

Immunomodulation during human pregnancy

Placental exosomes as vehicles of immune suppression

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To myself

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Abstract

The mammalian pregnancy comprises a challenge to the maternal immune system since the fetus is semi-allogeneic and could thus be rejected. Pregnancy success is associated with the placenta that is not only essential for oxygen supply, nourishment and pregnancy hormones but also plays a role in the protection of the fetus against maternal immunologic attack. The aim of the current studies was to elucidate the role of human placenta as an immunomodulatory organ with a special focus on placental exosomes as vehicles for establishment of maternal tolerance to the fetus.

We discovered that the syncytiotrophoblast in human normal pregnancy constitutively produces and secretes exosomes. Exosomes are 30-100 nanometer-sized membrane vesicles of endosomal origin that convey intercellular communication. Exosomes are produced and released through the endosomal compartment and reflect the type and the activation state of the cells that produce and secrete them. They carry cytosolic and membrane-bound proteins and nucleic acids and can influence and re-program recipient cells. Depending on their interactions with cells of the immune system they can be divided into immunostimulatory or immunosuppressive.

We developed methods for isolation and culture of trophoblast and placental explants from human normal first trimester pregnancy and isolated exosomes from the culture supernatants. These exosomes were characterized biochemically and functionally regarding mechanisms with potential importance in the establishment of maternal tolerance towards the fetus. The following aspects were studied: 1) exosomal modulation of the NKG2D receptor-ligand system, a major cytotoxic pathway for NK- and cytotoxic T cells and thus potentially dangerous to the fetus; 2) placental exosome-mediated apoptosis of activated immune effector cells; and 3) Foxp3-expressing T regulatory cells in human pregnant uterine mucosa, the decidua.

Using immuno electron microscopy we show that human early syncytiotrophoblast constitutively expresses the stress-inducible NKG2D ligands MICA/B and ULBP1-5, and the apoptosis inducing molecules FasL and TRAIL. While MICA/B were expressed both on the cell surface and intracellularly on the limiting membrane of multivesicular bodies (MVB) and on exosomes, the ULBP1-5, FasL and TRAIL were solely processed through the MVB of the endosomal compartment and secreted on exosomes. The NKG2D ligand-expressing placental exosomes were able to internalize the cognate receptor from the cell surface of activated NK- and T cells thus down

regulating their cytotoxic function. In our studies of apoptosis we found that placental exosomes carry the proapoptotic ligands FasL and TRAIL in their active form as a hexameric complex of two homotrimeric molecules, required for triggering of the apoptotic signaling pathways. This finding was supported by the ability of isolated placental FasL/TRAIL expressing exosomes to induce apoptosis in activated peripheral blood mononuclear cells (PBMC) and Jurkat T cells. Additionally, we studied Foxp3-expressing T regulatory (Treg) cells in paired human decidual and blood samples from pregnant women compared to non-pregnant controls. The CD4⁺CD25⁺Foxp3⁺ Treg cells were 10 fold enriched in the decidual mucosa compared to peripheral blood of pregnant women and non-pregnant controls. We discovered a pool of Foxp3-expressing, CD4⁺CD25⁻ cells in human decidua, a phenotype consistent with naïve/precursor Foxp3⁺ Treg cells. These results suggest local enrichment of Treg cells in decidua of normal pregnancy. Furthermore, we have results indicating that the exosomes, isolated from placental explant cultures, carry PD-L1 and TGFβ on their surface, molecules known to promote induction of Treg cells. Taken together, our results provide evidence that placental exosomes are immunosuppressive and underline their role in the maternal immune modulation during pregnancy. The constitutive production and secretion of immunosuppressive placental exosomes create a protective exosomal gradient in the blood surrounding the fetoplacental unit. This “cloud of immunosuppressive exosomes” conveys immunologic privilege to the developing fetus and thus contributes to the solution of the immunological challenge of mammalian pregnancy.

Populärvetenskaplig sammanfattning

Graviditeten hos däggdjur och människa innebär en stor immunologisk utmaning för modern. Fostret som uttrycker gener från både mamman och pappan riskerar att uppfattas som främmande av mammans immunförsvar och därmed bli attackerat. Att detta inte sker vid normal graviditet är mycket tack vare moderkakan/placentan - ett mycket specialiserat organ som bildas direkt efter befruktningen och innehar en nyckelroll för att säkerställa en lyckad graviditet. Moderkakan ansvarar för fostrets försörjning av syre och näring och avlägsnar slaggprodukter. Den producerar viktiga graviditetshormoner som styr graviditeten och den har även en avgörande roll som immunreglerande organ som skyddar fostret mot ett potentiellt angrepp från mammans immunförsvar.

I den här avhandlingen har vi tittat närmare på moderkakan som ett immunreglerande organ och vi har specifikt studerat moderkakans utsöndring av exosomer. Exosomer är små membranomgivna vesiklar/bubblor, endast 30-100 nanometer stora, som förmedlar kommunikation mellan celler. Exosomerna har både proteiner bundna på sin yta samt inuti bubblorna men innehåller också viktiga RNA molekyler som kan påverka och programmera om mottagarcellen. Genom att skicka exosomer mellan varandra kan cellerna kommunicera utan att behöva vara i direktkontakt med varandra.

Vi har visat att moderkakan kontinuerligt utsöndrar exosomer under den normala graviditeten. Dessa exosomer kan dämpa mammans immunförsvar på flera olika sätt. De kan påverka s.k. "mördarceller" av NK och T cells typ så att dessa, i närheten av fostret, temporärt förlorar sin förmåga att döda. Genom att bära signalmolekyler som binder till aktiverande mottagarmolekyler/receptorer (NKG2D) på mördarcellerna, agerar exosomerna som "lockbete" som stänger ner mottagarmolekylen och gör mördarcellerna oförmögna att reagera. Vidare har vi visat att moderkakans exosomer har en sorts "döds-molekyler" kallade FasL och TRAIL på sin yta. Dessa döds-molekyler binder till aktiverade immunceller och tvingar dem att starta sitt inbyggda självmordsprogram som kallas apoptos. Det här är två mekanismer som moderkakan använder via utsöndring av exosomer för att avvärja två potentiella hot mot fostret under graviditeten - aktiverade immunceller och mördarceller. Båda dessa celltyper kan angripa moderkakan, störa dess funktion och därmed orsaka fosterskada och/eller rubba det normala fortskridandet av graviditeten.

Vi har även studerat regulatoriska T celler i livmoderslemhinnan. Regulatoriska T celler är en specifik undergrupp av immunceller som kan dämpa immunförsvaret genom att styra/hämma andra immunceller på flera olika sätt. Regulatoriska T celler har visat sig ha stor betydelse för den immunologiska balansen under graviditeten. Vi kunde påvisa att det under graviditeten förekommer en ökad mängd av dessa regulatoriska celler lokalt i livmoderslemhinnan, just där moderkakan sitter fast, medan mängden av dessa celler ute i blodet är den samma som hos icke-gravida kvinnor. En intressant upptäckt vi gjorde var att det pågår en utmognad av dessa celler lokalt i livmoderslemhinnan under graviditeten. Moderkakans exosomer skulle kunna vara inblandade här genom att verka som startsignal för denna utmognad. Detta misstänker vi eftersom vi fann att exosomerna också hade två signalmolekyler, TGF β och PD-L1, på sin yta och dessa molekyler är kända för att delta i bildandet av regulatoriska T celler. Exosomernas inverkan på regulatoriska T cellers utmognadsprocess är något vi kommer undersöka närmare i våra fortsatta studier.

Sammanfattningsvis har vi visat att moderkakan skyddar fostret genom att skicka ut exosomer som hämmar mammans immunförsvaret genom minst tre olika sätt: 1) begränsning av mördarcellernas förmåga att döda, 2) avlägsnande av potentiellt farliga immunceller via en direkt effekt av dödsfaktorer som framtvänger celldöd och 3) genom att påverka den lokala utmognaden och därmed den ökade mängden av regulatoriska T celler som vi visat förekommer lokalt i livmoderslemhinnan under graviditeten. Den kontinuerliga utsöndringen av exosomer från moderkakans yttersta skikt ut i mammans blod ger upphov till en koncentrationsgradient av exosomer som är högst närmast fostret där behovet av immundämpning är som starkast, för att sedan avta ju längre bort från livmodern och ut i övriga kroppen man kommer där immunförsvaret behöver fungera som vanligt igen.

Resultaten som presenteras i denna avhandling bidrar till ökad förståelse för hur fostret skyddas under normal graviditet - en kunskap som är nödvändig som ett första steg till att kunna förstå de situationer där immunotoleransen mot fostret inte fungerar. Karakteriseringen av moderkakans exosomer och deras roll vid normal graviditet öppnar således möjligheter att utveckla nya behandlingsmetoder för sjukliga graviditetstillstånd som upprepade missfall, havandeskapsförgiftning och infertilitet. Kunskap om immunhämmande exosomer kan även tillämpas inom andra viktiga områden som organtransplantation och cancerdiagnostik/behandling.

Original Publications

Paper I

An efficient optimized method for isolation of villous trophoblast cells from human early pregnancy placenta suitable for functional and molecular studies

Stenqvist AC*, Chen T*, Hedlund M, Dimova T, Nagaeva O, Kjellberg L, Innala E, Mincheva-Nilsson L.

American Journal of Reproductive Immunology. 60(1):33-42, 2008

(*Equal contribution)

Paper II

Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function

Hedlund M, Stenqvist AC, Nagaeva O, Kjellberg L, Wulff M, Baranov V, Mincheva-Nilsson L.

Journal of Immunology. 183:340-351, 2009

Paper III

Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus

Stenqvist AC, Nagaeva O, Baranov V, Mincheva-Nilsson L.

Journal of Immunology. 191(11):5515-5523, 2013

Paper IV

Maternal FOXP3 expressing CD4+ CD25+ and CD4+ CD25-regulatory T-cell populations are enriched in human early normal pregnancy decidua: a phenotypic study of paired decidual and peripheral blood samples

Dimova T, Nagaeva O, Stenqvist AC, Hedlund M, Kjellberg L, Strand M, Dehlin E, Mincheva-Nilsson L.

American Journal of Reproductive Immunology. 66:44-56, 2011

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Abbreviations

| | |
|-----------------|--|
| Ab | antibody |
| APC | antigen presenting cell |
| BCR | B cell receptor |
| CTB | cytotrophoblast |
| CTL | cytotoxic T cell |
| DAMP | damage-associated molecular pattern |
| DC | dendritic cell |
| DISC | death inducing signaling complex |
| EV | extracellular vesicle |
| FasL | Fas ligand |
| Foxp3 | forkhead box p3 |
| GM-CSF | granulocyte/macrophage colony-stimulating factor |
| IEM | immuno electron microscopy |
| IFN | interferon |
| IHC | immunohistochemistry |
| IL | interleukin |
| ILV | intraluminal vesicles |
| LPS | lipopolysaccharide |
| MHC | major histocompatibility complex |
| MIC | MHC class I chain-related proteins |
| MMP | matrix metalloproteinase |
| MVB | multivesicular body |
| M Φ | macrophage |
| NK | natural killer |
| NKG2D | natural killer group 2 member D |
| NKT | natural killer T |
| PAMP | pathogen-associated molecular pattern |
| PBMC | peripheral blood mononuclear cells |
| PD-L1 | programmed-death 1-ligand 1 |
| STB | syncytiotrophoblast |
| TCR | T cell receptor |
| TEM | transmission electron microscopy |
| TGF β | transforming growth factor β |
| Th | T helper |
| T _{H3} | T helper type 3 |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Treg | T regulatory |
| T _{r1} | type 1 regulatory |

INTRODUCTION

The mammalian pregnancy is a challenge to the maternal immune system since acceptance of the semiallogeneic fetus during pregnancy can be viewed as the only naturally occurring transplantation. Due to genetic imprinting, the placenta, a fetal organ essential for pregnancy success and the utmost border between the fetus and the mother, selectively over-expresses paternal antigens and although recognized as foreign by the maternal immune system, is still not rejected. Multiple mechanisms have been found to operate at the feto-maternal interface contributing to the immunologic privilege of the fetus. However, the whole picture is far from clear and the survival of the semiallogeneic fetal allograft during pregnancy still remains an enigma that has been referred to by the famous Australian immunologist and Nobel Prize Laureate Sir Peter Medawar as “a paradox of Nature”.

The mammalian placenta is crucially important during pregnancy not only as a supplier of hormonal, nutritional, and oxygen support and waste remover but also as an important immunomodulatory organ. Many different placental mechanisms and pathways have been suggested for adjustment of the maternal organism for the benefit of the ongoing pregnancy. There is no doubt, however, that the important role of the placenta as an immunomodulatory organ is based on a fine-tuned intercellular communication between the mother and the fetus that moulds the “internal language and cross talk” of pregnancy. Revealing this language and cross talk would be a huge step to understanding how the immunological challenge of accepting a semiallogeneic allograft and supporting its development and growth is solved in the contest of mammalian reproduction.

We have discovered that the syncytiotrophoblast of human placenta, which freely bathes in maternal blood, communicates with the maternal immune cells by producing and secreting immunosuppressive exosomes. Exosomes are nanometer-sized extracellular membrane vesicles of endosomal origin, which act as messengers between cells without the need of cell-cell contact. They are formed in multivesicular bodies in a way that they carry membrane proteins expressed in the same manner and orientation as on the cellular membrane and also contain inside both cytosolic proteins and nucleic acids such as mRNA/microRNA and mitochondrial DNA allowing several different communication-possibilities at the protein signaling- and genetic reprogramming levels.

In this dissertation, a closer look is taken on fetal immune escape mechanisms in human pregnancy with a focus on the contribution of

placenta-derived exosomes. We chose to investigate two systems; 1) the NKG2D receptor-ligand system, a powerful cytotoxic system and thus a potential threat to the fetus, and 2) FasL- and TRAIL-mediated apoptosis, a mechanism used for protection at immune privileged sites. Furthermore, a third actor in immune modulation during pregnancy, T regulatory cells, has been investigated. We have asked the questions: What is the nature and role of placenta-derived exosomes during normal human pregnancy? How are they involved in the regulation of the above mentioned important immune mechanisms and cells during pregnancy? Initially, a brief summary of the immune system, human pregnancy and the placenta is given as a background to the discussion of the results obtained in the present study.

BACKGROUND

I. A brief overview of the immune system

Definition and general properties

The immune system in mammals is defined by its three main functions. First and foremost, it defends the organism in the battle against invading microorganisms, such as bacteria, virus, fungi and parasites. Second, the immune system provides a constant immune surveillance that recognises damaged or transformed cells thus contributing to prevention of tumors. Third, by using its defence and surveillance mechanisms the immune system plays an important role as a promoter and guardian of the homeostasis of the body, mainly at its mucosal sites, where the “outer world”, represented by food, air pollutants and the commensal microbial flora, intimately meets various mucosal surfaces that represent the boarder to the “inner world” of the body.

