EXOSOMES AND THE NKG2D RECEPTOR-LIGAND SYSTEM IN PREGNANCY AND CANCER: USING STRESS FOR SURVIVAL

By

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Cover: Electron micrograph of isolated exosomes from human early placenta.
Courtesy Dr.Vladimir Baranov.

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Nu ska en tornado gå fram

Gerd Lundquist

To my family and my Love

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ABSTRACT

Although not obvious at first sight, several parallels can be drawn between pregnancy and cancer. Many proliferative, invasive and immune tolerance mechanisms that support normal pregnancy are also exploited by malignancies to establish a nutrient supply and evade or edit the immune response of the host. The human placenta, of crucial importance for pregnancy success, and its main cells, the trophoblast, share several features with malignant cells such as high cell proliferation rate, lack of cell-contact inhibition and invasiveness. Both in cancer and in pregnancy, the immune defense mechanisms, potentially threatening the survival of the tumor or the fetus, are progressively blunted or even turned into tumor- or pregnancy-promoting players.

Amongst immune mechanisms that are meant to protect the host from cancer and can be a potential threat to the fetus, the NKG2D receptor-ligand system stands out as the most powerful, stress-inducible "danger detector" system that comprises the activating NK cell receptor NKG2D and its ligands, the MIC (MHC class I Chain-related proteins A and B) and ULBP (UL-16 Binding Proteins) families. It is the major cytotoxic mechanism in the body promoting surveillance and homeostasis. In the present thesis we investigate the NKG2D receptor-ligand system in human early normal pregnancy and in the leukemia/lymphoma cell lines Jurkat and Raji and ask the questions "How is the NKG2D receptor-ligand system functioning in pregnancy and tumor? How is the danger of cytotoxic attack of the fetus avoided? Why is the immunosurveillance function compromised in cancer patients?"

We developed a method to isolate and culture villous trophoblast from early human normal placenta and used it to study the NKG2D receptor-ligand system. We discovered that the NKG2D ligand families of molecules MICA/B and ULBP1-5 are constitutively expressed by the syncytiotrophoblast of the chorionic villi. Using immnunoelectron microscopy, we studied the expression of these molecules at the subcellular level and could show for the first time that they are preferably expressed on microvesicles in multivesicular bodies (MVB) of the late endosomal compartment and are secreted as exosomes. Exosomes are nanometer sized microvesicles of endosomal origin, produced and secreted by a great

variety of normal and tumor cells. The exosomes are packages of proteins and ribonucleic acids that function as “mail” or “messengers” between cells conveying different biological information. We isolated and studied exosomes from placental explant cultures. We found that they carry NKG2D ligands on their surface and are able to bind and down-regulate the cognate receptor on NK-, $CD8^+$ and γδ T cells. The down-regulation selectively caused impairment of the cytotoxic response of the cells but did not affect their lytic ability as measured by perforin content and gene transcription. Thus, the NKG2D ligand-bearing exosomes suppress the cytotoxic activity of the cells in the vicinity of the placenta, leaving their cytolytic machinery intact, ready to function when the cognate receptor is restored/recycled. These findings highlight the role of placental exosomes in the fetal-maternal immune escape and support the view of placenta as an unique immunomodulatory organ.

Next, we studied the expression and exosomal release of NKG2D ligands by tumor cells using the leukemia cell lines Jurkat and Raji as a tumor model. We found that NKG2D ligand-bearing exosomes with similar immunosuppressive properties as placental exosomes are constitutively secreted by the tumor cells, as a mechanism to blunt the cytotoxic response of the immune cells and thus protect themselves from cytotoxic attack by the host. Interestingly, we found that thermal- and oxidative stress up-regulates the exosome secretion and the amount of exosome-secreted NKG2D ligands. Our results imply that tumor therapies that cause stress-induced damage, such as thermotherapy and stripping of oxygen supply to the tumor, might have a previously unrecognized side effect causing enhanced exosome production and secretion, which in turn suppresses the natural anti-tumor immune response and thus should be taken into account when designing an optimal therapy of cancer patients.

In conclusion, we describe a novel stress-inducible mechanism shared by placenta and tumors as an immune escape strategy. We found that placenta- and tumor-derived NKG2D ligand-bearing exosomes can suppress immune responses to promote the survival and well being of the fetus or the tumor. Our work comprises an important contribution to the elucidation of the NKG2D ligand-receptor system and its mode of operation in the human body and opens new perspectives for designing novel therapies for infertility and cancer.

SAMMANFATTNING PÅ SVENSKA

Även om det kan verka paradoxalt, utmanar graviditet och cancer immunsystemet på liknande sätt. Dessa två så diametralt olika tillstånd representerar två mycket speciella situationer: graviditeten innebär utveckling och tillväxt av ett foster, en egen individ olik modern, tillfälligt ”transplanterad” i hennes kropp; cancersjukdomen innebär att kroppsegna celler, som har blivit olika/förändrade genom en process kallad malignifiering, växer och sprids genom att ”transplantera” dottersvulster i olika organ. Vid båda dessa tillstånd har fostret och cancern ett gemensamt mål, att överleva och parasitera i en annan kropp och de har utvecklat liknande strategier för att undvika angrepp från värdens immunsystem. Moderkakan, placentan, och dess trofoblastceller är livsviktiga för graviditeten och delar många egenskaper med många olika cancerceller såsom okontrollerad celldelning, tillväxt och invasion.

I vår strävan att förstå på vilket sätt tumörer och placenta lyckas undvika en immunologisk attack har vi valt att studera NKG2D receptor-ligand systemet. Detta system är ett mycket viktigt immunologiskt verktyg med vilket alla förändrade, infekterade och på olika sätt biologiskt stressade celler, inklusive cancerförändrade celler, avlägsnas från kroppen med hjälp av ”mördarceller”, så kallade cytotoxiska T celler och NK celler. Mördarcellerna uttrycker NKG2D receptorn på sin yta som binder till sina ligander, MIC och ULBP1-6, uttryckta på förändrade kroppsceller. När en bindning har skett överförs en aktiveringssignal till mördarcellen som dödar t.ex. cancercellen, märkt med MIC och/eller ULBP molekyler på sin yta. Vi har ställt oss frågorna: ”Hur fungerar NKG2D receptor-ligand systemet vid graviditet och cancer? Hur undviker fostret att attackeras av mammans immunförsvar? Varför lyckas inte NKG2D receptor-ligand systemet eliminera de förändrade tumörcellerna hos cancerpatienter?”

Vi upptäckte att moderkakans syncytiotrofoblaster utsöndrar liganderna till NKG2D receptorn, MICA/B och ULBP1-5, bundna till ytan av mycket små (nanometer-stora) membranomgivna blåsor som kallas exosomer, avbildade från en elektronmikroskopisk bild på omslaget av denna avhandling. Exosomerna är 30-100 nm stora, uttrycker många olika proteiner både på ytan och inuti och kan produceras och utsöndras i blodet av många olika

celler. Exosomerna används som ett sätt att kommunicera och kan betraktas som cellernas ”brev” till varandra. Vi upptäckte att moderkakans syncytiotrofoblastceller producerar exosomer som bär NKG2D liganderna MIC och ULBP på sin yta. Dessa exosomer binder med sina MIC och ULBP molekyler till NKG2D receptorn, trycker ner den från cellytan och på så sätt förstör mördarcellens avdödande förmåga. De NKG2D ligand-bärande exosomerna som moderkakan, placenta, producerar och utsöndrar används för att undvika attack från moderns immunsystem och på så sätt skyddas fostrets överlevnad och utveckling. Liknande mekanism används även av cancerceller för att etablera och sprida sig i värdens kropp.

I vår nästa studie undersökte vi NKG2D ligand-bärande tumörexosomer från leukemi och lymfomceller. En viktig upptäckt var att cancercellerna ökade sin exosomutsöndring och därmed sin nedreglering av mördarcellernas avdödande förmåga när de utsattes för cellulär stress. Våra resultat antyder att cancerbehandling som verkar genom stressinducerande mekanismer, såsom kemoterapi och/eller termoterapi och strypning av syretillförseln till cancerceller kan ha tidigare okända bieffekter som trycker ner patienternas immunförsvar via ökad produktion av tumörexosomer - en viktig aspekt som bör övervägas när man planerar optimal anti-cancer behandling.

Sammanfattningsvis använder placenta och cancerceller liknande strategi för fostrets överlevnad och cancerns etablering, tillväxt och spridning, baserad på utsöndring av NKG2D ligand-bärande immunosuppressiva exosomer. Våra resultat ökar förståelsen av NKG2D receptor-ligand systemets och exosomernas funktion och kan bidra till utvecklingen av nya strategier i behandlingen av infertilitet och cancer.

ORIGINAL PAPERS

Paper I

Ann-Christin Stenqvist, Ting Chen, **Malin Hedlund**, Tanya Dimova, Olga Nagaeva, Lennart Kjellberg, Eva Innala, Lucia Mincheva-Nilsson. An efficient optimized method for isolation of villous trophoblast cells from human early pregnancy placenta suitable for functional and molecular studies. *American Journal of Reproductive Immunology,* 2008; 60(1): 33-42.

Paper II

Malin Hedlund, Ann-Christin Stenqvist, Olga Nagaeva, Lennart Kjellberg, Marianne Wulff, Vladimir Baranov, Lucia Mincheva-Nilsson. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. *The Journal of Immunology,* 2009;183(1):340-351.

