with different trophoblastic cell lines for 1 and 5 days. The trophoblastic cell lines HTR-8 (benign trophoblasts) and Jeg-3 (choriocarcinoma) to explore possible differences in dNK-trophoblast interactions between benign and malignant contexts.

2.3 The alteration of CA 125 binding capacity of different leukocyte subtypes in epithelial ovarian cancer

Recently, a novel microscopic technique was described which enables us to evaluate the degree of MUC16 binding to PBMC(54). The new technology involves: 1) anti-CA125 antibody conjugated gold nanoparticles serving as optical nanoprobe; 2) high contrast fluorescent darkfield microscopy for the detection of PBMC-bound gold nanoparticles; as well as 3) a computational algorithm to automatically count the nanoparticles bound to the cell surface.

Plasmonic resonance is a special characteristic of gold nanoparticles. It permits augmented resolution and detection of reduced numbers of molecules bound to leukocytes. Preliminary tests involving a small number of EOC-diagnosed patients showed that their PBMC (naïve samples) had higher levels of MUC 16 bound to the cellular surfaces than the PBMCs of healthy patients. Longitudinal research provided data obtained from a single individual patient which also showed elevated CA 125 levels bound to different leukocyte subtypes. The level of binding generally correlated with serum CA 125 but during a 6-month period it exceeded the latter, preceding recurrence. In our research, we present the data based on an extended period of observation on Patient 2# using a new technique that allows a degree of qualification about types of PBMCs that have MUC 16 bound to them. We also correlated our data with the levels of complete blood counts, serum CA 125, and clinical details of the patient's care.

3 Methods

3.1 Decidual NK cell isolation from term, fresh placenta.

1.1. Approval from IRB

The present research was conducted with the approval of Partners Healthcare Institutional Review Board protocol 2016P001505. (Boston, Massachusetts) In accordance with an institutional discarded tissue protocol, the written informed consent requirement did not apply.

1.2. Sample collection

The selection of candidates to participate in the research project was performed by reviewing the schedule of the operating theatre for planned singleton cesarean sections that occurred at term either for breech presentation or for elective repeat delivery. Uneventful gestation was a prerequisite for inclusion in the study. Thus, gestational conditions such as gestational diabetes mellitus or preeclampsia and cases where the obstetrician requested placental pathologic evaluation represented exclusion criteria. As soon as the placenta was removed, the specimen was placed in a clean room located immediately adjacent to the operating room, where it was processed. Participants involved in the collection of molar tissue samples were chosen by means of the clinical, radiological and laboratory findings. Molar tissue evacuation was carried out in two phases. After vacuum aspiration, a gentle curettage of the uterine cavity that did not penetrate the myometrium was performed. The collection of the second fraction was performed by our lab member and supervised by the gynecologic pathologist. Time is a key factor in the loss of live leukocytes in human tissue samples, therefore these samples needed to be collected within 10 minutes of delivery or vacuum evacuation (58).

1.3. Dissection of the decidua basalis

Figure 1 demonstrates the essential phases of the protocol of NK cell isolation. We selected sections of the placenta minimum 3 cm from the site of umbilical insertion. We removed a 50-gram specimen (which measured approximately 5 x 5 x 3 cm) using sterile technique, with metal forceps and a scalpel. By means of the scalpel the amniotic

membrane was also removed and the decidua parietalis was separated from the decidua basalis. The latter was subsequently cut into 5 mm pieces, approximately. We gently rinsed the collected tissues in sterile Ca^{2+} and Mg^{2+} free 1X PBS Gibco® (Thermo Fisher, Waltham, MA) for the purpose of removing any overlying clot and afterwards placed them in ice-cold sterile Ca^{2+} and Mg^{2+} free 1X PBS in 50 mL Falcon® conical tubes (Corning, Tewksbury, MA). The tubes were transported for further dissection to the laboratory on ice.

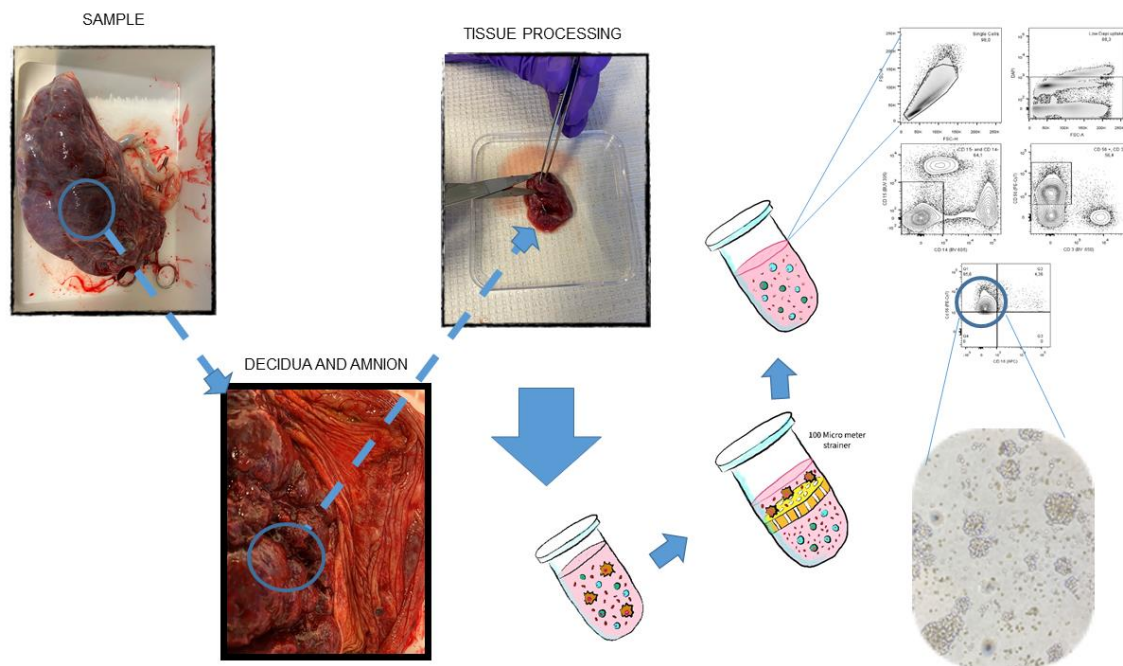


Figure 1 (59): The procession of the placental/ CHM (Complete Hydatidiform Mole) sample. After the sample is taken from the surgery room, it was dissected into smaller pieces. After tissue dissociation and enzymatic degradation, a 100 μm cell strainer is used. Red Blood Cell Lysis Buffer is added. After incubation with leukocyte marker antibodies, natural killer cells of decidual phenotype are selected and isolated. The dNK cells show good viability and preservation of their decidual phenotype after one week in culture.

1.4. Disaggregation of tissue and preparation of decidual cell suspension.

First we put the tissue samples on sterile tissue culture plates of 10 cm, inside a biosafety cabinet. After that, we separated the stromal tissue of the decidua basalis from blood vessels and blood clots with sterile forceps and metallic scissors. The sample was then moved into a 50 ml conical tube with abundant, cold and sterile Ca^{2+} and Mg^{2+} free 1X PBS and was subsequently shaken three to five or more times by hand. Next, we put the tissue sample into a new 50 mL conical tube along with Ca^{2+} and Mg^{2+} free 1X PBS at a

1:5 ratio by volume, then centrifuged it for 5 minutes at 300 x g. This was followed by the careful aspiration of the supernatant, which did not disturb the pellet.

After adding Accutase® enzyme (Accutase Cell Detachment Solution, Biolegend) to the pellet in the conical tube, we pre-warmed it to 37 °C at a 1:2 pellet to enzyme volumetric ratio. We subsequently transferred portions of approximately 10 ml in volume of the pellet-enzyme mixture to Gentle MACS C Tubes® (Miltenyi Biotec, Cologne, Germany). This was followed by the insertion of the C tubes into the gentleMACS Dissociator® (Miltenyi Biotec), programmed for (Spleen Program) a total of 668 rounds per run for 17 seconds. After dissociation, we moved the cell slurry into 50 mL conical tubes, where it was incubated on an incubator shaker (Boekel Scientific, Lower Southampton Township, PA) with a 1 mm orbit at 575 rpm at 37 °C for 45 minutes aiming at the detachment of the tissue resident natural killer cells from the decidua. It should be noted that using a single tube may not be sufficient in this phase of the study, since the amount of sample to be placed into the 50 ml conical tube must not exceed 25 ml.

1.5. Preparation of the cell suspension for cell sorting.

The reaction was quenched as we filled up the 50 ml tube with Ca²⁺ and Mg²⁺ free 1X PBS before passing the sample through a 100 µm cell strainer (Falcon, Corning, Tewksbury, MA) and into a new conical tube of 50 ml. The sample was then centrifuged for 5 minutes at 300 x g and at room temperature. While the sample was being centrifuged, we performed the preparation of 1X red blood cell (RBC lysis buffer, Biolegend) lysis buffer from 10X stock, diluting 1 mL in 9 ml of ice cold, deionized water. After this, the supernatant was carefully aspirated, and the ice cold, 1X RBC lysis buffer was added to the pellet (volume pellet: RBC lysis buffer 1:5), incubated on ice for 5 minutes and occasionally shaken. Timing had to be carefully considered, as too little time may result in considerable RBC contamination while too much time may have a negative effect on the leukocyte viability.

We diluted the lysis buffer with abundant Ca²⁺ and Mg²⁺ free 1X PBS to stop the lysis reaction. Then we discarded the supernatant after spinning the cells for 5 minutes at 350 x g. Next, we re-suspended the pellet in Ca²⁺ and Mg²⁺ free 1X PBS, then performed the 350 x g centrifuge again so as to remove any remaining cell debris and lysis buffer. Following the discarding of the supernatant, the pellet was re-suspended in 1 ml of ice-cold staining buffer and the sample was filtered through a 35 µm cell strainer (Falcon,

Corning, Tewksbury, MA). A manual hemocytometer was used to measure cell concentration. It is remarkable that RBC contamination is greatly reduced (though it does not disappear completely) following the RBC lysis, while the trophoblast and fibroblast cells are mostly excluded by the strainer because of their diameter, which exceeds 35 μM . After counting, we re-suspended the cells in FACS buffer (BD Biosciences) at a concentration of 10 million cells / ml before labeling the antibodies.