The immune system is traditionally divided into two branches, the innate, antigen-non-specific, and the acquired/adaptive, antigen-specific, which are interconnected to each other and cooperate to protect the organism from intruders. Phagocytic cells, such as granulocytes and macrophages (MΦ), antigen-presenting dendritic cells (DC), natural killer (NK) cells, natural killer T (NKT) cells, $\gamma\delta$ T cells and soluble proteins like cytokines, chemokines, complement factors and acute phase proteins comprise the innate, antigen-non-specific branch of the immune system, also known as first line of defense (1, 2). The main way of innate immune system activation is based on recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are molecules of exogenous type, such as lipopolysaccharide (LPS), lipoteichoic acid, zymosan, flagellin etc. present on various microbes. DAMPS are danger detection molecules of endogenous type, such as heath shock protein (HSP) 60, double stranded (ds) RNA, EDA domain of fibronectin and others, present in eukaryotic cells. PAMPs and DAMPs are recognized by pattern recognition receptors (PRR) expressed on a variety of immune cells, the Toll-like receptors (TLR) being a common example of PRR (1, 2). The acquired branch of the immune system comprises of T- and B cells, plasma cells and antibodies. It is an adaptive defence system, characterised by three main features – specificity, memory and self/non-self discrimination, based on antigen recognition, presented by proteins of the major histocompatibility complex (MHC) class I and II. Two of the specific responses generally evoked are a humoral response giving rise to specific antibodies and a cellular

response giving rise to various effector cells with helper or cytotoxic functions (1, 2). Both branches of the immune system cooperate intimately with each other to give rise to a competent immune response, able to protect the organism from various biological dangers such as microbial intruders, malignant transformation and disruption of mucosal homeostasis.

Immune cells

Granulocytes

The granulocytes belong to the innate immunity and as all immune cells they have their progenitors, the CD34⁺ cells, in the bone marrow. They belong to the myeloid lineage and mature under the influence of cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) -5. Granulocytes are the most abundant leukocyte type in the peripheral blood. They comprise about 75% of all white blood cell and are the most short-lived with renewal of the pool every third day. They are divided into three cell subtypes depending on the pattern of hematoxylin/eosin staining - neutrophilic granulocytes/neutrophils, eosinophilic granulocytes/eosinophils and basophilic granulocytes/basophils (1, 2).

The neutrophils are by far the most dominant population and the main phagocytic cell type in peripheral blood. Neutrophils are one of the earliest recruitments at inflammatory sites. They possess a migratory capacity, leave the blood circulation in a defined way by a set of adhesion molecules and move towards a chemical gradient of cytokines such as IL-1 β and IL-8, complement factors, e.g. C4a, C5a, leukotrienes and prostaglandins by a process called chemotaxis. Neutrophils have numerous granules that carry enzymes, antibacterial substances such as lactoferrin and defensins participate in phagocytosis of microbes by oxygen-dependent (respiratory burst) and oxygen-independent mechanisms (3). They can also form neutrophil extracellular traps (NETs) of DNA material that can entrap and immobilize pathogens facilitating the phagocytic process (4).

Eosinophils comprise 1-5% of the leucocytes and contain granules that carry histamine, leukotrienes, basic proteins and peroxidase able to produce reactive oxygen species (ROS). Eosinophils are recruited by IL-3, -5 and GM-CSF and can produce Th2 type of cytokines such as IL-4, -5, -6, and -13. Eosinophils increase in number in allergies and infections with parasites (1, 2).

Basophils are found in very few numbers in the peripheral blood (about 0.1%). They share features with mast cells such as expression of the IgE

receptor (FcεR) and degranulation by receptor-cross-linking. They might be involved in allergies and parasitic diseases but at present their exact function is still unclear (1, 2).

Antigen-presenting cells (APC)

The adaptive immune response commences by antigen presentation to T and B cells. The MHC class I and class II, expressed on APC, are involved in this presentation. The MHC class I molecules present intracellular proteins and are expressed on almost all cells in the body except erythrocytes and human trophoblast whereas the class II molecules present extracellular antigens and are expressed on cells with antigen presenting function, i.e. MΦ, DC and B cells (5).

The immature monocytes originate from a precursor in the bone marrow, enter the peripheral blood and migrate by specific chemokine and adhesion receptors to tissues where infection or inflammation takes place and there they mature into MΦ (1, 2). MΦ produce inflammatory cytokines such as interferon (IFN) γ and IL-12 and display many functions important in innate and adaptive immunity. They engulf and kill microbes, and promote inflammation by producing cytokines and chemokines that activate and recruit other immune cells to the inflammation/infection site. They express TLR, complement receptors, scavenger receptors and Fc receptors and efficiently mediate phagocytosis and clearing of infected or transformed cells and cellular debris (1, 2, 5).

The DCs are constitutively present in epithelia and most tissues and when activated they are able to migrate to lymphoid organs such as lymph nodes. In humans, they are divided into three types according to origin – lymphoid, myeloid and non-hematopoietic. They possess a variety of pattern recognition receptors such as TLR and the NOD-like receptors and are able to detect both PAMPs and DAMPs (1, 2). Like macrophages they engulf and degrade pathogens but mainly for antigen presentation at immune sites and not for clearance. At their immature stage DCs are mostly phagocytic but acquire cytokine producing ability at their maturation. DCs express co-stimulatory molecules and cytokines essential for the activation and differentiation of naïve T cells to effector cells and function as a link between innate and adaptive immunity (1, 2, 6).

B lymphocytes, plasma cells and αβT lymphocytes

B cells mature in the bone marrow and are lymphocytes characterized by clonally distributed B cell receptor (BCR) on their surface. BCR is a

membrane bound immunoglobulin (Ig) molecule that works as an antigen-binding receptor and is composed of two identical light and two identical heavy chains with variable (V) and constant (C) regions. The V regions determine the antigen specificity and the C regions are associated with effector functions. B cells are also expressing a cluster of co-receptor proteins known as CD19, -21 and -81. Naïve B cells express IgM and IgD. Activation by binding to BCR may induce changing the constant part of the Ig molecule to IgG, IgA or IgE, a process called isotype switching. B cells can present antigens and after an activation response turn into memory cells. Activated B cells differentiate to plasma cells, end-differentiated cells that lose their BCR expression on the cell surface and produce high amounts of antibodies (Ab) through their well developed endoplasmic reticulum and Golgi complex. Their specific function as Ab-producing factories is reflected in their morphology of eccentrically positioned nucleus and basophilic cytoplasm full of voluminous amount of antibodies (1, 2, 5).

T cells mature in the thymus and can be divided into several subsets depending on their marker expression and function. They express clonally distributed T cell receptors (TCR) on their surface and the CD3 complex of proteins that transmits the TCR signal upon activation. According to the protein chains expressed in their TCR, T lymphocytes can be divided into TCR $\alpha\beta$ and TCR $\gamma\delta$ lymphocytes that differ in their biology and function. The activation of $\alpha\beta$ T lymphocytes depends on antigen presentation by sensing of processed peptides in the cleft of MHC class I or class II molecules expressed on APC. Moreover, the $\alpha\beta$ T cells are MHC restricted, i.e. their response is depending on APCs originating from the same donor as the T cells. $\alpha\beta$ T cells are divided into CD4⁺ cells with helper function and CD8⁺ cells with cytotoxic function (1, 2, 7, 8).

The CD4⁺ cells, also known as T helper (Th) cells, play a central role in the initiation of the immune response. Under the influence of various cytokines they differentiate from T naïve cell into subsets of Th cells; Th2, Th17, iTreg and the most recently described Th9, which in turn regulate different immune responses by their own cytokine production (9-11). Table I summarizes the Th cell subsets known of today, their cytokine production and the immune responses they promote.

The CD8⁺ cells, known as cytotoxic T cells (CTL), are activated by T helper 1 cytokine response to kill infected, transformed or in other way damaged cells. CTLs kill/lyse their targets by two main pathways: 1) cytolytic granule exocytosis of perforin, granzymes and granulysin and 2) FasL/Fas interaction forming death inducing signaling complex (DISC). In both cases

| Th response | Differentiated under the influence of | T-helper cell subsets | Cytokines produced | Type of immune response promoted |
|-------------|---------------------------------------|-----------------------|--|--|
| *Th1 | IFN- γ , IL-12 | Th1 | IFN- γ , TNF- γ /lymphotoxin | Cytotoxic response |
| Th2 | IL-4 | Th2 | IL-4, IL-5, IL-13, IL-24, IL-25, IL-31 | Humoral response |
| Th17 | TGF- β , IL-1, IL-6, IL-23 | Th17 | IL-17A, IL-17F, TNF | Inflammatory response |
| Th3/**Tr1 | TGF- β , IL-10 | ***iTreg | TGF- β , IL-10 | Regulatory response |
| Th9 | IL-4, TGF- β , IL-25 | Th9 | IL-9 | Allergic- and autoimmune inflammation, anti-tumor immunity |

*Th - T helper; **Tr - T regulatory; ***iTreg - inducible T regulatory cells

Table I. T helper cell subsets and cytokines involved in the regulation of immune responses

the final steps leading to cell death are effectuated by the apoptosis-inducing caspase cascade reactions (12).

T regulatory cells

Regulation of the immune response is of key importance for executing immune surveillance while maintaining peripheral tolerance and homeostasis. There are many different types of regulatory T (Treg) cells and their common denominator is regulation of the immune response by immune suppression. Among the heterogeneous Treg population, two human Treg cell subtypes with the phenotype CD4⁺CD25⁺ stand out and comprise the vast majority of Treg cells and will be discussed here: the naturally occurring/innate/thymus-derived Treg cells and the inducible/adaptive Treg cells that mature in the periphery.

Definition, origin and frequency in the blood

The CD4⁺CD25⁺ Treg cells are cells with immunosuppressive function that play a critical role in the control of the immune response, the creation and maintenance of tolerance and the homeostasis of the organism. They are found in very small numbers in the peripheral blood where anything from 1-10% has been reported, the usual amount being ~2-4% of the CD4⁺ T cell population (13).

The natural Treg cells, also called innate, arise with self-antigen specific TCR in the thymic medulla at the same time as effector T cells develop in the thymic cortex, i.e. during the early stages of fetal T cell development. They

are proposed to prevent peripheral activation of self-reactive T cells that have escaped the negative selection in the thymus (14). Both their development in the thymus as well as their maintenance and self renewal in the periphery seem to depend on co-stimulation by CD28 (15, 16).

Adaptive Treg cells arise in the periphery from naïve T cells under certain conditions of antigenic stimulation in the presence of the immunosuppressive cytokines IL-2 in combination with IL-10 or transforming growth factor β (TGF β). Unlike the natural Treg cells, the adaptive Treg cells develop and function independently of CD28 co-stimulation. The maintenance of functional adaptive Treg cells instead requires continued antigen-exposure e.g. during an infection or organ transplantation (14). The adaptive Treg cells are thus suggested to be important for preventing chronic inflammation and contribute in maintaining tolerance to foreign antigens like in the establishment of oral tolerance (17).

Natural Treg cells seem to mainly exert their suppressive effect via cell-contact mediated mechanisms through membrane-bound molecules, while adaptive Treg cells instead use contact-independent suppression via release of cytokines (18).

Treg phenotype and Foxp3

Treg cells are commonly defined by a combination of CD4 and high levels of the high affinity IL-2 receptor α -chain subunit CD25. Other markers are necessary since the CD25⁺ population includes also activated cells lacking suppressive function. An additional marker that has turned out to be very valuable is the forkhead box P3 (Foxp3), a transcription factor that has been suggested as a key regulator for Treg cells (19). Foxp3 acts as a transcription repressor affecting hundreds of genes (20, 21). In mice, Foxp3 is exclusively expressed by Treg cells and is both necessary and sufficient for the development and function of Treg cells (22). In humans the situation is not quite as clear since other activated T cells also transiently express Foxp3 (23). However, a high and stable Foxp3 expression is coupled to a suppressive function also in human (24). Humans that lack a functional Foxp3 encounter severe consequences with a syndrome called IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome) with a fatal systemic autoimmunity, which highlights both Foxp3 as important for human Treg cells, and also the vital role for Treg cells in maintaining immune homeostasis (25). The generation of/switch into adaptive Treg cells from naïve T cells is suggested to occur through the induction of Foxp3 expression (22, 26).

Other commonly used Treg-associated markers include CTLA-4, LAG-3, HLA-DR, CD103, Neutropilin-1, CD62-L^{high} and CD127^{low}. These markers can also be found on other T cell subsets, and since there is no marker with exclusive Treg-specificity it is favorable to use a combination of several molecules when identifying Treg cells.

Cytokine production

The adaptive Treg cells can be divided into different subsets depending on their cytokine profile where Type 1 regulatory (T_r1) cells seem to be activated by IL-10 and produce IL-10 and TGFβ themselves while T helper type 3 (T_H3) cells are activated by TGFβ and produces predominantly TGFβ themselves (27). Several immune suppressive mechanisms have been shown for both IL-10 and TGFβ. IL-10 is a potent inhibitor of the production of pro-inflammatory cytokines, it downregulates MHC class II expression and co-stimulatory molecules reducing the antigen-presentation of APC and thus the activation of T cells. TGFβ inhibits the production of IL-12 and upregulates cell cycle inhibitors which together potently inhibit proliferation of T cells. TGFβ also inhibits transcription factors required for differentiation of Th1 and Th2 cells, and inhibit macrophage-activation as well as maturation of DC (28).

Function

As mentioned above, Treg cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. They also prevent sterilizing immunity and limit antitumor immunity. They suppress activation, proliferation and/or function of effector T cells, and they also affect immune responses by modulating the recruitment and function of other cell types (14, 17). Human Treg cells act via bystander suppression, meaning that while activation of Treg cells is TCR specific, their suppression is more general and influences immune responses against random antigens (29). Several different suppression-mechanisms have been described for Treg cells: 1) production of immunosuppressive cytokines where IL-10 and TGFβ are the key mediators of adaptive Treg cell function, 2) cytotoxicity through secretion of granzymes but also through the TRAIL-DR5 pathway, 3) metabolic disruption like depletion of IL-2 via their high CD25 expression and/or release of adenosine nucleosides activating the adenosine receptor 2A which in turn inhibits effector T cell function and 4) by targeting dendritic cells through CTLA-4 whereby Treg cells were suggested to attenuate DC activation of effector T cells, to stimulate DC-expression of indoleamine 2,3-dioxygenase (IDO) and to inhibit the maturation of DC (23).

Treg cells need to be properly regulated and able to discriminate desirable immune responses from those that are deleterious. A crosstalk between Treg- and its targets/effector T cells has been suggested with reduced Treg cell-suppressive function when the level of immune activating signals is very high as during an infection (30). A high TCR stimulation of effector T cell seems to render them refractory to the suppressive Treg cell-signals (31).