Paper III

Malin Hedlund, Olga Nagaeva, Dominic Kargl, Vladimir Baranov, Lucia-Mincheva-Nilsson. Thermal- and oxidative stress cause enhanced release of NKG2D ligand-bearing immunosuppressive exosomes in leukemia/lymphoma T and B cells. *Submitted.*

LIST OF ABBREVIATIONS

APCs	antigen-presenting cells
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad 3 related protein
BCR	B cell receptor
CD	cluster of differentiation
Chk1	checkpoint kinase 1
CTB	cytotrophoblast
CTLs	cytotoxic T cells
DCs	dendritic cells
ECM	extracellular matrix
ESCRT	endosomal sorting complex required for transport
EVT	extravillous trophoblast
FasL	Fas-ligand
Foxp3	transcribing forkhead box protein 3
GH	growth hormone
GPI	glycosylphospatidylinositol
hCG	human chorionic gonadotropin
hCS	human chorionic somatomammotropic hormone
HLA	human leukocyte antigen
hPL	human placental lactogen
HSP	heat shock protein
IDO	indoleamine 2, 3-dioxygenase
IEM	immnunoelectron microscopy
IFN	interferon
Ig	immunoglobulin
IGF	insulin growth factor
IHC	immunohistochemistry
IL	interleukin
ILV	intraluminal vesicles
JAK	janus kinase
KIR	killer cell-Ig-like receptors
LIF	leukemia inhibitory factor
МΦ	macrophage
MHC	major histocompatibility complex
MIC	MHC class I Chain-related proteins
MTOR	oxygen-sensitive mammalian target of rapamycin
MULT-1	murine UL16-binding-protein-like transcripts-1
MVB	multivesicular body
NK	natural killer
NKT	natural killer T
PAMP	pathogen-associated molecular pattern
PECAM	platelet endothelial cell adhesion molecule
PLAP	placental alkaline phophatase
RAET	retinoic acid early transcript
STAT	signal transducers and activators of transcription
STB	syncytiotrophoblast

ULBP	UL-16 Binding Proteins
uNK	uterine natural killer
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLRs	toll-like receptor
TNF	tumor necrosis factors
TRAIL	tumor necrosis related apoptosis inducing ligand
Tregs	regulatory T cells
TUN	trophuteronectin
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VT	villous trophoblast

INTRODUCTION

Although not obvious at first sight, several parallels can be drawn between pregnancy and a tumor condition. It might seem as a far-fetched comparison, but from an immunologic point of view pregnancy and malignancies comprise a similar challenge to the immune system.

The immune defense of the body is effectuated by a system of highly competent immune cells, signal substances and effector mechanisms that mediate immune protection and homeostasis. Cancer, a disease originating from alteration in the cellular genome, and the placenta, a semiallogeneic fetal organ, are genetically different from their hosts and, as such, would be sensed as foreign or ”non-self” by the immune system and would provoke an immune response that will threaten their survival. Despite the fact that placenta and cancer are both immunogenic tissues, they are both able to escape from the host immune surveillance. What is even more interesting, the mechanisms engaged in the immune evasion appear to be surprisingly similar. Both in cancer and pregnancy the immune defense mechanisms, potentially threatening the survival of the tumor or the fetus, are progressively blunted by the activation of immune suppressive pathways, or even turned into tumor- or pregnancy-promoting players. This is beneficial for reproduction and mammalian species’ survival but detrimental for the host/patient harbouring a tumor.

The ability of placenta and cancer to compromise the immune surveillance mechanisms in pregnant women and in cancer patients is highly complex and cannot be explained with a single unifying mechanism of immune escape. Instead, a jigsaw puzzle of molecules and mechanisms operate in concert to establish the immune privilege of the fetus or the tumor.

In this thesis, one of the most potent pathways for immune surveillance, the NKG2D receptor-ligand system, also known as a self-induced “danger detection system”, an instrumental mechanism for immune protection and homeostasis, is investigated in the context of these two conditions. How is the NKG2D receptor-ligand system functioning in pregnancy and tumors? How is placenta and tumor evading the NKG2D receptor-mediated immune attack? Why and how are intruders like the fetal semiallograft and the genetically-altered tumor accepted by the immune system and consequently by the body of the

pregnant woman and the succumbing body of the tumor host? We found an intricate way of using the ligands of the NKG2D receptor and involvement of placental and tumor exosomes. Initially, a brief background of the immune system, pregnancy, cancer and exosomes is given as a background to the discussion of the results obtained in the present study.

BACKGROUND

1. The immune system in general and in pregnancy

1.1 Definitions and general properties

The immune system of mammals is a defense system that provides protection against invading microorganisms and, by immunosurveillance, promotes homeostasis and prevents development of tumors. It comprises of two branches - the innate, antigen-non-specific, and the acquired, antigen-specific immune system. Different cell types and signal molecules act together to eliminate an unlimited variety of foreign invaders and preserve the homeostasis of the body.

The innate branch of the immune defense involves phagocytic cells, such as macrophages (MΦ), granulocytes, antigen-presenting cells (APCs)/dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, γδ T cells, and soluble proteins like complement factors, acute phase proteins, cytokines and chemokines. The acquired branch of the immune defense comprises of T- and B cells, plasma cells and antibodies. It is an adaptive process, characterized by specificity, memory and self/non-self discrimination based on recognition of antigens presented by the major histocompatibility complex (MHC) class I and II proteins. Two types of immune responses are generally evoked: a humoral response resulting in specific antibodies and a cellular response resulting in activation of cytotoxic effector cells such as cytotoxic T- and NK cells. In the different phases of an immune response, cells from both the innate and the adaptive immunity co-operate with each other to induce cell proliferation and differentiation leading to various effector functions. Thus, the innate and adaptive defense mechanisms are intimately connected with each other in their role to protect the organism from intruders.

1.2 Cells of the immune system

1.2.1 B lymphocytes

The receptor of B lymphocytes (BCR) consists of a membrane bound immunoglobulin (Ig) molecule that works as an antigen-binding unit. The receptor consists of two heavy chains and two light chains that are composed of a variable and a constant region. The constant

region of the heavy chain is responsible for the biological function and the variable region determines the antigen specificity. Moreover, B cells have a co-receptor complex consisting of cluster of differentiation (CD) 19, CD81 and CD21 that is activated by binding a protein that is part of the complement system. Naïve B cells express IgM and IgD. While activated, by direct binding of the BCR to epitopes of unprocessed antigens, the B cells may change the constant part of the Ig molecule, a term called isotype switching and produce IgG, IgA or IgE. Activated B cells can differentiate into plasma cells that produce antibodies. Moreover, B cells can present antigens or turn into memory B cells [1]. B lymphocytes are very rare or absent in the maternal-fetal interface [2].

1.2.2 Antigen-presenting cells

Antigen presenting cells present antigens to T and B cells and in this way initialize the adaptive immune response. Major histocompatibility complex molecules class I and II also called human leukocyte antigens (HLA) I and II, are involved in the antigen presentation. MHC class I presents intracellular proteins and is expressed on all nucleated cells in the body. MHC class II presents extracellular antigens and is expressed on APCs, including monocytes/MΦ, DCs and B cells [1]. Monocytes have chemokine- and adhesion receptors mediating migration from the blood flow to the tissue during infection and inflammation. They secrete inflammatory cytokines and are able to differentiate to MΦ or DCs [3]. Macrophages are equipped with pattern recognition receptors making them sufficient at phagocytosis and clearing of infected or transformed cells and cellular debris. Additionally, MΦ produce inflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-12 [1]. Dendritic cells are migratory cells distributed in the tissue, and when activated, they migrate to lymphoid organs. There are three types of DCs in humans: lymphoid, non-hematopoietic or myeloid. Besides their antigen presenting abilities, they display a phagocytic capacity in their immature stage and cytokine producing competence in their mature stage [1].

In pregnancy, the maternal mucosa-associated MΦ comprise 10-15 % of the leukocytes in the decidua. They engulf microorganisms and immune complexes, and play an important role in removal of apoptotic cells. Decidual MΦ may present maternal and/or fetal antigens to resident T lymphocytes. The maternal MΦ produce cytokines and have been shown to

enhance IFN-γ secretion by uterine NK cells (uNK) when cultured together. Dendritic cells in the maternal interface have myeloid origin and can be immunomodulatory [2, 4].

1.2.3 αβ T lymphocytes

T cells can be divided into diverse subsets according to their receptors, the markers they express and their functions. Depending on the T cell receptor (TCR), T cells are divided into TCRαβ or TCRγδ cells. Additionally, αβ T cells are divided into two subclasses defined by the expression of CD4 or CD8 molecules. The TCR of αβ T cells are composed of the α and β chain that forms the antigen-specific binding unit and the CD3 complex of molecules which transports signals into the cell upon activation. To be able to bind the TCR, the proteins need to be presented as peptides in a complex with class I or class II MHC proteins on the surface of an APC [5, 6].

$CD4^+$ T cells, also called T helper (Th) cells, hold a central position in the immune system. By producing a specific set of cytokines they promote humoral or cellular immune response. Naïve $CD4^+$ T cells can become two different types of Th cells: those who secrete IFN-γ and IL-2, called Th1 cells which evoke cellular immune response and those who secrete IL-4 and IL-5, called Th2 cells which evoke humoral immune response [5].

$CD8^+$ T cells, also called cytotoxic T cells (CTLs), mainly kill infected or transformed cells. Their activation and differentiation are promoted by IL-2 and IFN-γ i.e. by the Th1 immune response [5, 6]. The CTLs lyse their targets by cytolytic granule exocytosis or by apoptosis induced by cross linking of Fas/Fas-ligand (FasL) [7]. In the cytolytic granule exocytosis pathway, cytoplasmic granules containing perforin, granzymes and granulysin are secreted. Perforin forms pores in the plasma membrane, allowing the granzymes to enter in to the cell and cause cell death by apoptosis [8]. Apoptosis can also be induced by ligation of FasL on CTLs with the cell-death transducing receptor Fas on target cells [9]. Additionally, CTLs secrete cytokines and thus contribute to regulation of the immune response [6].

There are contradictory results concerning the amount of αβ T cells in blood during pregnancy. In pregnant women, the amount of αβ T cells is decreased or unchanged. In mice, silencing of antigen-specific T cell response towards paternal antigens has been reported. Various mechanisms are suggested for the control of the amount of maternal T cells at the fetal-maternal interface, i.e. expression of indoleamine 2,3-dioxygenase (IDO) in placenta which inhibits T cell proliferation, and/or clonal deletion of fetus-specific $CD8^+$ T cells through Fas-FasL system [2]. There is a reversal in the CD4:CD8 T lymphocyte ratio in the endometrium and decidua compared with peripheral blood, suggesting a suppression of Th cells. The role of the decidual αβ T cells in pregnancy is currently not completely understood [2].

1.2.4 Regulatory T cells

Another group of $CD4^+$ T cells expressing CD25 protein and transcribing forkhead box protein 3 (Foxp3) is called regulatory T cells (Tregs). The natural Tregs are generated in the thymic medulla and express and secrete the immunosuppressive cytokine TGF-β. Another group of Tregs, called induced- or adaptive Tregs develop in the periphery in response to stimulation with specific antigens and secretes TGF-β (Th3 cells) or IL-10 (Treg1 cells) [10]. It is not completely clear how adaptive Tregs inhibit T cell proliferation. Many mechanisms have been proposed, including cross talk with immature DCs and triggering DCs to produce IDO. Regulatory T cells have been found in human decidua during early pregnancy. In mice, maternal Tregs comprise approximately 20 % of decidual $CD4^+$ T cells and were able to suppress proliferation of autologous stimulated T cells and rescue pregnancy in abortion prone mice [11, 12]. In human pregnancy, it has been shown that the amount of Tregs was reduced in decidual samples from recurrent abortions [13].