1.6. Antibody staining and cell sorting.

We added fluorophore-labeled antibodies to the cell suspensions and incubated them as indicated (**Table 1**), applying CD 3, CD 14, CD 15, CD 56, CD 16 antibodies. Following labeling, the cells were twice washed in FACS buffer. A live/dead stain was added with DAPI at 0.01 $\mu\text{g/ml}$. Then we transported the samples to the cell sorting laboratory on ice. Agilent Cross Laboratory software on a BD FACS Aria cell sorter was used to carry out cell sorting. **Figure 2.** and **Figure 3.** illustrate the gating strategy for end term placenta and CHM, respectively. We selected CD 3 -, Cd 14 -, CD 15 - and CD 56 + cells as NK cells, and identified them as follows: CD 56 bright and CD 16 – NK cells were identified as being of decidual, CD 56 diminished and CD 16 + NK cells were identified as belonging to the peripheral phenotype. With the sorting finished, the uNK cells were placed in a Ca^{2+} and Mg^{2+} free 1X PBS + 2% FBS solution (a protein concentration higher than that could prove harmful to the cells, which typically become more vulnerable after sorting). Then the freshly sorted uNK cells were, on ice, transferred the laboratory, where the cell culturing took place.

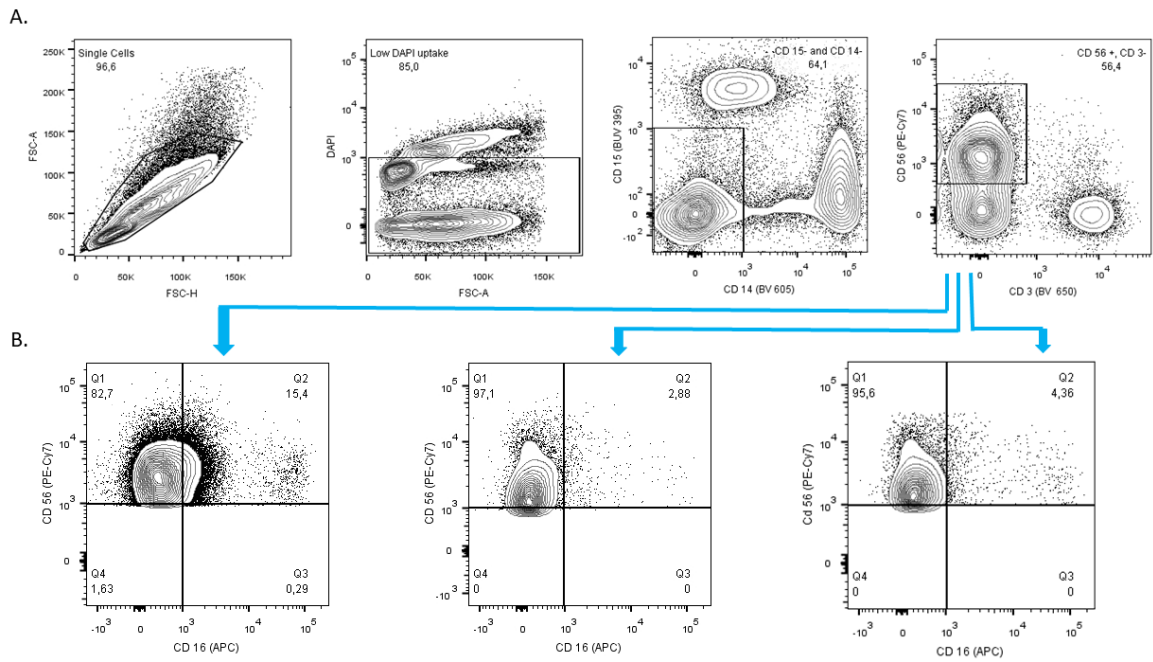


Figure 2 (59): A: NK cell isolation from the placenta (Gating Strategy). After the selection of single cells and the exclusion of DAPI + dead cells the CD 3+, CD 14+ and CD 15+ cells were gated out (macrophages and neutrophils). **B:** CD 56+ and CD 16- cells selected from different placental samples.

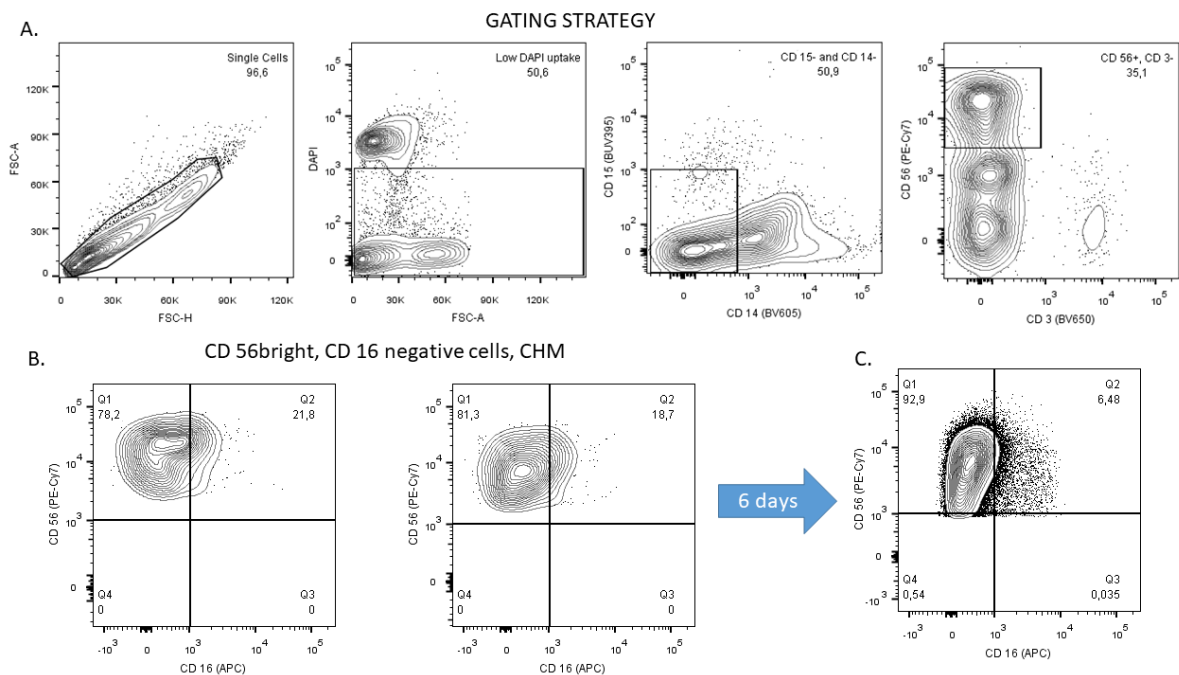


Figure 3 (59): A: NK cell isolation from Complete Hydatidiform Mole sample. Single cells selected followed by live dead separation using DAPI. CD 3- and CD 15+, CD 14+ cells are gated out. **B:** CD 56 bright and CD 16 negative NK cells selected after the procession of 2 Complete Hydatidiform Molar samples. **C:** The phenotype of NK cells after 6 days of culturing.

Antibody	Producer	Application
CD 56 (PE-Cy7)	BD Biosciences	30 minutes in darkness, at 4°C
CD 56 (FITC)	Biolegend	30 minutes in darkness, at 4°C
CD 16 (APC)	Biolegend	30 minutes in darkness, at 4°C
CD 3 (Brilliant Violet 650)	Biolegend	30 minutes in darkness, at 4°C
CD 15 (Brilliant Violet 605)	BD Biosciences	30 minutes in darkness, at 4°C
CD 14 (Brilliant Ultra Violet 395)	BD Biosciences	30 minutes in darkness, at 4°C
DAPI (4',6-Diaminido-2-Phenylindole, Dilactate)	Biolegend	10 minutes in darkness, at 4°C
CFSE (5(6)-Carboxyfluorescein Diacetate N-succinimidyl Ester)	Sigma Aldrich	10 minutes in darkness, at 37 °C, 5% CO ₂
PE- Cy7 Isotype control (Mouse IgG1 κ Isotype Control)	BD Biosciences	30 minutes in darkness, at 4°C
FITC Isotype control (Mouse IgG1, κ Isotype)	Biolegend	30 minutes in darkness, at 4°C
APC (Mouse IgG1 κ Isotype Control)	Biolegend	30 minutes in darkness, at 4°C

Table 1: *Fluorophore conjugated antibodies used*

1.7. Culturing of the dNK cells.

For 10 minutes we centrifuged the sorted cells at 300 x g at room temperature. Then we discarded the supernatant, and subsequently re-suspended the cells in the NK cell culture media (ATCC). A small dose of recombinant human IL-2 (500 IU IL-2/ 1 ml media; NK92 ATCC CRL 2407, ATCC, Manassas, VA) was added to the cell culture, starting with the first day and repeated every 3rd day. The media was changed also every 3rd day. The cells were incubated at 37 °C and 5% CO₂. A week later, we analyzed the cells using flow cytometer (DPX Analyzer, FlowJow® Software) and assessed them for expression of CD 56, CD 16, CD 14, CD 15, and CD 3.

1.8. NK cytotoxicity assay.

The uNKs and NKs which had been isolated from the CHM tissue were kept in culture for 7 days, under the same conditions as detailed above. On the 7th day, we conducted a flow cytometry based NK cytotoxicity assay on the basis of the protocol described by Kandarian et al (60). We incubated the NK cells (Effector cells) with a typical NK cell target, CFSE- labelled K 562 cells (ATCC). The 4-hour incubation took place at 37 °C, 5% CO₂ in the darkness. Different Effector: Target ratios were used. Following the 4-hour incubation, we placed the samples on ice and performed live/dead staining (DAPI).

Figure 4. illustrates the results of the analysis of the CFSE pre-labelled target cells concerning their dead/live ratio based on DAPI uptake.