$\gamma\delta$ T lymphocytes and NKT cells

The $\gamma\delta$ T cells and NKT cells are special cell subsets belonging to the innate immunity. The $\gamma\delta$ T cells can be divided into circulating $V\delta 2^+$ cells present in the peripheral blood and resident $V\delta 1^+$ cells present in various mucosa such as the intestinal and lung mucosa and also the pregnant uterine mucosa, the decidua (32). The great majority of $\gamma\delta$ T cells does not express CD4 or CD8 molecules and are not restricted to the conventional classical MHC class I and II molecules. Some subsets recognise CD1d. The $\gamma\delta$ T cells secrete a variety of cytokines, express NKG2D as a co-receptor and are able to kill targets. Their function is somewhat different from $\alpha\beta$ T cells. Besides being able to combat intracellular infections such as tuberculosis ($V\delta 2^+$ $\gamma\delta$ T cells) they can respond to cellular stress and transformed cells and exert killing by FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or NKG2D receptor-ligand interactions. The resident $V\delta 1^+$ T cells are involved in local immune regulation and protection of homeostasis at mucosal surfaces and due to these functions they are sometimes called adaptive T regulatory cells. Being cells expressing both TCR and NK cell receptors they are also considered by some to belong to the group of NKT cells (33).

Natural killer T (NKT) cells comprise a unique group of T cells that simultaneously express TCR and NK cell receptors. They are usually double negative, i.e. lack expression of CD4 and CD8, and recognize antigens in the context of CD1d. Their most prominent feature is their secretion of huge amount of cytokines, such as IL-4, TNF- α and IFN- γ , and their killing ability. There is a special group of NKT cells, considered by some as the classical ones, which were the first to be discovered and to be named NKT cells. These cells have an invariant α V chain in their TCR, $V\alpha 14$ in mice and $V\alpha 24$ in humans (34).

NK cells and their receptors

Natural killer (NK) cells are cytotoxic effector cells of the innate immunity engaged in the combat against viral infections and tumors. Compared to the adaptive T and B cells, the NK cells lack clonally distributed antigen-specific

receptors and are not MHC dependent for their activation as T and B cells are nor are they restricted to MHC in the manner T and B cells are. Instead they possess a set of various activating and inhibitory receptors that recognise and react on the presence or absence of MHC. The way a NK cell is activated either to react or to suppress its reaction is depending on an intricate interplay between the inhibitory and the activating receptors. In humans, the NK cells receptors are divided into three groups depending on their structure: 1) the killer immunoglobulin-like receptors (KIRs), 2) the natural cytotoxicity receptors (NCRs), and 3) the c-type lectin-like receptors (Table II). Most of the inhibitory receptors are among the KIR and Ig-like transcripts (ITL) belonging to the Ig superfamily and bind classical and non-classical MHC class I molecules. The inhibitory receptors react on the presence of “healthy” MHC class I molecules and transmit inhibitory signals through immunoreceptor tyrosine-based inhibition motif (ITIM) present in the intracellular tail of the receptor. The phosphorylated ITIM recruits SHP1/2 phosphatases that dephosphorylate and inactivate adaptors involved in NK cell activation resulting in inhibition of NK cell attack (35). Thus, normal cells are protected by from NK cell attack due to adequate expression of MHC class I molecules while altered and/or missing self on the target cells is inducing NK cell killing (36). In addition to altered or missing self, NK-cell activation requires a positive signal delivered by engagement of activating receptors such as NCRs – NKp30, NKp44, NKp46, NKG2D and co-receptors such as 2B4 (CD244) or NK, T and B lymphocytes-antigen (NTB-A). The activating receptors are transducing signals through tyrosine-based activation molecules (ITAM) resulting in up-regulation of the NK killing ability and/or their cytokine production (35). The major activating NKG2D receptor and its ligands are discussed separately in chapter II.

Some humoral components of the immune system

Antibodies

Antibodies are immunoglobulin-proteins produced and secreted by plasma cells. Their main functions are neutralisation of microbes, complement activation and opsonisation. Moreover, by opsonisation of target cells they also participate in the killing mechanism denoted antibody-dependent, cell-mediated cytotoxicity (ADCC). The basic structure of an antibody molecule consists of light and heavy chains bound together by disulphide bonds. These chains have variable regions where antigen binding takes place and constant regions that form the Fc part of the molecule, also called effector part that can bind to Fc receptors expressed by a variety of immune cells. The heavy chains are named α , γ , δ , ϵ and μ and define the Ig class. There are 5 type of

| Group by molecular structure of the receptors | Receptors | Signal | Ligand |
|---|---------------|--------|--------------------------|
| Ig superfamily-killer immunoglobulin-like receptors (KIRs), Ig-like transcripts (ILT) | KIR3DL1 | –* | MHC-B |
| | KIR3DL2 | – | MHC-A |
| | KIR2DL1, 2, 3 | – | MHC-C |
| | KIR2DL4 | – | MHC-A, -B, -G |
| | KIR2DS1, 2 | +** | MHC-C |
| Natural cytotoxicity receptors (NCRs) | ILT 2 | – | MHC-A, -B, -G |
| | NKp44 | + | unknown, influenza virus |
| | NKp30 | + | unknown |
| | NKp46 | + | unknown, influenza virus |
| C-type lectin like receptors | NKp80 | + | unknown |
| | NKG2A/B | – | MHC-E |
| | NKG2C/E | + | MHC-E |
| | NKG2D | + | MICA/B; ULBPs |

* –, inhibitory signals transduced by immunoreceptor tyrosine-based inhibition motif (ITIM)

** +, activating signals transduced by immunoreceptor tyrosine-based activating molecule (ITAM)

Table II. Some of the human NK cell receptors and their ligands (37).

Ig molecules; the pentameric IgM - the antibody produced before class-switches to other antibodies, IgG - the main type present in serum, IgA - the antibody operating at mucosal surfaces, IgE - involved in allergy and parasitic diseases and IgD whose function is still unclear (1, 2).

Cytokines

Cytokines are small glycosylated signalling proteins of low molecular weight produced and secreted in a highly regulated fashion by a variety of immune and non-immune cells. They have regulatory effects on hematopoietic, endocrine, neural and many other cell types and signal by binding to specific cytokine receptors on target cells in an autocrine, paracrine or endocrine manner. The number of cytokines is high and still rising. Most of them can be affiliated to one of the following families: hematopoietins, interferons, interleukins, chemokines or the TNF family. Cytokines can be both pleiotrophic, having different biological effects on different target cells, and redundant, i.e. different cytokines can mediate similar biological effect/function. They can act synergistic (potentiating each other) or antagonistic (inhibiting each other). These properties of cytokines contribute to the regulation and fine tuning of immune responses (38). The microenvironment in which the cytokine is present, i.e. the combination of tissues, cells and active substances such as other cytokines, hormones and

signal substances, defines the role and biological effect of a cytokine in a particular situation.

Complement

The complement system consists of a series of plasma proteins, most of them serine proteases that exist as proenzymes before activation. The complement has important effector functions in both innate and adaptive immune responses. It is activated in so called complement cascade reactions by 3 ways: the classical antibody-dependent, the alternative and the lectin pathway. The complement activation participates in important biological functions: cell lysis, inflammation, opsonisation, phagocytosis, and clearance of immune complexes (1, 2).

Modulations of the maternal immune response during pregnancy

The overall objective of the maternal immune system during pregnancy is to retain protection against infectious intruders and immune surveillance without damaging the fetus. The outmost border to the maternal organism, the placenta, contains both maternal and paternal genes but the paternal ones are preferentially expressed (39) making recognition and rejection of the placenta by the maternal immune system a potential risk. Despite this fact, the mammalian pregnancy defies the natural laws of transplant rejection and a peaceful feto-maternal co-existence prevails in normal healthy pregnancy.

In 1953, Medawar proposed three mechanisms for immune protection of the fetus: anatomical separation of the fetus from the mother; antigenic immaturity of the fetus and immunological indolence/inertness of the mother towards the fetus (40, 41). Although these concepts were instrumental for guiding reproductive immunology research for years they are only partly true in pregnancy and have been revised in recent years. The anatomical separation of maternal and fetal tissues is only partial and the chorionic villi are bathing in maternal blood. Moreover, there is both maternal and fetal chimerism which reflects the intimate maternal-fetal contact during pregnancy (42). It is well known that multiparous women have developed a variety of allogeneic antibodies arguing against fetal antigenic immaturity. There are many reports contradicting the concept of maternal inertness toward the fetus and indicating that the maternal immune system is not only aware of fetal antigens but also responds to them but in such a way that maternal-fetal immunotolerance is established (40).

The fetal tolerance-promoting modifications of the maternal immune system during pregnancy are not effectuated by a single mechanism. Instead, a variety of hypothetical mechanisms, investigated both in animal and human studies, are suggested to operate in concert at the local, *in uteri*, and systemic level. One theory is that there is a nonspecific immune suppression during pregnancy induced by pregnancy hormones, progesterone being the one most frequently investigated (43). Murine studies have shown that the maternal innate and adaptive immunity is transiently tolerant to fetal histocompatibility antigens during pregnancy (40, 44, 45). Regarding antigen presentation, only a selected repertoire of classical and non-classical MHC molecules is differentially expressed by certain trophoblast subpopulations. The syncytiotrophoblast lacks expression of both classical HLA class I and II molecules, impairing antigen presentation to CTL. The extravillous trophoblast expresses an unusual MHC combination of MHC class I proteins: a truncated form of the classical HLA-C molecules and non-classical HLA-G and -E that could locally modulate NK cell function (46). Moreover, we have discovered that human placenta expresses the MHC-chain-related molecules MICA and B (47). These MICA/B molecules, and ULBP1-5, the other family of NKG2D ligands, are one of the aims of this thesis and will be discussed in detail later. Another suggested mechanism of fetal tolerance is placental expression of IDO, an enzyme that catalyzes the degradation of tryptophan causing cell starvation that inhibits T lymphocyte proliferation (48, 49). Subsets of regulatory T cells operate in human and murine pregnancy (50-53). The extrinsic apoptosis-inducing molecules FasL and TRAIL are expressed throughout pregnancy and suggested to play an important role in implantation, placenta formation and immune protection of the fetus, and will be discussed in the results of this thesis as well (54-58). Furthermore, complement attack on pregnancy is avoided by placental expression of complement regulatory proteins (59, 60). Another immune modification at the systemic level is a cytokine shift from Th1 to Th2 that occurs in pregnancy, which directs the immune response towards antibody production and humoral immunity and away from the potentially dangerous cytotoxic responses. A cytokine shift towards Th1 during pregnancy has been linked to spontaneous abortions, premature delivery and preeclampsia (61-64).

The human pregnant uterine mucosa, the decidua, is rich in immune cells. In human early pregnancy decidua about 15-30% of all cells are leukocytes that are either randomly dispersed between the decidual stromal cells or localized in aggregates of cells, named lymphoid cell clusters (LCC), or subepithelially positioned in the vicinity of the endometrial gland epithelium (65, 66). The decidua-associated lymphoid tissue (DALT) comprises the dominant CD56^{bright}/CD16⁻ uterine NK-like (uNK) cells, $\gamma\delta$ - and $\alpha\beta$ T cells, dendritic

cells, macrophages and regulatory T cells. The majority of the immune cells in the LCC of DALT are activated and express a variety of phenotypic-, adhesion-, memory- and activation markers such as CD2,-3,-4,-7,-8, CD45RO, CD94, CD38, CD69, CD71, HML-1 and MHC class II suggesting that the LCC are sites for activation, cooperation and differentiation of lymphoid cells (65, 66).

The role of the most abundant immune cell population in early decidua, the uNK cells, is still unclear. Interestingly, these cells are only present in decidua during the first and second pregnancy trimester, decrease gradually in amount as the pregnancy progresses and are completely absent at term trimester and delivery. A part of them express RAG1 and RAG2 and there is evidence that they can locally mature to $\gamma\delta$ T cells (67, 68). Thus, these cells might comprise a heterogeneous population of precursors for T and NK cells. As pregnancy progresses, the uNK cells disappear and the $\gamma\delta$ - and $\alpha\beta$ T cells are relatively enriched and comprise the dominant lymphocyte population in decidua at the end of pregnancy. B cells are scarce/absent in decidua (40).

There are contradicting results regarding the composition of immune cells at the systemic level, i.e. in the peripheral blood of pregnant women as reviewed by Mincheva-Nilsson (40). However, regardless of controversies, leukocytes of the innate immunity seem to be the dominating cell populations in normal uncomplicated pregnancy. Flow cytometric studies of PBMC from pregnant women showed a leukocyte profile resembling the picture of a "mild sepsis" (69). Thus, there is an accepted consensus that in normal human pregnancy, both at the systemic and the local level, the adaptive immunity is suppressed and the innate immunity is relatively enhanced, in attempt to compensate for the down regulation of the adaptive immunity. In recent studies expression of TLRs by trophoblast cells was found, suggesting that they might function as components of the innate immune system in pregnancy (70, 71). In other words, from an immunologic point of view normal human pregnancy seems to be an innate immunity event (44). This is in line with the fact that pregnant women appear to be more sensitive to viral infections and other intracellular pathogens, for example varicella/zoster and malaria infections, but seem to cope better with extracellular infections (72). Further proof for the modulatory effect of pregnancy is the fact that pregnancy can alter the course of systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (73-75).

In summary, a variety of immune cells, pregnancy hormones, signal molecules and multiple mechanisms of peripheral and local tolerance cooperate to modulate the maternal immune system to maintain pregnancy

success. To these mechanisms and players we can now add exosomes and exosome-mediated immune suppression that will be discussed in the results of this thesis.

II. The NKG2D receptor-ligand system

The NKG2D receptor

The NKG2D receptor-ligand system comprises a potent cytotoxic system for elimination of foreign, stressed, transformed or infected cells. NKG2D stands out as a major activating NK cell receptor. Engagement of the NKG2D receptor with any of its ligands surpasses other inhibitory signals and leads to activation of the NK cell in two defined directions - cytokine production and perforin-mediated cytotoxicity resulting in killing of the target cell that expresses the ligands (76). The receptor, natural killer group 2 member D (NKG2D), is a type II transmembrane protein of the C-type lectin-like family that is expressed as a homodimer on the surface of most human NK-, NKT-, $\gamma\delta$ T- and CD8⁺ $\alpha\beta$ T cells where it serves as an activating receptor (77). The NKG2D do not have any signaling motifs within its short intracellular domain. The signaling in humans is transduced through interaction with the adaptor molecule DAP10 while in mice both DAP10 and DAP12 serve as adaptor molecules. Interactions with the adaptor molecules trigger the PI3K and Grb2 signaling cascade (78).

The NKG2D ligands

The ligands for NKG2D are divided in two families: 1) the MHC class I chain-related proteins A (MICA) and B (MICB), and 2) the UL16-binding protein (ULBP) 1-6 also known as retinoic acid early transcripts 1 (RAET1). Both families are distantly related to MHC class I molecules, but instead of presenting antigens they serve themselves as signals for cellular stress (79). MICA, MICB and ULBP4-5 are transmembrane proteins with various cytoplasmic tails, while ULBP1-3 and 6 are attached to the cell surface via glycosylphosphatidylinositol (GPI) anchors (78). Especially the GPI-anchored ligands seem to have a high affinity for- and are constitutively present in lipid rafts (80). The NKG2D-ligands are highly polymorphic, in particular the MICA/B (78). The sequence homology between the two ligand families are only 20-25% and they also vary in their expression pattern, domain structure and their affinity for NKG2D (81). A striking fact is that NKG2D, a single non-polymorphic receptor, has a great variety of ligands from two different families. The significance of this ligand polymorphism and diversity is not completely understood but when operating in redundancy the many NKG2D ligands provide an obvious advantage in the

battle against evolutionary evolved cancer- and viral immune evasion strategies, and likely, this evolutionary pressure is one of the reasons for the increased NKG2D ligand diversity(81).

