ABBREVIATIONS

αGalCer	α-galactosylceramide
AGE	advanced glycation end-products
APC	antigen-presenting cells
Ang	angiopoietin
BSA	bovine-serum albumin
CBA	cytometric bead array
CCR	chemokine (C-C motif) receptor
CSF	colony-stimulating factor
CBMC	cord blood mononuclear cells
cAMP	cyclic AMP
CMV	cytomegalovirus
conA	concanavalin A
CXCR	CXC receptor
dNK	decidual natural killer
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECS	elective caesarean section
ELISA	enzyme-linked immunosorbent assay
EVT	extra villous trophoblasts
FcR	Fc receptor
FcRn	neonatal Fc receptor
Flt3L	fms-like tyrosine kinase 3 ligand
GM-CSF	granulocyte macrophage colony-stimulating factor
HSC	haematopoietic stem cells
HMGB1	high mobility group box 1
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IHC	immunohistochemistry
IVF	in vitro fertilizations
LIR	leukocyte Ig-like receptor
ILT	immunoglobulin-like transcripts
IFN	interferon
IL	interleukin
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IMA	ischemia-modified albumin
KIR	killer immunoglobulin-like receptors
LIF	leukemia inhibitory factor
mHLA	membrane bound HLA
MHC	major histocompatibility complex
MIC	MHC class I chain-related proteins
MIP	monocyte inflammatory protein
NCR	natural cytotoxicity receptors
NK	natural killer
NKP	NK cell precursor
NKR	natural-killer cell receptors
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptors
PBMC	peripheral blood mononuclear cells
PGN	peptidoglycan
PGF	placental growth factor
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
RAGE	receptor for advanced glycation end-products
RNA	ribonucleic acid
RSA	recurrent spontaneous abortions
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
sFlt1	soluble fms-like tyrosine kinase-1
sHLA	soluble human leukocyte antigen
SCT	syncytiotrophoblast
Tc	cytotoxic T cell
TGF	transforming growth factor
Th	T-helper cell
TLR	toll-like receptor
TNF	tumour necrosis factor
TPA	phorbol ester
T_{reg}	regulatory-T cell
ULBP	UL 16-binding protein
uNK	uterine natural killer
VD	vaginal delivery
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

INTRODUCTION

The major task for the immune system is to recognize and eliminate non-self molecules and pathogens. Deficiencies in any part of the immune system result in a higher risk of infections, but thanks to the plasticity and redundancy of our immune system, some deficiencies could be compensated for by other immunological components. A strong evolutionary pressure from surrounding microbes and pathogens has resulted in the development of our immune system. To accomplish all of the different demands it must be tightly regulated, otherwise there can be inappropriate reactions to self antigens – autoimmunity, or overactive immune responses known as hypersensitivity reactions.

During pregnancy, there is a dual challenge for the maternal immune system. At the same time it has to tolerate the semi-allogenic foetus, and still keep the ability to combat pathogens. This maternal tolerance should be manifested both locally in the intrauterine environment, in the placenta and peripherally in the maternal circulation, where foetal cells are found during pregnancy. In addition, maternal immune cells contribute to a correct placental development.

The immune system

The immune system can be divided into two categories: the innate and the adaptive immune branches. Although they can be separated in terms of specificity, kinetics and memory development, they clearly influence and regulate each other.

The innate immune system needs no prior activation and is the first defence against pathogens. Cells belonging to the innate immune system are the natural killer (NK) cells, monocytes/macrophages, dendritic cells (DC) and granulocytes. They are recruited to the site of infection or inflammation and start to combat the pathogen by phagocytosis and the release of toxic substances, chemokines and cytokines. Further, they signal to the adaptive immune system by the release of different factors and monocytes/macrophages and DCs present antigens to adaptive cells through the major histocompatibility complex (MHC) II, expressed on their cell surface. Also, macrophages and NK cells are numerous in the placenta, where they have the ability to change in the hormonal rich environment. Instead of attacking foreign antigens they become tolerant to the foetus. These cells will be further discussed on page 22 and 32.

In addition, nearly all nucleated cells express MHC I, which can present endogenous and non-self molecules. The absence of MHC I on foetal cells found in close contact to the maternal blood in placenta are discussed to be one of the mechanisms for the foetus to avoid recognition by the maternal immune cells.

The adaptive immune branch includes B and T cells, which have highly specific antigen receptors on their surface and recognize specific epitopes on pathogens or on soluble molecules. B cells are particularly important in the response against extracellular pathogens by secreting antibodies. T cells can be further subdivided into three major groups. Cytotoxic T (Tc) cells are involved in killing of intracellular pathogens like viruses; helper T (Th) cells coordinate immune responses by cell-cell contact and the secretion of cytokines; and regulatory T (T_{reg}) cells are involved in the regulation of immune responses. T cells are also present in the placenta, while B cells are virtually absent from this environment (further discussed on page 23).

Accurate communication and balance of signalling molecules between the immune cells must occur. During the early parts of the immune response, macrophages, neutrophils and DCs are activated and start to produce different cytokines leading to the activation of $CD4^+$ Th cells. The Th cells polarize into Th1 or Th2 cells, a cell-mediated or humoral response, depending on what pathogen to eliminate. The Th cells produce different cytokines leading to the activation of different immune cells. Th1 cell typically produce interleukin (IL)-2, granolucyte macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ, while the Th2 subset produce, among others, IL-4, IL-5 and IL-13. For a long time the Th1 and Th2 cells have been considered to be the main cells to generate the two different cytokine profiles. Lately, other cytokine producing cells like NK cells and NKT cells have also been included in the discussion of the polarization towards a cell-mediated or humoral response. Therefore, the polarization of immune cells is now more often referred to as a type 1 or type 2-response.

IL-12 is a Th1 promoting cytokine, mainly produced by monocytes and dendritic cells. It consists of two subunits, p35 and p40. For many years it was considered to support inflammatory responses in several pathological conditions like autoimmunity. Later, it was discovered that the p40 subunit could associate with a p19 subunit, making up IL-23 [1]. IL-23 was then found to be involved in several pathological conditions, previously ascribed to IL-12. It is known that this cytokine can activate yet another T-cell subset, the Th17 cells,

responsible for IL-17 production. IL-17 can act on a variety of cells and is thought to be involved in autoimmunity [2].

The importance of a correct balance between the Th1/Th2-profile during pregnancy has been widely discussed. This is further discussed on page 17.

Human pregnancy

Human pregnancy is divided into three trimesters and reaches full term after 40 weeks of gestation. The first two trimesters are characterized by implantation, organogenesis, functional maturation and foetal development, while the third trimester is more focused on foetal maturation, growth and weight gain.

The placenta

In Greek, placenta means "flat cake" and it is a temporary organ consisting of embryonic and endometrial (maternal) tissue. The development of the placenta, referred to as placentation, is absolutely essential for a successful pregnancy, as the placenta provides the foetus with nutrients and oxygen. An insufficiently developed placenta can result in complicated pregnancies, like preeclampsia, and foetal growth restriction.

The placenta is also an immunologically unique organ where the maternal immune system interacts with foetal cells [3, 4]. How the allogenic foetus can avoid recognition by the maternal immune system is still an enigma, but many theories have evolved during time (further discussed on page 18).

Implantation

After several rapid cell divisions the fertilised egg becomes a blastocyst, which consists of an inner cell mass (which will form the embryonic disc) and an outer cell layer, called the trophoblast cells (fig. 1). The latter cell type takes part in the placenta formation. During the late blastocyst stage, the trophoblast cells start to form two different layers. The outer layer differentiates into multinuclear cells, the syncytiotrophoblasts (SCT), and the inner layer forms the cytotrophoblasts [3].

Syncytiotrophoblast (SCT) cells

The SCT cells will then start to secrete digestive enzymes, cytokines and growth factors. This makes it possible for the SCT cells to digest the uterine tissue and create a blood-filled space called lacunae, surrounding the blastocyst. The lacunae will form the intervillous space, where the chorion villi (finger-like structures containing foetal blood vessels) are surrounded by maternal blood. The SCT cells give rise to the outermost surface of the finger like chorion villi. The onset of the utero-placental blood circulation occurs around week 8-9 of gestation

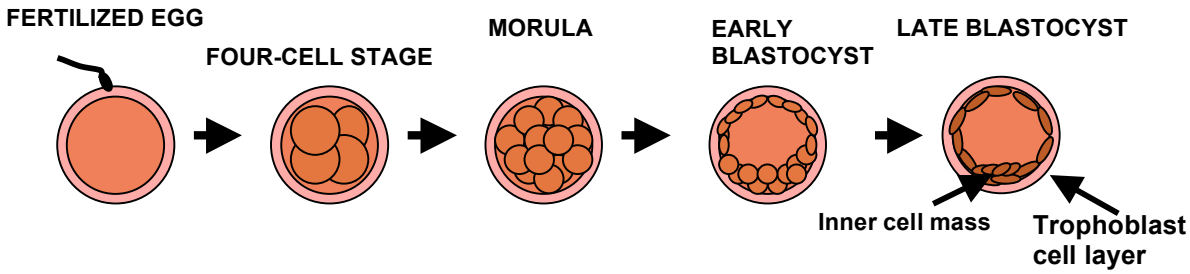


Figure 1. The fertilized egg divides rapidly and the late blastocyst will implant in the uterine wall.

and the villi are properly vasculated around 21-24th week of gestation. This is the site for oxygen, nutrition and waste exchange, and also where the maternal immune cells encounter the SCTs and create one of the maternal-foetal interfaces (fig. 2b). This maternal-foetal interface will become more important during the later part of pregnancy, as the blood supply of the foetus will become more demanding. The SCT cells are constantly renewed and shed into the maternal circulation throughout the whole pregnancy [4].

Cytotrophoblast cells

Early in the first trimester the cytotrophoblast cells, the inner layer of the trophoblasts surrounding the blastocyst, penetrates the overlaying SCT cells and start to invade the maternal tissue in a tumour-like fashion. This proceeds during weeks 6-18 of gestation [4]. The terminology for the invading trophoblast cells is somewhat confusing, and they are referred to as X cells, invasive or intermediate trophoblasts as well as extra villous trophoblasts (EVT), which is the name I have chosen to use here.

The EVTs penetrate and invade the maternal spiral arteries situated deep down in the endometrium, in order to establish a sufficient blood delivery to the foetus. This process is

strictly regulated. During embryogenesis, *i.e.* before week 9 of gestation, the spiral arteries are still blocked by trophoblast plugs. During this time the uteroplacental blood flow is minimal, which protects the sensitive foetal organogenesis from oxidative stress. After 9 weeks the uteroplacental blood flow is rearranged, and there is an excessive destruction of the muscular walls and the spiral arteries are opened up by EVTs. The increasing amount of blood is then canalized into the intervillous space in placenta. The endothelium of maternal arteries is replaced by trophoblasts that express different markers for endothelial cells. This artery remodelling is complete around week 20. This entire process of "endometrial remodelling" is called decidualization and the altered endometrium is then called decidua. The decidua is the maternal tissue attaching the placenta to the uterine wall. The invading EVT cells into decidua create the second maternal-foetal interface in placenta (fig. 2a) [4].

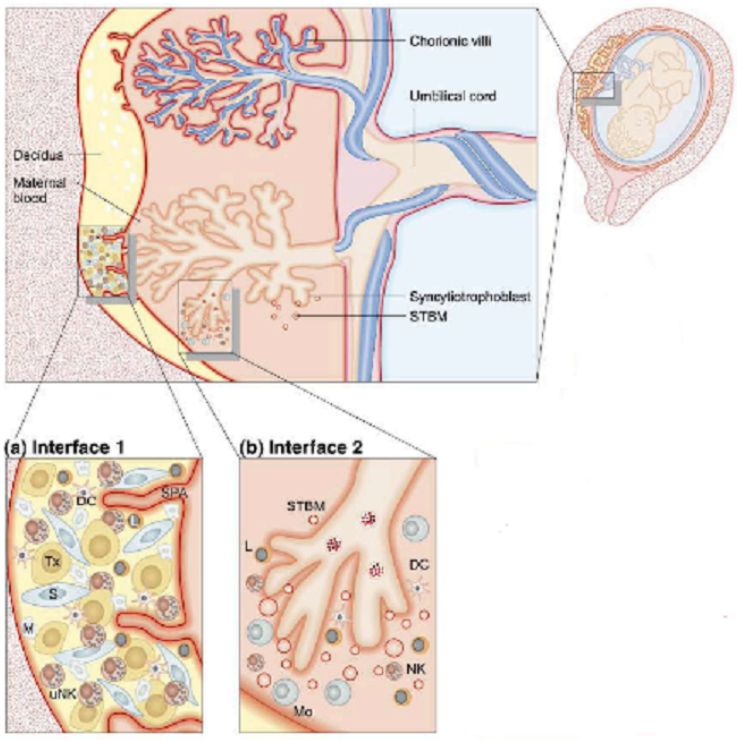


Figure 2. Placenta showing **a**. decidua with invading extra villous trophoblast cells and **b.** representing the syncytiotrophoblast surrounding the finger-like chorion villi. (Modified from TRENDS in Immunology 2006, Vol.27 p399-404). Reprinted by permission from Elsevier Ltd.