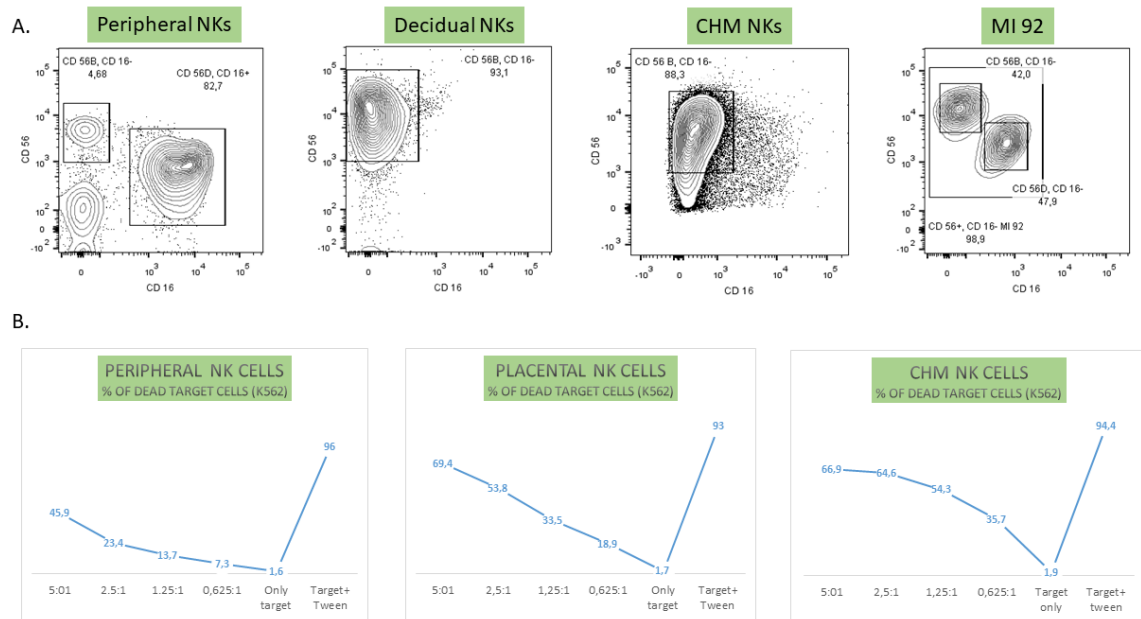


Figure 4 (59): **A:** The phenotype of different Natural Killer Cell populations after 1 day in culture. MI 92 was a commercially available cell line. **B:** Cytotoxic activity of different NK populations 6 days after isolation. On the x axis we can see the Effector (NK cells): Target (K562) ratios The cytotoxicity curve of the peripheral NKs and the CHM NKs are based on one sample. The cytotoxicity curve of the placental NKs (dNK) is based on 3 samples. SE (5:1: 4,67, 2,5:1: 5,99, 1,25:1: 4,46, 0,625:1: 2,13, Only target: 0,26 Target + Tween: 0,26).

3.2 Phenotypic and functional alteration of decidual NK cells after the exposure to different trophoblastic cell lines

2.1. IRB Approval

The present research was conducted with the approval of Partners Healthcare Institutional Review Board protocol 2016P001505. In accordance with an institutional discarded tissue protocol, the written informed consent requirement did not apply.

2.2. Collection of samples and cell lines used

The selection of candidates to participate in the research project was performed by reviewing the schedule of the operating theatre for planned singleton cesarean sections

that occurred at term either for breech presentation or for elective repeat delivery. Uneventful gestation was a prerequisite for inclusion in the study. Thus, gestational conditions such as gestational diabetes mellitus or preeclampsia and cases where the obstetrician requested placental pathologic evaluation represented exclusion criteria. We processed the placenta following our protocol to obtain high number of living dNK cells for the co-culturing (59). Briefly, placental tissue was manually morcellated and then digested with collagenase into a single cell suspension, followed by flow cytometry sorting. HTR-8 cells are derived from human, first-trimester invasive eVT cells transfected with the gene encoding for simian virus 40 large T antigen, while the Jeg-3 cells are choriocarcinoma cells derived from the Woods strain of the Erwin- Turner tumor by Kohler and associates (61, 62). Both cell lines had been obtained from American Type Culture Collection (ATCC, Manassas, VA).

2.3. Co-culturing

After isolation from the placenta, dNK cells were kept in NK media overnight at 37 C°, 5% CO₂. Both HTR-8 and Jeg-3 cells were seeded on a 6 well plate (30 000 cell/cm³ density) in RPMI + 20% FBS medium, 37C°, 5% CO₂ 24 hours before the co-culturing. dNK cells were transferred into RPMI medium and added to the already adherent trophoblastic cells to achieve a 1:10 ratio of trophoblastic cells to dNK cells. (See **Figure 5.**). Controls included dNK cells with no exposure to trophoblastic cell lines or exposure to a different tumor cell type (Ovarian Adenocarcinoma Cell Line OVCAR-3, also from ATCC). Low dose of IL-2 was added (500 IU IL-2/ 1 ml media) to the plates at this point and on day 3 to achieve dNK cell survival. For each round of experiments, three technical replicates with the same set up were examined simultaneously. The dNK cells were selected from the same, randomly selected placental sample. The experiment was repeated three times using three different placentas.

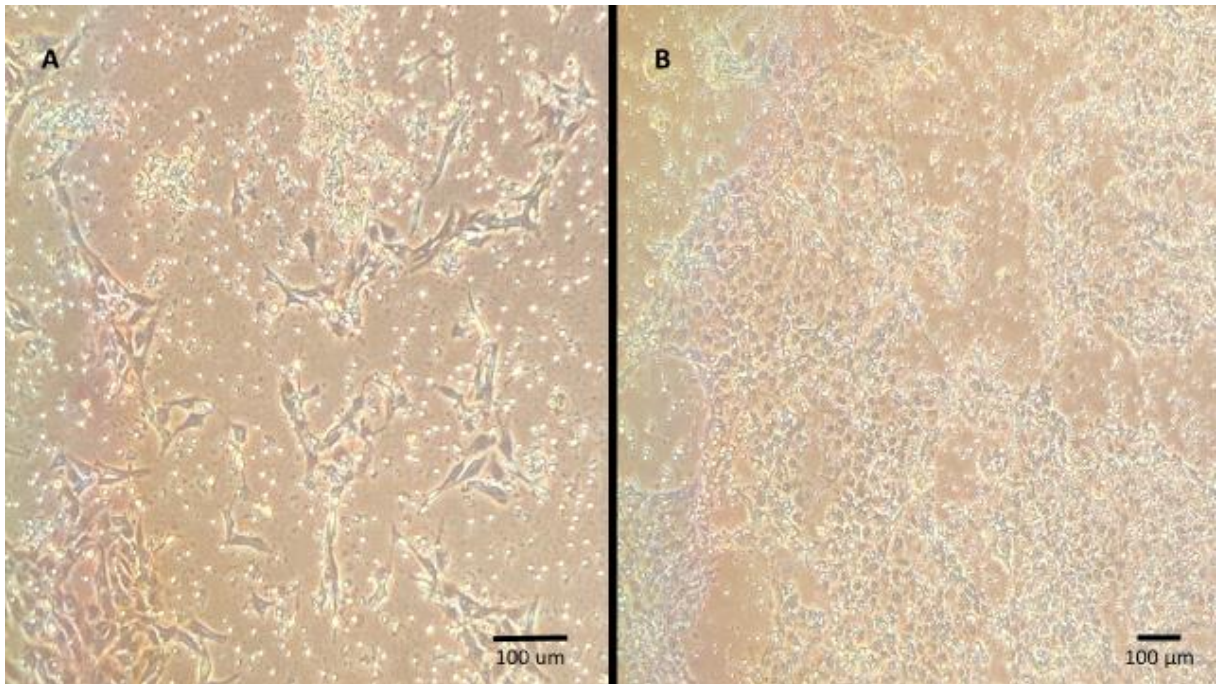


Figure 5: Microphotograph of the dNK cells and HTR-8 co-culture plate. A. HTR-8 cells and decidual Natural Killer Cells. B. Jeg-3 cells and decidual Natural Killer Cells. In both plates the confluent, adherent cells are the trophoblastic cells, the smaller, cluster forming cells are the Natural Killer Cells.

2.4. dNK isolation and cytotoxicity assay

On day 1 and day 5 both the trophoblastic and dNK cells were collected from the plates and dNKs were selected by flow cytometry. We applied Agilent Cross Laboratory software on a BD FACS Aria cell sorter to sort the cells. The gating strategy to separate the NK cells from the trophoblastic cells is illustrated in **Figure 6**. CD 3, CD 14, CD 15, CD 16, CD 56, DAPI staining was used to analyze NK cell phenotype. We conducted cytotoxicity assays in 96 well U bottom plates using the isolated dNK cells as effector and K 562 cells as target at 5 to 1 and 2.5 to 1 effector to target ratios. dNK cells and target cells were incubated for 4 hours in the dark at 37°C, 5 % CO₂. The percentage of dead cells was assessed by DAPI staining. We added tween (0,1 % Tween-20) to target cells, using it as positive control for NK killing function and target cells alone served as negative control (60).