The NKG2D ligands are expressed in low levels in normal cells, however, they are upregulated or expressed *de novo* by a great variety of biological stress signals such as DNA damage, irradiation, oxidative stress, inflammation (autoimmune diseases, viral and bacterial infections) and malignant transformation. A great variety of tumors constitutively express NKG2D ligands. Thus, these molecules act as signals for stress, danger and pathological changes of the cell (82). There are suggestions for some additional yet unknown tissue-specific function since some of the NKG2D ligands have been found at sites where they most likely are expressed for some other reason than to trigger cytotoxicity, like in the mouse embryonic brain and cells in the bone marrow (81). Some tumors release soluble forms of the ligands, both shed variants and ligands bound to exosomes. It has been shown that the soluble NKG2D ligands act as decoy, downregulating the cognate receptor and impairing cytotoxicity. This mechanism of release of soluble NKG2D ligands seems to be a tumor evasive strategy, protect the tumor cells from being attacked by the host immune system (83-85). We have found a similar strategy of releasing NKG2D ligands on placental exosomes as a way of protecting the fetus from immune attack (47).

The mode of NKG2D-mediated cytotoxicity

NKG2D serves as a primary granule-mediated cytotoxicity receptor. After engagement with any of its cognate ligands, cytotoxic granules are secreted from NK cells by exocytosis inducing a very rapid cell death, within just minutes. The cytotoxic granules are complex organelles containing mediators of a diverse range of cell-death pathways. Three of the major granule proteins are perforin, originally described as a pore-forming protein, granzymes, a family of structurally related serine proteases varying in their substrate specificities, and granulysin, a cytolytic protein of the saposin-like protein family. The exact cytotoxic mechanism of these granules is not yet completely defined, but granulysin seem to function as a lytic molecule while perforin seem to be an essential enabler of the function of the granzymes which can (in the presence of perforin) trigger apoptosis directly, via activation of caspases or via triggering nuclear/DNA damages (86).

III. FasL, TRAIL and apoptosis induction

Apoptosis

Apoptosis, also called programmed cell death, is a controlled non-inflammatory process for elimination of damaged cells, cells that are no longer needed or self reactive immune cells (87). It is an essential mechanism during development, aging and normal tissue homeostasis but can also occur as a defense mechanism in immune reactions or when cells are damaged by disease or other harmful events like irradiation (88).

There are at least three different apoptotic pathways; 1) the intrinsic pathway initiated via intracellular signals sensed by mitochondria, 2) the extrinsic pathway initiated via plasma membrane death receptors, and 3) the perforin/granzyme pathway induced by the delivery of cytotoxic granules from NK cells and cytotoxic T cells (87). They use different signaling routes but they all eventually lead to the activation of effector caspases (caspase-3, -6 and -7) guiding the termination of the cell. The result is proteolysis of the cytoskeleton, fragmentations of cellular organelles like ER and Golgi apparatus, nuclear fragmentation, DNA degradation and finally formation of apoptotic bodies that displays molecules promoting their engulfment by phagocytes (87). A short summary of the extrinsic pathway of apoptosis, which we have studied in early pregnancy placenta, is given below.

The extrinsic pathway

The extrinsic pathway initiates apoptosis through death-receptors, members of the TNF-superfamily characterized by a cytoplasmic domain of about 80 amino acids called the death domain (DD) (88). Engagement of the death receptors with their cognate ligands will recruit adaptor proteins like FADD which in turn recruits procaspase-8 and -10 and the death-inducing signaling complex (DISC) is formed leading to auto-catalytic activation of caspase-8 and -10 that initiate a rapid apoptotic cascade (87, 88). Being such a powerful system, there is an obvious need for a tight regulation. One way for this is that aggregation of death ligands must occur in order to promote apoptosis. Thus it was shown that two adjacent trimeric FasL represent the minimal ligand aggregate required for DISC formation and apoptosis signaling (89).

Two of the most important death ligands of the TNF superfamily are Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Previous studies have shown that FasL and TRAIL are expressed by the trophoblast of human placenta and it was suggested that apoptosis might be a mechanism

for protection of the fetoplacental unit against a potential maternal immune attack (54, 56-58). An important discovery was that the FasL expression in human placenta is localized in the endosomal compartment on intraluminal vesicles inside multivesicular bodies (54). However, the exact mechanism of apoptosis-mediated immune protection was not proven or completely clear. Therefore, one of the topics in this thesis is the question if and how apoptosis is involved and used by human placenta for protection against activated maternal lymphocytes potentially dangerous to the fetus. Below follows a short presentation of these two apoptosis inducing molecules.

Fas ligand (FasL)

Fas ligand (FasL, APO-1L, CD95-L) is a type II transmembrane protein important for tumor surveillance, regulation of immune responses, organ development and tissue homeostasis (90). FasL is expressed by activated T cells, some tumor cells and by epithelial and other cells at immune privileged sites, one example being the brain, where many cells in the CNS express FasL. FasL seems to impede immune responses by ligating its cognate death receptor Fas (APO1, CD95) on activated lymphocytes prompting them into apoptosis (91). Most of the current knowledge about FasL and its contribution to immune privilege comes from studies of the eye, which is the most well studied immune privileged site (91). The eye exhibits a constitutive expression of functional FasL, whereas a loss of FasL-expression was shown to provoke damage of the ocular tissue during immune responses (92). FasL has also been ascribed a key role for success in transplantation of allogeneic corneal grafts (91). Besides its proapoptotic properties, FasL has also been ascribed co-stimulatory function since FasL-mediated signaling was shown to be required for the optimal proliferation of CD8⁺ T cells (93).

As with all TNF family ligands, the extracellular domain of FasL is sensitive to cleavage by metalloproteases which then produces a 26kDa soluble form of FasL. The soluble FasL seem to lose its apoptotic activity and has even been shown to inhibit the apoptotic action of membrane bound FasL (94, 95).

TNF-related apoptosis-inducing ligand (TRAIL)

TNF-related apoptosis-inducing ligand (TRAIL, APO2L) is, as FasL, a type II transmembrane protein and is mainly expressed by cells of the immune system. It binds to several receptors where DcR1 and DcR2 act as decoy receptors while DR4 and DR5 are death-receptors activating the apoptotic pathway. A fifth receptor that also acts as a decoy receptor is the soluble osteoprotegerin (OPG) (96). When binding to any of its cognate death-

receptors it activates similar intracellular apoptotic pathways as FasL indicating a potential functional redundancy between these two ligands (97). Tumor cells have been shown to be more sensitive to TRAIL induced apoptosis than normal cells, possibly due to the high expression of decoy receptors in normal cells. This led to the suggestion for a pivotal role of TRAIL in tumor surveillance (98). But there are also reports of TRAIL-mediated apoptosis of activated T cells, suggesting co-operation between TRAIL and FasL (58, 99-101).

IV. Exosomes - endosomal nanovesicles for inter-cellular communication

General description of extracellular vesicles and definition of exosomes

Release and uptake of extracellular vesicles (EV) is a way of intercellular communication without the need of direct cell-cell contact. There are several different kinds of EVs that can be classified according to shape, size, composition, mechanism of release and cellular origin, a summary of the most common ones and their major characteristics are presented in Table III. Practically all cells release EVs and various EVs are present in the blood and many bodily fluids like saliva, plasma, breast milk, amniotic fluid, bronchoalveolar lavage fluid, urine and various malignant effusions such as pleural and ascitic fluids (102, 103).

The EVs of interest in this dissertation are the exosomes, some of the smallest EVs that originate in the late endosomal cellular compartment and have a defined pathway of generation and secretion. The current definition of exosomes is secreted membrane nanovesicles with the following characteristics: 1) 30-100 nm size; 2) a buoyant density of 1.13-1.19 g/ml when isolated by ultracentrifugation on a sucrose gradient; 3) presence of tetraspanins in their lipid-rich detergent resistant membrane; 4) a characteristic cup-shaped form observed by electron microscopy of ultracentrifugation/sucrose gradient-isolated exosomes and 5) endosomal origin (103). Exosomes carry membrane-bound and cytosolic proteins and nucleic acids like mRNA, miRNA and mitochondrial DNA providing them with a great potential in cell-to-cell communication and ability to transfer genetic information and reprogram recipient cells (102, 104-107).

Biogenesis of exosomes

The formation of exosomes, through the endosomal compartment, is a key feature of exosomes whereby they diverge from all other EVs that are

| Characteristics | Exosomes | Microparticles/ microvesicles | Shed microvilli | Apoptotic bodies |
|---------------------------------|--|--|--|---|
| Size | 30-100 nm | 0.1-2 μ m | >400 nm | 100-700 nm |
| Floatation density | 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 -translucent | Various shapes, round, elongated and cylinder-like | Irregular and heterogenous |
| Lipid membrane composition | Cholesterol-, sphingomyelin- and ceramide rich lipid rafts, exposed phosphatidylserine | Exposed phosphatidylserine, some enriched in cholesterol diacylglycerol, some undetermined | Undetermined | Undetermined |
| Specific identification-markers | Tetraspanins (CD63, CD9, CD83), ESCRT complex members (Tsg101, Alix) | Integrins, selectins, CD40 and others depending on the cell type | Various depending on the cell type | Histones, DNA |
| Intracellular storing | Yes | No | No | No |
| Origin in the cell | Endosomal compartment/MVBs | Plasma membrane | Plasma membrane | Fragments of dying cells, undetermined |
| Mechanism of sorting | Ceramide and ubiquitin dependent | Unknown | Unknown | Fragments of dying cells, undetermined |
| Mechanisms of release/secretion | Exocytosis by fusion of MVBs with the plasma membrane | Plasma membrane blebbing | Plasma membrane blebbing | Plasma membrane blebbing and cellular fragmentation |

Table III. Some of the main characteristics of different extracellular vesicles (adapted from ref. (104).

formed by blebbing or shedding of the plasma membrane. As illustrated in Figure 1, intraluminal vesicles (ILV) are formed by inward budding of the late endosomal limiting membrane leading to the formation of multi vesicular bodies (MVB) - vacuoles filled with ILV. The MVB either fuse with the lysosome for protein degradation or fuse with the plasma membrane and release its contents (the ILV now called exosomes) into the extracellular space. Proteins can be sorted to the limiting membrane of MVB in two ways, by direct transportation of *de novo* synthesized protein from the Golgi

complex or by endocytosis of plasma-membrane proteins aimed to be recycled and therefore transported via the early endosome. By this way of vesicle formation, the proteins carried on the exosomal membrane end up with the same topological orientation and preservation of their three-dimensional structure as the ones expressed on the cellular membrane thus keeping their biologic activity. Various membranal and cytosolic proteins as well as nucleic acids can be enriched selectively or randomly in the exosomes compared to the cells that produce them. Generally, the composition of the exosomes emanates from the protein and nucleic acid composition of their mother cells and reflects in any given time the state of the cells that produce them, e.g. activation, signaling, quiescence etc. (103, 104).

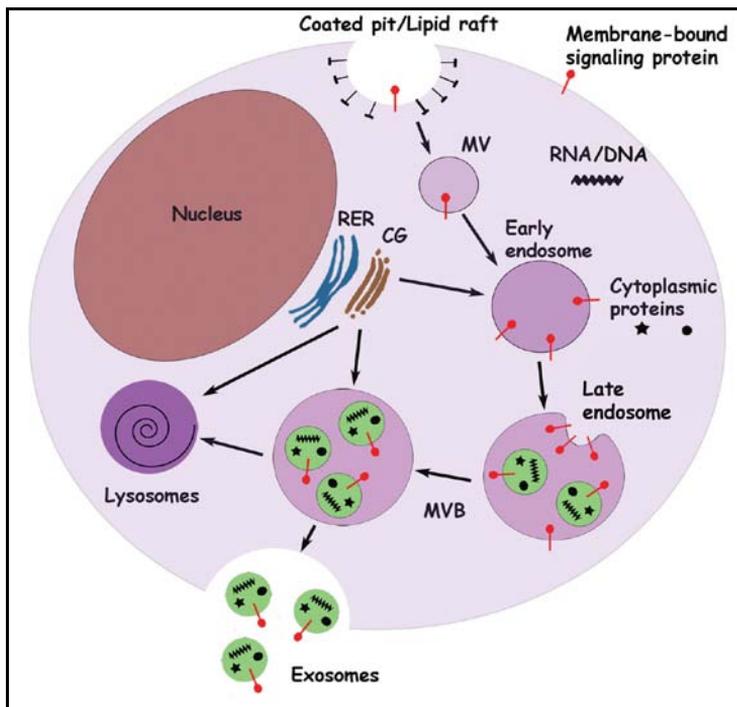


Figure 1. Biogenesis of exosomes. Schematic illustration of the generation and secretion of exosomes through the endosomal pathway and how exosomes can be loaded with *de novo* synthesized proteins or with recycled proteins from the cell membrane (104).

CG: Complex Golgi; RER: rough endoplasmic reticulum; MV: microvesicle; MVB: multivesicular body.

How the composition of the ILV is decided and which proteins/nucleic acids are enriched is far from completely understood. The RNA content of exosomes differs from the donor cell, indicating a specific targeting of mRNA

into exosomes through yet unknown mechanisms (107, 108). One piece of machinery involved in the specific sorting of proteins to the limiting membrane of MVBs and further in the actual formation of the ILV is the Endosomal Sorting Complex Required for Transport (ESCRT). This system of four different ESCRT complexes recognizes ubiquitylated transmembrane proteins and sorts them into the forming ILV (109-112). Another suggested, ESCRT-independent, sorting mechanism involves lipid rafts rich in sphingomyelin metabolites such as the sphingolipid ceramide (113, 114). The exosomal membrane is highly enriched in tetraspanins, a family of molecules with four transmembrane domains. The tetraspanins are suggested to stabilize the exosomal structure and to participate in targeting specific proteins to the exosomes, GPI-linked proteins being one type of proteins specifically enriched on the exosomal membrane (115). Tetraspanins in association with lipids seem to stabilize other proteins and form complexes with a variety of both membrane and cytosolic proteins (116). These complexes form tetraspanin-enriched domains (TEM) which, similar to rafts and clathrin-coated pits, facilitate vesicular fusion/fission (117, 118).

As mentioned above, MVB filled with ILV can be either transported for degradation to the lysosomal compartment (degradative MVB) or move to the cellular membrane where they open and secrete the ILV in the extracellular space as exosomes (exocytic MVB). The mechanisms deciding the fate of the MVB are predominantly unknown but Rab GTPases seem to be important determinants. Rab7 has been suggested to mediate the transport to the lysosome for degradation while Rab11, Rab35, Rab27a and Rab27b have all been suggested to be of importance in the docking of MVBs to the plasma membrane (119-122).