Another subpopulation of cells derived from the cytotrophoblast cells covers the chorionic villi underneath the layer of SCTs and a special kind of cytotrophoblasts develop into anchoring villi. The anchoring villi form a bridge and connect the trophoblasts to decidua and myometrium of the uterus [3, 4].

Regulation of EVTs

The placentation in humans is unique and differs from that in other mammals. Humans have a haemochorial placenta, like many other mammals, recognized by the development of the decidua and remodelling of the spiral arteries where foetal and maternal immune cells are in intimate contact. Although rodents also have a haemochorial placenta it is not as invasive with the excessive EVT penetration of the endometrium as is seen in humans. Also, the distribution of (mouse) maternal immune cells in the placenta is different from that in humans [5].

It is important that the trophoblast invasion is highly regulated and thereby creating optimal conditions for the foetus [5, 6 7]. Uncontrolled invasion of EVTs can happen when the blastocysts are implanted outside uterus, which can lead to maternal death. The opposite scenario, when the trophoblast invasion is inhibited, can give rise to foetal growth restriction, stillbirth and preeclampsia [5].

Pregnancy until labour

During gestation, the placenta continues to mature and increase in size, until approximately week 36 of gestation. The maturation of the villi, from primary, secondary to third trimester villi, contributes to the increasing placental size. The shapes of the terminal villi are round, uniform in size and they have reached their final size between weeks 28-36. Except for the foetal vessels they also contain fibroblasts and Hofbauer cells (macrophage-like cells). After birth, the placenta will detach and shed off from the uterus wall. It is not fully understood how labour is initiated, but hormones like estrogen and oxytocin are involved together with production of cytokines [4].

The placenta: a maternal-foetal interface

The placenta is an organ that serves as an interface between the mother and foetus. It is not a complete barrier because various small-weight molecules diffuse/are passively transported through the placenta. The transport across the placenta is bidirectional *i.e.* nutrition and oxygen pass through from the maternal circulation, while foetal waste material enters into the maternal circulation. Molecules with a high molecular weight usually do not pass through from mother to foetus, but one exception is the antibodies, which are actively transported [8-10].

Antibodies

The transport of maternal antibodies starts around week 16 during gestation, and increases and continues until term. By the time of birth, most foetal immunoglobulin (Ig) G subclass levels exceed the maternal levels [10, 11].

It is not yet fully clear how IgG antibodies and their subclasses cross the placental barrier. The transport of antibodies involves a passage through trophoblast cells, stromal villi (which include Hofbauer cells, macrophages found in the stroma of the villi) and finally through the foetal vessel endothelium. Fc-receptors for the different IgG subclasses, FcγRI, FcγRII, FcγRIII and FcRn (neonatal Fc-receptor) are expressed in the placenta. The Fc-receptors can be found on foetal Hofbauer cells, placental stroma and foetal endothelial cells [11], but they differ in their placental locations.

Apart from IgG, also IgA and IgM are likely to pass, although to a much lower degree, from mother to foetus [12]. IgA [12] and IgE [13] are found in foetal tissue from healthy pregnancies, while also IgM (apart from IgG and IgA) is found in placentae in moderate and severe inflammations [12].

Cytokines

It is debated whether cytokines can cross the placenta [14, 15]. Based on placental perfusion models with placentae collected from healthy term pregnancies, a bidirectional transfer of IL-6, but no transfer of IL-1α and tumour necrosis factor (TNF)-α have been reported [15]; while others found no transfer of IL-6 [14]. Nevertheless, very little is known regarding the possible cytokine transport in pathological pregnancies where the morphology can be noticeably altered [16].

Foetal and neonatal immune system

It is believed that the foetal/neonatal immune system is biased towards a type 2-cytokine-profile together with an impaired production of pro-inflammatory cytokines [17, 18]. The *in vivo* cytokine profile of the newborn is difficult to estimate, since available information is mostly based on results from *in vitro* stimulated cord blood mononuclear cells (CBMC). Depending on the experimental set-up, the stimuli used, different results are obtained. An increased neonatal IL-10 production has been described after stimulation of CBMCs compared to adult peripheral blood mononuclear cells (PBMCs) [19], while levels of pro-inflammatory cytokines

IL-12 and IFN-α were very low [19, 20, 21]. Conflicting data about IFN-γ production in cord blood have been reported, being higher [22] or lower [23, 20] than adult production. Contrasting results are also obtained regarding TNF-α, with significantly higher levels in cord blood compared to PBMC after treatment with inactivated respiratory syncytial virus (RSV) [23], while stimulation with LPS, gave much lower TNF-α production in cord blood compared to adults [24]. CBMCs produce high levels of IL-23 and IL-17 [25] and levels of IL-6 have also been found to equal adult levels after stimulation [23]. These cytokines are thought to compensate the low production of other pro-inflammatory cytokines and protect the newborns from infections.

The decreased CBMC production of IL-12, IFN-α and IFN-γ is suggested to be due to impaired neonatal intracellular events [17, 18]. Cyclic AMP (cAMP) is a second messenger reported to be increased in neonates. It is thought to inhibit intracellular pathways involved in IFN-α, IFN-γ and IL-12 transcription [17], although IL-6 transcription is preserved [17]. Reduced IL-12 production is also explained by a defect in the transcription of the subunit p35 in neonatal DCs, which together with p40 constitutes IL-12p70 [26].

Neonatal age also appears to influence IFN-γ concentrations in serum. While serum collected from cord had undetectable IFN-γ levels, the IFN-γ levels dramatically increased after 5 days of age [27]. Additionally, children delivered vaginally are more triggered to produce IL-12 and IFN-γ than children delivered by un-laboured elective caesarean section (ECS) [28].

Labour also seems to favour increased numbers of NK cells in full-term delivered neonates [29]. The phenotype and functions of NK cells in cord blood differ from those of adult NK cells. There is a decreased expression of the inhibiting receptor leukocyte Ig-like receptor (LIR) 1/ immunoglobulin-like transcripts (ILT) 2 on NK cells in cord blood, while the activating receptors, NKG2D and NKp30, are up-regulated compared to 2 and 5 year old children [30]. Cord blood NK cells express the activating receptor CD69 [22], although at low levels, which seem to decrease with age and reach the same levels as in adults [30]. Further, resting NK cells collected from cord blood show a lower cytotoxicity compared to adults, however after stimulation with IL-12 the cytotoxicity was increased in CBMC to the same levels as adult PBMC [22].

Foetal lymphocytes and antibody production

Like the foetal/neonatal APCs and NK cells, T cells from foetus/neonates differ from adult T cells. In general the neonatal T cells show defects in their cytokine production [31], although the impaired activation of neonatal T cells can be reversed depending on what stimulus is used. IFN-γ production by neonatal T cells treated with phorbol 12-myristate 13-acetate (PMA) or a combination of phytohaemagglutinin (PHA) and phorbol ester (TPA) result in high levels [32, 33], while there is a poor IFN-γ production after stimulation with concanavalin A (conA) [33]. Further, numbers of memory T cells in newborns are much lower when compared to adults [31].

Antibodies are produced in foetal spleen and start to be synthesised during the 10th week of gestation. The IgG levels found in foetal sera increases with gestational age with its peak at birth, although the main part of IgG is of maternal origin. Further, there are low levels of IgM and even lower levels of IgA and IgE found in newborns. This is believed to be due to a defect in Ig isotype production by neonatal B cells [31] but their potential to class switch may differ [33, 34]. The CD40-CD40L interaction is important for proper isotype class switch and the impaired production of Ig isotypes is thought to depend on altered CD40L levels on cord blood T cells. If this is true or not is hard to tell, as there are different reports regarding the CD40L expression levels on cord T cells with either lower [35] or equal levels [36] compared with adults. Further, cord blood B cells show poor abilities to switch to IgG or IgA after CD40 ligand stimulation [35].

Preeclampsia

Preeclampsia is diagnosed in around 5-10% of all pregnancies worldwide. It is a disorder seen in the later part of pregnancy; symptoms, like hypertension and proteinuria arise after 20 week of gestation. Further, different maternal organs, such as kidney and liver, can be affected [37] and in severe cases both mother and foetus can die.

The disease is differentiated into early or late onset of preeclampsia; the former develops before 30 weeks of gestation, while the latter develops after 30 weeks of gestation. The early onset preeclampsia is often the more severe type [38].

There are several risk factors for preeclampsia such as a previous history of preeclampsia, nulliparity, multiple pregnancy, age (>40 years), obesity, pre-existing hypertensive diseases

and other diseases like diabetes, renal disease, existence of anti-phospholipid antibodies and autoimmune disease [39]. Viral infections have also been proposed as a risk factor for preeclampsia. Antibody levels to both cytomegalovirus (CMV) and to *Chlamydophila pneumoniae* are increased in early onset preeclampsia compared to late onset preeclampsia and healthy pregnancies [40].

The disease is dependent upon the existence of a placenta, as the removal of placenta and foetus stops the disease [37]. As the disorder is already established early during placentation but symptoms are not discovered until after 20 weeks of gestation, the disorder is difficult to cure. There is often an altered placental morphology in third trimester placentae from preeclamptic pregnancies [4].

The children of preeclamptic women are often born premature with a low birth weight and they often require neonatal hospital treatment. They also have an increased risk of getting coronary heart diseases, hypertension, osteoporosis and diabetes later in life [41, 42]. However, very little is known regarding the state of their immune system.

Possible mechanisms behind the development of preeclampsia

It has been speculated that preeclampsia is a consequence of the rejection of the semi-allogenic foetus by the maternal immune system. In preeclampsia, the utero-placental circulation is reduced and the EVT cells have often failed to penetrate deep down into the endometrium to remodel the maternal spiral arteries in a proper way, which is referred to as a poor placentation (fig. 3). The placental arteries in preeclampsia are also often fewer in numbers. The poor blood supply in a preeclamptic placenta often leads to hypoxic placental conditions [43-45].

The mild inflammation in the circulation seen in healthy pregnant women is further increased in preeclampsia. It is thought that the preeclamptic placenta releases a higher amount of different factors into circulation than observed in healthy women, due to its hypoxic conditions, which could be responsible for the increased inflammatory response [44-48]. Several factors are produced from placental cells, like placental growth factors (PGF) and the soluble receptor for vascular endothelial growth factor (VEGF)-1, speculated to contribute to the systemic inflammatory response in these women [49].

In addition, there is a continuous shedding of SCTs due to renewal of the outermost layer of the villi during pregnancy. It is known that there is a higher amount of cell debris, like trophoblast cells, foetal deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), in the circulation of women suffering from preeclampsia, compared with healthy women [47]. The higher load of foetal cell debris is thought to interact with the maternal immune system and thereby give rise to the clinical symptoms during preeclampsia [44, 46].

It is further possible that the immune regulation of the placentation process is altered in preeclampsia. It has been proposed that an aberrant immune regulation occurs, with an increased risk for preeclampsia if the mother is dominant for a haplotype of the inhibiting killer immunoglobulin-like receptor (KIR) together with foetal expression of the human leukocyte antigen (HLA)-C2, which is more favourable to bind the inhibiting haplotype [50]. It is also found that CMV infection can reduce the invasive activity of EVTs *in vitro* [51] and to alter trophoblast expression of the HLA-G molecule [52, 53] which could alter the susceptibility of the trophoblast to maternal immune cells.

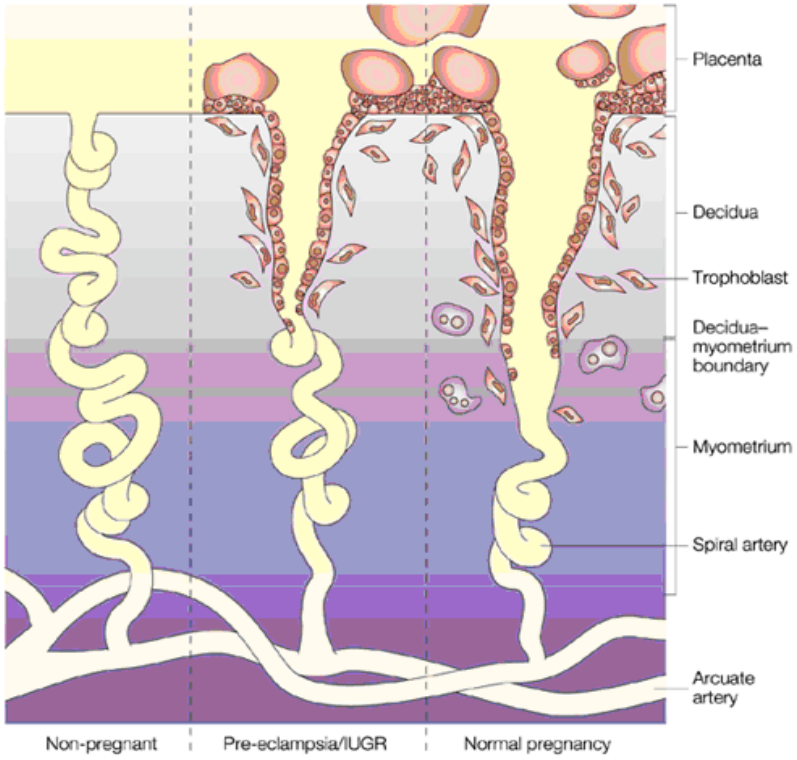


Figure 3. The figure represents a non-pregnant uterus, a preeclamptic pregnancy with its shallow extra villous trophoblast invasion and a healthy pregnancy. (Nature Reviews Immunology, 2002, Vol.2, p656-664). Reprinted by permission from Nature Publishing Group.