1.2.5 γδ T lymphocytes

γδ T cells with a γ chain and a δ chain in their receptor have the capacity to respond quickly without the need of expansion of their specific clone. In contrast to αβ T cells, most of the γδ T cells do not express CD4 or CD8 molecules. The γδ T cells respond to cell stress and can kill transformed or infected cells through the FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or NKG2D receptor-ligand system. In addition, they

produce immunomodulatory cytokines that can work both suppressive and stimulatory on the immune system [14]. γδ T cells are present in decidua of all mammals and are increased during pregnancy in the decidua as well as in the peripheral blood. The expression of TCRγδ in the pregnant uterus is hormonally controlled. γδ T cells in the peripheral blood of pregnant women express progesterone receptors [2, 15].

1.2.6 NKT cells

Natural killer T cells are a unique group of T lymphocytes that express both the TCRαβ chain and the NK cell receptors. A special group of NKT cells that was the first to be discovered and described is the NKT cells carrying an invariant α chain in their TCR, Vα14 in mice and Vα24 in humans. These cells are usually CD4 and CD8 negative, although some express CD4. They produce a huge amount of cytokines, such as IL-4, TNF-α and IFN-γ, and have cytotoxic ability [16]. NKT cells are present both in peripheral blood and the decidua of pregnant women. The NKT cells recognize antigens in the context of CD1d. CD1d is expressed by VT and EVT [2, 4].

1.2.7 NK cells

The NK cells, one of the major cellular components of the innate branch of the immune system, possess the ability to lyse target cells in a MHC-independent manner and to produce cytokines and chemokines. They participate in the early innate immune responses and, by immunosurveillance, may play an important role in homeostasis and anti-tumor defense. The NK cells recognize and kill abnormal cells, like virally infected- and tumor cells by the "cytotoxic hit". The recognition of targets by NK cells is described by the so-called "missing self" hypothesis proposed by Kärre et al. [17]. According to this hypothesis the NK cells recognize and react to the presence/absence of MHC molecules on the target cells. Recognition of intact MHC molecules inhibits NK cell cytotoxicity, while absence or abnormal MHC stimulates their killing capacity. Thus, stressed, infected and transformed cells that down-regulate their MHC class I antigen expression to escape detection by cytotoxic $CD8^+$ T cells, will instead be recognized and killed by activated NK cells [18].

NK cells recognize their targets by a set of activating and inhibitory NK cell receptors that regulate their lytic and/or cytokine producing capability. There are two major types of NK receptors that include activating and inhibitory receptors: the immunoglobulin superfamily and the C-type lectin-like family. There are inhibitory receptor subfamilies: the killer cell-Ig-like receptors (KIR), the CD94/NKG2A lectin-like receptor and, the murine ly49 lectin-like receptors that are not found in humans. The activating receptor subfamilies that trigger NK cell-mediated cytotoxicity consist of activating members of KIR, CD94/NKG2C and the activating killer cell receptor NKG2D [19].

The uNK cells, $CD56^{+bright}/CD16^{-}$, in contrast to peripheral blood $CD56^{+dim}/CD16^{+}$ NK cells, are the dominating leukocyte population in the fetal-maternal interface during early pregnancy and in the endometrium before implantation. Uterine NK cell population decreases during pregnancy and is absent at pregnancy termination. Their cytotoxic granules containing perforin, FasL and granzymes, suggest a cytotoxic potential. Although the $CD56^{+bright}/CD16^{-}$ uNK cells have dominated the reproductive immunology research for many decades their precise function is still not known. The role of NK cells in peripheral blood of pregnant women is not clear. There are contradictory reports, showing decreased or increased number of NK cells in the peripheral blood of pregnant women and in women with pregnancy failure [2].

1.3 Antigen-presenting molecules

1.3.1 Major histocompatibility complex

The major histocompatibility complex, also called HLA in humans, encodes 2 types of polymorphic proteins, the MHC class I and class II molecules. Class I and II function in antigen presentation to T cells. In general, class I molecules, expressed on nucleated cells, present processed endogenous antigens to $CD8^{+}$ CTLs, while class II molecules, expressed on APCs, including MΦ, DCs and B cells, present processed exogenous antigens to $CD4^{+}$ Th cells [1].

1.3.1.1 Classical HLA molecules

The class I molecules consist of a large glycoprotein α chain and β_2 microglobulin and are encoded by three different loci on human chromosome 6, HLA-A, HLA-B and HLA-C.

The endogenous antigens presented by class I molecules are degraded into peptides intracellularly, assembled together with the class I molecule in the endoplasmatic reticulum, transported through the complex of Golgi and presented on the cellular surface of nucleated cells together with β_2 microglobulin. The MHC class II molecule, expressed on B cells, MΦ and DCs are composed of two glycoproteins, the α and β chains. There are three major class II proteins designated HLA-DR, HLA-DQ and HLA-DP encoded by their polymorphic loci on chromosome 6. Exogenous peptide presentation by class II molecules involves: i) internalization and degradation of proteins within the endosomes and lysosome of the cell by digestive enzymes, and ii) binding of the exogenous peptides with class II molecules, for subsequent presentation to $CD4^+$ T cells, that takes place in the endosome [1]. The VT in human placenta does not express MHC class I and II molecules [2].

1.3.1.2 Non classical HLA molecules

The non classical MHC class I molecules are a diverse group of proteins including HLA-G, HLA-E, HLA-F and CD1. The HLA-G, E and F, structurally related to the classical MHC class I molecules, are not polymorphic as the classical ones [20]. The CD1 molecules do not pair with β2-microglobulin. The EVT cells express a unique combination of HLA-E, HLA-C, HLA-G and CD1d that has an important immunomodulatory function in the human placenta [21-23].

1.4 Toll-like receptors

Toll-like receptors (TLRs) are transmembrane proteins with extracellular domains. Today, there are ten TLRs known to be expressed in humans and they are mainly expressed on MΦ but can also be found on neutrophils, eosinophils, epithelial cells and keratinocytes. The TLRs recognize pathogen-associated molecular patterns (PAMP) such as LPS on bacteria, peptidoglycans, bacterial flagellar proteins and viral double-stranded RNA. Toll-like receptors also recognize endogenous molecules such as heat shock proteins (HSP) and dsDNA [24]. Activation of most TLRs programs $CD4^+$ T cells to Th1 response, although they can also induce Th2 response [25]. All ten TLRs are expressed by trophoblast cells in human placenta. The expression pattern varies by gestational age and trophoblast type. In first trimester placenta, the cytotrophoblast (CTB) and EVT express TLR-2 and TLR-4. In

contrast, the syncytiotrophoblast (STB) lacks expression, indicating that the placenta will respond to invading microorganisms only if they pass through the STB [25].

1.5 Cytokines

Cytokines are small proteins of low-molecular weight produced and secreted by a variety of cells. They play a major role in the induction and regulation of different cellular responses by activating intracellular signal transduction pathways that lead to various functions. There is a high number of different cytokines and most of them fall into one of the following families: hematopoietins, interferons, interleukins, chemokines or tumor necrosis factor family. The cytokines are receptor dependent and can only act on cells that have their cognate receptors. Most cytokine receptors signal through the Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) proteins. Antigen-stimulation of Th cells in the presence of cytokines can stimulate cellular immunity and Th1 response, including IFN-γ, IL-2, TNF-α, TNF-β, IL-12 and IL-15 secretion, or humoral immunity and Th2 response, including IL-4, IL-5, IL-9, IL-10 and IL-13 secretion [1]. During pregnancy, there is a shift towards anti-inflammatory Th2 response in the systemic maternal immunity, although there is no consensus as to whether it is due to an increase in Th2 cytokine production or a decrease in Th1 cytokine production [2].

1.6 Complement system

The complement system consists of a series of plasma and cell surface proteins with important effector functions in innate and adaptive immune responses. Activation of the complement cascade results in cell lysis, opsonization of bacteria, inflammation and clearance of immune complexes. The complement system is induced by three different pathways: the classical, the alternative or the lectin pathway that all activate the same attack membrane complex of proteins [1]. During pregnancy, a complement-attack of the semiallogeneic placenta is prevented by expression of complement regulatory proteins on trophoblast cells [2].

1.7 Immunosurveillance

The immunosurveillance theory was first described by Burnet and Thomas [26, 27], who proposed that tumor cell-specific antigens provoke an effective immunological reaction that would eliminate cancer development. Today the immunosurveillance theory is suggested as a process consisting of three phases: elimination, equilibrium and escape. Elimination represents the classical concept of immunosurveillance. The equilibrium phase refers to the immune-mediated latency after incomplete killing of cancer cells when the remaining cancer cells continue to proliferate. The escape phase is the period when the cells that have avoided the immune system expand and become clinically detectable [28].

Both the innate- and the adaptive immune responses are involved in immunosurveillance. They are modulated by the cellular origin of the tumor, mode of transformation, anatomical location, natural immunogenicity and the tumors ability to produce cytokines. The anti-tumor immune response occurs as a consequence of activation of DCs, αβ T cells, γδ T cells, NK cells and NKT cells. IFN-γ secretion by these cells is an important factor in anti-tumor defense. This secretion has two effects: i) increased expression of MHC class I on cancer cells enhancing their immunogenicity and ii) promotion of the cytotoxic immune response thus eliminating cancer cells. Regulatory T cells are immunosuppressive by nature and thus have the capacity to protect cancer cells from immune attack via secretion of inhibitory cytokines such as TGF-β and IL-10 [28, 29].

Other effector mechanisms involved in immunosurveillance are the inducers of apoptosis: TRAIL and the Fas-FasL system. It has been shown that TRAIL expression protects from cancer development and that the protective effect was dependent on IFN-γ [30]. “Soluble” FasL can be found in two different biological forms - a soluble form produced by proteinase-cleavage of its membranal form, and a membranal “soluble” form on secreted exosomes. Microvesicles and exosomes bearing FasL, shed by human placenta and cancer cells, have been shown to promote a state of immune privilege and induce apoptosis of immune cells through Fas-FasL interactions [9, 31-34]. However, the NKG2D receptor-ligand system, which is in focus of this thesis, plays the most central role in immunosurveillance.