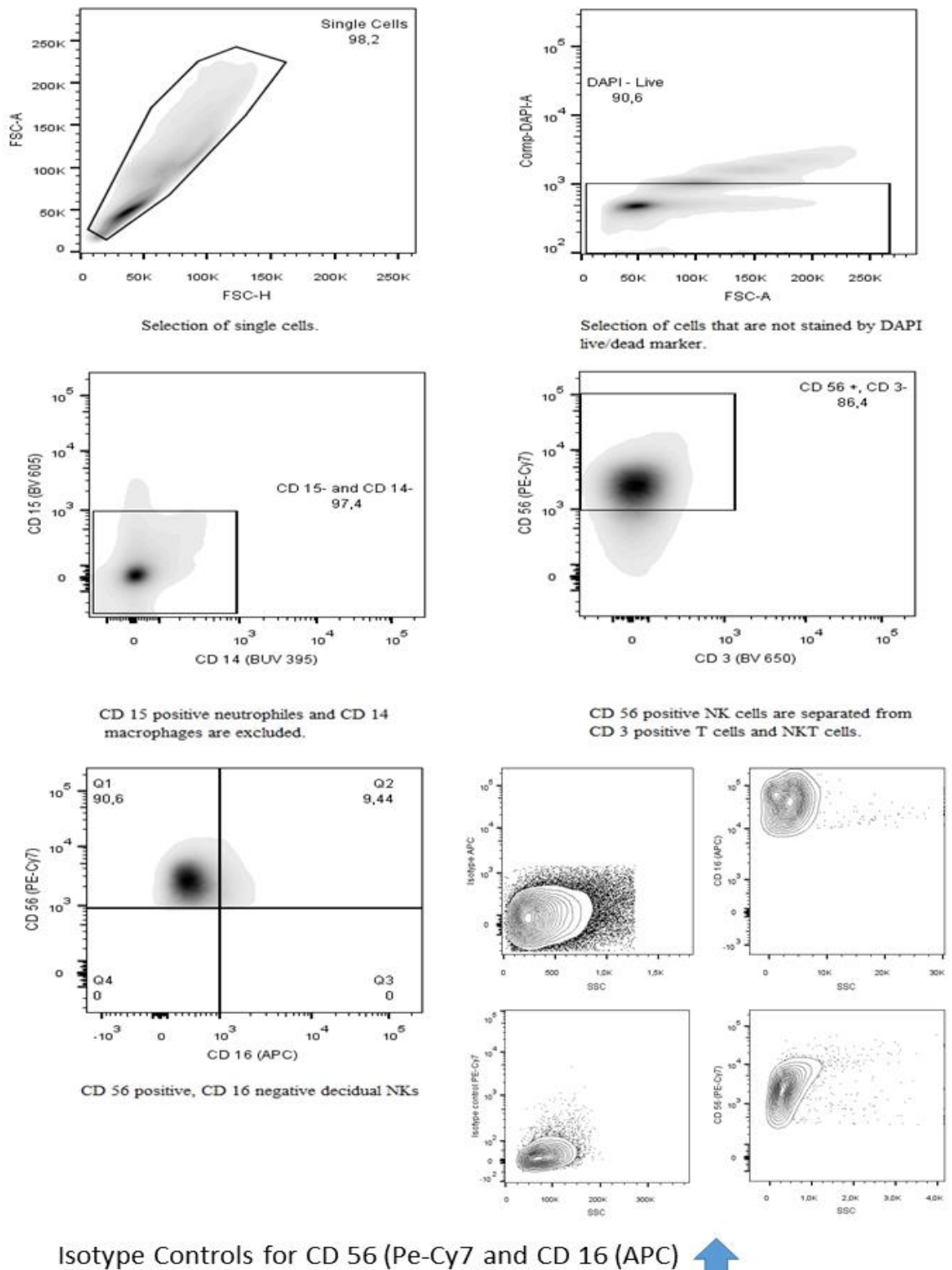


Figure 6: A: The gating strategy of dNK cell selection. Fresh, term placentas were collected from the surgery room. After the procession of the fresh, term placenta, the leukocytes were separated from the cell suspension using a tissue processor (gentleMACS Dissociator® (Miltenyi Biotec) and Accutase® enzyme (Accutase Cell Detachment Solution,

Biologend). The dead cells are excluded by DAPI staining. Among the leukocytes the CD 3-, CD 14-, CD 15- negative cells are identified as NK cells. The CD 56 bright, CD 16- cells were selected as dNKs. We can also observe the isotype controls for the two main antibody used for CD 56 diminished/ bright and CD 16+/- distinction.

3.3 Measurement of Ca -125 binding capacity of leukocyte subtypes during epithelial ovarian cancer

3.1. Clinical Specimens

Under approved protocols, whole blood specimens were collected beginning in 2016 in heparinized or Calcium EDTA tubes from female patients who were presumed to have ovarian cancer but had not started treatment yet (DFCI 16-255) or from healthy women (BWH 2018P001677). The patient providing the longitudinal specimens was diagnosed with a high-grade serous ovarian cancer in 10/2012 and underwent suboptimal debulking (stage IIIC) followed by six cycles of carboplatin and Taxol chemotherapy. In 2/2014, the patient was started on carboplatin and Doxil for a platinum-sensitive recurrence and in 2/15 begun on Avastin and oral cytotoxin after a failed clinical trial of Olaparib vs. placebo clinical trial 10/2015. In 2016 when this research began, the patient, a friend of one of the authors, made a request for her blood to be included in the study. She received information that it would not be possible to utilize the results in her clinical care, signed an informed consent document, and was granted a single subject exemption under the BWH IRB. An extra Calcium EDTA tube of blood was drawn for the purpose of the research when clinical bloods were ordered. In this paper our attention is concentrated on the 14-month timespan between 2/2018 and 4/2019. There are several reasons why we are especially interested in this period: the first is the high frequency of the sampling; the second is the presence of clinical evidence of the patient's increasing tumor burden; and the third is the fact that several novel therapies were being tried. All specimens during this period were being processed in the same laboratory to preserve viable PBMC using Ficoll density gradient centrifugation density per standard protocol(63). 0.1 ml aliquots were created with cell concentrations of 1 million and stored at -80 before being transferred to liquid nitrogen.

3.2. Quantification and Qualification of MUC16 binding to PBMCs

Methodology for quantifying the degree of MUC16 binding to PBMC is described in detail in reference 9. Briefly, quantification of the degree of MUC16 binding to PBMC means counting that considers both the number of PNPs bound to the cell in different surface planes as well as the color (wavelength) of the nanoparticle, which reflects overlaying of 1, 2, or 3 PNPs. This work adapted an existing fluorescent darkfield microscope. Refinements to allow not only counting of bound PNPs to all PBMCs but also qualification of PBMC type required a new instrument described in detail in this publication PLACEHOLDER (64). The new instrument has the following features: air rather than oil optics; completely automated scanning; refinements to the algorithm for detecting cells with bound PNP; and addition of fluorescent markers able to quantify five PBMC cell types (See **Figure 7.**). Under this protocol the frozen PBMC were thawed and treated with fluorescent antibodies (CD45, CD19, CD14, CD56, CD3—various combinations of which and expression levels can identify T-cells, B-Cells, NK cells, NK-T cells, and monocytes (63). After this step, the sample was incubated with the anti-CA125 gold PNPs and binding to five different cell types quantified. (See **Figure 8.**)

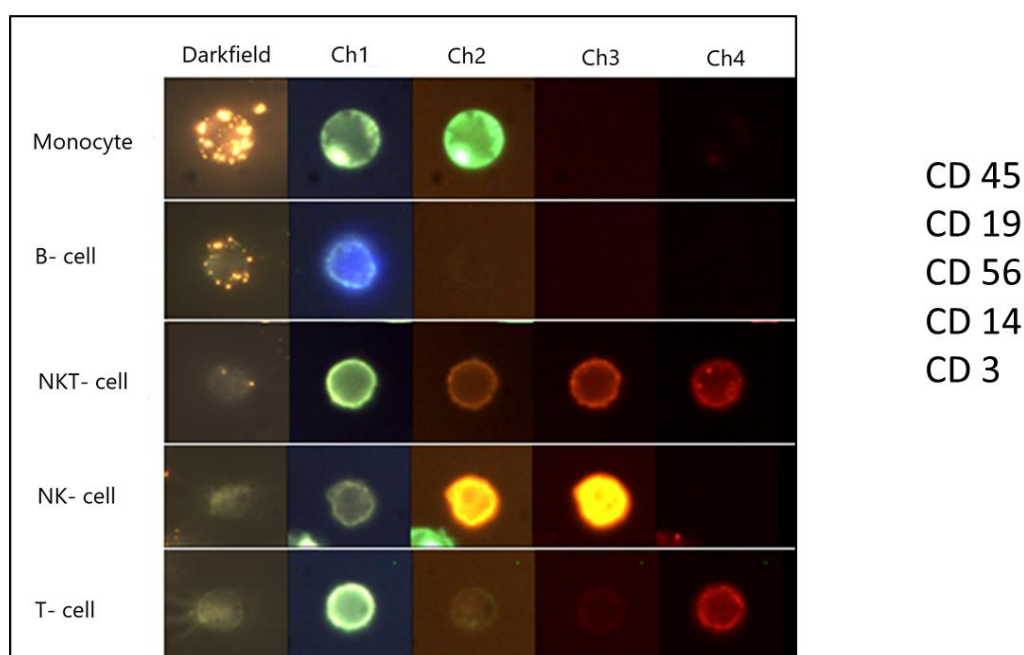


Figure 7 (54): Different leukocyte subtypes in different light channels. Gold nanoparticles are shown in the first column.

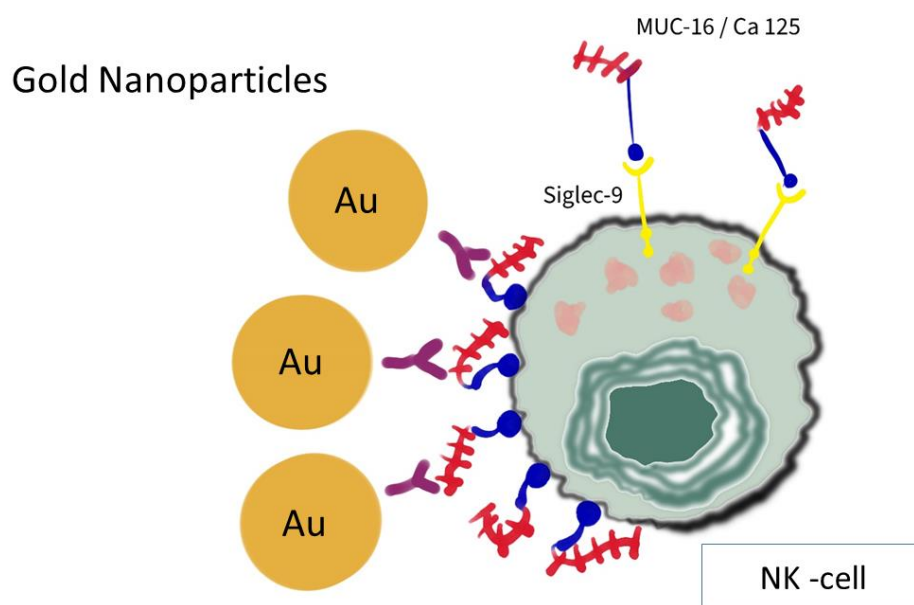


Figure 8: The anti- CA conjugated gold nanoparticles bound to the surface of an NK-cell.