To learn about the mechanisms behind preeclampsia it is needed to study the early immunological events during pregnancy, which is difficult when studying the disease in humans. Recently a mouse model for preeclampsia has been developed [54], a valuable tool that can shed some light into the early events during placentation.

Preeclampsia markers

An early marker for preeclampsia, preferably one that could distinguish between mild and severe preeclampsia, would be a valuable tool to trace or/and treat preeclamptic women in an early stage of the disease. Different markers, many of which are involved in immunological events, angiogenesis and artery remodelling have been proposed as candidates. As preeclampsia is a two-stage disease, with poor placentation and later escalated inflammatory responses seen in circulation, it is a challenge to find one marker responsible for the disease, and most probably there are several factors involved. Different disease phenotypes and individual differences further complicate the search for a uniform marker for preeclampsia. Below follows a presentation of some suggested prognostic/diagnostic markers.

Cystein C is an inhibitor for proteases considered to be important for trophoblast invasion. Protein levels of placental cystein C are increased in severe preeclampsia [55].

Further, the angiogenesis-inhibitor soluble fms-like tyrosine kinase-1 (sFlt1) is elevated in preeclampsia [44]. Levels of sFlt1 have been studied as a possible candidate to predict early and late onset of preeclampsia. Although levels of sFlt1 were found to be higher in preeclampsia compared to healthy pregnant controls, levels did not differ between the two stages of preeclampsia [56].

Trophoblast invasion occurs during placental oxidative stress, which is further pronounced in preeclampsia [57]. Ischemia-modified albumin (IMA), a protein elevated in cardiac ischemia, is studied as an early marker for preeclampsia. Sera from early pregnant women were collected before the occurrence of any symptoms, and women consequently developing preeclampsia showed higher concentrations of IMA in sera than healthy pregnant women [58].

Cytokines have also been extensively investigated in the search for a preeclampsia marker. Still, no single cytokine has shown to be a reliable marker.

The immunological paradox of human pregnancy

During the last decade(s), new immunological factors have been discovered to shed light into how the maternal immune system changes during pregnancy to create tolerance to the foetus. Naturally, it is difficult to study all aspects of human pregnancy *in vivo*, which has resulted in many studies based on animal models. In the 1950's the placenta was thought to be a cellular barrier, which inhibited contact between the maternal immune system and the semi-allogenic foetus [59]. During many years it was thought that the entire maternal immune system had to be suppressed to be able to accept the foetus.

Indeed, the maternal immune system changes during pregnancy, both locally in the placenta and in the circulation. Today, it is suggested that immune alterations involve both the adaptive and the innate immune system, inducing a balance between these to systems to create the optimal milieu for a successful pregnancy. Alterations of maternal immunity fluctuate during the course of pregnancy and also differ between the circulation and the intrauterine environment. The immunological changes involve functions of cells and also changed production of different factors by the maternal immune cells. Further, pregnancy is influenced by hormones, *e.g.* progesterone and oestrogen, which in turn can influence the immune responses.

Several cytokine-producing cells, represented by both maternal and foetal cells, present in placenta contribute to the cytokine milieu locally during pregnancy. It is important to remember that also non-immune cells in the placenta are major producers of immunological factors like cytokines.

The Th1/Th2 theory

In the end of the 1980's, the so-called Th1/Th2 theory evolved, which argued in favour of a strong cytokine shift towards Th2 during successful pregnancies [60]. Several studies conducted in animals in the 1990's further strengthened the acceptance of the theory. These studies showed that injection of Th1 cytokines in pregnant mice resulted in resorption of embryos. This could be reversed when antibodies against these Th1 cytokines were administered. The inhibition of foetal resorption could also be observed by simultaneous administration of IL-10, a cytokine classified as a strict Th2 cytokine at that time [61]. The Th1/Th2 theory was extrapolated to be valid also in humans [62, 63] and it gained further support from studies

showing that women suffering from Th2-dependent diseases, like systemic lupus erythematosus, deteriorated during pregnancy, while women suffering from Th1-mediated diseases, like rheumatoid arthritis, improved during pregnancy [64]. There have also been reports of a dominance of Th1 cytokines, like IFN-γ, in women suffering from recurrent spontaneous abortions [65].

The importance of the Th1/Th2 theory is still discussed in pregnancy, but it is more and more considered as an oversimplification. IL-10 should not be considered as a strict Th2 cytokine in humans, rather it is a regulatory type of cytokine. Further, there have been suggestions that neither IL-4 nor IL-10 are absolutely required for successful pregnancies as IL-4 and IL-10 deficient mice are able to complete pregnancy [66]. Also, today it is known that a healthy pregnancy also involves mild inflammatory responses [67, 68]. Furthermore, the Th1 cytokine, IFN-γ has been discussed to be important for artery remodelling during early pregnancy [69, 70].

Immunological mechanisms establishing maternal tolerance

Many different mechanisms are involved to support maternal-foetal tolerance. Here some of them are presented.

The maternal-foetal interface lacks the expression of the classical class Ia MHC-molecules HLA-A and HLA-B. Although, the classical HLA-C is present on EVTs in placenta, and the foetal HLA-C haplotype appears to influence pregnancy outcome, as discussed above [50]. SCTs do not express any MHC-molecules at all, which is believed to be one way of escaping T-cell recognition. Moreover, the EVTs also express the non-classical Ib molecules HLA-E, HLA-F and HLA-G, which probably are central to inhibit NK cell-mediated lysis. The interactions between uterine NK (uNK) cells and placental cells expressing non-classical HLA-molecules can result in different immunological responses (further discussed on page 35).

T_{reg} cells can suppress alloresponses in mice. T_{reg} depleted mice do not maintain a normal pregnancy, while abortion-prone mice are able to sustain pregnancy when T_{reg} are administered, stressing the importance of a role for T_{reg} in achieving tolerance [71]. The role of T_{reg} in human pregnancy has been less clear. However, it has been reported that the decidual and peripheral blood T_{reg} frequency increased during early pregnancy [72]. Recently it was also

demonstrated that women undergoing spontaneous recurrent abortions (RSA) have decreased numbers of T_{reg} that are also functionally deficient [73].

Several T_{reg} mechanisms to suppress potentially dangerous $CD4^+$ and $CD8^+$ T cells are proposed. One includes the ability of T_{reg} to regulate indoleamine 2,3-dioxygenase (IDO) production by antigen presenting cells (APC) [74, 75]. IDO is responsible for local reduction of the amino acid tryptophan. T cells are susceptible to altered concentrations of tryptophan hence T-cell proliferation is suppressed when IDO is produced. IDO is also produced by cells in decidua [76].

Another way for T_{reg} to inhibit T-cell proliferation, is by the production of IL-10 and transforming growth factor (TGF)-β [77], both cytokines being produced at the maternal-foetal interface [78, 79].

Other mechanisms for the foetus to avoid maternal T-cell recognition include the expression of Fas ligand (CD95L) by trophoblast cells. Fas ligands are secreted from first trimester trophoblasts in microvesicles and induce T-cell apoptosis by activation of the Fas pathway [80]. However, the importance regarding Fas/Fas ligand expression at the maternal-foetal interface is unclear. It is speculated that the expression of these molecules contribute more to the prevention of autoimmune activation by immune cells instead of being involved in foetal tolerance [81]. Placental cells are also efficient cytokine producers; *e.g.* IL-10 is highly expressed in 1st trimester SCT, which could further regulate activated maternal immune cells.

Further, CD55 and CD46, two proteins regulating the complement system, are expressed in first and third trimester placenta [82]. Together with CD59, they are proposed to regulate the complement system during pregnancy and protect the foetus from maternal complement attack [83].

There is a constant shedding of trophoblast cells into the maternal circulation during third trimester in healthy pregnancies. Accordingly, the circulating maternal immune cells encounter foetal cells and this is thought to be one mechanism by which maternal tolerance is establish towards foetal antigens [84].

The immune system in the maternal circulation during pregnancy

T cells were early brought into focus when discussing the immunological shifts during pregnancy, as discussed above. Many studies have been conducted regarding the amounts of circulating T cells, both $CD4^+$ and $CD8^+$. The results have been contradictory, increased as well as decreased numbers of both T-cell populations have been reported [8586, 87]. However, it has been shown that the expression of different $CD4^+$ cell markers do not change, indicating that there is no altered activation of these cells [88]. T_{reg} are also present in the circulation [72] and there are increased numbers during early human pregnancy [89], but the significance of this increase is not known. B cells do not appear to change in amounts during pregnancy compared to non-pregnant women, and antibody levels are more or less unaltered [85].

Circulating NK cells decrease in numbers during pregnancy [85, 86]. Both NK and NKT cells have been proposed to be important for the cytokine shifts observed in healthy as well as preeclamptic women [46, 90].

There is a mild inflammatory response during pregnancy in healthy women, and an activation of the innate immune system. There are increased numbers of neutrophils in the circulation as pregnancy proceeds, and these cells also appear to be more activated [91]. Monocytes do not seem to fluctuate in numbers but appear to be in a more activated state than in non-pregnant women and they have an increased production of IL-1β and IL-12 [67, 91].

IL-10 is an anti-inflammatory cytokine suggested to be important for healthy pregnancies [92] to undermine the inflammatory response. However mice lacking IL-10 are still fertile [66]. The exact function of IL-10 during pregnancy is not known. Many studies have reported reduced levels of IL-10 both in circulation and placenta in pathological pregnancies like preeclampsia [92, 93].

HLA-G belongs to the non-classical 1b MHC-molecules, which have a low polymorphism and are found in the placenta. It includes four membrane bound proteins (G1, G2, G3 and G4), and three soluble isoforms (G5, G6 and G7, of which G5 and G6 also are referred to as sHLA-G1 and sHLA-G2, respectively) [94, 95, 96]. The role for HLA-G in pregnancy is still not known, but it is thought to be important for pregnancy outcome. Enhanced soluble (s) HLA-G secretion by the embryo appears to result in a higher chance of a positive pregnancy outcome

[97]. Increased serum levels of sHLA-G are found in early pregnant women when compared to non-pregnant women [98, 99]. The sHLA-G levels are reported to stay the same [100] or decrease as pregnancy advances [98].

Reduced sHLA-G levels have been reported in preeclampsia [98, 101]. The sHLA-G5 can induce endothelial apoptosis and the lower levels of sHLA-G in preeclampsia, shown in some studies, are suggested to be one of the reasons for the shallow EVT invasion known to occur in preeclampsia [102].

Immune system during labour

The precise mechanisms that trigger labour are not yet identified. Labour itself is described as an inflammatory response and several pro-inflammatory cytokines like IL-6, IL-8, IL-1β and TNF-α are increased in placenta [103], but also plasma HLA-G levels are elevated, (Roberta Rizzo Sverremark Ekstrom, personal communication). IL-6 has been suggested to stimulate the release of oxytocin [104], a hormone essential for initiating labour [3]. Furthermore, toll like receptors (TLR) are suggested to be involved in the processes of labour [103]. They are expressed in placenta at different sites, *i.e.* in cervix, amnionic epithelial cells, trophoblasts and decidua. These receptors, when engaged to their ligands, can induce pro-inflammatory responses including the production of many of the cytokines known to be elevated at labour. The inflammatory response during labour is also reflected in circulation. IL-18 is also proposed to be an inducer of labour [105]. IL-18 has also been found to be elevated in miscarriages [106].

The immune system in the local intrauterine environment during pregnancy

During implantation the endometrium thickens and assumes the characteristics of an inflammatory response together with changes in the constellation of immune cells, their phenotypes and activities.

NK cells in decidua

There is a great influx of NK cells to the pregnant uterus. These NK cells represent approximately 70% of all immune cells in the early decidua. They are in close contact with the invading EVTs during the early part of pregnancy and are reduced in numbers in second

and third trimester. The NK cells found in decidua have been suggested to be important in trophoblast regulation due to their location and their ability to interact with EVTs via receptor-ligand interactions, as well as their capacity to produce cytokines considered promoting angiogenesis. These cells are further discussed on page 32.