1.8 The NKG2D receptor-ligand system

NKG2D receptor-ligand interaction is one of the major cytotoxic effector mechanisms critically important in elimination of infected, stressed and/or transformed cells. The importance of the NKG2D receptor in NK cell activation is illustrated by the fact that engagement of NKG2D with one of its many ligands bypasses any inhibitory signal from other NK receptors, leading to killing of the NK cell target [35]. Moreover, in mice, NKG2D receptor-ligand system deficiency promotes the development of spontaneous tumors [36].

1.8.1 The NKG2D (KLRK1) receptor

NKG2D was first identified in 1991 as "natural killer group 2, member D". It has a low homology to the other receptors in this group with only 21 % sequence identity. In contrast to the other members, which are heterodimers and pair with CD94, the NKG2D receptor forms a homodimer at the cell surface [37, 38].

In humans, NKG2D is expressed on all NK cells, some γδ T cells and $CD8^+$ αβ T cells [39]. NKG2D serves as a primary activating receptor of NK cells triggering cytotoxicity and cytokine production. On T cells, it acts as a co-stimulatory receptor that can stimulate the activation of naïve T cells or trigger cytotoxicity [40]. In contrast to murine NKG2D, which expression is not affected by cytokines, human NKG2D is up-regulated by IL-15, IL-10, IL-12, IFN-α and downregulated by TGF-β and IL-21 [41].

NKG2D is a type II transmembrane glycoprotein and a member of the C-type (calcium-dependent) lectin family [39]. In mammals, the signalling of the receptor is mediated by signaling adaptors, DAP10 and DAP 12. Human NKG2D uses DAP10 as the only adaptor. Mouse NKG2D can use both DAP10 and DAP12. This is determined by alternative splicing which generates two different transcripts, a long and a short isoform of NKG2D. The long isoform, NKG2D-L, is related with DAP10 and is constitutively expressed on all human NK cells while the short form, NKG2D-S, is related to DAP10 and DAP12 and expressed only on murine activated NK cells [35, 42, 43]. Engagement of the NKG2D receptor with its ligand causes cellular internalization of the receptor-ligand complex which leads to down-modulation of NK cell cytotoxicity [44].

1.8.2 The NKG2D ligands

Although related to the MHC class I antigens, NKG2D ligands do not present antigens but serve as antigen themselves, and upon cross linking to the NKG2D receptor trigger a range of immune effector functions such as cytotoxicity, cytokine production and cell proliferation [45]. In mammals, the NKG2D receptor recognizes groups of ligands that are distantly related to the MHC class I molecules. In humans there are eight proteins that serve as ligands of the NKG2D receptor, they are grouped into two families: the MHC class I Chain-related proteins A (MICA) and B (MICB) and the cytomegalovirus UL16-binding proteins (ULBP) 1-6, named so because some of the ULBP molecules have the ability to bind cytomegalovirus UL16 protein. The ULBP are also known as retinoic acid early transcript1 (RAET1) proteins [39, 46-51]. In mice, NKG2D binds to five retinoic acid early transcripts (RAE-1α-ε), three H60 minor histocompatibility antigen and murine UL16-binding-protein-like transcripts-1 (MULT1) [52]. The NKG2D ligands share little sequence similarity, only 20-25 % between the two families, and they vary in expression pattern, domain structure, cellular localization and binding affinity to NKG2D [53, 54]. NKG2D ligands are highly polymorphic, particularly MICA and MICB, for which 70 and 31 different alleles have been described, respectively [55, 56].

MIC proteins A and B comprise three extracellular domains (α1-3), a transmembrane region and a cytoplasmic tail, like classical HLA class I heavy chains, but do not associate with β_2-microglobulin or carry peptides [49]. ULBP1-3 are GPI anchored proteins while ULBP4/RAET1E and ULBP5/RAET1G are type I transmembrane proteins. In contrast to MIC the ULBP family lacks the α3 domain and contains only MHC class I-like α1 and α2 domains [45].

MIC molecules are stress-induced molecules since it has been shown that they could be induced by heat shock [57]. MICA and MICB have heat shock promoter elements, in contrast to ULBP that lack these motifs. MIC and ULBP molecules are up-regulated by DNA damage, oxidative stress, inflammation (autoimmune diseases, viral and bacterial infections) as well as in a broad range of different cancers [58]. MIC and ULBP are expressed in normal bronchial cells, intestinal epithelium, placenta, muscle cells and the skin [47, 57, 59-62].

A discrepancy between mRNA and protein expression of NKG2D ligands suggest transcriptional- and post transcriptional regulation. For example, microRNAs, which have been shown to bind 3'UTRs of MICA and MICB, effectively suppress the expression levels of MIC molecules [63]. The regulation of expression of NKG2D ligands in response to stress is mediated, in part, through the DNA damage pathway, which involves: i) ataxia telangiectasia and Rad 3 related (ATR) protein, primarily involved in sensing cells that do not proliferate appropriate; ii) ataxia telangiectasia mutated (ATM) protein, detecting double stranded DNA breaks; and iii) checkpoint kinase 1 (Chk1), a kinase involved in the signal cascade triggered by these two molecules [64]. NKG2D ligands are expressed both on the cell surface as well as intracellularly. For example, in normal bronchial epithelium, MIC and ULBP1-4 were expressed intracellularly. However, when the cells were stimulated by oxidative stress, the NKG2D ligands were expressed on the surface. These results suggest that there is a post translational regulation of the NKG2D ligand expression [60]. The intracellular fate of the NKG2D ligands is not well known. Recently it was shown that NKG2D ligand proteins in mice undergo ubiquitination, resulting in rapid degradation [65].

1.8.3 The lytic machinery and the cytotoxic hit mediated by the NKG2D receptor

As mentioned before, activation of NKG2D in NK cells results in cytokine secretion and/or killing by the “cytotoxic hit”. In the cytolytic granule exocytosis pathway, after NKG2D receptor-ligand ligation, cytoplasmic granules containing perforin, granzymes and granulysin are secreted. Perforin assists the granzymes to enter the target cells where they cleave different targets, including procaspases, inducing cell death by apoptosis [8, 66].

2. Mammalian pregnancy

2.1 Placenta is a unique temporary organ for pregnancy success

2.1.1 General description

Placenta is essential for mammalian pregnancy. It is a temporary organ, produced during embryogenesis that mediates the physiological and functional connection between the mother and the fetus. Placenta functions as a nutrition and waste exchanger and has important endocrine properties crucial for the pregnancy success. The placenta contains both maternal and paternal genes. However, due to genomic imprinting, the paternal genes are preferentially expressed in placenta [67-69].

The fetus is never in direct contact with the maternal blood or tissue, instead the placenta works as a barrier between the fetal and maternal blood. The mammalian placentas are classified into four categories: 1) epitheliochorial, 2) synepitheliochorial, 3) endotheliochorial and 4) hemochorial. In the epitheliochorial placenta, present in pigs and horses, the trophoblast is in direct contact with uterine epithelium. In the synepitheliochorial placenta, present in sheep, cows, goats and deer, the maternal epithelium is partly preserved and the uterine mucosa is in contact with the invasive trophoblasts. The placenta of dogs and cats is called endotheliochorial because of direct apposition between the trophoblast and the endothelial cells of the maternal blood vessels. The most invasive placenta is the hemochorial, present in humans, rodent and primates. The trophoblast cells invade the maternal tissue, reaching the decidua, the inner third of the myometrium and the maternal spiral arteries [70].

2.1.2 Morphological organisation of human early pregnancy placenta

The chorionic villi, irrigated by maternal blood, are the main functional unit of the human placenta (fig.1). The pluripotent CTB differentiates into two distinct cell types: i) the EVT invading the maternal pregnant uterine mucosa, the decidua, and participating in the endovascular remodelling of the spiral arteries during placenta formation, ii) the STB, lining the outermost layer of the chorionic villi and in contact with the maternal blood. Extravillous trophoblast differentiates into two subpopulations: interstitial EVT that invades as far as the inner third of the myometrium and differentiates into giant multinucleated

cells, and endovascular EVT. Increased blood flow into the placental intervillous space, needed to successfully support the growing fetus, is achieved by remodelling of the spiral artery wall and invasion of the EVT. The invasion results in disruption of the ECM until the trophoblast reaches the vessels and replaces the endothelium with a trophoblast layer [71]. Shallow EVT invasion and defective vascular remodelling of the spiral artery may result in impaired placental blood perfusion and can lead to complications of pregnancy, such as pre/eclampsia [72]. Syncytiotrophoblast is composed of multinucleated syncytium without cell borders. Usually, the chorionic villi consist of one layer of CTB, covered by a layer of the multinucleated STB, stromal mesenchymal cells and fetal blood vessels. Thus, human early pregnancy placenta consists of four cell layers separating the fetal and the maternal blood. It is referred to as the *placental barrier* and counts from the maternal blood: syncytiotrophoblast-, cytotrophoblast-, mesenchymal- and endothelial cell layers [2, 73].