Composition of exosomes

The exosomal membrane is a lipid raft-rich bilayer built up by cholesterol, sphingolipids and tetraspanins (123). The exosomes carry membrane proteins as well as cytosolic proteins. Exosomes from different cellular origin seem to share one set of common proteins while they also express a more unique set of cell-type specific proteins reflecting the function of the original cells. The common set of proteins deals with their biogenesis, trafficking and structure and include ESCRT proteins (like Alix and Tsg101), Rab proteins and tetraspanins (like CD63, CD81, CD9). Since exosomes reflect the cells that produce them there is no true exosome-specific marker but the tetraspanins are the group of proteins that is most commonly found associated with exosomes and are generally used as exosomal markers (103, 123, 124). In addition to proteins, exosomes also contain ribonucleic acids

mRNA, microRNA and other non-coding RNA and mitochondrial DNA and have the ability to reprogram recipient cells (107, 125-127).

Functional properties of exosomes

Secreting proteins via exosomes has several advantages; i) a preserved three-dimensional structure and thus the biological activity of the proteins carried by exosomes; ii) signal delivery independent of cell-cell contact; iii) lower mobility and higher concentration of the carried molecules; iv) independence of *de novo* protein synthesis; v) biological effects at a distance (124). As well as other EVs mentioned above the exosomes are also produced and secreted by a great variety of cells like fibroblasts, syncytiotrophoblast, immune-, epithelial- neural- and tumor cells; and are present in various bodily fluids like blood, urine, saliva, breast milk, malignant and pleural effusions of ascites, bronchoalveolar lavage and synovial fluid (102). Their function somewhat depends on the origin cell but a substantial amount of the exosomes seem to have a major role in influencing the immune system (105). In general, exosomes derived from antigen presenting cells are antigen-loaded and have immunoactivating properties while exosomes from most tumors and epithelial cells, including syncytiotrophoblast-derived exosomes from human normal placenta, are tolerogenic or immunosuppressive (103, 124, 128, 129). Exosomes from human intestinal epithelium are often referred to as tolerosomes as they are suggested participants in oral tolerance (128, 130). Tumor exosomes carry molecules and signal substances that disturb and downregulate immune effector mechanisms or enhance the suppressive function of regulatory T cells, promoting the protection of the tumor from host immune responses (85, 131, 132). Placental exosomes resemble tumor exosomes in avoiding the host-/maternal immune defense, and use similar immune suppressive mechanisms (47, 54, 55, 133). As mentioned above, exosomes also contain a variety of nucleic acids. It has been shown that exosomal mRNA could be translated in target cells indicating ability of exosomes to transfer genetic information (107). In another report, miRNA transferred by T-cell exosomes inhibited target genes in dendritic cells (126).

In summary, exosomes seem to play a substantial role in intercellular communication and target cell reprogramming. An important key feature is their ability to modulate the immune system in activating or inhibitory direction. Placental exosomes and their contribution as immune modulators during human early pregnancy is the main theme of the current dissertation.

V. Pregnancy and the human placenta

General description of the placenta

The mammalian placenta is a temporary organ with several essential tasks promoting a successful pregnancy; it separates the fetal and the maternal blood from each other, secures the exchange of nutrient, gas and waste, and is of crucial importance as an endocrine organ producing hormones for the successful ongoing and outcome of pregnancy.

The formation of the placenta starts at the implantation of the blastocyst into the uterine mucosa. The blastocyst consists of two cell types: the inner cell mass which will become the fetus and the outer cell mass of undifferentiated trophoblast stem cells, the cytotrophoblast, which will give rise to several different subtypes of trophoblast such as the invasive extravillous trophoblast, the endovascular trophoblast and the villous trophoblast comprising the syncytiotrophoblast (STB) and cytotrophoblast (CTB) of the chorionic villi; all these types of trophoblast, described below and illustrated in Figure 2, will be temporally and spatially developed at different stages of placenta formation and will thus participate in the building of the most essential and still enigmatic temporary organ for mammalian pregnancy, the placenta. At the early stage, all mammalian pregnancies appear similar, but further on in the development there are considerable anatomical and histological differences in the mammalian placentae of different species.

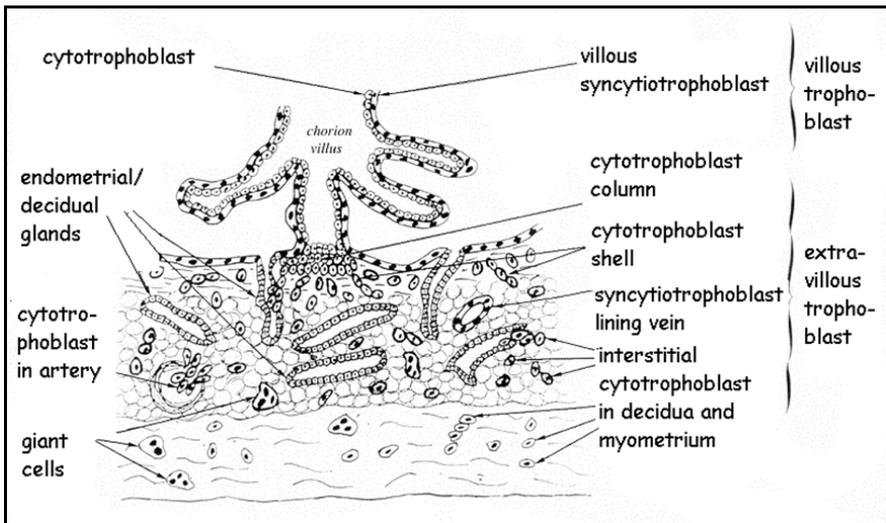


Figure 2. Trophoblast subpopulations. Schematic illustration of the different trophoblast subpopulations in the chorion villus and in the placental bed(134).

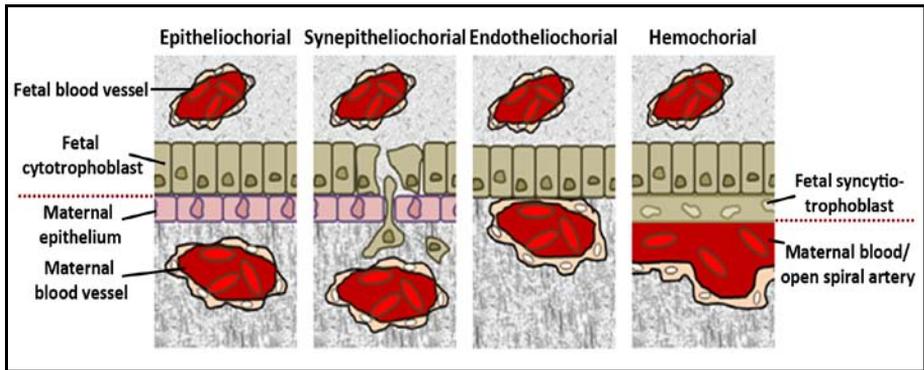


Figure 3. Different types of placentation in mammalian pregnancy.

Illustration of the trophoblast invasion in different species. In the epitheliochorial placenta (horse and pig), the trophoblast is adjacent to an intact uterine epithelium. In the synepitheliochorial placenta (ruminants) the trophoblast partially invades through the epithelium and into the uterine stroma. In the endotheliochorial placenta (dog and cat) the epithelial layer is completely destroyed and the trophoblast is adjacent to the endothelial layer of the maternal vessels. In the hemochorial placenta (human, primates and rodents), even the endothelial layer is destroyed and the trophoblast is in direct contact with the maternal blood.

Depending on how the maternal and the fetal tissues that comprise the placenta are opposed to each other and how far the invasiveness for the trophoblast is, there are four types of mammalian placentae: 1) the epitheliochorial placenta as in horse and pig, where the trophoblast and the uterine epithelium are opposed to each other in a direct contact but with no erosion of the epithelium; 2) the synepitheliochorial placenta characteristic for ruminants, where the uterine epithelium is only partially conserved and the trophoblast partially invades past the epithelium into the connective stroma of the uterine mucosa; 3) the endotheliochorial placenta as in dog and cat, where the uterine epithelium is destroyed completely and although the maternal vessels are kept intact, the trophoblast invasion goes until meeting and opposing the endothelium of the maternal uterine vessels, and finally 4) the hemochorial placenta as the one in humans, primates and rodents, which is the most invasive of all placenta types since the trophoblast invades through all cellular layers in the maternal uterine mucosa including the endothelial layer of the maternal vessels. In the hemochorial placenta the maternal vessels are opened and transformed by the endovascular trophoblast into lacunae, i.e. “lakes” of maternal blood that surround the chorionic villi of the placenta. The hemochorial placenta provides the most intimate contact between the mother and the fetus in that the chorionic villi lie in direct contact with the maternal blood. The different types of mammalian placentae are illustrated in Figure 3.

During human pregnancy there are thus two closely interconnected types of transitory tissues that are both shed after parturition, the fetal trophoblast and the maternal counterpart the decidua. A brief description of both will follow below.

Decidua

The decidua is the mucosal layer of the endometrium of the pregnant uterus. It can be classified anatomically into decidua basalis at the implantation site that is invaded by trophoblasts, decidua capsularis covering the embryonic membranes and decidua parietalis covering the rest of the uterine cavity, as illustrated in Figure 4. The decidual tissue is comprised of endometrial/decidual glands, blood vessels and the decidual stroma composed of large loosely connected stromal cells. Dispersed between these cells are numerous immune cells. Decidua is a tissue rich in leukocytes, about 15-30% of all cells in human early pregnancy decidua are leukocytes (65, 135). The decidua-associated lymphoid tissue (DALT) has a distinct histological organization: 1) lymphoid cell clusters (LCC), a structure unique for the decidua, which are aggregates of activated cells found adjacent to vessels and decidual glands; 2) subepithelial lymphoid cells with close contact to the basal part of the glandular epithelium (in contrast to other mucosal sites which have intraepithelial lymphocytes); and 3) individual immune cells randomly scattered between stromal cells. B cells are scarce or absent in the decidua, instead there are abundant CD56^{+bright}/CD16⁻ uNK cells, $\alpha\beta$ - and $\gamma\delta$ T cells, dendritic cells and macrophages as described in chapter I.

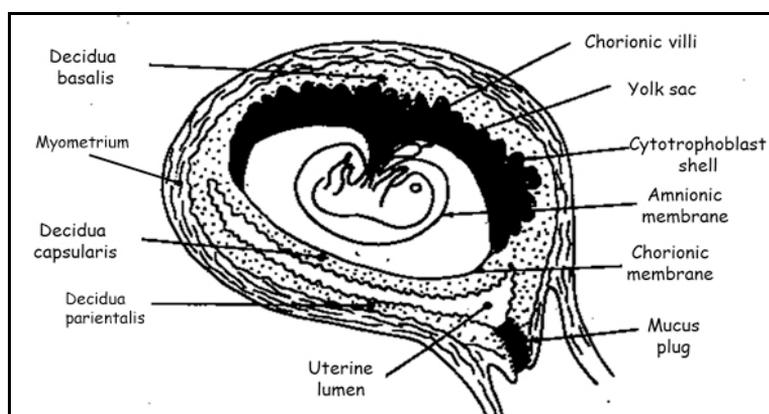


Figure 4. The anatomic classification of decidua in early human pregnancy. Illustration of the human pregnant uterine cavity showing the different parts of the decidua and the relationships between fetal membranes, trophoblast and decidua (134).

Trophoblast

During placenta formation, the pluripotent cytotrophoblast cells can take two differentiation pathways and become villous- or extravillous trophoblast. As mentioned above, Figure 2 shows a schematic presentation of the trophoblast subpopulations. Even though the trophoblast subpopulations show phenotypic variations, one shared feature of all trophoblast cells is that they express cytokeratin 7, 8 and 18, indicating their epithelial origin (136).

The villous trophoblast

The main functional unit of the placenta, intimately connected with the placental physiology, is the chorionic villi, floating in maternal blood and participating in the nutrition, gas- and waste exchange. The chorionic villi are covered by an outer layer of STB that are in direct contact with the maternal blood and an inner layer of CTB, which is prominent in the early pregnancy but gradually disappears and is completely absent in the last trimester and term placenta where the chorionic villi have a single layer of STB. Under the covering STB/CTB facing the maternal blood, the chorionic villi comprise of fragile mesenchymal tissue in which the fetal blood vessels transporting oxygen nutrients and waste are situated. Thus in early pregnancy there are four layers of tissues in between the fetal and maternal blood, i.e. vessel endothelium, mesenchymal cells, CTB cells and STB, which are referred to as the placental barrier. In term pregnancy the CTB disappears and the placental barrier comprise of only three layers.

The human STB, comprise terminally differentiated CTB cells that have fused to form a multinucleated syncytium covering the chorionic villi. The free apical part of the STB that is in direct contact with the maternal blood is covered with numerous highly pleomorphic microvilli and surface projections that are in permanent movement. Morphologic studies by electron microscopy have shown that there is a continuous and constitutive shedding from the apical STB membrane of plasma membrane-derived microvesicles and whole microvilli (137), a shedding activity that can be enhanced by biological stress and various pathological pregnancy conditions, the most well known and studied being pre-/eclampsia (138). Moreover, our electron microscopy studies discovered a vigorous exosome biogenesis and secretion, which is the main topic further described and discussed in this dissertation (paper II and III).

STB is the most important of all cell types of the human placenta with several functions as: 1) the chief regulator of oxygen, nutritional and energy transport; and waste remover between the fetus and the mother essential for

the fetal survival, development and growth; 2) the site of synthesis of a variety of proteins such as steroid and placental protein hormones, signal substances, immunomodulatory proteins, adhesion molecules and matrix metalloproteases, all of crucial importance for a successful pregnancy (139, 140).

Extravillous trophoblast

The essential role of the extravillous trophoblast is to participate in the early stages of placenta formation where one of the most important tasks is to remodel the uterine vascular system in order to achieve a placental bed with an adequate blood supply ensuring the huge need of oxygen, nutritional and energy supply for the growing fetus. At the top of some villi, CTB cells proliferate through the STB layer and anchor the villi to the uterus through formation of cytotrophoblast column and shell. This anchoring trophoblast cells make a specialized fibronectin-related extracellular matrix substance called fetal fibronectin or trophuteronection (TUN) that mediates the adhesion. The interaction between trophoblast and the extracellular matrix is a critical feature for pregnancy success since as invasive trophoblast cells they now differentiate to become either endovascular- or interstitial extravillous subpopulations. The endovascular extravillous trophoblast finally penetrates and remodels the maternal spiral arteries to control the blood flow in the placental intervillous space. It replaces the endothelium with a single endovascular trophoblast layer expressing the vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell adhesion molecule 1 (PEACAM-1) (141). The interstitial extravillous trophoblast invades as far as the inner third of the myometrium and further differentiates to form giant multinucleated cells. Abnormalities like shallow trophoblast invasion and/or inadequate vascular remodeling have been correlated to pregnancy complications and pre-/eclampsia (142).