Antigen-presenting cells in the decidua

Close to the implantation site macrophages are also found, although they are not as abundant as the NK cells. Around 20-30% of all leukocytes in early decidua are macrophages [6] and the number remains high throughout pregnancy. Decidual macrophages are suggested to be important for protecting the foetus against infections during pregnancy as well as to have a role in different processes during implantation [107]. They are $CD14^+$ and express HLA class II and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [6]. They also express the inhibiting receptors ILT2 and ILT4 [108], which can interact with HLA-G expressed on the EVT. Their close contact with uNK cells and the possible interaction between macrophages and EVTs, could indicate of a role during trophoblast invasion [6].

The correct balance between apoptosis and survival of trophoblasts is important for a proper EVT invasion. Apoptotic trophoblast cells are cleared by decidual macrophage phagocytosis and inhibitory factors for apoptosis, like X-linked inhibitor of apoptosis (XIAP), promote trophoblast survival. If the amount of apoptotic trophoblasts is high, like in preeclampsia, it is believed that the macrophages release an inactive form of XIAP together with pro-apoptotic factors and thereby induce increased apoptosis of trophoblast cells [107]. They are also producers of IL-10 and TGF-β1 [108].

DCs represent around 1% of all leucocytes in first trimester decidua [6], this includes both mature ($CD83^+$) and immature ($CD83^-$) DCs. The immature decidual DCs include populations both positive for $CD83^-DC\text{-}SIGN^+CD14^+$ [109] and $CD83^-DC\text{-}SIGN^-CD14^+$ [110].

Different roles of the mature and immature DCs in pregnancy have been suggested. The immature DCs are scattered around in decidua and are in close contact to the uNK cells and trophoblast cells, in contrary to the mature $CD83^+$ DCs found in myometrium of decidua [109, 111]. The close contact between $CD83^-$ DCs and uNK and trophoblast cells makes them possible candidates in maternal artery remodelling processes.

The mature DCs in decidua show different properties when compared to peripheral counterpart. They produce less amounts of IL-12 compared to the peripheral DCs [112]. Further, culturing naïve T cells and decidual DCs, results in higher IL-4 production from T cells, than in co-cultures of naïve T cells and peripheral DCs [112].

Lymphocytes in decidua

There are only a small number of B cells in decidua, which are located deep down in the basal layer of the endometrium [6]. Around 10% of all immune cells in early pregnant uterus are represented by T cells, both $CD4^+$ and $CD8^+$, together with γδ T cells [113, 114]. Regulatory T (T_{reg}) cells ($CD4^+/CD25^{high}/Foxp3^+$) [77] and NKT cells [115] are also present.

NKT cells are $CD56^+$ T cells expressing the CD3 receptor complex [116, 117]. They are cytokine-producing cells and play an immunoregulatory role in several immunological events [118]. There are a higher numbers of NKT cells in decidua compared to the numbers of peripheral NKT during early pregnancy [119, 115]. Although an endogenous NKT cell ligand has not yet been found, it is known that they can be activated when the CD1d molecule presents the glycolipid α-galactosylceramide (αGalCer) to NKT cells. Interestingly, CD1d is expressed on villous trophoblasts as well as on EVTs and can induce proliferation *in vitro* of NKT cells [115]. Additionally, they appear to produce more IFN-γ when compared to circulating NKT cells [119, 115].

Natural Killer (NK) cells

NK cells are granulated lymphocytes that belong to the innate part of the immune system. They have various functions and bridge innate and adaptive immunity. They are the first defence against tumour and virus infected cells [120] and are producers of different cytokines that can regulate the adaptive immune system. They are also proposed to be involved in autoimmunity and tissue inflammation [121]. To avoid tissue damage and autoimmunity, NK-cell regulation is accomplished by complex receptor interactions. NK cells are present in blood, and lymphoid tissues, and can migrate into non-lymphoid tissue when needed. They are abundantly present in placenta during early pregnancy.

NK cells in the circulation

Around 10-15% of all blood peripheral lymphocytes are NK cells. They are defined phenotypically by the surface expression of the adhesion molecule CD56 and lack of the CD3 molecule [120, 122]. Further, they are divided into two subpopulations ($CD56^{bright}$ and $CD56^{dim}$), which differ in surface receptor expression and function. Approximately 90% of human NK cells are $CD56^{dim}/CD16^{bright}$; this subpopulation is more cytotoxic and produces a negligible amount of cytokines. They also express high amounts of CD16, an Fcγ-receptor. The remaining 10% are $CD56^{bright}/CD16^{dim}$ or $CD16^{neg}$ are less cytotoxic and produce large amounts of cytokines. Furthermore, the two different NK cell populations also have different activating and inhibitory receptor repertoires and adhesion molecules [123].

NK cell maturation

It is not fully understood how NK cell maturation occurs in adults or in neonates; and if the maturation is similar in adults and newborns. Most of the present knowledge about NK-cell maturation is based on *in vitro* studies with adult peripheral blood cells or in immune-deficient mouse models [124]. Here the development of human (adult) NK cell maturation will be described in brief.

NK cells most probably derive from the $CD34^{+}$ haematopoietic stem cells (HSC) present in the bone marrow [125]. Even though the bone marrow is considered as the main NK-cell maturation site, HSCs are also found in thymus, spleen, liver and omentum in adults, but if HSCs can differentiate into NK cells in these environments is still not known. They are also found in foetal liver and yolk sac [126]. Depletion of the bone marrow environment resulted in defected NK-cell function and homeostasis, stressing its crucial role for NK cell maturation [127].

The bone marrow contains stromal cells, cytokines and growth factors able to mature the HSC into NK-cell precursors (NKP) [126]. When the multipotent HSCs are stimulated *in vitro* with cKIT or (fms-like tyrosine kinase 3 ligand) FLt3L, they continue to mature into NKPs. NKPs are destined to develop into NK cells. They are suggested to either further mature in the bone marrow or in other secondary lymph tissues, as they have been found in human lymph nodes [128].

The different maturation states of NK cells are acknowledged by their phenotype. NKPs express IL-2R and IL-15R but lack typical NK-cell markers, (such as CD56, NKG2A and CD16) [129]. These NKPs mature into immature NK cells and further into mature NK cells. The immature NK cells are $CD56^{-}CD3^{-}CD161^{+}$. It is still not clear whether the immature NK cells have an immunological function or not. $CD56^{-}CD3^{-}CD161^{+}$ cells are not cytotoxic, but can develop into cytotoxic cells expressing CD56 *in vitro* [130]. The immature NK cells do express NKG2D receptors and other receptors important for growth and survival [127] but they do not express killer immunoglobulin-like receptors (KIRs) [131].

Overall, stromal cells, cell-cell contact and IL-15 seem to be very important for NK-cell development. For example, human NKP cells can give rise to $CD56^{+}CD3^{-}$ cells when simulated with different factors such as IL-2 and IL-15 [127]. Also, NK cell numbers drastically decrease in IL-15 depleted mice and humans [126].

NK cell subpopulations

The $CD56^{bright}$ and $CD56^{dim}$ cells differ in several aspects. IL-15 stimulated NKP cells can mature into $CD56^{bright}$ cells and further into $CD56^{dim}$ cells, suggesting that the $C56^{bright}$ cells are immature NK cells [132]. $CD56^{bright}$ cells have a low or absent expression of KIRs, but a high expression of CD94/NKG2. The opposite receptor pattern is true for $CD56^{dim}$ cells [123]. Further, the high and intermediate affinity-IL-2 receptors are constitutively expressed on $CD56^{bright}$ cells, which therefore respond quickly to low doses of IL-2, in contrary to $CD56^{dim}$ cells, which only express the intermediate affinity IL-2 receptor. Further, these two subsets also differ in their chemokine receptor repertoire. $CD56^{bright}$ cell express high amounts of chemokine (C-C motif) receptor (CCR) 7 and CXC receptor (CXCR) 3 and migrate rapidly to their ligands, whereas the $CD56^{dim}$ cells lack the expression of CCR7 but do express high levels of CXCR1 and CX3CR1 [123, 133].

NK0, NK1, NK2 and NK3 subpopulations

T cells are roughly divided into T1and T2 cells based on their cytokine profile, and NK cells have been further subdivided according to the same principle. Whether these cells are two different lineages of NK cells or cells in different maturation stages are not known. NK cells cultured with IL-4, produce high amounts of IL-15 and IL-3 and low amounts of IFN-γ, *i.e.* the NK2 cells, while NK cells cultured with IL-12 produce IFN-γ and IL-10, *i.e.* the NK1 cells. No differences in cytotoxicity have been found between NK1 and NK2 [134].

NK2 cells are also proposed to be immature NK cells, with the ability to mature into NK0 cells when stimulated with IL-4. The NK0 cells would produce IL-13 and IFN-γ, and further mature into NK1 cells, which are cytotoxic and produce IFN-γ [132]. Furthermore, $CD56^-$ cells stimulated with Flt3L and IL-2 produce higher levels of IL-13 and IL-5, while $CD56^+$ cells produce more IFN-γ, indicating that the NK-cell maturation status influence their cytokine profile [135].

The NK3 cells produce TGF-β [136]. The distribution of NK1, NK2 and NK3 cells has been studied during early human pregnancy. The dominant decidual NK-cell subset in healthy pregnancies was the NK3 subpopulation, while the NK1 cells were increased in miscarriage cases. The dominant NK-cell population in the periphery was the NK1 subpopulation [136].

IL-18R and ST2L are described as reliable markers for human type 1 and type 2 cells, respectively [137]. With the use of these two markers the ratios of NK1/NK2 in successful pregnancies compared to preeclampsia have been investigated [90]. A higher ratio of NK1/NK2 was observed in preeclampsia. The NK1 and NK2 cells where further divided into $CD56^{bright}$ and $CD56^{dim}$ cells, and higher amounts of $NK2^{bright}$ cells were detected in healthy pregnancies [90].

Regulatory NK cells

Although, no surface markers are yet found for the regulatory NK-cell population, they are believed to exist [133] and considered as regulatory NK cells on the basis of what cytokines they produce. Their role has also been discussed during early pregnancy, and they have been divided into IL-10 producing NKr1 cells and TGF-β producing NK3 cells [136].

NK cell tolerance and "education"

NK cells express a large range of different germ-line encoded receptors. This means that the receptors are not unique for each individual NK cell and they do not harbour receptors recognizing a specific antigen as T cells and B cells do. Target recognition involves the interaction between inhibitory and activating receptors on the NK cell, and target cell ligands which are both MHC class I and non-MHC molecules. Each NK cell usually expresses several different receptors with similar function and the receptors can have overlapping specificities. To explain how NK cells are regulated, several theories have been proposed.

As the NK cells only express a selection of stimulatory and inhibiting receptors, the inhibitory receptor(s) expressed on the NK cell must be compatible to the MHC class I ligand expressed, for each individual. This raises the question if NK cells are going through a selection/education process, assuring that only the NK cells that recognise self-MHC class I molecules mature.

Several years ago the "missing-self" theory was proposed [138]. Here, NK-cell killing of target cells would occur if the expression of MHC class I molecules was altered due to transformed or virus-infected cells. Today, we know that NK-cell regulation is much more complex. A normal amount of MHC class I molecules is not always protective [139], and the finding of NK-cell receptors binding non-MHC class I ligands, also indicated other mechanisms of regulation. Further, the "missing-self" theory could not explain the fact that individuals with MHC class I deficiency maintained NK-cell tolerance [140].

The "at least one" theory suggests that NK cells express at least one inhibiting receptor for a self-MHC class molecule. Another proposal is that NK cells express a lesser amount of stimulatory receptors [141]. Further, it is also suggested that regulatory cells could interfere with self-destructive NK cells [141]. Yokoyama *et al* have proposed a mechanism for NK-cell tolerance called licensing [142]. If the inhibiting NK-cell receptor recognizes self-MHC class I it becomes licensed, *i.e.* a functionally competent NK cell. If the NK-cell MHC class I receptor does not recognise the self-MHC class I molecule, this NK cell remains unlicensed or functionally incomplete [142].

The finding of NK cells not expressing any inhibitory receptors for self-MHC class I ligands in normal mice, made scientists rethink regarding the "at least one" model as these NK cells seemed to have normal levels of other receptors [143]. Further, these cells are called hyporesponsive cells due to their poor response towards MHC-deficient cells [143]. Whether the hyporesponsive NK cells are immature or not are debated, but they do express other receptors characteristic for mature NK cells [141, 143]. Nevertheless, it is thought that NK-cell tolerance can be achieved although no inhibiting receptor for MHC class I is expressed and that the hyporesponsiveness is thought to be one of the mechanisms in which the NK cells become tolerant [143].

NK cell receptors

NK cell receptors (NKR) include three major families; the KIRs, C-type lectin superfamily (CD94/NKG2) and natural cytotoxicity receptors (NCR). The role of NCRs is still uncertain but they are thought to be involved in lysis of tumour cells. NK cells also express receptors included in the ILT-family; some of the ligands for these receptors are still unknown. The expression pattern of NKRs on a certain NK cell, clearly defines the function of that particular cell (fig. 4).