3.3. Clinical Data

Information abstracted from the patient's medical record included serum CA125, complete blood count with differential, chemotherapy regimens, as well as imaging studies and clinical notes assessing disease status. While it was possible to link a CBC to each of the 30-study blood measured in this longitudinal set, it was possible to associate a CA125 value on the same day for 17 of the study bloods. The CBC differential count include hematocrit, platelet count, absolute counts for neutrophils, monocytes, eosinophils, and basophils. Three ratios involving leukocyte types were also calculated: the neutrophil-to-lymphocyte ratio (NLR); the platelet-to-lymphocyte ratio (PLR); and the lymphocyte-to-monocyte ratio (LMR).

3.4. Statistical Methods

For each sample, images of cells are acquired using darkfield and fluorescence microscopy. Fluorescent images are used to identify cell lineage. 3D darkfield images are used to count PNPs with an automated algorithm. A Poisson distribution is fit to the binding events of each cell type. On average, counting of PNP considered 312 cells of the same type per samples (minimum: NK-T-Cells 15 cells / sample, maximum T-Cells with

1054 cells / sample). The mean of such distribution (lambda parameter) corresponds to the average number of PNPs per cell and is the data shown in **Figures 11., 12., 13.** We use the exact method to compute 95% CI for Poisson distributions, as explained in (65). This method yields non-symmetrical confidence intervals. All computations are performed in python using the scipy library.

A Spearman correlation matrix was calculated with the purpose of assessing the relationship regarding the average number of MUC16 molecules bound to the five PBMC cell types (B-cells, monocytes, NK-cells, T-cells, and NK-T-cells) and other markers (CA125, blood count differential, blood count ratios, hematocrit and platelets). This analysis was conducted in SAS v.9.4 and all p-values are two-sided.

4. Results

4.1 Decidual Natural Killer cell isolation from fresh, term placentas

We obtained uNK cells from the fetal- maternal interface and CHM tissue. On the basis of at least 10 attempts, about 65 % of all DAPI- (alive) single events were NK cells of uterine phenotype in the case of end-term placenta. Due to the purity of the cell suspension, it was possible to acquire 160,000 uNK cells/ minute on average. After the cells had been sorted, we acquired 140 000 uNK cells per gram of starting placental material on average. As for the CHM tissue, on the basis of 2 attempts, about 33 % of all DAPI- (alive) single events were NK cells of uterine phenotype. Cell suspension purity enabled us to obtain, on average, 80 000 uNK cells/ minute. Following the sorting of cells, we acquired, on average, 70 000 uNK cells per gram of starting CHM tissue. (**Table 2.**)

Tissue sample	Sorting	Cell yield
Fresh, end term placenta (x10)	160,000 NK/ 1 minute	140,000 NKs/1 gr. of sample
CHM (x2)	80,000 NK/ 1 minute	70,000 NKs/1 gr. of sample

Table 2: Different tissue samples and the mean NK cell yield respectively. (CHM: Complete Hydatidiform Mole).

It was possible to keep the uNK cells in cell culture for 1 month by regularly exposing them to low doses of human IL-2. It was up to the 1st week of culturing that their original uterine phenotype was maintained. Following 1 week of culturing, we evaluated the cytotoxicity of placental NKs and CHM NKs. The peripheral NK cell cytotoxicity curve is concordant with the literature (60, 66). The cytotoxicity of the uterine NK cells was elevated in comparison with the peripheral NKs. More research and data are necessary, at this point, in order to understand the precise cause of this increase in cytotoxicity values - whether it results from the isolation protocol or from certain other factors, including the culturing conditions. Currently, there are soon-to-be published phenotypical and functional analyses providing more detail. The assessment of the cytotoxicity of the CHM NKs was based on 1 sample, which increases the efficacy of our protocol in acquiring

viable cells, however, it is not sufficient to make further deductions concerning the cytotoxicity of NKs isolated from CHM.

4.2 Alteration of the phenotype and cytotoxicity of dNK cells after the exposure to different trophoblastic cell lines.

In each experiment, approximately 3 million dNK cells were acquired from 20 grams of fresh, end term placenta. The selected NK phenotype was assessed before addition to the previously seeded trophoblastic cell lines and after 1 day and 5 days of co-culturing. The phenotype of the dNKs before the co-culturing and after 1 and 5 days is shown in **Figure 9**. We found that the original CD 56 bright, CD 16- phenotype was preserved after one day of co-culturing with both trophoblastic cell lines and or after 5 days of exposure to HTR-8 cells or OVCAR3 cells. In contrast, there was an appearance of a CD 56 diminished, CD 16+ NK population (44.1%) next to the CD 56 bright, CD 16- pool (55.1%) after the 5-day long exposure to Jeg-3. The dNK cells that were re-sorted after 1 day from the co-culture showed decreased cytotoxicity compared to the control of dNK cells with no exposure (**Figure 10**). After 1 day the percentage of dead target cells were 29.4% at a 5 to 1 ratio and 16.8% at a 2.5 to 1 ratio in the case of dNK cells co-cultured with HTR-8 cells and 29.6% at 5 to 1 ratio and 17.3% at 2,5 to 1 ratio when dNK cells were exposed to Jeg-3. On the 5th day, the percentage of dead target cells were 13.4% at a 5 to 1 ratio and 6.83% at a 2.5 to 1 ratio in the case of dNK cells co-cultured with HTR-8 cells, but 5.48% at a 5 to 1 ratio and 4.84 % at a 2.5 to 1 ratio when dNK cells were exposed to Jeg-3. Controls with no exposure to any trophoblastic cell lines showed dead target cells at 70.21 % at a 5 to 1 and 54.9 % at a 2.5 to 1 ratio on day 1 and 68.43 % at a 5 to 1 and 50.1 % at 2.5 to 1 on day 5.

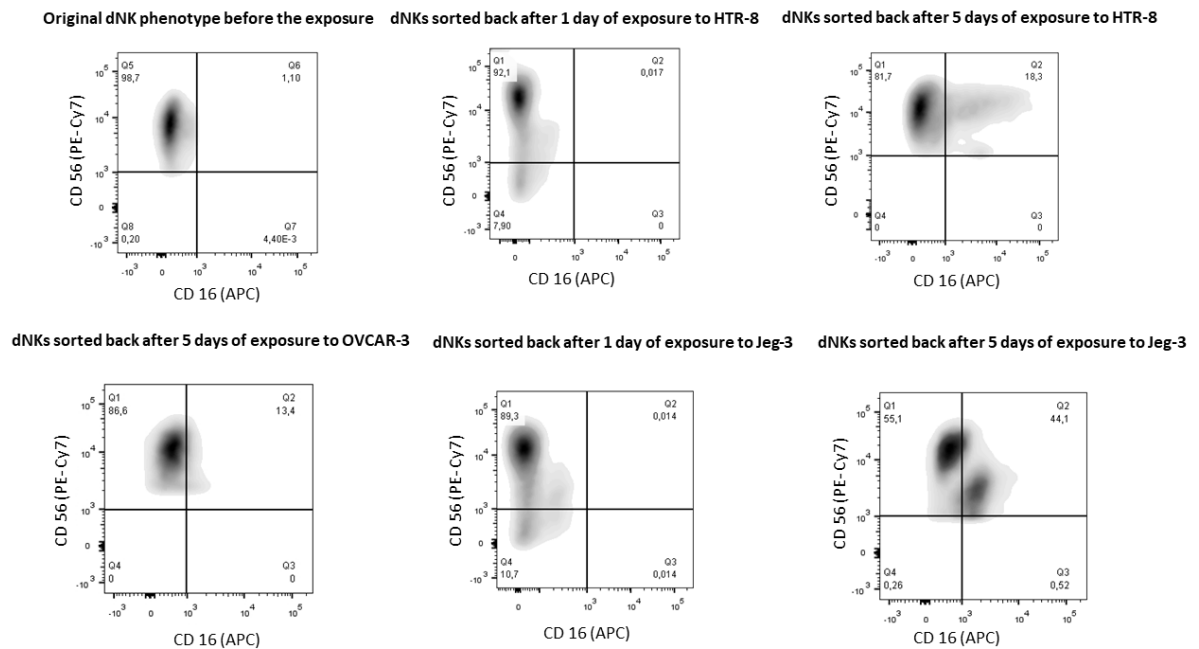


Figure 9: The phenotype of dNKs before and after the exposure to trophoblastic cells. Live/dead distinction was made by DAPI staining. We can see no significant changes in the dNK phenotype after 1 and 5-day long exposure to HTR-8 cells or OVCAR cells. The 1-day long exposure to Jeg-3 didn't alter the dNK phenotype but after 5 days a CD 56 diminished, CD 16+ population appeared with a similar phenotype to the peripheral NKs.