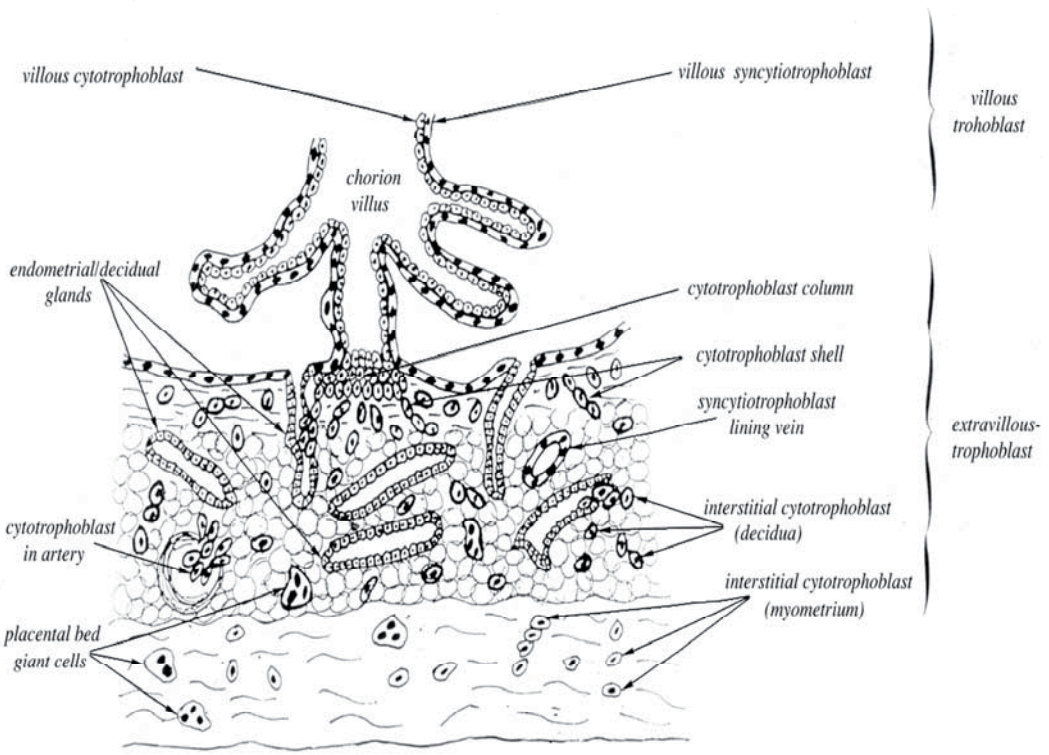

Figure 1. Schematic presentation of different trophoblast subpopulations in the chorionic villi and in the placental bed (illustration from ref. [74])

2.2 Human trophoblast - the main cells of the placenta with key importance in pregnancy

2.2.1 Phenotypic and functional characteristics of villous trophoblast

All trophoblast cells express cytokeratines 7, 8 and 18, indicating their epithelial origin [75]. Adhesion molecules are widely expressed by the trophoblast cells. The calcium dependent molecule E-cadherin, mediating homotypic adhesion between cells, is specific for CTB. Other adhesion molecules such as the integrins are expressed by different subsets of trophoblast cells and seem to be associated with their invasive behaviour. The CTB expresses integrins $\alpha v\beta 5$ and $\alpha_6\beta_4$ [76-78].

As previously described, in humans, the chorionic villi covered by the STB are in direct contact with the maternal blood and participate in the fetal nutrition, gas- and waste exchange. Respiratory gases diffuse from the maternal blood through the entire STB plasma membrane. This diffusion is dependent on the flow rates of the umbilical- and uterine circulation [79]. The STB is the major producer of placental hormones and, thus is an important factor for pregnancy success. Villous trophoblast secretes polypeptide hormones, like human chorionic gonadotropin (hCG), human placental lactogen (hPL) (also called human chorionic somatomammotropic hormone (hCS)), placental growth hormone (GH), and the steroid hormones progesterone and estrogen [80, 81].

Human chorionic gonadotropin is crucial for human pregnancy and works as an agonist for luteinizing hormone (LH), rescues the corpus luteum from involution ensuring the maintenance of ovarian progesterone secretion [80]. Besides being essential for the successful pregnancy, progesterone has a suppressive effect on the immune response [82]. Interestingly, it has been shown that progesterone has other effects as well, such as modifying the $GABA_A$ receptor in the central nervous system [83, 84]. The role of hPL in the placenta is not completely clear. Normal pregnancies have been described in the absence of hPL secretion. Growth hormones, produced by the placenta, are proposed to have a metabolic role on the maternal organism during pregnancy, e.g. involvement in the insulin resistance [80].

The villous trophoblast cells are semiallogeneic and should be attacked by the maternal immune cells. To avoid the immune response, the STB cells do not express classical MHC

molecules, but express important molecules that are involved in immune modulation. Two of them are PD-L1, also called CD240 and B7-H1, which is detected on the STB, and the related type I membrane protein CD200 [22]. Moreover, STB expresses a variety of complement regulatory proteins such as decay accelerating factor, CD46 and CD59, protecting placenta from complement attack [22]. In addition, STB expresses FasL. In humans, FasL are expressed within STB as endosomal vesicles [9, 31].

2.2.2 Phenotypic and functional characteristics of extravillous trophoblast

Extravillous trophoblast invasion is dependent on detachment of CTB cells from the basement membrane. The CTB cells undergo a proliferative burst and differentiate into cells of the CTB column, anchoring the peripheral villi to the decidual bed. This anchoring cell subpopulation and the transition from CTB to the migratory EVT are mediated by contact between the migratory EVT and the decidual extracellular matrix (ECM). This adhesion is due to fibronectin-mediated extracellular matrix binding. Fetal fibronectin or trophuteronectin (TUN) are produced by the EVT. Transforming growth factor β and leukemia inhibitory factor (LIF) have been shown to inhibit the trophoblast-differentiation into an invasive phenotype. However, there are other reports showing that LIF increases the invasion of first trimester EVT and mediates adhesion to ECM. Extravillous trophoblast expresses receptors for laminin, fibronectin and integrins. The integrin expression changes when CTB differentiates from a villous to an extravillous phenotype, with down-regulation of $\alpha_6\beta_4$ and up-regulation of $\alpha_5\beta_1$ integrin [85-87]. Interestingly, when the EVT has invaded the spiral arteries, it mimics the endothelium by expression of the vascular cell adhesion molecule 1 (VCAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) [76, 88]. As described earlier, EVT expresses a unique combination of HLA-E, HLA-C and HLA-G [21-23]. Like STB, EVT expresses the immune modulatory protein PD-L1 [22].

3. Immune escape - a common strategy for pregnancy and cancer

Although the placenta is a normal tissue, its principal cells, the trophoblasts, share several features with malignant cells. Cancer is a disease originating from alteration in the cellular genome resulting in an invasive and proliferating tumor that, like placenta, moulds its own environment to favour its survival and expansion. Despite the fact that trophoblast and cancer cells are both immunogenic tissues, they are both able to escape from the host immunosurveillance [2].

3.1 Trophoblast and cancer cells share many biological features

In common, cancer cells and trophoblast share many biological characteristics, including their capacity for proliferation, migration, invasion and establishment of blood supply (table 1). Both cancer cells and trophoblast cells have increased telomerase activity, reflecting their high proliferative capacity [89, 90]. Other mediators that promote proliferation and inhibit apoptosis, are survivin, which is overexpressed by cancer- and trophoblast cells [91, 92], and the insulin growth factor (IGF). Additionally, IGF protects cancer cells from destructive effects of chemotherapy and radiation [93, 94]. Several proto-oncogenes encoding growth-factors are expressed by cancer- and trophoblast cells [95].

Functional intrinsic capabilities required for malignancy trait	**Cancer cells**	**Trophoblast cells**
Self-sufficiency in growth signals	Yes	Yes
Insensitivity to anti-growth signals	Yes	Yes
Resistance to apoptosis	Yes	Yes
Limitless replicative potential	Yes	Yes
Sustained angiogenesis	Yes	Yes
Tissue invasion and metastasis	Yes	Yes, invade and disseminate

Table I. Human cancer- and normal trophoblast cells share six intrinsic characteristics

Many similarities can be observed between invasive EVT and cancer cells, including altering cell adhesion molecules, secretion of proteases and growth factors. Changes in adhesion molecule expression such as integrins, secretion of E-cadherin and epithelial-mesenchymal transition, a cellular program that allows polarized, immotile epithelial cells

to convert to motile mesenchymal cells, are mechanisms that trophoblast cells and cancer cells use to lose polarity and enhance motility [96-98]. Epidermal growth factor (EGF) and the Wnt signaling pathway are involved in switching cancer cells and trophoblast cells from proliferative to invasive phenotype [99-102].

Blood supply is crucial for survival of the cancer and the fetus. The process that is responsible for this is called *vasculogenic mimicry*, in which cells, other than endothelial cells, form vascular structures [103, 104]. Vascular endothelial growth factor (VEGF), angiopoetins and the oxygen-sensitive mammalian target of rapamycin (MTOR)-pathway are other substances and mechanisms important for the angiogenesis in many tumors and crucial for the spiral artery remodelling during placenta formation [105-108].

3.2 Trophoblast and cancer use similar immune escape mechanisms

Trophoblast and cancer cells do not only share many proliferative and invasive features, additionally they actively modulate the host immune response. Uterine NK cells are the most abundant immune cells at the fetal-maternal interface. One mechanism of recruitment of uNK cells from the blood is IL-15 secretion by endometrial stromal cells [4]. The same mechanism has been shown in numerous malignancies where NK cells infiltrate in response to IL-15 [109].

Cells that infiltrate the fetal-maternal interface and play important roles in pregnancy and cancers are MΦ, Tregs and DCs. Macrophages in the decidua secrete IL-10 and contribute to a tolerogenic Th2 milieu [4] while MΦ associated with cancer can be both immunosuppressive and inflammatory [110]. The amount of Tregs, expressing CD4, CD25 and FOXP3, are significantly increased in decidua [4, 11]. A similar expansion of Tregs can be seen in cancer, contributing to impaired antitumor immunity [111].

HLA-G expression on EVT suppresses killing by both NK- and cytotoxic T cells, regulates cytokine production in blood mononuclear cells, induces apoptosis of immune cells, and impairs maturation of DCs. The immune inhibitory effect of HLA-G is due to binding to the inhibitory receptors, immunoglobulin-like transcripts (ILT-2 and ILT-4), expressed on myeloid and lymphoid cells. There are several reports showing HLA-G expression in a

wide variety of cancers, although there are some controversies about these findings [21, 23, 112, 113]. A similar immune-inhibitory effect of HLA-G in cancer has been suggested. A soluble form of HLA-G has been found in peripheral blood of pregnant women impairing NK/DC cross-talk, promoting inflammation and apoptosis. Similarly, soluble HLA-G has been reported in serum of cancer patients. Additionally, HLA-G has also been found on exosomes in melanoma patients [23, 114-117]. Another soluble immunomodulator, CD30, a marker for Th2 polarization, is overexpressed by B cells in pregnant women as well as by B cells in cancer patients. Reduced expression of CD30 is related to pathological pregnancies suggesting a role in immunomodulation during pregnancy [118, 119].