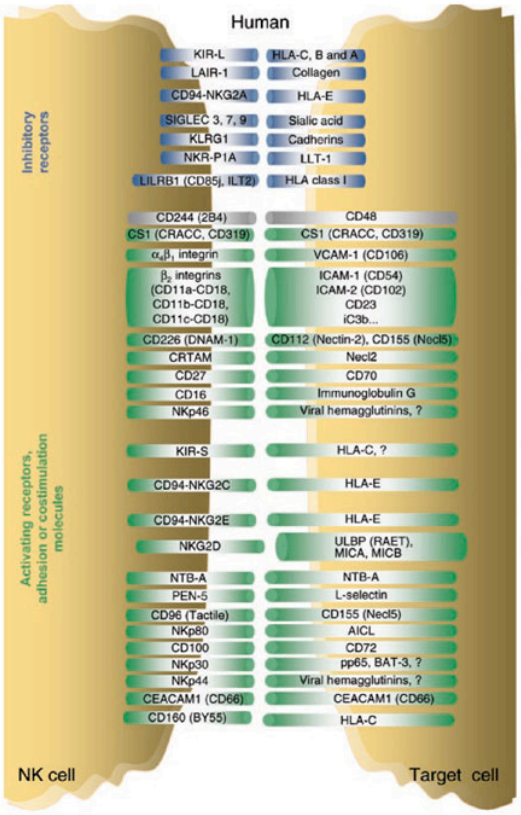


Figure 4. NK cells express numerous inhibiting and activating receptors. (Modified from Nature Reviews Immunology, 2008, Vol.9, p503-510). Reprinted by permission from Nature Publishing Group.

Killer immunoglobulin-like receptors

KIRs are expressed on NK cells and on a minor subpopulation of $CD8^+$ cells [144, 145]. They primarily recognise classical MHC molecules like HLA-A, HLA-B, HLA-C but also the non-classical HLA-G distributed in tissue [146, 147]. There are 16 different KIR genes encoding inhibiting and activating receptors, and these receptors are classified by three different criteria. Firstly, the number in the name of each KIR represents the number of extracellular Ig-like domains (for example, in KIR2DL1, 2D stands for two domains). Secondly, they are divided depending on the length of the cytoplasmatic tail (S for short and L for long), and

finally they are grouped for sequence similarity. One individual has a unique setup of KIR genes, which results in different KIR combinations on NK cells within the same individual [148, 147].

KIRs are divided into two different haplotypes, A and B. Both of these are highly polymorphic. The haplotype A mainly contains genes encoding inhibitory KIRs, while haplotype B contains inhibitory KIRs as well as genes encoding for activating KIRs [148]. The individual setup of KIR genes is not changed within an individual over time and KIRs do not seem to be influenced by cytokine stimulation [148].

CD94/NKG2 receptors

CD94/NKG2, belonging to the C-type lectin receptor family, is expressed on NK cells and $CD8^+$ T cells [149]. There are several NKG2 receptors, one being inhibitory while the others are activating. The only receptor possessing an inhibitory function is CD94/NKG2A (with its splice variant NKG2B). Activating receptors belonging to this family are CD94/NKG2C and CD94/NKG2E (with its splicing variant CD94/NKG2H). As CD94 lacks signalling capacities, the intracellular events are dependent on the NKG2 molecule. The inhibitory receptor CD94/NKG2A contains an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) motif, while the activating receptors CD94/NKG2C and CD94/NKG2E posses an immunoreceptor tyrosine-based activation motif (ITAM) motif [150]. The ligand for both receptors is HLA-E [151], and NKG2C binds HLA-E with a 10-fold lower affinity than NKG2A [152]. Further, the binding of NKG2 to HLA-E is peptide dependent [152]. These receptors are involved in immune responses to infectious diseases and cancer and the expression of these receptors can be regulated by cytokine stimulations (further discussed below).

The activating receptors NKG2D and NKG2F do not associate with CD94. Recognized NKG2D ligands are the MHC class I chain-related proteins (MIC)-A and MICB [153], while the ligand for NKG2F is still unknown [154]. Apart from MICA and MICB, the ligands for the activating receptor NKG2D are the virus derived UL 16-binding protein (ULBP) -1 and ULBP-2 [155156]. MICA and MICB are upregulated in stressed, virally infected and transformed cells. They associate with two transmembrane bound DAP10 homodimers [157]. Apart from ligand-receptor interactions, NK cells seem to need cytokine signals before NKG2D can trigger cytotoxicity [153].

NKG2 receptors are expressed at high levels on the $CD56^{bright}$ subset in contrary to $CD56^{dim}$ cells [123]. Also, NKG2D ligand-stimulated NK cells produce several cytokines like, IFN-γ and GM-CSF [158].

Cytokines can further influence NKG2-receptor expression on NK cells. IFN-γ stimulated NK cells result in higher expression of NKG2A and a down-regulation of NKG2D, while IFN-α stimulation results in the opposite receptor expression pattern [159]. Further, IL-21 can down-regulate NKG2D expression on both NK cells and T-cells [160].

NKG2A, NKG2C and NKG2D in disease

Altered expression of NKG2-receptors on NK cells has been reported in different diseases. Human CMV infected fibroblasts treated with IL-15 result in higher expression of CD94/$NKG2C^{+}$ NK cells [161], while there is a proportional decrease in NK cells expressing NKG2A in HIV infection [162]. CD94 expressing NK cells are increased in haemodialysed uraemic patients (=renal failure) [163] and in HIV-1 infected patients [164]. Furthermore, tumour infiltrating NK cells collected from patients with renal cell carcinoma express enhanced levels of CD94/NKG2A receptor [165].

CD69

The CD69 receptor is expressed on activated NK cells after stimulation [166]. CD69 triggers cytotoxicity [166, 167], proliferation and TNF-α release from NK cells [166]. Additionally, CD69 mediated cell degranulation is suppressed by CD94/NKG2A through inhibiting intracellular ERK activity [168]. CD69 is also expressed on B and T cells, monocytes and neutrophils [169].

Natural-cytotoxicity receptors and immunoglobulin-like transcript receptors

The activating NKp30, NKp44 and NKp46 receptors belong to the NCR family and are expressed on NK cells [170, 171, 172]. While the NKp30 [170] and NKp46 receptors [172] are expressed on resting as well as on activated NK cells, the NKp44 receptor is only expressed on activated NK cells [171]. The human CMV protein pp65 is a ligand for Nkp30 [173], while NKp46 and NKp44 recognize influenza haemagglutinin [174175]. These receptors are involved in cytotoxicity against certain tumours, although tumour lysis requires the cooperation of NCR and NKG2D [176]. NKp80 is a novel activating NK cell receptor that has recently been discovered [177].

ILT2 and ILT4, also called LIR1 and LIR2, are expressed on NK cells, as well as on monocytes/macrophages and T and B cells [178]. These receptors are involved in cytokine production and inhibition of cell lysis [179, 180] and interact with classical HLA-A, HLA-B, HLA-C and non-classical HLA molecules, HLA-E, HLA-F and HLA-G [181, 178, 182, 183, 145].

NK cells are major cytokine producers

NK cells, primarily the $CD56^{bright}$ cells, produce a variety of cytokines, like IL-1, IL-10, IL-13, IFN-γ, TNF-α, TNF-β, TGF-β and GM-CSF [123, 184]. In turn, NK cells are also activated by cytokines and they constitutively express cytokine receptors like IL-1R, IL-2R, IL-10R, IL-12R, IL-15R and IL-18R. $CD56^{bright}$ NK cells have a higher expression of IL-1R, IL-18R and an IL-2R (CD25) than $CD56^{dim}$ NK cells [123, 185].

Accessory dependent NK-cell activation

Although no prior activation of NK cells is necessary, they do require additional stimuli to become maximally activated by soluble factors and cell-to cell contact [186]. Monokines are important NK cell activators and IL-12, IL-15 and IL-18 have the ability to stimulate NK-cell development and/or trigger NK cell IFN-γ production [123, 187, 188]. IL-12 is mainly produced by monocytes and macrophages stimulating the IFN-γ production of NK cells [123]. IL-15 is important in NK-cell development, while IL-18 can either trigger a Th1 or Th2 response [189], depending on the surrounding cytokine environment. Studies have shown that a combination of IL-12 and IL-18 give the maximal NK cell production of IFN-γ, while IL-12 and IL-15 give optimal NK cell production of IL-10 [123].

NK cells can form immunological synapses with both DCs and monocytes, which will create the optimal conditions for NK cell activation. For example, optimal IL-12 signalling occurs when an immune synapse is formed between an NK cell and an accessory cell [190]. Further, IFN-γ production by NK cells is diminished after $CD14^{+}$ cell depletion and the blocking of various co-stimulatory molecules on activated monocytes also result in decreased IFN-γ production [191-193].

Uterine NK cells

The uNK cells are a special subset of NK cells found in the human uterus. The majority of uNK cells are $CD56^{bright}/CD16^{neg}$ and express up to a five-fold higher level of CD56 compared to the $CD56^{bright}$ peripheral cells. The uNK cells have phenotypic similarities with the $CD56^{bright}$ cells found in blood; they are skewed towards cytokine production and express the CD94/NKG2A receptor at high levels [194]. Further, both $CD56^{bright}$ cell populations show low cytotoxicity, but the uNK cells are large and contain many cytolytic granules, while the $CD56^{bright}$ blood NK cells are small with few granules [6].

Uterine NK cell recruitment

The origin of uNK cells is debated. It has been suggested that they are recruited from the periphery into the uterus. Alternatively they could be derived from stem cells situated deep down in the endometrium [6]. The former theory has gained most attention and it is further supported by studies performed in mice. Uterine grafts from mouse, with NK and uNK cells, were transplanted into uNK/NK cell deficient mice. No self-renewal of uNK cells were found in the recipient mice, *i.e.* no survival or renewal of uNK cells could occur, supporting the idea that uNK cells cannot be renewed within the placenta [195]. To further challenge the theory of uNK cells being recruited from peripheral tissues, bone marrow, thymus, spleen cells and lymph nodes containing pre-NK cells from immunocompetent mice were transplanted into uNK/NK deficient mice. This resulted in high amounts of uNK cells in the NK/uNK cell deficient mice, supporting that uNK cells are recruited from these lymphoid tissues [195].

Distinct chemokines expressed on the $CD56^{bright}$ and $CD56^{dim}$ populations in blood reveal different migration capabilities of the two subsets [196]. Trophoblast cells, expressing chemokine ligands, have the capability to attract peripheral $CD56^{bright}$ cells expressing receptors for the same chemokine ligands [197]. Further, monocyte inflammatory protein (MIP) 1α, produced by cytotrophoblast cells, has been found not only to attract monocytes but also $CD56^{bright}$ cells [198]. uNK cells are thought to be further differentiated in the hormone rich and specific cytokine milieu of the uterus, if recruited from the periphery [6]. Chemokine- receptor expression differs between peripheral and NK cells found in decidua. To address whether specific cytokines present in placenta could shape the peripheral NK cells expression of chemokine receptors, peripheral $CD16^{-}$ NK cells were stimulated with IL-10, IL-12, IL-15 and IL-18. In particular the addition of IL-15 resulted in a chemokine receptor expression similar to NK cells in decidua [197].

Uterine NK cells – their non-pregnant functions

The uNK cells appear during puberty and are present in cycles during menstruations. These cells increase in the secretory phase of the menstrual cycle when they differentiate and become active [199, 200]. It is also during this time decidualization begins if fertilization occurs. Several cytokines appear in the uterus during this time, like IL-12, IL-13, IL-15 and IL-18 [201, 202, 203]. If menses occurs, the uNK cells amount will decline [199], but during fertilization, the amount of uNK cells increases during early pregnancy and does not start to decline until later during pregnancy [204].

Differences between uNK, decidual NK and peripheral NK cells

During pregnancy, the uNK cells are often referred to as decidual (dNK) cells. As mentioned above, the u/dNK cells and peripheral NK cells differ in phenotype and functions. The differences between dNK from first trimester pregnant women and peripheral NK cells collected from non-pregnant subjects have been studied using microarray analysis [205]. As many as 197 upregulated genes were found in dNK cells when compared to circulating NK cells, among these were several KIR receptors and also NKG2C and NKG2E, the latter two being activating receptors [194, 205].

dNK cells have the necessary machinery, like perforin, granzymes A and B, required to kill target cells [206]. Despite this, their cytotoxic capabilities are reduced [207]. They also express NKG2C and NKG2E [205] but they fail to kill the MHC I-negative NK cell line K562 [207]. Kopcow *et al* showed that the dNK cells fail to polarize the perforin-containing granules towards the synapse formed between the dNK cells and the target cells [207].

Decidual NK cell receptors

In humans dNK cells are believed to be involved in the regulation of trophoblast invasion and the placentation process during early pregnancy. Current theories indicate the importance of a proper dNK-cell receptor expression, as both too much inhibition and activation have been suggested to contribute to pathology [5, 50].

Killer immunoglobulin-like receptors expression in placenta

Several KIRs are expressed by dNK cells [205, 208]. KIR expression on uNK and peripheral NK cells can vary within the same individual [209].