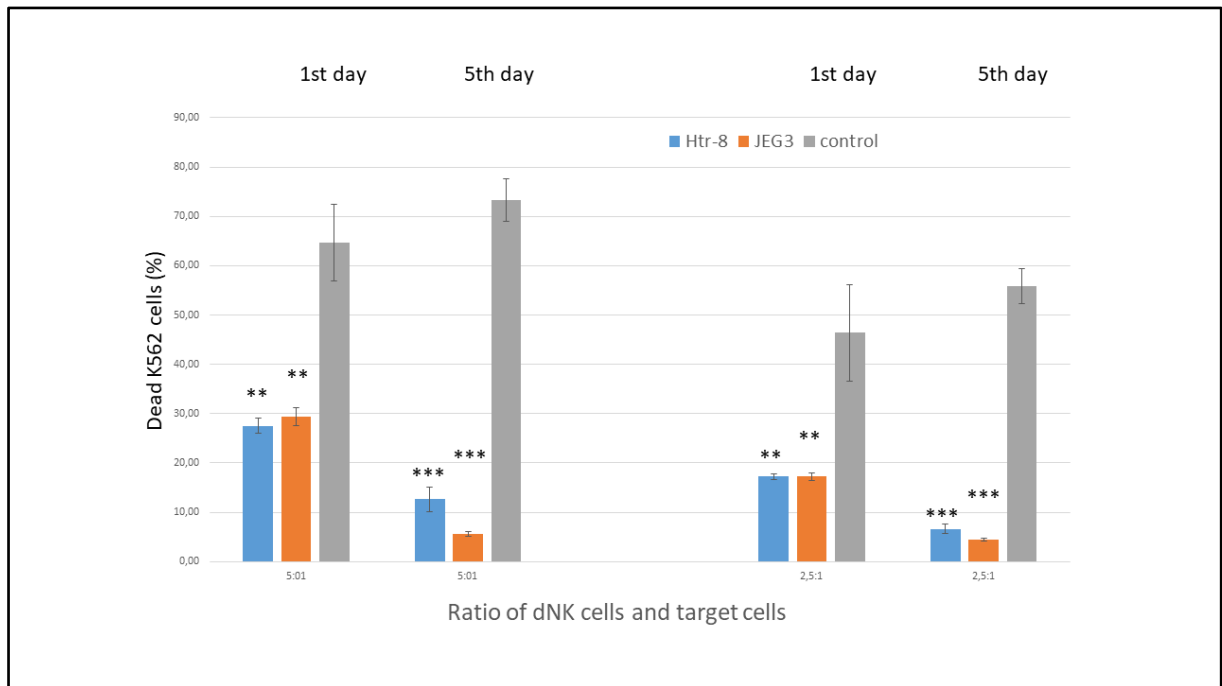


Figure 10: The changes in dNK cytotoxicity after exposure to different trophoblastic cells. The changes are similar after the 1-day long exposure in case of both cell lines. After 5 days, a significant depression of dNK cytotoxicity is shown after exposure to Jeg-3 compared to HTR-8 at 5 to 1 ratio. The controls are: K 562 cells in Tween -20 0,1% culture media. Standard error (SE) is indicated.

4.3 The alteration of CA 125 binding capacity of different leukocyte subtypes in epithelial ovarian cancer

(Figure 11.) shows average number of PNPs bound to B-cells, NK cells, NK-T cells, T-cells, and monocytes in the timespan between 8/2017 and 4/2019 and reflects the average number of MUC16 molecules bound to these cell type. In this patient, monocytes had the highest number of PNP-binding over the period with counts that ranged from approximately 30 to 65. Cell types with the fewest levels of binding were NK and T-cells with an average PNP-binding range of 5 to 20.

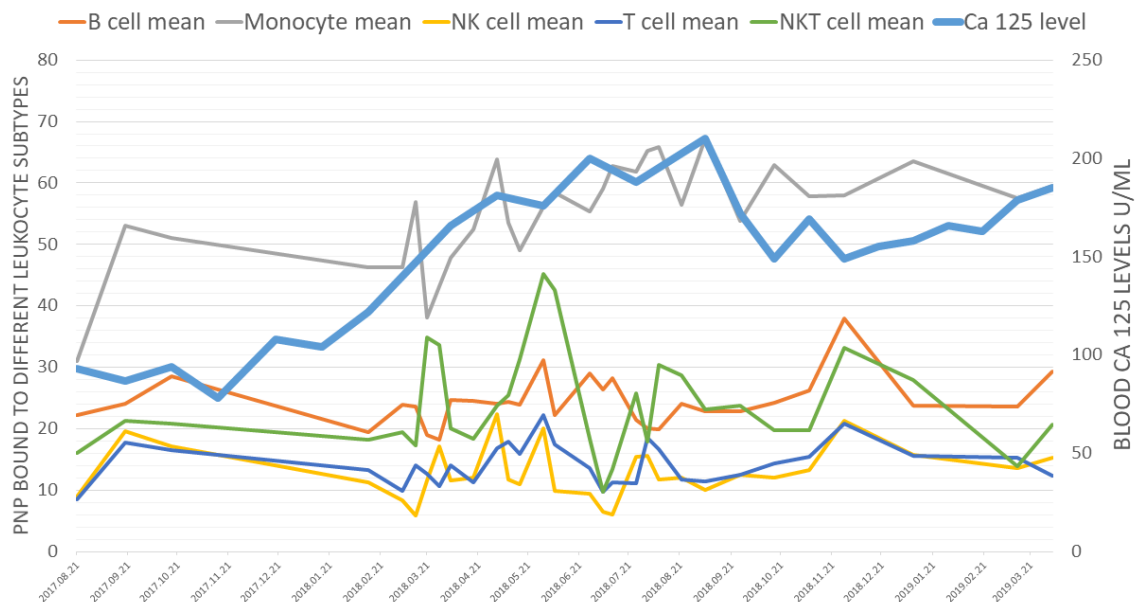


Figure 11 (51).: Average number of PNPs bound to B-cells, NK cells, NK-T cells, T-cells, and monocytes between the time period of 8/2017 and 4/2019 and reflects the average number of MUC16 molecules bound to these cell type. On the right, y axis we can see the serum levels of CA 125 (U/ml).

A number of fluctuations can be discovered in the PNP-binding (Plasmonic Nanoparticle) over time to cell type. Figure 12 shows the potential linkage of these fluctuations with clinical events for NK cells. The average number of MUC16 molecules bound to the five PBMC cell type, illustrated in Figure 1, and the associated serum CA125 were correlated with each other, with differential blood count data. and with 3 ratios calculated from

leukocyte types, PLR, NLR, and LMR (Platelets to Lymphocytes, Neutrophils to Lymphocytes, Lymphocytes to Monocytes Ratio). **Table 3.** highlights the significant correlations among these parameters.

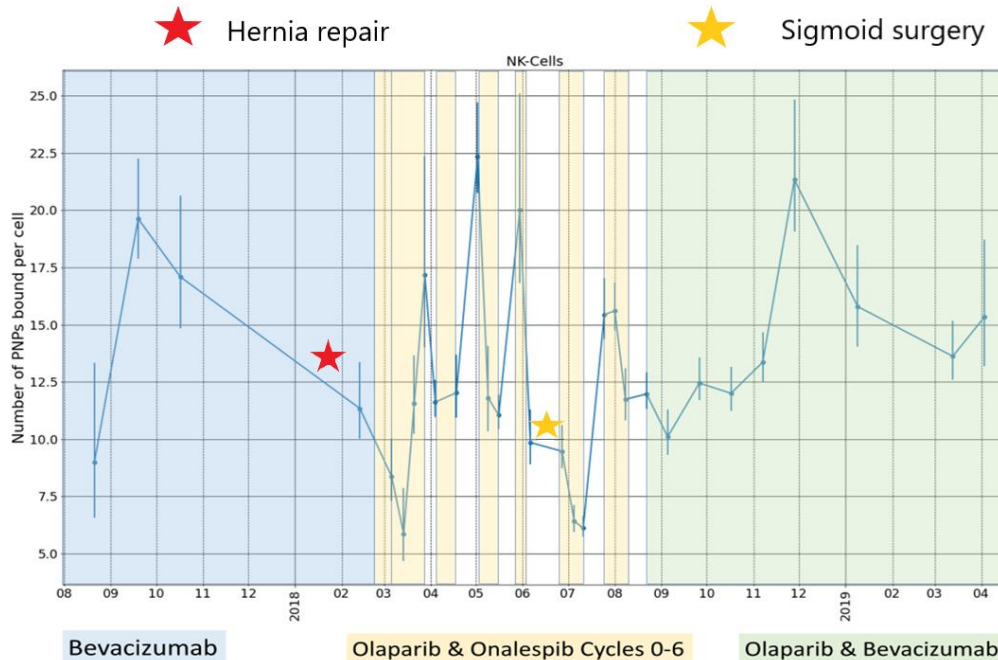


Figure 12 (51): We can observe two peaks in the binding capacity of Natural Killer Cells at 10/2017 and 12/2018 that occurred 2 months after starting or restarting Avastin therapy. Between 3/2018 to 9/2018 the patient was treated with oral Olaparib continuously and Onalespib infusions (anti- HSP 90 inhibitor). Sharp declines in the level of CA 125 bound to Natural Killer Cells were seen on the first day of the infusion cycle (except the cycle marked C2), with rebounds 14 days after the administration of the infusion and before the next infusion. There were drops in NK cell binding after two surgeries (hernia repair on 2/7/2018 and sigmoidectomy for residual disease on 6/11/2018).

CA125 was positively correlated with average monocyte count ($r=0.58$, $p=0.01$), with NLR ($r=0.76$, $p=0.001$), with PLR ($r=0.67$, $p=0.003$) and inversely and correlated with hematocrit ($r=-0.54$, $p=0.026$). Mean level of monocyte binding was inversely correlated with circulating monocytes ($r=-0.44$, $p=0.015$) and lymphocytes ($r=-0.57$, $p=0.001$) and positively correlated with NLR ($r=0.48$, $p=0.007$) and PLR ($r=0.62$, $p<0.001$). Mean NK cell counts were positively correlated with mean T-cell count ($r=0.49$, $p=0.005$) and NK-T cell count ($r=0.43$, $p=0.017$) and inversely correlated with circulating platelets ($r=-0.54$, $p=0.002$). Mean T-count was positively correlated with NK-T cell count ($r=0.39$, $p=0.32$) and inversely correlated with platelets ($r=-0.39$, $p=0.035$). Finally, NK-T cell counts were inversely correlated with eosinophil count and platelets ($r=-0.54$, $p=0.002$). No significant correlations with the mean B-cell counts and with circulating neutrophils were

discovered. Correlations among all parameter measured included those among the CBC counts themselves and the exact correlations and p values are shown in **Table 3**. Longitudinal data illustrating the inverse correlation between mean monocyte binding and circulating monocytes and lymphocytes are presented in **Figure 13**.