Pregnancy as a challenge for the immune system

The human placenta contains both maternal and paternal genes, but due to genomic imprinting paternal genes are preferentially expressed making the fetoplacental unit vulnerable for being recognized as foreign and attacked by the maternal immune system (143). Evidence shows that fetal alloantigens are indeed recognized by the maternal immune system, but this recognition seems to actually lead to a state of tolerance instead of rejection (144). The fetus is not in direct contact with the maternal tissues, instead the physical and functional connection is mediated by the placenta. There are two immunologically important fetomaternal interfaces as illustrated in

Figure 5; 1) at the systemic level where the villous syncytiotrophoblast are in direct contact with the maternal blood and thus the circulating maternal immune cells and 2) at the local level where the extravillous trophoblast invade and meet the local maternal immune cells resident in the decidua. Numerous mechanisms exist that cooperate to prohibit maternal immune attack. The placenta itself possesses several such mechanisms and can thus be entitled as an immunomodulatory organ and the pregnant uterus as an immune privileged site. The term immune privilege refers to the state of some regions in the human body where immune-mediated inflammation and allograft rejection is reduced. It does not imply a total occlusion of the immune system but instead there is a tightly regulated adaption of the immune system to a more tolerant state. Different regions have different degree of immune privilege but at some extent they seem to apply several similar mechanisms for maintenance of the tolerance state (101). Three regions with particularly well developed immune privilege are the brain, the eye and the pregnant uterus. The cells of the brain and the eye have limited

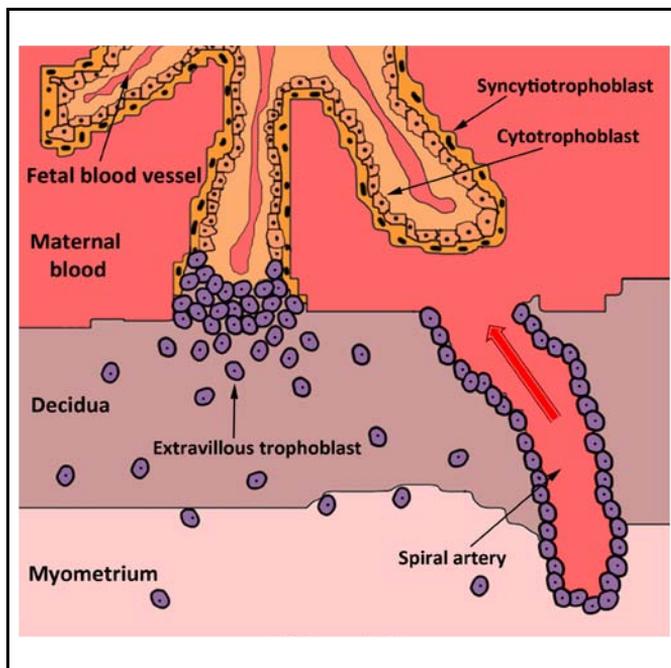


Figure 5. Two feto-maternal interfaces where trophoblast cells meet the maternal immune system. One intimate interface is between the villous trophoblast and the maternal blood and thus the systemic immune cells. Another intimate interface is between the extravillous trophoblast and the local maternal immune cells resident in the decidua.

capacity of regeneration and thus need to be particularly protected. In the uterus the immune system is obliged to tolerate the genetically different cells of the fetus and the placenta to assure the survival of the offspring and thus the species.

The syncytiotrophoblast of human placenta is a rigorous producer of various extracellular vesicles

Placenta exhibits a continuous shedding of microvesicles and even of whole microvilli from the apical ST membrane during pregnancy. Such placenta-derived microvesicles, named syncytiotrophoblast microparticles (STBM), were found to be significantly elevated in pre-eclamptic women with early onset preeclampsia compared to normal pregnancy (145, 146).

STB holds a great potential of extensive protein synthesis and the syncytioplasm is rich in free ribosomes and rough endoplasmic reticulum (RER) with Golgi complexes at certain intervals throughout the syncytium (104). Beside the signs of the profound *de-novo* protein synthesis, there is also an indication for recirculation of membrane bound proteins. Numerous bristle-coated pits/caveolae rich in lipids and associated with uptake of membrane molecules, are scattered between the ST microvilli (140). The features of ST with both recycling and new synthesis of proteins together with the MVB rich endosomal compartments indicate a potential for ST as an active producer of exosomes. The ST could thus be regarded as a good model for exosome studies including their biogenesis, protein sorting and function.

AIMS OF THE INVESTIGATION

The overall objective of this thesis was to study immunomodulatory mechanisms protecting the fetus from maternal immune attack during human normal pregnancy with a main focus on the contribution of placental exosomes.

The specific aims were:

- To isolate and characterize the biogenesis, morphology and phenotype of exosomes secreted in explant cultures of human normal early pregnancy placenta
- To study maternofetal tolerance by investigating the protective contribution of placental exosomes carrying:
 - The NKG2D receptor ligands MICA/B and ULBP1-5
 - The proapoptotic molecules FasL and TRAIL
- To study the CD4⁺CD25⁺FOXP3⁺ T regulatory cells of normal pregnancy in paired samples of isolated decidual mononuclear cells and peripheral blood mononuclear cells from pregnant and non-pregnant women
- As a prerequisite and corollary of the placental exosomal studies above, an optimized isolation method for villous trophoblast from human normal early pregnancy placenta was developed.

METHODOLOGICAL CONSIDERATIONS

Animal models cannot replace human placenta

The structural and functional uniqueness of the human placenta makes it very difficult to replace with other animal models. Working with non-human primates involves greater ethical concerns than using the more established laboratory animals, and also bigger requirements for their housing and the long gestation time could also be a problem. Furthermore, in most of them, the implantation is more superficial with a more restricted trophoblast invasion than in humans (147). The placentae of rodents and primates are indeed hemochorial like humans, but there are nevertheless significant differences in the anatomy and cellular composition of the murine placenta and decidua. Mouse models have contributed to an increased understanding about murine pregnancy, and have been of benefit for studies in humans since there are several similarities. But in line with this gained knowledge, the differences between the species have also become more pronounced which clarify the uncertainty in extrapolating results from one model to the other. In mice it seems that the implantation event induces decidualisation whereas the first signs of decidualisation in humans can be hormonally achieved before conception has even occurred (148). The time point when the placenta achieves its definite structure also significantly diverge with as early as at day 21 of pregnancy in humans while a comparable structure in mouse is not seen until halfway through gestation. The endocrine function of the mouse and human placenta is also very different. The mouse pregnancy is totally dependent on corpus luteum for production of progesterone through the whole gestation, while it is the syncytiotrophoblast that is the main hormone producer in human placenta (149).

When considering the use of other alternatives like cell lines, it is important to bear in mind that these cells have adapted to grow in culture without the influence of surrounding tissues and thereby they risk to lose essential tissue-specific functions and acquire a molecular phenotype quite diverged from the genuine *in vivo* cells. The major drawback regarding trophoblast cell lines is however that it is only possible to culture CT, and thereby, one misses out to study the most interesting cell type of the placenta, the fully differentiated ST situated in direct contact with the maternal blood and thus the maternal immune cells.

Collection of human placental and decidual samples for studies of human normal reproduction

Consequently, we chose to work exclusively with placental and decidual tissue, donated by healthy women undergoing elective termination of pregnancy (8-16 weeks of gestation) after ethical committee approval and informed consent. The early pregnancy, when placenta is being formed and the pregnancy is established in the maternal organism, was our main choice since it would be the most interesting to study and it would in a best way reflect the questions we wanted to pursue. Samples from normal mid pregnancy were not possible to get for obvious ethical reasons. Term placenta, after delivery, is a completely “different” organ compared to early placenta and was only used in comparative purpose in our studies. To ensure that the tissue is minimally manipulated only tissue from terminations performed by vacuum aspiration and term placenta from normal uncomplicated pregnancy and delivery were included and used in the studies. All samples were collected in ice-cold RPMI 1640 immediately after extraction and decidual tissue and chorionic villi were separated and processed within maximally 3-4 hours after extraction. The samples used for explants culture, cell isolation, exosome isolation etc. were not mixed but kept separate to minimize MHC-dependent *in vitro* effects and avoid *in vitro* activation due to alloreactivity.

Isolation of decidual mononuclear cells

Isolation of immune cells from human decidual tissue of early pregnancy was done using an optimized isolation technique for decidual mononuclear cells developed in our group (150). In brief, the decidual tissue sample were thoroughly washed in Hank's solution to remove contaminating blood before cut into small pieces and filtered through a 60 μ m stainless steel mesh to make single cell suspension. The cell suspension was then subjected to discontinuous density gradient centrifugation of 20-, 40- and 80% Percoll. Mononuclear cells and epithelial cells were collected from the interface between 40% and 80%. The epithelial cells were then removed by incubation with mAb BerEP4 and goat-anti-mouse coated magnetic beads (Dynabeads) and magnet treatment.

There are several advantages of our method. Firstly, only mechanical disruption is used in the preparation of single cell suspension thus the degradation of surface molecules on the decidual leukocyte cell population by enzymatic treatment was avoided. Secondly, Percoll was used, known to be gentle to cells and cellular components and the gradient was optimized to maximize the yield of decidual leukocytes with high viability (150). From

these cells, T regulatory cells, used in our study in paper IV, were separated with the CD4/CD25 T Regulatory Cell Isolation Kit (Miltenyi Biotech; Bergish Gladbach, Germany) according to the manufacturer's instructions.

Isolation of villous trophoblast

The human villous trophoblast of early pregnancy comprises an inner layer of CTB and an outer layer, syncytium, composed of the mature and fully differentiated STB. The STB is the main producer of pregnancy-related hormones, cytokines, and growth factors and is responsible for important placental functions such as nutrition, and gas and waste exchange. The STB is also in direct contact with the maternal blood and a major source for immunomodulatory molecules. As a prerequisite for our further studies we optimized a method for isolating villous trophoblast cells from early pregnancy human placenta since most of the previously described methods were adopted from term placenta and/or did not consider the problem of contamination by non-trophoblastic cells in the obtained cell fractions (151-153). Our focus was purity, viability and preserved morphology.

Our isolation technique is based on three steps: treatment by a mild enzymatic cocktail, optimized by us, to detach the cells from each other and make a single cell suspension, a Percoll-based density gradient centrifugation for enrichment of trophoblast cells and, finally, a purification step by depletion of contaminating leukocytes by treatment with immunomagnetic anti-CD45 antibody-coated Dynabeads. A detailed description of the method is given in paper I.

With this technique, we achieved a good yield of villous trophoblast ($1.62 \pm 0.35 \times 10^6$ cells/g tissue) with a high viability ($93.6 \pm 2.3\%$). The expression of cytokeratin 7 (CK-7) was used to demonstrate the trophoblast origin of the isolated cells. More than 95% of the isolated cells were positive for CK-7 indicating a pure trophoblast cell fraction. When examined by electron microscopy, a mixture of pure CTB and STB could be seen. Both cell types were in very good condition showing a well preserved morphology. There was previously no method described for separating the two isolated subpopulations. We showed however that the STB could be captured on magnetic beads coated with either anti-PLAP or anti-MIC mAbs and removed by negative selection, leaving a pure CTB population to be examined further and which could also be used in primary cultures. The captured STB could be used for molecular studies after RNA or DNA extraction.

In summary, our method is gentle and easy to perform and gives pure and highly viable villous trophoblast, both CTB and STB with well preserved morphology, from human first trimester placenta. CTB and STB obtained by this method were used in the studies presented in papers II and III.

Placental explant culture

Several cell types secrete exosomes into the human blood stream making blood very inconvenient to work with since it requires separating the different exosomes from each other, and this procedure would also require a large volume of blood in order to get any qualified amount of exosomes. Culturing of placental explants enabled us to obtain rather large quantities of exosomes exclusively produced by the placenta. This was not optimal since the exosomes were produced under *in vitro* conditions, but so far this has been the only way to obtain a medium from which substances secreted from human placenta can be collected. To minimize experimental influences on the tissue and to avoid excessive cell death, we only used freshly extracted placentas that were handled very carefully, and we limited the culture time to 24h.

Human early pregnancy placental samples were washed thoroughly in Hank's solution to remove contaminating blood. Small pieces, ~5-10 mg wet weight, of chorionic villi were cut out and cultured in RPMI 1640 supplemented with 0.5% BSA and antibiotics at 37°C and 5% CO₂ in humidified air. After maximum 24h of culture, the supernatant were collected and cleared of cell debris by centrifugation at 4000 x g for 30min. Exosomes were either isolated immediately from the supernatant, or the supernatant was stored at -80°C for later use.

Isolation of exosomes

The syncytiotrophoblast from human placenta does not only secrete exosomes, but also a great variety of other microvesicles/microparticles are shed from the apical part of the syncytiotrophoblast membrane. A big challenge in isolating exosomes is therefore to minimize contamination of other microvesicles to obtain as pure exosome fraction as possible. This was achieved through the use of differential centrifugations in combination with a continuous sucrose gradient (1.02-1.19 g/ml) or a discontinuous sucrose gradient/cushion (1.08/1.18 g/ml). We optimized every step in this procedure and the purity of our exosome fractions was continuously examined by transmission electron microscopy. In our isolation procedure we combined 30 min centrifugation at 17,000 x g and filtration through a 0.22 µm filter to remove larger vesicles and protein precipitates. Thereafter,

two succeeding ultracentrifugations for 1 h at 110,000 x g were applied, the second one either with a continuous or discontinuous sucrose gradient/cushion. The obtained exosomal fraction was thereafter washed by PBS and finally ultracentrifugated and kept at -80°C until use. To avoid the cleavage of membrane bound proteins from the surface of exosomes we used a protease inhibitor cocktail (Complete Mini; Roche Diagnostics). Since the size of exosomes are below the cut-off size of most flow cytometers, in our analyses by flow cytometry we used antibody capture by loading the exosomes (carrying our molecule of interest) on mAb/Ab coated latex beads revealed by direct conjugated anti-CD63 secondary mAbs, CD63 being a well accepted tetraspanin marker for exosomes.

RESULTS AND DISCUSSION

VI. Rationale of the project

As mentioned earlier the maternal immune system must alter its responses during pregnancy to meet the challenge of accepting and supporting a semiallogeneic fetus. The fetus, viewed as a transient transplant in the uterine cavity, has to face a variety of threats and develops strategies to avoid them. We have chosen to study three immune mechanisms: 1) the maternal cytotoxic system of the NKG2D receptor and its ligand families MHC class I chain related antigen A and B (MICA/B) and UL16-binding proteins (ULBP) 1-5; 2) the apoptosis-inducing molecules Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) and 3) the maternal T regulatory cells during early normal pregnancy at the local (in decidua) and systemic level. Our objective was to elucidate the involvement of these mechanisms in creating maternal tolerance towards the fetus.