KIR2DL4 is expressed on dNK cells and its ligand, HLA-G, is expressed on the trophoblast cells. KIR2DL4 is divergent from the other KIRs, and it is not present on the cell surface but mainly localised intracellulary. Further, it is the only KIR expressed in all NK cells [148]. Its ligand, HLA-G, is found on EVTs in placenta and their interaction is thought to induce IFN-γ production [146]. As IFN-γ has been implicated in the implantation process, the KIR2DL4-HLA-G interaction could play a role during placental development. Furthermore, KIR2DL4 interactions inhibit NK-cell cytotoxicity and promote cytokine production [210, 211]. Higher amounts of KIR2DL4 have been found in healthy planned abortions compared to RSA [212] but this receptor does not seem to be absolutely necessary for successful pregnancies. A mother giving birth to several children has been found to totally lack the KIR2DL4 gene [213].

CD94/NKG2 in placenta

dNK cells also express the inhibiting receptors CD94/NKGA [194] and the activating receptors NKG2C and NKG2E [194, 205]. The dNK cells express these receptors in higher levels compared to peripheral NK cells [205, 7]. The CD94/NKG2 receptors interact with HLA-E [214, 215], which is expressed on EVT cells. The NKG2 and HLA-E interactions in placenta are thought to include inhibition of cell lysis [7].

Expression of NKp30, 44, 46 and NKG2D in placenta

Like the peripheral NK cells, first trimester dNK cells express the activating receptors NKp30, NKp44, NKp46 and NKG2D [207, 216]. The exact function of the NCRs in decidua is not known today.

The ligands for NKG2D, MICA and MICB, are present in both early and late placenta and MICA and MICB mRNA is expressed in SCT [217]. Further, sera from pregnant women have high levels of the MICA and MICB, and have the capability to down-regulate NKG2D expression on PBMCs from non-pregnant subjects [217].

Immunoglobulin-like transcript receptors in placenta

ILT receptors are expressed in placenta and they interact with HLA-G and possibly with HLA-C and HLA-F [178, 179, 181-183], which all are expressed in placenta [7, 218, 219]. Both ILT2 and ILT4 are expressed on decidual macrophages as shown by reverse transcriptase polymerase chain reaction (RT-PCR) [220] and a minor part of the dNK cells express ILT2 [221]. The

involvement of the ILT2 receptor includes inhibition of cell lysis as well as cytokine production [179, 180]. This could indicate a regulatory role of this receptor during pregnancy.

NK cells in the decidua and the intrauterine cytokine environment

The dNK cells are cytokine-producing cells and contribute to the cytokine milieu in placenta. They produce cytokines like colony-stimulating factor-1 (CSF), GM-CSF, IFN-γ, TNF-α, leukaemia inhibitory factor (LIF) [222] and angiogenetic factors such as placental growth factor (PGF), VEGF-C and angiopoietin (Ang) 2 [223]. NK cells are also major producers of IFN-γ. IFN-γ is considered to be a cytokine important for angiogenesis [69, 70] and *in vitro* studies have suggested a contact-independent regulation of trophoblast cells by IFN-γ [69].

Many of the NK-cell activating cytokines, like IL-12, IL-15 and IL-18, are also present in the placenta [224-226]. IL-18 stimulates cytolytic capabilities of decidual lymphocytes when stimulated with different combinations of IL-12 and IL-15 [226, 227]. IL-15 is present in the endometrium, where it follows the cycles of uNK cells, [202] and in first trimester decidua until the end of pregnancy [224]. Findings in both humans and mice suggest IL-15 to be an important cytokine by contributing to dNK-cell proliferation and differentiation [228, 229].

The uNK cell receptor ligands

Many ligands for these NK cell receptors are also found in placenta, indicative of an important interplay between foetal cells and maternal NK cells.

HLA-G

The membrane bound HLA-G (mHLA-G) molecule is expressed on EVTs, amnion epithelial cells, foetal endothelial cells and macrophages within the placental villi [218], while the soluble isoforms of HLA-G is found in various compartments in placenta such as SCTs, placental macrophages and amniotic fluid, and also in maternal and cord blood [218]. HLA-G is the ligand for the receptors KIR2DL4 and ILT2 expressed in placenta. The proposed functions of mHLA-G include inhibition of NK-cell mediated cell lysis; cytokine production [180, 230, 231] and NK-cell proliferation [232]. Cultured purified uNK cells from non-pregnant women stimulated with mHLA-G resulted in high IFN-γ production [232]. mHLA-G expressed on EVTs is proposed to modulate cytokine production and thereby regulate the EVT invasion and angiogenesis [218]. Lower expression levels of mHLA-G in preeclamptic [101, 233] and RSA [234]

have been described. In contrast, others have reported higher levels of mHLA-G expression in villi and decidua from women suffering from pregnancy loss compared to planned pregnancy terminations [235].

HLA-E and HLA-F

HLA-E is one of the non-classical MHC molecules expressed on EVTs [214] and it interacts with CD94/NKG2 [214]. The interaction between HLA-E and its receptors have different affinity depending on what peptide is bound to HLA-E [152]. One of the strongest binding affinities to CD94/NKG2 is reached when HLA-E is loaded with the HLA-G derived leader sequence [152, 236]. Further, newly synthesised HLA-E cannot be correctly folded if not a leader sequence is present. In this way HLA-G can influence HLA-E binding capacity as well as maturation [237]. HLA-E can also bind and present peptides from Hsp60 as well as viral and bacterial components [238]. In addition, human CMV regulates HLA-E cell surface expression on virus-infected cells [239], an interesting aspect as preeclampsia is associated with increased CMV antibody levels [40].

The expression of HLA-E on endothelial cells increases when they are stimulated *in vitro* with IFN-γ and TNF-α, two cytokines known to be present at the maternal-foetal interface during early pregnancy [240]. The increased expression of HLA-E was further demonstrated to be protective from NK cell lysis [240].

HLA-F is also expressed on EVT in term placenta [236] but its function during pregnancy remains to be elucidated.

HLA-C

HLA-C belongs to the classical Ia HLA-molecules and can interact with KIR receptors in decidua [7] and possible ILT2 and ILT4 [178, 179]. HLA-C is expressed on EVT cells [241, 7] and is a polymorphic protein divided into two HLA-C groups, C1 and C2, which bind different inhibiting or activating maternal KIR receptors [242, 243]. Depending on the combination of foetal HLA-C and maternal KIR haplotype, the outcome of the interaction could either be more inhibiting or activating. Women suffering from preeclampsia as well as women with recurrent miscarriage more often have a KIR AA haplotype [50, 244]. Together with these findings it was also discovered that the foetal HLA-C in preeclamptic cases more often expressed the HLA-C2 group, indicating an over-inhibition of KIR^+ NK cell receptors in

preeclampsia [50]. Couples with recurrent miscarriages also more often expressed the HLA-C2 group [244].

High Mobility Group Box 1 (HMGB1)

HMGB1 was first discovered as a DNA-binding protein and a transcription factor, but is now also considered to be an alarmin [245]. It is an endogenous molecule involved in inflammation and has been implicated in many different tissue-specific activities. HMGB1 also has the ability to activate systemic and local inflammatory responses. During the course of an inflammatory response, HMGB1 induces NF-κB activation and the release of pro-inflammatory cytokines. In contrast to other cytokines, like TNF and IL-1, HMGB1 is released rather late during an inflammatory reaction - after several hours [246]. However, it also possesses diverse functions such as promoting angiogenesis [247], initiating tissue repair [248] and can regulating cytokine production [249]. HMGB1 is a leaderless cytokine, meaning that it is not processed through the Golgi apparatus. Instead it is secreted via a non-classical pathway involving exocytosis of lysosomes. HMGB1 can be passively secreted from necrotic cells [250] or actively secreted from activated monocytes, mature DCs and NK cells [246, 251, 252]. Recently there have been reports regarding the involvement of HMGB1 in several diseases, like cardiovascular disease, autoimmunity and inflammatory conditions [246, 253, 254].

HMGB1 receptors

Receptors described to interact with HMGB1 are the Receptor for Advanced Glycation End-products (RAGE), TLR2 and TLR4 [255].

Receptor for Advanced Glycation End-products

The first proposed receptor for HMGB1 was RAGE which is a pleiotropic receptor expressed on macrophages/monocytes, endothelial cells, vascular smooth muscle cells and neurons [255]. It exists as a trans-membrane receptor as well as in a soluble form, then acting as a regulator/inhibitor of HMGB1 actions [246]. HMGB1 interaction with RAGE involves NFκB-mediated inflammatory responses including TNF-α, IL-1β and IL-8 production [256, 257], cytokines known to be elevated during labour [103].

Toll-like receptors

TLRs belong to the pattern recognition receptors (PRR), which are expressed on different cell types belonging to the innate, adaptive immune system as well as non-immunological cells. The PPRs recognize molecular structures, pathogen-associated molecular patterns (PAMPs), expressed by different microorganisms. Up till today there are 10 functionally active TLRs found in humans [258].

TLR2 binds gram-positive bacterial components such as bacterial lipoproteins, like peptidoglycan (PGN) and also fungal zymosan [259]. Additionally, TLR2 can form heterodimers together with either TLR1 or TLR6 [258]. TLR4 interacts with bacterial gram-negative lipopolysaccharide LPS [258] and associates with CD14 and MD-2 to be fully activated [260, 261].

Lately both TLR2 and TLR4 have been suggested to be receptors for HMGB1 [262]. This suggests that at least some TLRs can respond not only to microbial stimuli, but also to endogenous "stress" ligands. TLR4 also binds heat shock proteins [263], which also are the endogenous ligands for TLR2/TLR6 [258]. Many of the cytokines secreted after TLR activation are able to activate the innate immune system including IFNs, TNF-α, IL-6, IL-10 and IL-12 [258].

TLRs expression and function in placenta

TLRs are present in placenta and their expression appears to be trimester dependent. Most probably TLRs function as a defence against infections during pregnancy, initiators of labour [103] and to influence placental tissue/trophoblast cytokine production [264, 265].

mRNA have been found from ten TLR in first trimester decidua, while high mRNA levels of TLR2, TLR7 and TLR9 were found in third trimester decidua [266]. From these, TLR2 and TLR4 are found at protein levels in extra villous tissue and in the villous cytotrophoblasts in first trimester [264] while they are found in EVTs and SCTs in third trimester [265]. Stimulation of first and third trimester placental trophoblasts with LPS, resulted in release of IL-6, IL-8, TNF-α and IFN-γ [265, 264]. Further, TLR2 has been shown to induce apoptosis of PGN cultured trophoblast cells in contrary to TLR4 ligation by LPS that failed to induce apoptosis [264].

A role for HMGB1 in pregnancy?

Little is known regarding the expression or involvement of HMGB1 or its primary receptor, RAGE, in pregnancy. In contrast, the expression of TLR2 and TLR4 has been studied both in healthy pregnancies [265] and pathological pregnancies [267, 268]. Since both the early stages of implantation as well as labour are considered as controlled inflammatory processes, a role for HMGB1 during both processes could be envisioned.

i) HMGB1 during implantation

HMGB1 has been identified as an angiogenesis switch molecule, and initiates endothelial growth, cell migration and tissue repair [247, 248, 255, 269]. In addition, HMGB1 can enhance IFN-γ release from macrophage stimulated NK cells [270]. Although a local, controlled IFN-γ could be crucial during the implantation process as indicated in mouse models, an excessive release of HMGB1 during implantation might stimulate exaggerated IFN-γ production resulting in a hampered placentation and preeclampsia.

ii) HMGB1 in labour

An increase in gene expression of multiple cytokines and chemokines known to be involved in acute inflammation has been observed in chorioamniotic membranes from patients in labour compared to membranes from those without labour [271]. HMGB1 is a potent inducer of NF-κB activation and of the release of pro-inflammatory cytokines and chemokines such as TNF, IL-1, IL-6 and IL-8 [249], suggesting a role for this cytokine during active labour.

iii) HMGB1 and its receptor in pathological pregnancies

TLRs have been suggested to contribute to the pathogenesis of preeclampsia, by responding to endogenous "danger" signals *in utero* [268].

Also, RAGE has been implicated in preeclampsia. Non-pregnant women have a very low expression of RAGE in uterine tissue when compared to pregnant and preeclamptic women [272]. Apart from HMGB1, RAGE also interacts with Advanced Glycation End-products (AGE) and both the ligand and receptor is upregulated in sera from preeclamptic women compared to successful pregnancies [273].

Severe cases of preeclampsia are often ended prematurely. Further, another frequent explanation of premature births is infections [274], which in turn, is one of the proposed causes for preeclampsia [40, 275]. Increased expression of TLR4 has been reported in preeclamptic placentae when compared to women with or without chorioamnionitis [268]. TLR2 expression in placentae with chorioamniotitis and from pregnancies with stillborn children is reduced compared to controls [276].

PRESENT STUDY

AIMS

1. There are many studies indicating the importance of functional dNK cells during placental formation and function. The aim in paper I was to study dNK cells with regard to numbers and receptor expression as well as the NK cell promoting cytokines IL-12 and IL-15, and the anti-inflammatory cytokine IL-10 in term placenta from healthy and preeclamptic women. The same cytokines, together with IL-18, were also measured in serum from the same women.