3.3 Exosome secretion is a way of intercellular communication and generation of "soluble" bioactive ligands

Membrane vesicles are classified based on their cellular origin, shape, and presence of a surrounding membrane. Membrane vesicles are produced by a vast majority of cells such as reticulocytes, mast cells, T and B cells, platelets, DCs, neurons and microglia, intestinal epithelia, uroepithelia, bronchial epithelia, hepatocytes, syncytiotrophoblast and tumor cells [61, 120-134]. Furthermore, membrane vesicles have been found in physiological fluids, such as saliva, urine, plasma, synovial fluid, amniotic fluid, malignant effusions, bronchial lavage fluid and breast milk [135-142]. There is a number of different types of membrane vesicles: plasma membrane microvesicles/microparticles, shed microvilli, apoptotic bodies, and exosomes. A summary of some of their properties is given in table 2 [143].

Characteristics	Exosomes	Microvesicles/ Microparticles	Shed microvilli	Apoptotic bodies/vesicles
Size	30-100 nm	0.1-2 μm	> 400 nm	100–600to700 nm
Density in sucrose	1.13 – 1.19 g/ml	Undetermined	Undetermined	1.16-1.28 g/ml
Sedimentation (g)	100,000 -110,000	10,000 -100,000	10,000	1,500 – 100,000
Morphological shape	Cup shaped, electron translucent	Various shapes, electron-dense and/or electron translucent	Various shapes, round, elongated and cylinder-like	Irregular and heterogeneous in shape
Lipid membrane composition	Cholesterol-, shingomyelin-, and ceramid-rich lipid rafts, expose phosphatidylserine	Expose phosphatidylserine, some enriched in cholesterol and diacylglycerol, some undetermined	Undetermined	Undetermined
Specific marker(s) for identification	Tetraspanins (CD63, CD9, CD83), ESCRT complex members (Alix, TSG101)	Integrins, selectins, CD40 and others, depending on the cell type	Various, depending on the cell type	Histones, DNA
Origin in the cell	Endosomal compartment - multivesicular bodies (MVB)	Plasma membrane	Plasma membrane	Fragments of dying cells, undetermined
Mechanism of sorting	Ceramid and ubiquitin dependent	Unknown	Unknown	Fragments of dying cells, undetermined
Intracellular storage	Yes	No	No	No
Mode of release/secretion	Exocytosis by fusion of MVB with the plasma membrane	Plasma membrane blebbing	Plasma membrane blebbing	Plasma membrane blebbing and cellular fragmentation

Table 2. Some characteristics of different microvesicles (table from ref. [144])

3.3.1 Definition of exosomes

Exosomes are small membrane-bound vesicles defined by the following characteristics: 1) cup-shaped form; 2) 30-100 nm in size; 3) density of 1,13-1,19 g/ml on sucrose gradient; 4) endosomal origin; 5) presence of tetraspanins in their lipid raft-rich membrane [132]. Exosomes were first described in 1983 by Johnstone and Stahl [120, 121] and their role in immunity was first suggested in 1996 by Raposo [124].

3.3.2 Biogenesis of exosomes

Exosome formation starts with endocytosis of proteins from the plasma membrane. This process occurs in different ways: clathrin dependent (e.g. transferrin receptor) or clathrin independent (e.g. glycosylphospatidylinositol (GPI)-anchored proteins). Then endocytosed molecules enter the early- and then the late endosome [145]. In early endosomes, membrane receptors are separated from their ligands. The endocytic vesicles then either recycle back to the plasma membrane or are transferred to the late endosomal compartments where intraluminal vesicles (ILV) are formed by inward budding forming multivesicular bodies (MVB). ILV can also be formed by direct transportation of proteins from the Golgi complex to MVB and insertion in the MVB limiting membrane. MVB can fuse with the plasma membrane and release ILV as exosomes, or fuse with the lysosome for protein degradation (fig.2) [132].

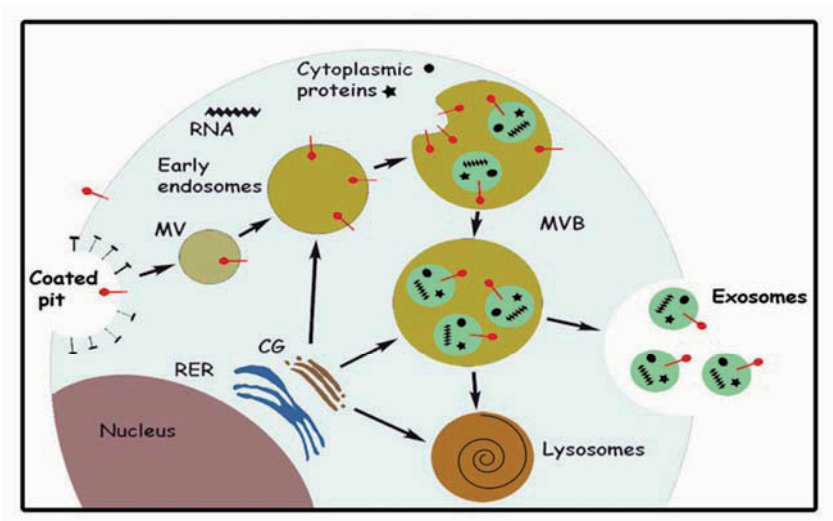

Figure 2. Generation of exosomes (from ref. [146]).

The mechanisms that determine the formation and fate of the ILV are not completely understood [147]. Lipids and tetraspanins in the limiting membrane of the MVB form microdomains, called lipid rafts, which seem to be involved in the sorting of proteins to the endosomal membrane. Other mechanisms, involved in the sorting to the endosomal limiting membrane and the forming of ILV, are the Endosomal Sorting Complex Required for

Transport (ESCRT) proteins. The ubiquitinylation process is tagging proteins of the endosomal membrane, targeting them for the ESCRT machinery. The ESCRT complex, which includes four protein complexes: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, sorts ubiquitinated transmembrane proteins into ILV. Additional mechanisms for sorting of non-ubiquitinated proteins through the ESCRT machinery has also been suggested [44, 129, 146, 148-150]. Recently, another sorting mechanism, independent of the ESCRT proteins, was described, involving sphingolipid ceramide [147, 151].

The mechanisms responsible for exocytosis of MVB and release of ILV as exosomes into the extracellular environment are not well known, although it has been shown that Rab11, Rab27a and Rab27b are involved in the docking of the MVB to the plasma membrane in a Ca^{2+} dependent manner. Additionally, the p53 protein and the transmembrane TSAP6 have been suggested to be involved in the regulation of exosome secretion [152-155].

3.3.3 Secreted exosomes - general characteristics and functions

Electron microscopy is still the most reliable method to study exosome morphology and biogenesis. Isolated exosomes are cup-shaped and heterogeneous in size, varying between 30-100 nm. Their membrane is composed of cholesterol, sphingolipids and tetraspanins. Today, there is no specific marker for exosomes, although they are typically enriched in proteins from endosomes such as CD63, Alix, TSG101, CD9, CD81 and CD82 [156, 157]. In addition, exosomes contain mRNAs, microRNAs and a high variety of different membranous and cytoskeletal proteins [156, 158]. Exosomes also contain cell specific proteins which enable tracking of the producing cells [122, 125, 126, 129, 146, 159].

The secretion of exosomes is a potent way of communication between cells. The benefits of secretion of proteins via exosomes are numerous: i) preservation of the three-dimensional structure of the protein, and thus their biological activity; ii) independence from cell-to-cell contact for signal delivery; iii) lower mobility and higher concentration of the carried molecules; iv) independence from *de novo* protein synthesis; v) biological effects at a distance [146].

Exosomes can be divided into immune activating and immune suppressive. Most of the exosomes produced by immune cells such as DCs, MΦ or B cells are immune activating, i.e. antigen presenting, acting directly or indirectly to activate immune effector mechanisms such as cytokine and antibody production, cytotoxicity and activation of T cells [143]. Whether exosomes can stimulate T cells directly or need the presence of DCs has been debated. Several studies have demonstrated that exosomes can stimulate T cells directly [160, 161] while others have shown that exosomes exert their effect through APCs [162]. Recent publications show that antigen-loaded exosomes derived from DCs alone augment the specific T cell response and that this effect was depending on B cells [160, 161]. Exosomes have been suggested to be an immune stimulatory factor in allergic immune response. B cell-derived exosomes that present peptides causing allergy can stimulate peptide-specific T cells to produce Th2-like cytokines [163].

The majority of exosomes, released by normal intestinal epithelia and cancer cells are immune suppressive. Exosomes produced by human intestinal epithelium have been suggested to play a role in oral tolerance and referred to as tolerosomes [134, 164]. Exosomes, most likely released from intestinal epithelia of antigen-fed rats can induce tolerance to the antigen when injected in naïve recipients and this tolerance is MHC class II dependent [134, 165, 166]. Cancer exosomes and placental exosomes suppress the host's immune defense by decoy-mechanisms of receptor down-regulation, apoptosis and Treg induction [9, 31, 33, 61, 134, 167-170]. Taylor et al. have shown that the amount of placenta-derived exosomes found in sera of pregnant women was significantly higher in those delivering at term compared to those delivering preterm. These exosomes, carrying biologically active components, such as FasL, induce T cell suppression via CD3-ζ and JAK3 [33]. We and others have shown that placenta releases exosomes, carrying immune suppressive molecules, e.g. NKG2D ligands, and FasL that suppress the maternal immune system [9, 31, 61, 171].

AIMS OF THE INVESTIGATION

The overall objective of this investigation was to study the expression, regulation and function of the NKG2D receptor-ligand system in pregnancy and cancer. We hypothesized that placenta and tumors escape NKG2D receptor-mediated immunosurveillance by generation of an exosomal form of NKG2D ligands.

The specific aims were:

- To investigate the mRNA transcription and protein expression of the NKG2D ligands MIC and ULBPs in normal human placenta and tumors.

- To isolate and characterise NKG2D ligand-bearing exosomes secreted from human placental explant cultures and T and B leukemia/lymphoma cells.

- To examine the effect of biological stress on the production and secretion of NKG2D ligand-bearing exosomes in cancer cells.

- To study the effect of NKG2D ligand-bearing placental and tumor exosomes on the down-modulation of the NKG2D receptor and its consequence for NK cytotoxicity.

- As a prerequisite and a corollary of the above investigation, an optimized technique for isolation of human early villous trophoblast was developed as a potential method for *in vitro* production of exosomes.

RESULTS AND DISCUSSION

In this section, I will present and discuss the main results of my work. The papers are referred to in the thesis by their Roman numbers (I-III).