Figure 13 (51): Average PNP-binding to monocytes during 8/2017 to 4/2019 with corresponding blood monocyte and lymphocyte counts

	Statistic	B cell mean	Mono- cyte mean	NK cell mean	T cell mean	NK T cell mean	Neutro- phils	Mono- cytes	Lympho- cytes	Eosino- phils	Baso- phils	NLR	PLR	LMR	Hemato- crit	Platelet
CA125	r	NS	0.58	NS	NS	NS	NS	NS	NS	NS	NS	0.76	0.67	NS	-0.54	NS
	p		0.015									0.001	0.003		0.026	
	n		17									17	17		17	
B-cell mean	r		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	p															
	n															
Monocyte mean	r			NS	NS	NS	NS	-0.44	-0.57	NS	NS	0.48	0.62	NS	NS	NS
	p							0.015	0.001			0.007	0.000			
	n							30	30			30	30			
NK cell mean	r				0.49	0.43	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.54
	p				0.005	0.017										0.002
	n				30	30										30
T cell mean	r					0.39	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.39
	p					0.032										0.035
	n					30										30
NK T cell mean	r						NS	NS	NS	-0.47	NS	NS	NS	NS	NS	-0.54
	p									0.008						0.002
	n									30						30

Table 3. (51): Correlations among all parameter measured included those among the CBC counts themselves and the exact correlations and p values. (NLR: Neutrophyl to lymphocyte Rate, PLR: Platelet to Lymphocyte Rate, LMR: Lymphocyte to Monocyte Rate).

5. Discussion

5.1 Isolation of dNK cells from fresh, term placenta

A certain elementary level of anatomical and surgical knowledge may be necessary to perform the dissection of the decidua basalis from the decidua parietalis and other tissues of the placenta. Although it is possible to carry out the mechanical dissection manually, using scissors and forceps, the time required for the processing of tissue can be decreased by the application of the gentle MACS Dissociator machine. In either case, washing and rinsing the tissue samples in abundant PBS is indispensable due to the high

risk of contamination with fetal blood (from the umbilical cord). It is likewise crucial to prevent contamination from maternal blood since the peripheral natural killer cells differ from the decidual ones.

A vitally important phase in the process is the preparation of the cell suspension. Although it is possible to process cell suspensions of different concentrations by means of cell sorting technology, the efficiency of sorting was enabled by the fact that tissue processing increased the purity of the sample. Decreasing the high number of RBC, trophoblastic, and fibroblastic cells leads to considerable reduction in the time and cost spent on cell-sorting. In the case of this protocol, we preferred the application of RBC lysis buffer to that of density gradient media and centrifugation. This allowed us to save time and to improve the quality of samples. Notably, although longer exposure to RBC lysis buffer might lead to increased uNK purity and cell acquisition, the decreased viability of the cells should also be taken into consideration.

In addition, cell strainers of different filtering diameters (100 μm and 35 μm) were applied. It is necessary to employ strainers since bigger particles may persist resulting from the fact that the enzymatic digestion of the tissue sample may sometimes be unable to dissolve the connective tissue.

Cells become more vulnerable after they have been sorted. The risk of cellular lysis may be higher as a result of the higher concentrations of proteins in the sorting buffer. It is not recommended to perform sorting in cell culture media since its pH is unstable at atmospheric CO_2 concentration. Moreover, should the cell culture media contain Ca^{2+} or Mg^{2+} ions, they might react with the phosphate component of some cell sorter device's sheath buffer forming precipitates (67). For this reason, we applied Ca^{2+} , Mg^{2+} free 1X PBS (Phosphate Buffer Saline) with a 2% FBS (Fetal Bovine Serum) as a sorting buffer.

We cultured the cells in a suitable cell culture media, typically used for NK cell culture (ATCC). We changed the culture media every 3 days. Low doses of IL-2 were also needed. It should be mentioned that it is essential to use high quality, recombinant human IL-2 in order to maintain the NK cell culture. Earlier studies indicate that different cytokines are required by endometrial NK cells (eNK) for phenotypic transformation to dNK (or uNK cells) (27, 68). Apparently, IL-15 may be the most significant factor (3, 27,

69, 70). Based on the flow cytometric comparison of the uNK cells, they kept their decidual phenotype for a week or longer after being regularly exposed to IL-2.

Regarding the limitations of this protocol, it must be noted that we collected all the healthy placental samples from term placentas. Thus, placental samples from earlier stages of pregnancy as well as from aborted pregnancies were excluded. Another limitation is that we conducted cytotoxicity assay of the acquired NK populations so as to confirm their viability and capacity to preserve their killing function, however, it must be stated that our study design does not enable us to make further deductions or comparison of the different cytotoxicity curves.

The surgical technique applied by the surgeon may have a significant impact on the viability of the NK cells in the case of CHM tissue collection since heat damage is possible if electrocauterization is used.

5.2 Alteration of the phenotype and cytotoxicity of dNK cells after the exposure to different trophoblastic cell lines.

In this research, we attempted to provide a more accurate *in vitro* model for the interaction of dNK cells with different types of trophoblastic cells. *In vitro* studies on trophoblastic cell lines and NK cell co-culturing usually use peripheral NK cells or NK cell lines (e.g. MI92) (42, 71). Our protocol for dNK cell extraction from end term, healthy human placenta provided us with a high number of live NK cells with a decidual phenotype (59). We selected the HTR-8 and Jeg-3 cell lines for the *in vitro* model of normal eVT and choriocarcinoma. Healthy trophoblastic cells present a wide variety of cell surface antigens to modulate the host's (mother) local immune response. Some of them are direct immune suppressors and some activate specific immune cell functions such as cytokine production. These suppressive and activator mechanisms permit the invasion of the eVT into the myometrium. This is an "organized and regulated" invasion in contrast to the spreading of choriocarcinoma. Our data suggest that choriocarcinoma may exaggerate these features.

CD 16 is a member of the immunoglobulin superfamily involved in the cell mediated antibody- dependent cellular cytotoxicity. It has a pivotal role in initiating lysis by NK

cells but it can be also found on the surface of neutrophils, monocytes, macrophages and T- cells (72). NK cells are associated with the CD 16A subtype of this cluster of differentiation. We hypothesized that there would be an alteration of dNK cell phenotypes after the exposure to different trophoblastic cell lines as the immunological interaction between the trophoblastic cells and the NK cells might alter the cytotoxicity and so the phenotype of the latter. We found increased CD 16 expression in NK cells in the group that was exposed to Jeg-3 for 5 days. Pongcharoen et al. found in their 2016 study that Jeg-3 cell culture supernatant decreased the IFN- γ production and expression in leukocytes (73). Previous studies also showed the modulation of IFN- γ production of leukocytes including NK cells after exposure to trophoblastic cells (74). It is also known that IFN- γ production and CD 107a up regulation is associated with the loss of CD 16 expression in NK cells(75).

The lack of IFN- γ may not lead directly to the increased expression of CD 16 but it does not inhibit the appearance or reappearance of a CD 16 + population of NK cells. Our results showed similar suppression of dNK cell cytotoxicity after 1 day of exposure to both trophoblastic cell types. After 5 days, more significant suppression of the dNK cytotoxicity was observed in dNK cells exposed to Jeg-3 than to HTR-8. In this case there was an appearance of a new ‘peripheral NK like’ phenotype in the dNK cells sorted back from the plate after the 5th day. While the aggressiveness of NK cells is generally linked to CD 16 positivity, it is important to remember that their overall cytotoxic activity is only partly based on the number of their expression of CD 16.

We recognize limitations in the study design. The conditions of the set up would have been closer to *in vivo* conditions if first trimester trophoblastic cells had been isolated rather than using cell lines. During our study we were unable to access healthy placental tissue samples from the first trimester due to institutional restrictions. On the other hand, healthy, end term placental tissue was available but were unable to reproduce a reliable protocol for primary trophoblastic cell isolation and culture from fresh, term placenta. Moreover, the structure and physiology of the term placenta differ from the early placenta that is still under the process of placentation(76), which is why we used trophoblastic cell lines available from commercial cell banks. During the physiological invasion by eVTs, the trophoblastic cells do not form a monolayer as they do in cell culture. These changes may affect the dynamics of the immunological interaction between dNKs and

trophoblastic cells. Finally, we were only able to perform cocultures for 5 days. The 5-day long exposure time was established because both trophoblastic cell lines can overgrow the 6 well plate on the 6th day affecting the cell function and viability.

We also feel the study has several unique strengths. Regarding the choriocarcinoma cell lines, we selected Jeg-3 because both Jeg-3 and the eVT derived HTR-8 have the same HLA expression pattern as they both express HLA G, C and E on their surface in contrast with other non eVT derived cell lines (77). HTR-8 cells are not identical to the invading eVT cells as they are immortalized by simian viral transfection but they have been isolated from explant cultures of first trimester human placenta in the 8th-10th weeks of pregnancy (61). We also used a different cancer cell line, OVCAR-3 cells (derived from ovarian adenocarcinoma) as a negative control to compare this co-culture with our cases of trophoblastic cells. Based on our results, reduced cytotoxicity of dNK cells induced by Jeg-3 appears to be unique to choriocarcinoma and not a generalized cancer phenomenon.

5.3 The alteration of CA 125 binding capacity of different leukocyte subtypes in epithelial ovarian cancer

The research value of longitudinal data on patients being treated for cancer depends upon: the quality of the clinical data recorded, research specimens collected and preserved, and what testing done on the specimens. For ovarian cancer, data from the chart will include pathology reports, CA125, CBC with differential, and imaging data. Blocks from surgeries will likely be available for research; but other specimens, like serum or plasma, are likely to be available only in the setting of clinical trials or a funded research protocol. Less frequently PBMC will be isolated and stored for research, although PBMC have been used mostly in real time for antigen or molecular genetic profiling.