2. Our aim in paper II was to investigate the expression of the pro-inflammatory cytokine HMGB1 and its potential receptors in healthy and preeclamptic third trimester placentae. Further, we investigated the HMGB1 expression in placentae from women who delivered vaginally and from women undergoing elective caesarean sections.

3. In paper I, we found enhanced serum levels of p40/IL-12/IL-23 and IL-15 in preeclamptic women. As IL-12 and IL-15 are NK-cell activating cytokines, the aim in paper III was to study if the increased levels of these cytokines influence peripheral NK-cell receptor expression and function in women with preeclampsia.

4. Preeclampsia is often accompanied by a disturbed placental morphology. It is not known if/how these morphological changes contribute to an altered maternal-foetal contact *in utero*, and in this way influence/skew the foetal immune system. Therefore, we studied if the altered maternal immunity in preeclampsia could affect the cord blood cytokines and cord blood NK cells and their function *(Study IV, preliminary data)*.

Table I. Demographic data of all healthy and preeclamptic mothers included in the different studies. Median (range) values are shown in the table.

Group	cases (n)	Maternal age at delivery (years)	Mode of delivery (VD/ECS)*	Gestational age (weeks)
Healthy mothers	31	33 (22-42)	9/22	39 (35-43)
Preeclamptic mothers	39	33 (22-44)	9/30	37 (26-42)
Mild cases of preeclampsia	17	33 (25-44)	5/12	39 (35-42)
Severe cases of preeclampsia	22	32.5 (22-43)	4/18	34 (26-40)

Several women were included in more than one study. * VD=vaginal delivery, ECS= elective caesarean section.

Table II. Demographic data of all neonates born from healthy and preeclamptic mothers included in study IV. Median (range) values are shown in the table.

Group	cases (n)	Maternal age at delivery (years)	Mode of delivery (VD/ECS)*	Gestational age (weeks)	Weight (children) (g)
Neonates from healthy mothers	18	33 (24-38)	7/11	39 (37-43)	3540 (2810-4900)
Neonates from preeclamptic mothers	19	33 (22-44)	7/12	38 (31-41)	3270(1158-4125)
Mild cases of preeclampsia	11	33 (27-44)	5/6	40 (37-41)	3295 (2510-4096)
Severe cases of preeclampsia	8	33 (22-41)	2/6	36.5 (31-40)	2580 (1158-4125)

* VD=vaginal delivery, ECS= elective caesarean section.

Overall, a total of 70 women (Table I) and 37 children (Table II) were included in the studies in this thesis. Material from the same women has been used in more than one of the studies as described below.

Study population (paper I)

A total of forty-six women, recruited at the delivery unit at the Karolinska University Hospital, were included in the study. Twenty-four of these women were healthy controls. Out of the 22 preeclamptic women, 8 were diagnosed with moderate preeclampsia and 14 with severe preeclampsia. Preeclampsia cases were divided into moderate (>0.3 g proteinuria,

systolic/diastolic blood pressure >140/90) and severe (>3 g proteinuria, systolic/diastolic blood pressure >160/110). Fourteen women had vaginal deliveries (VD) (9 healthy controls, 5 preeclampsia cases) and 32 women delivered by ECS (15 healthy controls and 17 preeclampsia cases). There were no significant differences in age between healthy and preeclamptic women (p=0.8). Women with preeclampsia delivered earlier than the healthy control women (week 35.5 vs. week 38.5, p=0.006). All women gave their informed consent to participate in the study. The Ethics Committee of Karolinska Institute, Stockholm, Sweden, approved the study.

Study population (paper II)

Twenty-five women participated in the study and were recruited at the delivery unit at the Karolinska University Hospital, Stockholm, Sweden. From these, 12 women had healthy term pregnancies while 13 women suffered from preeclampsia (5 with moderate preeclampsia and 8 with severe preeclampsia). Preeclampsia was defined as described for patients in paper I. Twelve women had VD (7 healthy controls, 5 preeclampsia cases) and 13 women were delivered by ECS (5 healthy controls and 8 preeclampsia cases). There were no significant differences in age between healthy and preeclamptic women. Women with preeclampsia delivered earlier than the healthy control women (week 36 vs. week 40, p=0.006) and null parity was more common in this group. All women gave their informed consent to participate in the study. The Ethics Committee of Karolinska Institute, Stockholm, Sweden, approved the study.

Study population (paper III)

Thirty-three women were recruited to the study at the delivery unit at Karolinska University Hospital. Seventeen women suffered from preeclampsia and 16 women with normal term pregnancies were included as healthy controls. Women suffering from preeclampsia were divided into moderate cases (n=10, >0.3 g proteinuria, systolic/diastolic blood pressure >140/90) and severe cases (n=7, >3 g proteinuria, systolic/diastolic blood pressure >160/110) of preeclampsia. Twelve women had VD (6 healthy controls, 6 preeclampsia cases) and 21 women delivered by ECS (10 healthy controls and 11 preeclampsia cases). All women gave their informed consent to participate in the study. Further, buffy-coats from 10 healthy blood donors were obtained from the blood bank at the Karolinska University Hospital, Stockholm. The Ethics Committee of Karolinska Institute, Stockholm, Sweden, approved the study.

Study population (Study IV, preliminary data)

Cord blood and sera were collected at the time of delivery at the delivery unit, Karolinska University Hospital. Eighteen children from healthy mothers and 19 children from preeclamptic mothers were included in the study. Preeclampsia was divided into moderate (n=11, >0.3 g proteinuria, systolic/diastolic blood pressure >140/90) and severe preeclampsia (n=8, >3 g proteinuria, systolic/diastolic blood pressure >160/110). The study included 14 mothers delivered by VD (n=7 healthy and n=7 preeclamptic mothers) and 23 mothers delivered by ECS (n=11 healthy and n=12 preeclamptic mothers). All women gave their informed consent to participate in the study. The Ethics Committee of Karolinska Institute, Stockholm, Sweden, approved the study.

Methods

The material and methods are described in each separate paper, except for the preliminary data in Study IV, which is described below.

Collection and preparation of CBMC

CBMC were collected and prepared within 24 hours after collection and isolated using Ficoll-Paque^{TM}PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). All CBMCs were stored in RPMI 1640 supplemented with 10% heat-inactivated FCS, L-glutamine (2mmol/L), penicillin G sodium (100 units/ml) and streptomycin sulphate (100µg/ml) (complete tissue culture medium, CTCM) containing 10% Dimethyl sulphoxide (DMSO) at -135°C until use.

For the preparation of serum, blood from cord was centrifuged at 1500 rpm for 10 minutes within 24 hours after collection. Serum samples were stored at -20°C before use.

Preparation of CBMC

All CBMCs were thawed quickly and washed twice in CTCM. CBMCs included in the phenotypic study were aliquot in $0.5x10^6$ cells/well and incubated for 20 minutes at 37°C prior to marker staining. For stimulation experiments, CBMCs at a concentration of $1x10^6$ cells/mL were stimulated with IL-15 (20ng/mL) and peptidoglycan (PGN) (1 µg/ml) in combination at 37°C for 24 hours.

Cytometric Bead Array

IL-12p70, TNF, IL-1β, IL-6, IL-8 and IL-10 in sera from children and mothers were measured with Cytometric Bead Array (CBA) (BD Biociences, San Diego, CA, USA) and performed according to the manufacturer's instructions. Calibration of the flow cytometer was performed using BD FACSComp™ and BD CaliBRITE™ Beads and analyzed using BD CBA Software (all from BD Biociences, San Diego, CA, USA). The detection limits for the cytokines were, IL-12p70 (1.9 pg/ml), TNF (3.7 pg/ml), IL-1β (7.2 pg/ml), IL-6 (2.5 pg/ml), IL-8 (3.6 pg/ml) and IL-10 (3.3 pg/ml).

Processing of placental tissue

The formalin fixed, paraffin embedded sections were processed as in material and methods described in paper I.

Immunohistological staining of placental tissue

Paraffin embedded sections were used for placental IgG stainings (mouse anti human IgG, 1/100, clone A57H, Abcam, Cambridge, UK) and stained as in paper I, with the exception of an incubation for 1 hour in RT regarding the primary antibody.

Flow cytometry

APC-conjugated anti-CD56, Per-Cp-conjugated anti-CD3 and PE-conjugated anti-CD16 monoclonal antibodies were used to identify NK cells (all from BD Biosciences, San Diego, CA). NK-cell receptor expression was studied by the following antibodies: Anti-CD69, anti-NKG2D (both PE-conjugated from BD Biosciences), PE-conjugated anti-NKp30 and anti-NKp46 (both from Beckman Coulter, Immunotech, Marseille, France), un-conjugated anti-NKG2A, NKG2C (RnD Systems, Minneapolis, MN, USA) detected with PE-conjugated goat anti-mouse (DakoCytomation). As negative controls, IgG1 (DakoCytomation, Glostrup, Denmark) and IgG2a (RnD Systems) were used.

Soluble HLA-G

Soluble HLA-G was measured using a sHLA-G-kit (BioVendor, Czech Republic) and performed according to manufacturer's instructions. The detection limit was 1.95 U/mL and optical densities were measured at 450 nm.

IFN-γ Enzyme-Linked Immuno Sorbent Assay (ELISA)

IFN-γ production in culture supernatants, after 24 hours of stimulation with IL-15+PGN, was studied by ELISA. Ninety-six well plates (Costar 3690, Corning Inc., Corning, NY, USA) were coated with anti-IFN-γ mAbs (2 μg/ml, Mab 1-DIK, from Mabtech, Stockholm, Sweden) and incubated ON at 4°C. Prior to adding the samples in duplicates blocking with 0.5 % bovine serum albumin (BSA) in PBS was performed. Biotin labelled mAb (1 μg/ml, 7-B6-1-Biotin, Mabtech, Stockholm, Sweden) and streptavidin-alkaline phosphatase (Mabtech, Stockholm, Sweden) were added followed by the substrate (SIGMA-Aldrich, Stockholm, Sweden). All incubations were performed for 1 h at 37°C, except when adding the substrate, when plates were incubated in room temperature. Standard curve was used with calculated dilutions of recombinant IFN-γ (NIBSC, Hertfordshire, UK). Detection limit was 3.9 pg/mL and optical densities were measured at 405 nm.

Intra-cellular staining of IFN-γ and IL-4

To study intracellular cytokine production from NK cells, staining for IFN-γ (FITC-conjugated from BD Biosciences) and IL-4 (PE-conjugated from BD Biosciences) antibodies were used. To prevent non-specific binding of antibodies to FcR, the cells were incubated in 1% BSA and 0.02% NaN_3 in PBS (wash buffer) supplemented with 5% human serum (blocking buffer) prior to staining with specific antibodies. Flow cytometry was performed on FACScalibur (BD Biosciences, Mountain View, CA, USA) and analyses were made using Cellquest Pro software (BD, version 5.2.1).

Statistics

Statistical differences between the healthy and preeclamptic children were evaluated by Mann-Whitney U non-parametric analysis. Correlations between mother and child were calculated with Spearman Correlation test. Statistical significance was assumed when $p<0.05$ and performed on Statistica 7.1 (STATISTICA Statsoft Inc., Tulsa, USA).

Results and Discussion

Paper I

The dNK cells are proposed to have an important role during placentation, although their exact role is not yet established. Considering the poor placentation known to occur in preeclampsia, it is important to investigate whether the dNK cells, or factors known to influence NK cell functions, are altered during this pregnancy disorder. The immunological changes during pregnancy are visible both in placenta and circulation; therefore we wanted to investigate NK cells and cytokines with a potential influence on NK cell activity and function, locally and peripherally, to see whether the immunological profiles were the same at both sites.

We studied dNK-cell numbers and receptor expression (CD56, CD94, CD16) in placenta. Further, we investigated the NK-cell activating cytokines IL-12, IL-15 and the anti-inflammatory cytokine IL-10 in placenta. This was performed with immunohistochemistry (IHC). The same NK-cell promoting cytokines, together with IL-18, were also studied in serum from healthy and preeclamptic pregnancies collected at delivery, by ELISA.

Since labour is considered to be an inflammatory response that could influence placental cell and cytokine expression, we only included women undergoing ECS in the IHC studies of the placentae. The number of $CD56^+$ cells was significantly increased in placenta from preeclamptic women, when compared to controls. Also, receptor expression of CD94 followed the same trend, with more $CD94^+$ cells in preeclampsia cases. Further, staining of consecutive sections showed that CD56 and CD94 were co-localized to the same cell. The increased number of $CD56^+$ cells in placenta from preeclampsia agrees with previous studies where increased amounts of $CD3^-CD56^+CD16^+$ cells also were observed in preeclampsia [277]. We could however not, detect any disease-related changes in the number of $CD16^+$ cells (data not shown).