4. Methodological considerations

4.1 Isolation of villous trophoblast cells from human early pregnancy placenta

Human placenta is a unique organ that governs pregnancy and as such a focus for research in reproduction. Studies of human placenta must be done on the organ itself and/or cells isolated from the placenta and cannot be replaced by animal models for biochemical and functional studies. The most important cell type responsible for nutrition and gas exchange to the fetus, and production of bioactive molecules such as pregnancy-related hormones, cytokines, chemokines and other immunomodulatory molecules, metalloproteases, adhesion molecules and growth factors is the STB that comprises the outermost cell layer of the placental villi which is in direct contact with the maternal blood. To isolate STB from early pregnancy placenta for our molecular and functional studies was a prerequisite for our further studies.

There are several reports describing methods for isolation of trophoblast cells from term placenta [172]. In these methods, using combinations of digestive enzymes and density gradient centrifugation, term CTB cells were obtained. However, there were very few methods for isolation of VT from human early placenta and these methods gave yields of throphoblast cells heavily contaminated with leukocytes [173]. Thus, there was a need to optimize a method for isolation of VT from early pregnancy placenta to get a pure trophoblast. At the same time, we needed a gentle isolation method that could allow us to (1) use isolated STB in molecular studies for transcription and expression of different genes; (2) obtain CTB for establishment of primary human trophoblast cultures; and (3) establish long term trophoblast cultures for harvesting of exosomes for future studies. In paper I we present an optimized and easy technique for isolation of VT from human early (8-14 weeks) normal placenta.

4.1.1 Description and advantages of our isolation procedure

The procedure includes three steps: (1) tissue disruption by treatment with a mild enzymatic cocktail, (2) Percoll gradient centrifugation for enrichment of trophoblast cells and (3) depletion of contaminating leukocytes using immunomagnetic beads coated with anti CD45 andibodies. The isolation procedure is illustrated in fig.1 paper I. Our isolation method gives a good yield of isolated trophoblast cells with preserved morphology and high viability (fig.2 and fig.4 paper I). The trophoblastic origin of the isolated cells was proved by cytokeratin 7 staining. We found that more than 95% of them were positively stained, showing a high purity of isolated cells, composed of both CTB and STB (fig.3 paper I). To obtain a single CTB population, a negative selection with magnetic beads coated with specific antibodies to surface molecules expressed on the STB cells, e.g. anti-MICA [61] or anti-PLAP, can be used. The positively selected STB, bound on magnetic beads can be used for RNA or DNA extraction and molecular studies. In summary, we have developed an easy and time-saving method that give us good yield of pure VT cells with preserved morphology, well suited for phenotypic, morphological and functional studies of the VT cells in early human placenta. Isolated VT cells were used in our molecular studies with quantitative RT-PCR technique, in immunoflow cytometry experiments for assessing the expression of NKG2D ligands and for western blot analyses (papers I and II).

4.2 Placental explant cultures

Culture of placental explants was used to obtain supernatants from which placental exosomes were isolated. Our placental explant cultures were performed for two reasons. From one side, we wanted to get exosomes produced by placenta only, but we could not use blood from pregnant women because several organs contribute to exosome secretion in peripheral blood. From another side, we wanted to obtain exosomes from an experimental setting that resembles, or comes as near as possible, to the *in vivo* situation. Although far from perfect, placental explant cultures are so far the only way to "mimic" an *in vivo* situation where placenta secreted substances can be collected in a culture medium. Dissected chorion villi of 5-10 mg wet weight from early normal human placenta were cultured in RPMI 1640 supplemented with 0.5 % BSA and antibiotics at 37^{o}C in 5 % CO_2 and humidified air. The supernatant was collected after 24-hour culture and kept frozen until exosome isolation. Since we were interested in isolation of secreted exosomes,

precautions were taken to avoid microvesicles released by dead cells. To minimize cell death, the time between extraction of placenta and setting of explant cultures was kept very short, the tissue was handled with great care, the explant dissection was done with gentle techniques and the culture time was limited to 24 hours. All isolated placental exosomes used in our experiments were produced by explant cultures (paper II).

4.3 Isolation of exosomes

Exosomes are present in human blood, saliva, urine, breast milk and other bodily effusions together with other microvesicles, shed from the cellular plasma membrane, and apoptotic bodies produced by dying cells. An important issue when studying exosomes is to be able to obtain a pure population of exosomes separated from other contaminating microvesicles. Many physical and chemical properties of exosomes and shed microvesicles are close to each other (table 2) and this demands stringent purification procedures to ensure that pure exosome population is obtained. This is even more important in studies of placental exosomes since it is known that the STB constitutively releases not only exosomes but also large amounts of microvesicles/microparticles shed from the apical part of the plasma membrane. Our method for exosome isolation, described in paper II and III, comprises a combination of ultracentrifugation and a continuous sucrose gradient (floating density 1.02–1.19 g/ml) or a sucrose cushion, thus ensuring exosome purity and minimizing contamination by other microvesicles. We have also continuously examined the purity of our exosome isolations by electron microscopy. Further, in all immunoflow cytometric work presented here, exosomes loaded on latex beads directly or via antibody capture, are used according to recommended protocol [174].

4.4 Quantification of exosome secretion

Today, there is no well-established and reliable method for exosome quantification. The most frequently used methods are based on total exosomal protein measurements by BCA- or Bradford assays and densitometric analysis of western blot bands for exosomal markers [175]. Recently, fluorescence intensity measurements of exosomes labelled with lipophilic fluorescence dyes has also been used [176]. To enhance the reliability of the quantification measurements of isolated exosomes we used these three different methods; BCA protein

measurement, lipid staining with Vybrant DiI and densitometric analysis of western blot bands of the exosomal marker CD63 (paper III).

5. The NKG2D receptor-ligand system in human pregnancy

We studied the NKG2D receptor-ligand system in human pregnancy for two main reasons: 1) the interaction of the activating NK cell receptor NKG2D and its inducible ligands is a central perforin-mediated cytotoxic pathway by which damaged-, transformed-, or infected cells are eliminated. Therefore, the NKG2D receptor-ligand system might be a potential threat to the fetus [177, 178], and 2) in tumors, soluble NKG2D ligands can bind to NKG2D and systemically down-regulate its expression on cytotoxic T cells and NK cells, providing a mechanism for tumor immune escape [40, 168, 179, 180]. Therefore, we asked the question: "Does placenta, similarly to tumors, generate and secrete soluble NKG2D ligands for immune escape?"

5.1 Expression of NKG2D ligands by human placenta

We investigated NKG2D ligand expression in human placenta by quantitative real time-PCR, flow cytometry, immunohistochemistry (IHC) and immunoelectron microscopy (IEM). Our molecular studies showed a constitutive gene expression of all NKG2D ligands (MICA/B and ULBP1-5). Moreover, all mRNA transcripts were translated into proteins. Flow cytometry analysis of isolated VT cells revealed MICA/B expression on the surface and intracellularly while ULBP1-5 expression was solely inside the cytoplasm (fig.1 paper II). These results were confirmed and further extended by IEM, which showed that all NKG2D ligands were exclusively expressed by STB (fig.2 paper II).

5.2 The NKG2D ligand molecules are processed, stored and secreted through the late endosomal compartment of syncytiotrophoblast

Our novel findings of NKG2D ligand expression in placenta raise the question: "Why are these molecules expressed in placenta and how are they processed, stored and secreted?" The precise mechanism that regulates the NKG2D ligand expression is still unknown. The shedding of NKG2D ligands from the cells as soluble molecules represents an additional level of complexity in this system. Recent reports demonstrate that the expression of MICB

molecules on the cell surface is accompanied by an intracellular accumulation of the molecules in the *trans*-Golgi network and late endosome-related compartments. MICB has a very short half-life at the cell surface due to clathrin-dependent endocytosis and/or shedding [181]. MICA allele 008, the most common MICA allele in Caucasians including Swedes, is released via exosomes from tumor cell lines, down-regulates the NKG2D receptor and impairs the cytotoxic response. The trafficking of MICA 008 protein to exosomes seems to depend on the truncated carboxy terminus of the MICA 008 molecule which is associated to an altered distribution to lipid rafts and ILV in MVB, in contrast to other alleles that are shed by proteolysis [40]. The GPI-anchored ULBP2 and 3 are released from the cell by different mechanisms: ULBP2 is mainly shed by metalloproteases while ULBP3 is mainly released on exosomes. Interestingly, exosomal ULBP3 was more effective for down-modulation of the NKG2D receptor than soluble ULBP2, released by proteolytic shedding [182].

Our IEM investigation showed different expression of the NKG2D ligands. MICA/B protein expression was concentrated to STB and existed in two forms: as a bipolar surface expression on the apical villous membrane of the STB that bathes in the maternal blood, on the basal membrane that faces the CTB, and in cytoplasmic vacuoles as MIC-stained microvesicles/exosomes [61]. In contrast, ULBP1-5 expression is restricted to the limiting membrane of numerous cytoplasmic vacuoles and tubule-like structures (fig.2 paper II). The vacuoles had the morphology of MVB of the late endosomal compartment that contained numerous tightly packed microvesicles. To further confirm that these ULBP-positive MVB are late endosomes, the placenta tissue was stained for TSG 101 [183] and the tetraspanin CD63 [184], as markers for the late endosome (fig.3 paper II). The limiting membrane as wells as the internal vesicles of MVB were positively stained for the ULBPs, indicating that the intraluminal microvesicles were formed by inward budding from the limiting membrane. The MVB were observed at different locations in the syncytioplasm, both at the perinuclear area and at the apical microvillous membrane, releasing their microvesicular content to the intervillous space (fig.2 paper II). Thus, it seems that newly synthesized ULBPs were directly transported from the *trans*-Golgi network to the MVB where they were located on the membrane of intraluminal vesicles/exosomes.

5.3 Placental explant cultures secrete NKG2D ligand-bearing exosomes that impair NK cell cytotoxicity

IEM studies revealed expression and storage of NKG2D ligands in MVB on intraluminal vesicles/exosomes, a finding that might indicate exosome secretion by STB. To confirm this suggestion, we cultured explants from human early placenta and isolated exosomes from the culture supernatant. The placenta-derived exosomes have a size between 40-90 nm with a characteristic cup-shape. They express MICA/B, ULBP1-5 and the tetraspanin CD63. Furthermore, they carry placental alkaline phophatase (PLAP) on their surface that confirms their placental origin (fig.4 and fig.5 paper II).