VII. Convincing evidence reveals that exosomal biogenesis and secretion takes place constitutively in the syncytiotrophoblast of human normal pregnancy placenta

Our quantitative real-time RT-PCR analyses showed a constitutive mRNA expression of NKG2D ligands, FasL and TRAIL in STB of human first trimester placenta and term placenta. We could demonstrate that the mRNA is constitutively translated into proteins as shown by IHC, flow cytometry and immuno electron microscopy (IEM) (*Fig. 1 paper II, Fig. 1 paper III*). The IEM studies revealed that all our molecules of interest were expressed solely by the STB and not by the CTB. Furthermore, by elaborate IEM studies we could show that FasL, TRAIL and ULBP1-5 are absent on the apical surface membrane of STB and instead expressed exclusively intracellularly on the limiting membrane of MVBs and on the membrane of intraluminal vesicles situated inside the MVBs (*Fig. 2 paper II, Fig. 2 paper III*). MICA/B had been shown in a previous paper to be expressed both on the MVB's limiting membrane and its intraluminal vesicles, but also on the apical surface membrane of STB (47). The localization of the ULBPs coincided with the expression of CD63 and TSG101, markers characteristic for the late endosomal compartment and for exosomes (*Fig. 3 paper III*). Similarly, we showed that FasL and TRAIL are also expressed on the limiting membrane of MVBs and on the ILVs inside. Thus, our IEM studies gave us invaluable data proving beyond doubt that exosome biogenesis constitutively takes place in the syncytiotrophoblast of human normal pregnancy. This is, to our

knowledge, the first detailed study on ultrastructural level establishing the endosomal expression of the NKG2D ligands *per se* and in human normal ST. Pregnancy imposes a strain on the maternal organism and is highly dependent on large amounts of oxygen and nutrients for its normal development. It is indeed a stressful situation to grow and develop in the maternal body under these conditions and thus that could be the reason for expression of the NKG2D ligands. But since NKG2D ligands on the trophoblast cell surface would comprise an obvious risk of activating NKG2D receptors and cytotoxic attacks resulting in elimination of the STB, it is most logical to avoid exposition of NKG2D ligands on the cell surface. Directing the ligands to MVBs and getting rid of them by secreting them as exosomes could be a way to prevent the expression on the cellular membrane. Another explanation could be additional tissue-specific regulation of NKG2D ligand expression.

Apoptosis and the apoptosis-inducing molecules FasL and TRAIL have been detected in the placenta throughout pregnancy, and have been shown to participate in implantation and placenta formation (154, 155). Our previous and current publication importantly shows the exosomal expression and secretion of these molecules in an oligomerized active form. The apoptotic ability of FasL and TRAIL as well as other members of the TNF family is only possible when they are expressed in a membrane-bound form. These molecules are MMP-cleavage sensitive since their molecules contain specific cleavage sites for MMPs that generate proteolytic shedded soluble proteins (156). The 26kDa soluble form of FasL (sFasL) is 1000 times less active in inducing apoptosis than the membrane bound mFasL. Moreover, sFasL has even been shown to counteract and inhibit the apoptotic action of mFasL (94). In contrast, mFasL, when expressed on the cellular membrane, has been shown to induce inflammation and promote allograft rejection (95, 157, 158) and could thus be potentially harmful for the placenta and the fetus. Thus, as reported by us in paper III and others (54, 55) it seems that FasL expression on the exosomal membrane is the type of membranal FasL form that promotes apoptosis without evoking harmful inflammatory response. Earlier reports of the placental location of FasL was quite contradictory however today it has been well established by us and others that FasL is solely expressed in the cellular endosomal compartment of ST on the membrane of exosomes (*paper III*)(54, 55). Similar to FasL, differences of TRAIL signaling between sTRAIL and mTRAIL have also been observed, where mTRAIL seem to be the most efficient in inducing apoptosis (159-161). Previous reports suggested TRAIL to be expressed in the placenta on the cellular membrane of both STB and CTB and to have additional functions besides apoptosis e.g. participation in the maturation of the trophoblast (58). However, besides apoptosis, other TRAIL functions have not been able to be

confirmed and are therefore no longer considered. Our detailed IEM work is, to our knowledge, the first demonstration of intracellular expression of TRAIL in STB on the endosomal MVB membrane and on intraluminal microvesicles/exosomes and throws a light on TRAIL as an additional pro-apoptotic molecule expressed on placental exosomes that works in concert with FasL in creating an immunologic privilege of the fetus.

VIII. Phenotypic studies of human placenta-derived exosomes suggest immune suppression

As previously described we discovered that human placenta constitutively secretes exosomes produced in the syncytiotrophoblast layer of the chorionic villi. Our IEM for ULBP1 and TRAIL staining captured direct illustrations of MVBs fusing with the plasma membrane to form an opening where the contents of exosomes are released into the intervillous space (*Fig. 2 paper II, Fig. 2 paper III*). Therefore, we established placental explant cultures to collect and characterize secreted placental exosomes. To be certain that the great majority of vesicles in the supernatant were secreted exosomes and to minimize release of vesicles due to cell death of necrosis we chose to perform short term cultures and harvested the culture supernatant after 24 hours. Placental exosomes were isolated with a sucrose gradient ultracentrifugation method. The yield was estimated by the BCA protein assay kit (Pierce) and varied between 17-34 μ g/g of early placental tissue and the purity, estimated by ocular analyses with transmission electron microscopy (TEM) of negative contrast stained exosome isolations, was very high, >98%. The obtained exosomes were analyzed for expression of NKG2D ligands, FasL, TRAIL, placental alkaline phosphatase (PLAP) and the exosome-specific markers CD63 and TSG101 by three independent methods - Western blot, flow cytometry of exosomes captured on mAb-coated latex beads and IEM of exosomes stained with specific antibodies and silver enhanced nanogold particles.

Our results, presented in paper II and III, clearly showed that placental exosomes carry on their surface the NKG2D receptor ligands MICA/B and ULBP 1-5, the apoptosis inducing molecules FasL and TRAIL and placental alkaline phosphatase that can be viewed as an “address” molecule, showing their placental origin (*Fig. 4 and 5 paper II, Fig. 3 paper III*). The expression of NKG2D ligands in placental exosomes is in agreement with tumor-derived exosomes shown to exhibit immunosuppressive properties (85, 162). Interestingly, we also found that placental exosomes expressed PD-L1 and TGF β (*not shown, unpublished*) and aim to proceed with these findings in future studies, testing the exosomes’ ability to induce Treg cells. Taken together our phenotypic studies revealed that placenta-derived exosomes

carry proteins on their surface that, similarly to other findings in tumors, define them as immunosuppressive (85, 129, 162).

An intriguing discovery was that the Western blot analysis for FasL and TRAIL showed exclusively larger protein bands, 75kDa and 70kDa respectively, for the exosomal fractions compared to Jurkat cell lysate and placental tissue, which had some amount of the protein in a larger bands but the main part of the protein was present in smaller bands of 37kDa and 34kDa respectively (*Fig. 3 paper III*). In a comprehensive paper Holler et al. reported that oligomerization consisting of at least two homotrimeric FasL molecules is required for the successful formation of DISC and the transmission of an active apoptosis-inducing signal (89). The protein bands of 75kDa and 70kDa obtained in our protein analyses correspond to the oligomerized form of FasL and TRAIL, indicating that the exosomes carry FasL and TRAIL on their surface aggregated in a bioactive apoptosis-promoting form. The exosomal membrane, rich in sphingolipids, cholesterol and tetraspanins, resembles the lipid rafts of the cellular membrane, which are known to promote both attachment and aggregation of signaling proteins as well as recycling (163). Thus the exosomal membrane is exceptionally suitable for enrichment of proteins in oligomerized form and our results indicate that FasL and TRAIL are stored in the syncytiotrophoblast on exosomes. This storage could have several advantages: 1) by storing FasL and TRAIL molecules in the endosomal compartment in MVBs of the cells these ligand would be protected from MMP cleavage that would destroy their apoptotic ability and alter their biological function, and 2) the storage of these molecules on the exosomal membrane could facilitate their oligomerization and thus, when secreted they have the ability to form DISC and trigger apoptosis. An interesting question is if these molecules are expressed on the same exosomes or on separate exosomes. We performed a double staining using 5nm- and 10nm-sized gold particles to be able to distinguish between FasL (revealed by 5nm gold) and TRAIL (10nm gold). In this staining we could see that FasL and TRAIL were expressed on different exosomes (*Fig. 3 paper III*). This might indicate that the exosomes are generated in separate MVBs, a finding of interest for further studies of exosome biogenesis. Similar observations, with only partial endosomal co-localization of FasL and TRAIL expression, have been reported in human melanoma (160). Thus, it is possible that there are placental exosomes expressing both molecules and more investigations are needed to rule out this possibility.

In summary our phenotypic studies provided convincing data of production and release of placental exosomes carrying on their surface MICA/B, ULBP1-5 (*Paper II*), oligomerized FasL and TRAIL (*Paper III*), and PD-L1 and TGF β

(*unpublished data*). Taken together these results suggest an immunosuppressive phenotype of placenta-derived exosomes.

IX. Functional characterization of placenta-derived exosomes

Having shown that placental exosomes possess an immunosuppressive phenotype, in the next step, we proceeded with testing if they indeed were immunosuppressive. Our functional experiments were performed in two directions: 1) assessment of the cytotoxic ability of freshly isolated PBMC from healthy donors in the presence of NKG2D ligand-expressing placental exosomes and 2) assessment of the apoptosis-inducing ability of placental exosomes on two different targets - Jurkat T cells, a common model cell line for testing apoptosis, and *in-vitro* activated human PBMC from healthy donors.

Exosomal NKG2D-ligands inhibit cytotoxicity by downmodulation of NKG2D

The NKG2D ligand-receptor system has been shown to be used by tumors in such a way that the tumor actually escapes immune attacks. Tumor derived exosomes carrying NKG2D ligands have been suggested to downmodulate NKG2D and impair the cytotoxicity of the host immune cells (85). We wanted to test whether placental exosomes display similar features since they also carry the NKG2D-ligands.

In paper II we assessed immunomodulation of cytotoxicity by NKG2D ligand-bearing placental exosomes. In receptor studies we showed that the NKG2D ligand-bearing placental exosomes could serve as a membrane bound “soluble” form of ligands with ability to reduce the surface expression of NKG2D receptor on NK cells, CD8⁺ cytotoxic T lymphocytes and $\gamma\delta$ T cells. This seemed to occur through internalization of the receptor, with a dose dependent effect of the placental exosomes (*Fig. 6, paper II*). The functional consequences of the exosome-mediated down modulation of NKG2D was reduced cytotoxicity as measured *in vitro* in the presence/absence of placental exosomes in cytotoxic assays where PBMC from healthy donors were used as effector cells against the MHC class I-negative, Fas-negative human erythroleukemia K562 as a target cells (*Fig. 7 paper II*). The reduced cytotoxicity was proved to depend on the exosomal NKG2D-ligands since exosomes with blocked ligands did not affect the cytotoxic ability of the PBMC. Further, we could show that the reduced cytotoxicity of the effector cells did not affect the perforin production at the protein and mRNA level arguing for intact cytolytic machinery. Thus the exosomal receptor down

regulation impairs the cytotoxic response but does not alter the cells activation status and their perforin-mediated lytic potency. This indicates that this is a local temporary suppression at the fetal-maternal interface. When the receptor will be recycled on the cell surface again, the cells are able to regain their normal cytotoxic function as they leave the placental bed and enter the peripheral blood circulation. This exosome-mediated NKG2D receptor down regulation is similar to the one recently described for tumors. Tumor-derived exosomes carrying NKG2D ligands were shown to down modulate NKG2D receptor and consequently impair the cytotoxicity of the host immune cells (85, 164). Human placenta and tumors have many biologic similarities such as high rate of cell division, invasiveness, and similar expression of various signaling molecules, adhesion proteins and growth promoting factors. Thus, another similarity is that both produce exosomes with immunosuppressive phenotype that carry NKG2D ligands and effectively down-regulate the cognate receptor. The reason for these similarities is obvious, both the tumor and the fetus have a common goal of escaping immune attack from the host/mother. Suppression of the host immune responses will promote establishing of the tumor and spreading of metastases while the down regulation of the maternal immune system will promote the ongoing pregnancy. The immune compromise necessary during pregnancy comprises a risk for tumor development and it is a well known fact that there is an increased risk for breast cancer during and in close proximity after a pregnancy (165-167).

It has been described that soluble NKG2D ligands, generated by MMP-cleavage, for example MMP-cleaved soluble MIC (sMIC), are also able to down-regulate the NKG2D receptor (168). The question is if there are any functional differences between exosome- and sMIC-mediated down-regulation of NKG2D. Previous reports have shown that NKG2D ligands, expressed on exosomes, are far more potent as receptor down modulators compared to the truncated MMP-cleaved soluble ligands (162, 169). The greater potency of exosome-mediated receptor down regulation lays in that, besides preservation of the molecular structure and biologic activity of the ligands, exosomes can enrich several molecules of the same ligand and/or simultaneously carry other NKG2D ligands as well and serve as multipotent carriers of NKG2D ligands able to impair the cytotoxic response to a greater extent (162, 169). It has been proposed that the sMIC in the serum of cancer patients was generated by MMP-cleavage only (168). However, the method used for detection in these reports did not distinguish between MMP-cleaved and exosomally carried MIC molecules, thus the protein moiety of MIC in the serum of cancer patients comprises a mixture of both. Moreover it is important to remember that the most abundantly expressed MIC protein, MICA*008, found in the cancer patients' sera is entirely released on

exosomes (162). Taken together, our data (Paper II) and the data presented above (85, 162, 169) suggest a new, more potent mechanism of down regulation of cytotoxicity based on immune suppression by exosomes carrying NKG2D ligands.

FasL- and TRAIL-carrying exosomes induce apoptosis in activated PBMC and Jurkat cells

Our phenotypic studies of placental exosomes showed that FasL and TRAIL seemed to be present on the exosomal membrane exclusively in oligomerized form, where, according to previous studies the minimal ligand structure of two homotrimers is required for the formation of DISC and apoptosis signaling (89). The next step was to test the apoptosis-inducing ability of the placental exosomes. For this purpose we used a commercial kit (Annexin V:PE Apoptosis Detection Kit, BD Bioscience) and two targets for apoptosis - activated PBMC, isolated from healthy donors and the human T-cell leukemia line Jurkat, a widely used target in apoptosis-experiments. We could show that placental exosomes induced apoptosis in Jurkat cells and activated PBMC in a dose-dependent manner (*Fig. 4 paper II*). In the presence of placental exosomes, activated PBMC displayed several classical signs of early apoptosis such as nuclear fragmentation, vacuolization of the cytoplasm and blebbing of the plasma membrane when analyzed by TEM. Placental explants supernatants depleted of exosomes and microvesicles did not show any apoptosis inducing capacity, indicating that the apoptotic effect was exosome specific. FasL and TRAIL are known to be easily cleaved off from their membranal attachment by MMP. Exosome samples unprotected by MMP inhibitors did not express FasL and TRAIL and did not mediate apoptosis. Our functional experiments correlate well with other reports of apoptosis triggered by exosomes from DC cells and various tumor-cells (170-173). Since soluble and membranal forms of FasL and TRAIL have separate and sometimes contradictory biologic activities, exosomally expressed and released FasL and TRAIL could be viewed as a “soluble form” with preserved membrane attachment, protected from MMP cleavage inside the endosomal compartment and able to induce apoptosis upon exosomal secretion.