In this report, we have described the use of saved viable PBMC and a novel method for quantifying the amount of MUC16 binding to five different types of peripheral blood mononuclear cells (T-cells, B-Cells, NK cells, NK-T cells, and monocytes). The testing was done on longitudinal specimens from a single patient suffering from high grade serous ovarian cancer in the course of a 21-month period of treatment. Results were then

correlated with observations from her clinical course. This paper will focus on some of the correlations that we find essential.

In Figure 1, we illustrated variation over time in the levels of MUC16-binding by cell type with higher level of MUC16-binding to monocytes and lower levels in other cell types, especially NK and T-Cells. As described by Belisle et al., the principal binding partner for MUC16 on leukocytes is Siglec-9 (sialic acid binding Ig-like lectin). Siglec-9 expression on types of leukocytes has been found to be highest on monocytes, intermediate on neutrophils, and weaker on lymphocytes types, including NK and T-cells (78). Thus, the high degree of siglec-9 expression on monocytes explains why this cell type had a relatively greater degree of MUC16 binding while binding to NK and T-cells was lower and consistent with the lower level of Siglec-9 expression reported for the latter cell types (78). The need to work with frozen viable PBMC meant that MUC16 binding to neutrophils could not be studied here since these are removed in the process of isolating PBMC for storage.

NK cells were selected to correlate with clinical details of the patient's treatment for ovarian cancer because of the previously referenced paper describing potential functional consequences of MUC16 binding to NK cells (49). A more recent publication from the same group suggests that MUC16 from the tumor may shift the CD56 dim, CD16+ NK phenotype to CD56 dim, CD16- which correlates with lower anti-tumor toxicity (48). Perhaps, the most interesting aspect of Figure 2 is the troughs and rebounds in MUC16-binding to the NK cells that seemed, in most cases, to coincide with the infusions of the anti-HSP-90 inhibitor, Onalesbib. It is known that the Anti-HSP 90 therapy modifies the expression of activating receptors on NK cells (79), while no specific effect of anti-HSP90 inhibitors on siglec-9 receptors has been published. Another detail worth noting is that peaks in MUC16 binding to NK cell binding were detected around 10/2017 and 12/1—both could be observed approximately 2 months following the commencement (or continuation) of the Avastin therapy. An increase in NK cells was observable in one clinical trial involving the effects of Avastin on in renal cell carcinoma (80). Lastly, a decrease in MUC16-binding to NK cell was detected following hernia repair on 2/7/2018 and a sigmoidectomy on 6/11/2018. Abdominal surgery seems to reduce NK cell count experimentally (81) on the one hand, and following primary surgery for ovarian cancer (82) on the other hand. It should be noted that each of these three reports indicates that

the increase (80) or decreases (81, 82) apply to NK cell numbers but not directly to the central topic of the present study, which is MUC16 binding to NK cells.

We believe some correlations were identified in Table 1 that suggest avenues for future studies. The only correlation discovered between serum CA125 and binding of MUC16 to the various PBMC cell types was a positive correlation with monocyte binding ($r=0.58$, $p=0.01$). This may well reflect the high level of Siglec-9 binding on monocytes previously referred to (78). Serum CA125 also found to correlate with the leukocyte ratios, NLR and PLR). Both of the latter correlations have earlier been reported to occur in female ovarian cancer patients in cross-sectional data (83); however, our study is probably the first to indicate the existence of the correlations in longitudinal specimens on the same patient as well. Indeed, the correlation existing between serum CA125 and NLR of $r=0.76$, $p=0.001$ proved to be astonishingly strong based on not more than 17 observations. CA125 was also weakly and inversely correlated with hematocrit. To some extent, this likely reflects anemia consequent to chemotherapy targeted to lower CA125 and occurred despite the patient receiving red cells during this period.

The positive correlation of MUC16 binding to monocytes with serum CA125 pointed out above likely explains positive correlation between mean monocyte binding with NLR and PLR. Of greater interest we believe, are the inverse correlations existing between monocyte binding and the peripheral monocyte and lymphocyte counts, as shown in figure 3. Monocytes, having reached the blood stream from the bone marrow, generally infiltrate a broad variety of tissues and develop into dendritic cells or macrophages (84). Some of them, however, return to the lymph nodes carrying antigens they had been exposed to, retaining their monocyte form (85). In either case, these would lead to a decrease in circulating monocytes if MUC16 binding to monocytes promoted migration to lymph nodes or cancer metastases. Also relevant to this observation is that M2-polarization of macrophages and migration to lymph nodes with metastatic disease occurs with certain cancers including pancreatic (86). An explanation for MUC16-binding to monocytes and the even stronger inverse correlation with circulating lymphocytes is less clear; but a review describing macrophage plasticity and interaction with lymphocyte subsets may be relevant (87).

Modest positive correlations were seen among the levels of MUC16 binding to NK cells, T cell, and NK cells and likely reflect some overlap between the markers for distinguishing these subsets. All three were inversely correlated with the platelet count which suggests there is some degree of platelet-lymphocytes “cross-talk” described by Li (88). Equally intriguing is the inverse correlation between MUC16 binding to NK-T cells and eosinophils. The significance of this observation is not clear although “cross-talk” between NK cells and eosinophils is also described (89). The therapeutic blockage of the Ca 125- Siglec-9 receptor ligand connection can also be in the focus of future studies.

6. Conclusions

6.1 Isolation of dNK cells from fresh, term placentas

The protocol synthesizes other methodologies in order to acquire a specific population of lymphocytes from a designated anatomic area (i.e., fetal- maternal interface). This has been of particular interest, in recent decades, to a number of research teams with a view to isolating specific leukocyte pools from the decidua and GTD tissue, which is an essential means of designing dynamic immunological studies both *in vitro* and *in vivo* (90-93). Our collection of findings indicate that this protocol can be relied on as a technique for acquiring high quality and viable leukocyte populations from the placenta. It has been demonstrated that the protocol can be effectively used for the acquisition of a high number of viable NK cells from CHM tissue. We maintained the NK cell populations in culture for a minimum of 1 week, preserving their original phenotype. The cells were sustainable for 1 month in culture, but their phenotype shifted into a similar form, consisting of CD 56 Bright, CD 16- and CD 56 Diminished, CD 16+ populations.

6.2 Alteration of the phenotype and cytotoxicity of dNK cells after the exposure to different trophoblastic cell lines.

While dNK cells undergo various changes in their function after being exposed to the invading eVTs, alteration of their cytotoxicity is only one aspect of it. The alteration of the NK phenotype, combined with decreased cytotoxicity in the group that was exposed to Jeg-3 supports our original hypothesis that the chorionicarcoma tissue creates a more suppressive micro environment for NK cytotoxicity. Unraveling the nature and exact mechanism of this effect will be an important area for future study.

6.3 The alteration of CA 125 binding capacity of different leukocyte subtypes in epithelial ovarian cancer

To conclude, we have presented the use of a new darkfield microscopic technique to quantify binding of MUC16 to longitudinally collected PBMC types in a single patient. A notable finding was that monocytes had the most elevated level of MUC16 -binding concordant with their high expression of a MUC16-binding partner, Siglec-9; and monocyte binding was the only cell type that correlated directly with serum CA125. The monocyte count in peripheral blood correlated inversely with MUC16-binding on monocytes suggesting depletion of circulating monocyte to reside in lymph nodes or metastatic sites. We should also highlight that the level MUC16-binding to NK cells probably best correlated, if only on a descriptive basis, with features of the patient's treatments including avastin, an HSP-90 inhibitor, and two abdominal surgeries. NK cell binding also correlated inversely with platelet count. Finally, NK_T cell binding correlated inversely with eosinophils. In the context of a case report our observations must be viewed as descriptive. Nor can we argue that this technology for clinical use. Nevertheless, we believe that the technology described will be a valuable research tool and that PBMCs are underutilized specimens for longitudinal research.

7. Summary

In my thesis I tried to reveal many aspects of the physiology and immunology of the fetal-maternal interface. Understanding the biology of the trophoblastic invasion may help us to comprehend the mechanisms how tumor tissue evades the immune surveillance. Isolation and analysis of the decidual NK cells has been the goal of many scientific projects in the past decades however the use of freshly isolated dNK in functional studies is still an un-common experimental set-up.

A new protocol was introduced for the isolation and culturing of dNK cells from fresh, term placentas and complete hydatidiform moles. The dNK cells became subsequently expanded in cell culture. Flow cytometry and functional assays were used to reassess their surface markers and cytotoxicity. The protocol yields high quantities of enriched dNK cells which can be sustained in cell culture for at least a month, while their phenotype and functionality are preserved for a week, thus providing a valuable tool for immunological and gynecologic- oncological studies.

In the second part of my thesis, we created a novel experimental set-up for modelling the immunological interaction between dNK cells and different trophoblastic cell lines. 5 days of exposure changed the phenotype of the dNK population exposed to Jeg-3 to be similar to the peripheral NK cell phenotype. We also found decreased cytotoxicity of NK cells co-cultured with malignant cells compared to the NK population exposed to benign trophoblastic cells and the controls. Understanding the ways of interaction between dNK cells and the trophoblastic cells may reveal similar immunological interactions between the host's NK cells and tumor cells.

My third project presents a different aspect of gynecologic- oncology, focusing on novel diagnostics of ovarian cancer. We tested this newly developed microscope system's research value applying specimens collected serially from a female patient who was currently undergoing therapy for high grade serous EOC. It is possible to assess MUC16-binding among cryopreserved PBMC cell types by means of darkfield and fluorescence microscopy. Correlations observable between the level of binding by cell type with serum CA125, CBC data, and treatment details indicate that these novel techniques can provide fresh insights into the clinical course of EOC.

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9. The candidate's own bibliography

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González G, Lakatos K, Hoballah J, et al. Characterization of Cell-Bound CA125 on Immune Cell Subtypes of Ovarian Cancer Patients Using a Novel Imaging Platform. *Cancers (Basel)*. 2021;13:2072. doi:10.3390/cancers13092072
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