Taken together, these results clearly demonstrate that human placenta constitutively expresses NKG2D ligands and that these ligands are secreted on exosomes. A logical question is: "Why are the NKG2D ligands expressed in human normal placenta?" With a constitutive expression of NKG2D ligands in the STB, the chorionic villi are at risk to be attacked by maternal cytotoxic lymphocytes expressing NKG2D. From the fetus point of view, NKG2D ligand expression should be avoided as a potential threat to its existence. The presence of these molecules in normal tissue, like the placenta, suggests that there must be additional benefits for pregnancy of NKG2D ligand expression.

Our studies in paper II show i) that NKG2D ligand-bearing exosomes released from human early placenta down-regulate the NKG2D receptor on NK-, $CD8^+$-, and γδ T cells in a dose dependent manner (fig.6 paper II) and ii) that the exosome-induced internalization of the NKG2D receptor impaired the receptor-mediated cytotoxicity of peripheral blood mononuclear cells (PBMC) isolated from healthy donors (fig.7 paper II). The impairment of cytotoxicity is due to down-regulation of the NKG2D receptor alone, since it did not affect the lytic potential of effector cells, as measured by mRNA and protein expression of perforin (fig.7 paper II).

Based on our results, we present a model for an immune escape strategy where the placenta secretes NKG2D ligand-bearing exosomes that bind the NKG2D receptor and block the maternal cytotoxic response against the fetus (fig.3). The exosomes, directly secreted by the STB to the maternal blood, are logically at the highest concentration in the intervillous

space of the chorionic villi that are “bathing” in the maternal blood in the placental lacunas. The concentration of placental exosomes in the blood decreases with increasing distance away from the placenta. Thus, the continuous release of exosomes by STB creates an exosomal concentration gradient, where the protection against maternal immune attack is strongest at the chorionic villi.

To our knowledge, pregnancy is so far the only example of a normal physiological condition that takes advantage of this adverse phenomenon and uses it to promote fetal allograft survival. However, the cost of using this escape mechanism might be the impairment of the systemic maternal immune response and might be one of the mechanisms accountable for the observed fact that pregnant women are partly immunocompromised and more susceptible to infections and cancer.

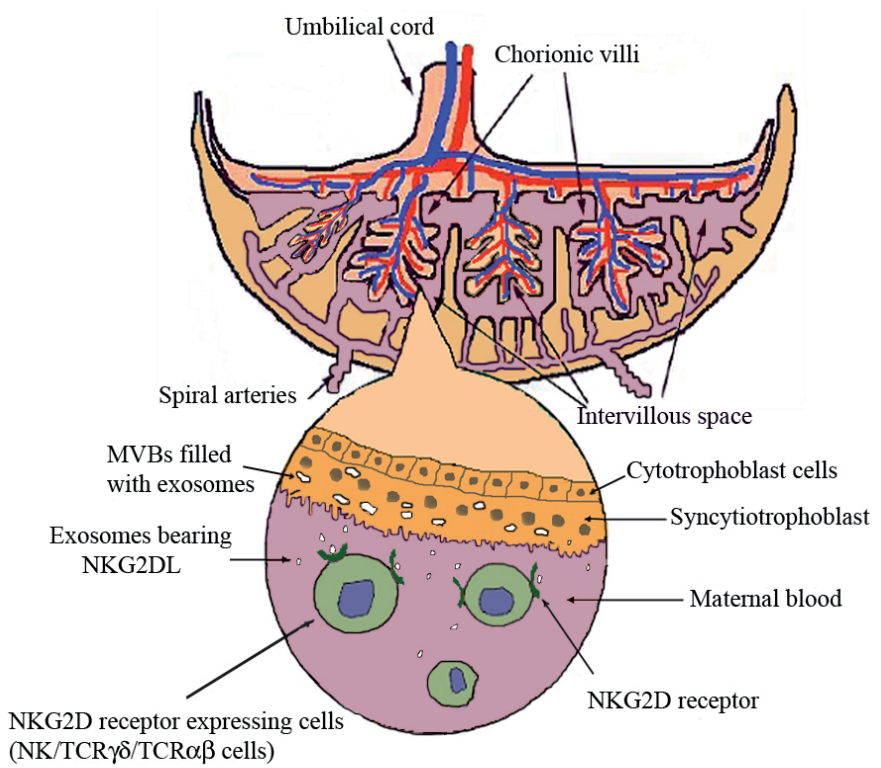

Figure 3. Schematic presentation of how the NKG2D receptor-ligand system works at the local and systemic level in human normal pregnancy.

6. NKG2D ligand expression and secretion by cancer cells

We have used Jurkat and Raji cell lines as a cancer model for studies of exosome secretion and NKG2D ligand expression by T- and B cell leukemia/lymphoma cells under normal and stress conditions. This model was chosen for the following reasons: i) these rapidly progressing malignancies have poor prognosis and documented NK cell dysfunction [185], ii) exosome secretion is a constitutive feature of many human malignancies and tumor-derived exosomes are known to express NKG2D ligands and interfere with the NKG2D receptor causing impairment of NK cell cytotoxicity, iii) NKG2D ligands are induced by biologic stress. The treatment regimens of these leukemia/lymphoma malignancies include heavy cytostatic treatment, sometimes combined with thermotherapy both of which expose the body to massive cellular stress, and iv) a comprehensive clinical study by Nückel et al [186] showed that soluble MICA, MICB and ULBP2 were present in the peripheral blood of patients with chronic B cell leukemia and related to a prognostic significance.

6.1 Stress-induced up-regulation of NKG2D ligands in leukemia cell lines

As mentioned earlier, NKG2D ligands are poorly expressed by normal cells but up-regulated in transformed cells. NKG2D ligands are also up-regulated by diverse cellular stress signals, such as heat shock, oxidative stress, irradiation, anti-cancer drugs and genotoxic agents, all of those causing DNA damage [187]. We stressed the leukemia/lymphoma cell lines Jurkat and Raji with thermal- and oxidative stress, and used up-regulation of HSP70 transcripts as a positive control to estimate the effectiveness of stress induction. Despite the accumulated reports about the nature of stress signals inducing NKG2D ligand expression only limited information about the precise mechanisms that lead to up-regulation of the ligands in cancer are available. The promoter elements for transcriptional control of these ligands are not yet fully understood. MICA/B molecule expression is regulated by promoter elements similar to those of heat shock HSP70 gene, while the transcriptional regulation of other NKG2D ligands remains unclear. It has been reported that heat shock-induced transcriptional activation has not been observed for ULBPs [177, 187]. We found mRNA and protein expression up-regulation for MICA/B and ULBP1-2 in response to thermal- and oxidative stress by H_2O_2. ULBP3 was not expressed in the cell lines tested (fig.1 paper III).

6.2 Thermal- and oxidative stress up-regulates secretion and expression of NKG2D ligand-bearing exosomes that enhance the suppression of NK cell mediated cytotoxicity

We isolated exosomes from supernatants of Jurkat and Raji cell cultures and found that leukemia/lymphoma T and B cells constitutively secreted exosomes and that the secretion was significantly increased after thermal- and oxidative stress (fig.2 paper III). Our IEM, dot blot and flow cytometric analysis revealed that the cell-derived exosomes carry the NKG2D ligands MICA/B and ULBP1 and 2 on their surface (fig.3, 4A, B paper III). Moreover, thermal- and oxidative stress significantly increased the total amount of secreted exosomes and thus, NKG2D ligands (fig.3 paper III).

As a next step, we investigated if tumor exosomes secreted under normal- and stress conditions could alter NKG2D receptor-mediated killing *in vitro*. Our results show that NKG2D ligand-bearing exosomes secreted from leukemia/lymphoma cell lines had a suppressive effect on the NK cell-mediated cytotoxicity and that this suppressive effect was enhanced by stress culture conditions (fig.4C paper III).

Our investigation (paper III) demonstrates that thermal- and oxidative stress can enhance the exosome secretion, thus generating an increased amount of soluble exosomal NKG2D ligands. As a consequence, the suppression of NKG2D mediated cytotoxicity is aggravated, which might promote immune escape of the leukemia/lymphoma cells. This suggestion is supported by Nückel et al. who have found that soluble MICA/B and ULBP2 were present in serum of patients with chronic B lymphocytic leukemia and all of them, especially ULBP2, were strongly associated with poor survival of patients [186]. Oxidative stress is induced by cancer chemotherapy, and hyperthermia is usually used as an adjunctive therapy alongside conventional cancer treatments. It has recently been reported that hyperthermia can suppress the lytic potential of NK cells via down-regulation of perforin/granzyme B expression [188]. We suggest that in addition to the suppressed cytolytic machinery of the effector cells, thermal stress might further augment the dysfunction of the NK cells by down-regulating their killing ability via increased secretion of immunosuppressive NKG2D ligand-bearing exosomes, and this should be taken into account when designing cytostatic and hyperthermal anti-cancer therapy.

CONCLUSIONS

- The MICA/B and ULBP1-5 proteins, stress-inducible ligands of the NKG2D receptor, are constitutively expressed by the syncytiotrophoblast of human normal early placenta. Immunoelectron microscopy studies of syncytiotrophoblast demonstrated expression of ULBP1-5 proteins exclusively in the multivesicular bodies on internal vesicles/exosomes. Placental explant cultures revealed secretion of NKG2D ligand-bearing exosomes.

- The NKG2D ligand-bearing placental exosomes down-regulate the cognate NKG2D receptor on peripheral blood NK- and T cells and impair NK cell-mediated cytotoxicity *in vitro* without altering of activation status or lytic potency.

- Leukemia/lymphoma cells constitutively express the NKG2D ligands MICA/B and ULBP1 and 2 and release them on exosomes. Interestingly, the exosome release could be up-regulated by thermal- and oxidative stress. The increased tumor exosome secretion provided an abundant moiety of soluble, membrane-bound form of NKG2D ligands, which interfered and suppressed the NK cell-mediated cytotoxicity *in vitro*.

- The exosome-mediated release of ULBP and MIC molecules by placenta and tumor is a novel way to generate a soluble form of NKG2D ligands.

- We suggest that syncytiotrophoblast- and leukemia/lymphoma cell-generated, NKG2D ligand-bearing exosomes are protective for placenta and tumors. This protection seems to be based, at least partly, on immune suppression of T- and NK cells by systemic down-regulation of the activating NKG2D receptor.

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