Exosomal release of FasL and TRAIL from human normal placenta has at least three beneficial effects for pregnancy: 1) no expression of mFasL and mTRAIL on the cell surface that could stimulate inflammation and be cleaved off to a soluble form that loses its apoptosis-inducing activity, 2) oligomerization of FasL and TRAIL on the exosomal membrane thus promoting DISC formation and 3) a powerful tool to induce apoptosis of activated maternal lymphocytes, potentially dangerous to the fetus both in the vicinity of the chorionic villi but also at a distance without the

requirement for cell-cell contact. In summary, our results indicate that FasL- and TRAIL-bearing placental exosomes comprise a mechanism helping the fetus evade attack of the maternal immune system and play an important role in the creation of immunological privilege of the fetus in the uterine cavity.

X. T regulatory CD4⁺CD25⁺FOXP3⁺ cells and their precursors, CD4⁺CD25⁻FOXP3⁺ cells, are enriched in decidua suggesting local Treg cell induction in human early normal pregnancy

In our phenotypic studies of placental exosomes we found expression of PD-L1 and TGF β on the exosomal membrane (*unpublished results*). Since these molecules are known to promote induction of Treg cells we plan, in the future, to investigate if placental exosomes can induce Treg cells in pregnancy. As a first step towards realizing this plan we set out to analyze the distribution, phenotype and cytokine mRNA profile of Treg cells in human early pregnancy. Treg cells comprise a small subset of CD4⁺CD25⁺ T cells with ability to maintain tolerance and homeostasis by immune suppression (13, 14, 23). Treg cells have emerged as key players in the control of the maternal immune responses and have been suggested to play a decisive pregnancy-promoting role both in human and murine pregnancies (51, 52, 174, 175). Foxp3 is a transcriptional repressor essential for Treg cell commitment, phenotype development and immunosuppressive function. Transient low-level Foxp3 expression can occur in effector T cells, whereas high and stable Foxp3 expression marks the Treg cell lineage (22, 176, 177). We therefore used Foxp3 as the lineage-specific marker in our investigations of Treg cells in human normal early pregnancy.

Our study assessed Treg cells in paired decidual and peripheral blood samples, a design that enabled us to measure local enrichment of Treg cells in decidua. Further, we compared Treg cells from the peripheral blood samples of pregnant women to those of non-pregnant women. The merits of the study lie in the comparing of tissue-derived and peripheral blood-derived lymphocytes from the same donor and that the analyses were performed in every step by both flow cytometry as well as microscopy, ensuring that the characteristically very small numbers of positively stained flow cytometric “events” we observed were indeed viable Treg cells with the characteristic morphology of small quiescent lymphocytes with a large nucleus and a narrow brim of cytoplasm. However, there are also draw backs of the study that were outside our ability to resolve. Firstly, we could not obtain tissue of normal endometrium together with the peripheral blood from healthy non-

pregnant women to match the decidua of early pregnancy thus only peripheral blood samples between pregnant and non-pregnant women were compared. Secondly we could not do a temporal study since, due to obvious ethical reasons, no samples from second trimester of normal human pregnancy were available to us. We did not include term placenta samples in the analyses since term placenta is an organ in its late senescence, affected by the delivery process with a very little amount of decidual tissue on the site facing the muscular layer of uterus and thus not suitable for comparison with the decidua of early pregnancy.

First we double stained decidua for CD4 and Foxp3 markers to see the local tissue distribution of Treg cells. We could present the first demonstration of CD4⁺Foxp3⁺ Treg cells in human decidua stained by IHC and visualized in light microscopy (*Fig. 1 paper IV*). We then used the three markers CD4, CD25 and Foxp3 in a simultaneous three color staining for flow cytometric analysis of Treg cells in isolated decidual mononuclear cells and peripheral blood lymphocytes. Interestingly we identified three decidual CD4⁺ T cell populations with Foxp3 expression; CD4⁺CD25⁺⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻, and all three populations were significantly enriched in DMC compared to PBMC in the paired samples from pregnant women, especially the CD4⁺CD25⁻ population that exhibited a 10-fold increase in decidua (*Fig. 2 paper IV*). An enrichment of decidual Treg cells during human pregnancy has previously been suggested (53, 178-180), however, the presence of CD4⁺CD25⁻Foxp3⁺ cells in human normal early pregnancy decidua is a novel discovery.

The decidual CD4⁺CD25⁺Foxp3⁺ cells expressed CD45RO, CTLA-4, Neuropilin-1, LAG-3, CD62L and CD103 consistent with Treg cell phenotype and they displayed a T_{H3} cytokine profile with TGFβ mRNA expression as the dominant cytokine (*Fig. 5 and table II paper IV*). The CD4⁺CD25⁻Foxp3⁺ displayed a high and stable level of Foxp3 comparable to the Foxp3 level of the CD4⁺CD25⁺ Foxp3⁺ Treg cells (*Fig. 4 paper IV*). High Foxp3 expression in human CD4⁺CD25⁻ cells has been suggested to be consistent with acquisition of Treg cell phenotype and function (24). Another study concluded that in the murine system, the CD4⁺CD25⁻Foxp3⁺ cells comprise a reservoir of naïve cells that was rapidly recruited to the CD4⁺CD25⁺Foxp3⁺ pool of Treg cells upon homeostatic expansion/activation (181). Our results showed an enrichment of Foxp3-expressing CD4⁺CD25⁺ and CD4⁺CD25⁻ cells in DMC compared to PBMC, which indicate that an enrichment of Treg cells is taking place locally in the decidua during human pregnancy, thus the pool of CD4⁺CD25⁻ cells in decidua might be cells of committed Treg precursors.

TGF β is a cytokine with the ability to convert naïve T cells into Foxp3⁺ Treg cells with concomitant TCR stimulation, as shown in both mice and human (182, 183). The exact mechanism for how TGF β turns on the Foxp3 gene is still to be elucidated. Programmed-death 1-ligand 1 (PD-L1 or B7-H1) is a type I transmembrane protein that has been shown to synergize with TGF β (independently of each other) in inducing Treg cells in the periphery (184, 185). PD-L1 has been suggested to play a role in both the induction of Foxp3 in the conversion of naïve T cells to Treg cells, but also in maintaining the Foxp3 expression and thus regulating the stability of adaptive Treg cells (184).

As mentioned above, we found that placenta-derived exosomes carry both TGF β and PD-L1 on their surface (*unpublished results*) and plan for future studies to investigate if placental exosomes are possible providers of the signal for the local induction of Treg cells occurring in the pregnant decidua. Support for our hypothesis is a recent murine study that showed the presence of TGF β on thymic exosome-like particles that were able to induce Treg cells in the lung and liver when injected i.v. (186). Another report of Treg induction has shown that microvesicles derived from ovarian cancer cell lines carry TGF β , and when cultured together with CD4⁺CD25⁻ cells, an increase in CD4⁺CD25⁺ Treg cells was noted. Furthermore, the tumor derived microvesicles enhanced the proliferation and the suppressive ability of the Treg cells (132).

In contrast to murine pregnancy and to some reports of human pregnancy, we did not find any significant differences in the amount of circulating CD4⁺CD25⁺Foxp3⁺ Treg cells when we compared peripheral blood between pregnant and non-pregnant women, an observation similar to the one reported by Mjösberg J et al. (187).

In summary our studies confirmed an enrichment of Treg cells in decidua and moreover, a presence of cells with a Treg cell precursor phenotype, suggesting that in the observed Treg cell increase in decidua, a local Treg cell maturation, at least for a part of the decidual Treg cells, should be considered.

XI. On the role of placental exosomes in the immune protection of the fetus - synthesis of facts and hypothetical assumptions

A successful outcome of human pregnancy is dependent on a complex regulation of the immune system in order to downregulate specific immune responses potentially harmful for the genetically divergent fetal cells without

compromising the ability to control infections. As mentioned earlier, the placenta is a key organ during pregnancy both for ensuring proper development of the fetus, but also for protecting the fetus not only by providing a physical barrier keeping maternal lymphocytes at a distance but the placenta also produces various immunomodulatory factors.

In this thesis we present three immunomodulatory mechanisms, mediated by placental exosomes, which contribute to the protection of the fetus 1) down modulation of NKG2D-mediated cytotoxicity and 2) FasL- and TRAIL-mediated extrinsic apoptosis of activated T lymphocytes and 3) enrichment of Foxp3-expressing T regulatory cells in the decidua, which possibly involves exosomes.

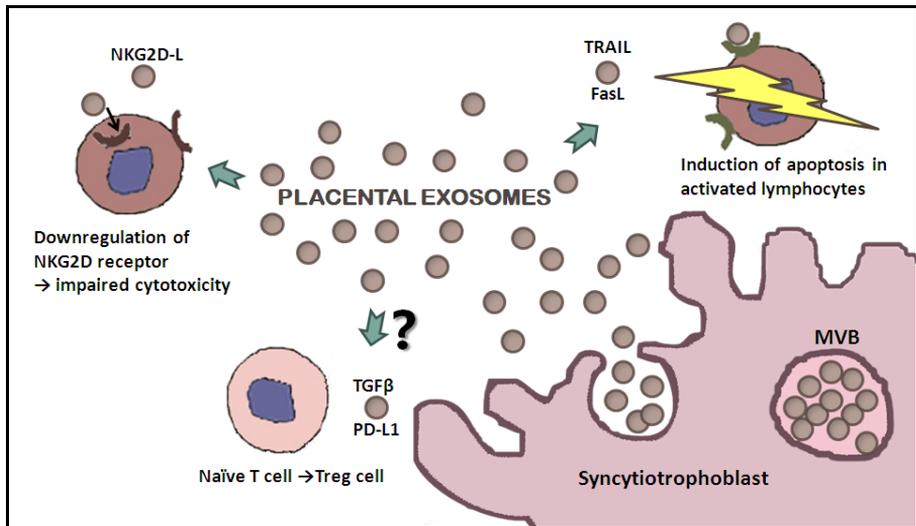


Figure 6. Human placenta produces and secretes immunosuppressive exosomes. Illustration summarizing the obtained results of the different immunomodulatory features of placental exosomes with impaired cytotoxicity of NK- and cytotoxic T cells, apoptosis-induction of activated lymphocytes and the possible priming of Treg cells.

Using TEM and IEM we brought evidence on the ultrastructural level that human placenta secretes exosomes. We show that they carry NKG2D ligands as well as the proapoptotic molecules FasL and TRAIL contributing in protecting the fetus from potentially dangerous activated lymphocytes and cytotoxic cells. Further we could show that there are elevated levels of both Foxp3-expressing CD4⁺CD25⁻ Treg-precursors and CD4⁺CD25⁺ mature Treg cells in the decidua indicating an occurrence of a local Treg-induction during

pregnancy. Treg cells suppress immune responses in several different ways and are thus of obvious benefit for the fetus. We found PD-L1 and TGF β to be present on placental exosomes and both these molecules are known to be involved in priming Treg cells. Thus, we strongly suspect that placental exosomes might be important players in the observed Treg-priming occurring in decidua. We have already initiated a study where we will test the *in vitro* ability of exosomal TGF β and PD-L1 to induce Foxp3-expressing CD4⁺CD25⁺ in PBMC and thus increase their number.

Taken together, our results clearly show that the placental exosomes are able to exert immune suppression through several different mechanisms are thus pluripotent. Our hypothesis of how placenta derived exosomes function as vehicles of immune suppression is illustrated in Figure 6.

The secretion of placental exosomes most logically comprise a gradient, with the highest concentration in the intervillous space of the chorionic villi where the immune suppression is critical since the chorionic villi are bathing in maternal blood and there is a risk of a potential attack on the ST by maternal immune cells. The exosome-mediated suppression of cytotoxic cells affects receptor expression but leaves the cytotoxic machinery of the cell intact. Thus, as the concentration of placental exosomes fades out in the periphery, the suppressed NKG2D receptor-bearing cells will recover their receptor and will be able to fulfill their cytotoxic function and fight infections.

The suppression of the adaptive immune system during pregnancy, although somewhat compensated by the innate immune branch, is necessary for the survival of the fetal allograft. However it leaves pregnant women partially immunocompromised, thus more susceptible to infections and cancer. This seems to be the price our species have to pay, i.e. to establish the very intimate hemochorial contact between the fetus and the mother in order to provide the specialized microenvironment and meet the high demands for oxygen and nourishment needed for the development of the human fetus with our highly developed human brain.

Syncytiotrophoblast secretes, as mentioned earlier, not only the endosome-derived exosomes, but also several other kinds of microvesicles released by budding off from the cell surface. The exact role for the different kinds of microvesicles is far from completely understood. The concentration of exosomes in the maternal peripheral blood was found to be lower at preterm pregnancy compared to normal term delivery pregnancy (188). Pre-/eclamptic women however instead show highly elevated levels of the larger microvesicles which are suggested to be involved in the emergence of the enhanced inflammation, changed cytokine production and the endothelial dysfunction characteristic for pre-/eclampsia (146, 189, 190). These findings

make us speculate that the proper tuning of placental function to some extent depend on a balance between the generation of shed microvesicles and the generation of exosomes. Over- or underproduction of either kind during pregnancy may have pathological consequences but of course much, much more and thorough studies are needed to elucidate this question.

In summary, we present in this thesis evidence that placenta-derived exosomes are immunosuppressive and affect immune cells through several different mechanisms contributing to the establishment of maternofetal tolerance. Hereby, we can add some more pieces to the overall complex puzzle of the multifactorial immunomodulation that occurs during human pregnancy allowing the fetus to defy the natural laws of transplantation and ensure survival of the human species.

CONCLUSIONS

- Human normal pregnancy placenta constitutively produces and secretes exosomes, nanosized vehicles for intercellular communication
- The placental exosomes exhibit immunosuppressive phenotype by carrying on their surface:
 - NKG2D-ligands MICA/B and ULBP1-5
 - apoptosis inducing molecules FasL and TRAIL in their active oligomerized form
 - TGF β and PD-L1, molecules involved in T regulatory cell priming
- The placental exosomes convey immunosuppressive functions such as downregulation of cytotoxicity, apoptosis of activated immune cells and possibly priming of T regulatory cells
- CD4⁺CD25⁺FOXP3⁺ regulatory cells and CD4⁺CD25⁻FOXP3⁺ precursor cells are enriched in decidua suggesting local maturation of T regulatory cells during human normal pregnancy
- Taken together our results suggest that the immune privilege of the fetus is mediated by multiple mechanisms where exosome-mediated immunosuppression plays a pivotal role and tie up the immunomodulatory and protective role of human placenta to its exosome-secreting ability

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