CD94/NKG2A is expressed during pregnancy [221] as well as CD94/NKG2C [194]. These receptors can interact with HLA-E on EVTs during early pregnancy, suggesting a possible role in trophoblast regulation. Here, we were not able to investigate the NKG2 expression in placenta due to the absence of suitable NKG2 antibodies for IHC; hence the increased

expression of CD94 in preeclampsia could either depend on to much activation or too much inhibition [50]. Flow cytometric analysis reveals that both NKG2A and NKG2C are expressed on dNK cells in early healthy pregnancy [194, 205]. Also, there is an increased expression of NKG2C mRNA in early pregnant women compared to peripheral NK cells collected from non-pregnant subjects [205]. This indicates an increased activation during healthy pregnancy in first trimester. However, these studies were conducted in early pregnancy using isolated decidual cells, whereas we have studied dNK cells *ex vivo* in tissue during third trimester. The isolation of cells from tissue is always accompanied with a risk of an alteration receptor expression on the isolated cells.

Expression of the NK-cell activating cytokines IL-12 and IL-15, together with the anti-inflammatory cytokine IL-10, was also studied in third trimester placentae from healthy and preeclampsia pregnancies. We found significantly decreased expression of IL-12 in villous trophoblasts from women suffering from preeclampsia, whereas in healthy placentae a strong staining of IL-12 was seen both in nucleus and cytoplasm. Our results agree with previous studies where decreased IL-12 production from stimulated decidual cells during preeclampsia was reported [277]. IL-12 can influence NK cells and their production of IFN-γ [69, 70, 278]. The low IL-12 expression in preeclampsia observed in our study could result in a suboptimal IFN-γ production, which is unable to properly support placentation. However, it is important to remember, that placentation takes place early during pregnancy and our results reflect the situation in the third trimester and at term.

IL-15 and IL-10 expression was also studied in relation to preeclampsia, both cytokines have been reported to be present in placenta [78, 228]. No significant differences regarding IL-10 and IL-15 expression were observed between the two groups, but there was a slight decrease of IL-15 and IL-10 expression in villous trophoblasts from the preeclampsia cases, in line with previous studies [93, 224].

The levels of IL-10, IL-12/IL-23 p40 and IL-15 were also studied in sera together with IL-18. Notably, IL-12/IL-23 p40 and IL-15 showed a reversed pattern in circulation when compared to their placental expression. Significantly higher IL-12/IL-23 p40 concentrations were detected in sera of the preeclamptic group, and higher concentrations of IL-15 could be observed in severe cases of preeclampsia when compared to healthy women. Gestational age and mode of delivery showed no correlation with IL-12/IL-23 p40 and IL-15 in serum from

these women. These results could indicate altered NK-cell functions in the circulation of women with preeclampsia or/and reflect the escalated inflammatory response. The escalated inflammatory response could depend on the higher load of cell debris found in preeclamptic women [44, 47] or could be involved in the vascular changes observed in these women [279]. However, we measured the IL-12/IL-23 p40 subunit, which unables us to separate IL-12 from IL-23. Previous reports, show no consensus with regard to IL-12 in preeclampsia [280-282]. In most of these studies IL-12 were measured following *in vitro* cultures and not directly in serum, which makes a direct comparison with our results difficult.

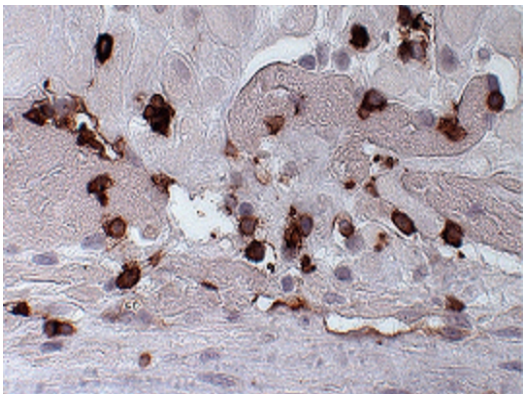
Previous studies regarding IL-18 in serum from women suffering from preeclampsia are contradictory [283, 284]. We could not detect any differences in IL-18 serum levels between healthy and sick women. However, IL-18 is suggested to be elevated during labour [105], which we also could confirm in our study. Higher levels of IL-18 were found in women undergoing VD.

We observed no differences in IL-10 concentrations in serum from healthy and preeclamptic women. This result could indicate that other anti-inflammatory factors are involved in pregnancy to dampen inflammatory responses [285].

Recent unpublished data from our group confirm and extend the findings in Paper I. IHC was performed with another anti-CD56 antibody using paraffin embedded formalin-fixed tissue from placentae (n=32) not included in Paper I. Again, we observed increased numbers of $CD56^+$ cells in preeclamptic third trimester placentae.

Interactions between HLA-G and HLA-E, the ligands for KIR, ILT2 and NKG2 receptors, and dNK cells are suggested to regulate trophoblast invasion [218, 7]. Decreased numbers of HLA-G- expressing cells in preeclamptic cases have previously been reported by others [101, 233]. Therefore, we further stained for HLA-G and HLA-E. No differences were found in HLA-G^+ or HLA-E^+ cell numbers between the two groups. We further studied if the dNK cells and the HLA-G expressing cells were differently located in healthy and sick placentae. This was done by double staining for CD56 and HLA-G. CD56 and HLA-E double staining could not be performed due to different antigen retrieval protocols for the antibodies in question. The HLA-G^+ cells were found in the region of decidua close to the intervillous space, while most $CD56^+$ cells were found in the basal layers of decidua (fig. 5 a, b and c) and just a few dNK

cells and HLA-G expressing cells were in close proximity (fig. 6). We could not see any differences in the locations or contacts between these two cell types in placentae from the two groups of women.



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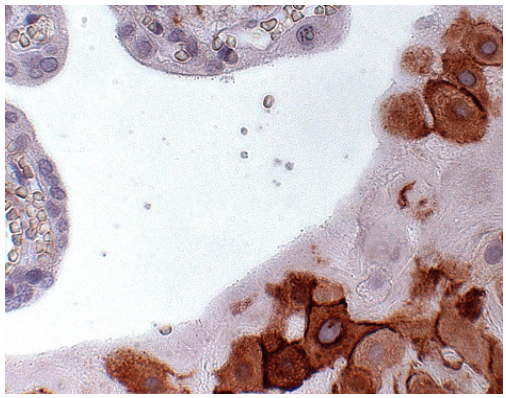

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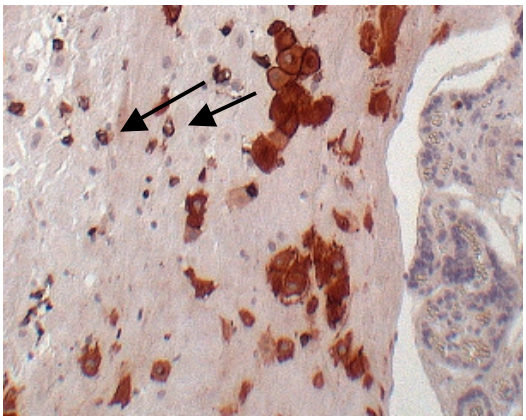

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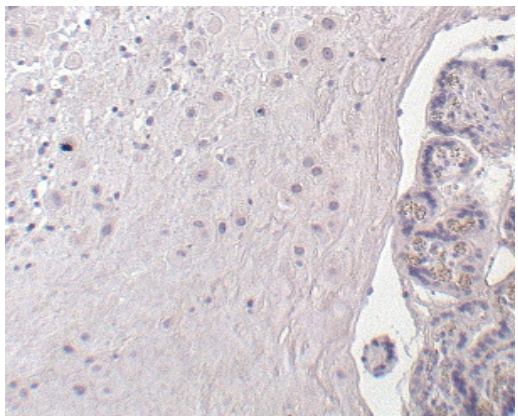

5d.

Figure 5a. CD56$^+$ (grey-black stained cells) and **(b)** HLA-G$^+$ (brown stained cells) cells found at different locations in a healthy third trimester placenta. The CD56$^+$ cells (**a)** are found in the basal layers of the decidua, while the HLA-G$^+$ cells (**b)** are found close to the intra villous space. Fig **c** showing the different locations of CD56$^+$ (grey-black stained cells) and HLA-G$^+$ (brown stained cells) in a healthy third trimester placenta in a smaller magnitude (the CD56$^+$ cells are pointed out with arrows). Negative, isotype matched control **(d)**, in a consecutive section to **c.** Similar location patterns of CD56$^+$ and HLA-G$^+$ cells were found in preeclamptic placentae.

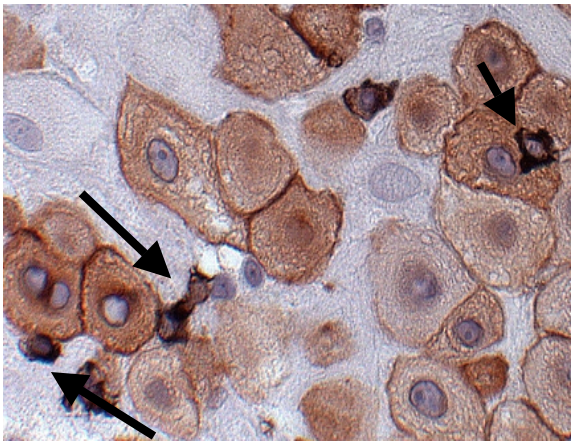

Figure 6. $CD56^+$ and HLA-G^+ cells in a healthy third trimester decidua. Few $CD56^+$ cells (stained grey-black, pointed out with arrows) were located in close contact with the HLA-G^+ cells (stained brown) in decidua. This was also found in preeclamptic placentae.

Early in pregnancy, when the placentation is ongoing, the regulation of EVT invasive activity is important. At this time, the dNK cells are abundant in the decidua and in close vicinity with the HLA-G expressing EVTs, suggesting a regulatory role for the dNK cells. As pregnancy proceeds, the decidua shrinks and is more or less like a thin membrane at term. The invasive capacity of the EVTs is reduced or absent, and perhaps the need for a close contact between regulatory cells like dNK cells is less important.

Paper II

In the second paper we studied HMGB1 and its receptor expression in third trimester placentae from healthy and preeclamptic women. Labour is known to be an inflammatory like process and differences in HMGB1 expression were also studied between VD and ECS. This was done using the IHC method. To verify the protein expression in SCT and decidua, HMGB1 mRNA was measured by *in situ* hybridization. We found distinct staining of HMGB1, both in the nucleus and cytoplasm of SCTs in the region of chorionic villi and EVTs in the decidua. To exclude that the cytoplasmic staining was due to differences in handling of the material *ex vivo*, we performed a kinetic study. No correlation between the staining result and the time span from delivery to fixation of the tissue could be observed. Further, the results from the *in situ* hybridisation experiments correlated well with results from IHC. We could not detect any differences with regard to mode of delivery but a tendency towards a higher cytoplasmic expression of HMGB1 in preeclamptic decidua could be noted. The receptors, RAGE, TLR2 and TLR4 were strongly expressed in trophoblast cells in both preeclamptic and healthy placentae, with no obvious differences between the two groups of women.

The presence of its suggested receptors RAGE, TLR2 and TLR4, indicates that the placenta can be a target for its own HMGB1 production. The tendency towards a higher cytoplasmatic expression of HMGB1 in the decidua from preeclamptic placentae is intriguing. An excessive release of HMGB1 in the placenta could have hazardous consequences, by triggering an exaggerated inflammatory response causing EVT apoptosis and hampered placentation. But HMGB1 has also been identified to be involved in angiogenesis, to initiate endothelial growth and endothelial cell migration [247, 269]. All of these processes also occur early in pregnancy, during placenta formation. An increased cytoplasmic expression in the placenta in preeclampsia could reflect a compensatory mechanism, to handle the reduction in placental blood supply.

In addition, HMGB1 has the ability to influence other immune cells and their cytokine production. It is proposed that HLA-G expressed on the EVT cells, can interact with both dNK cells and decidual macrophages mediating production of both cytokines and angiogeneic factors [232]. It is further shown that HMGB1 together with IL-12 can influence IFN-γ production by macrophage-activated NK cells [270]. As mentioned previously, IFN-γ is suggested to be involved in trophoblast invasion during early pregnancy [69, 229, 278].

We show increased levels of HMGB1 and have previously reported low levels of IL-12 in preeclamptic placentae. Thus, it seems utterly important that there is a fine balance between cytokines to achieve a succesful implantation. Maybe an altered HMGB1 production together with reduced IL-12 production (and perhaps also reduced placental IL-15) affect NK cell function, angiogenesis and subsequently also placentation and therebye contribute to development of preeclampsia.

Paper III

In the third paper we studied the circulating NK cells in women with preeclampsia, as we previously found increased numbers of phenotypically altered decidual NK cells in these women. Now we were interested in investigating if these alterations were also true in the periphery. Also we had noticed elevated levels of IL-12/IL-23 p40 and IL-15 in serum from preeclamptic women, and these cytokines, or monokines, could have the ability to influence NK cell phenotype and function. First, we investigated the effects of these cytokines *in vitro*, on peripheral NK-cell numbers, subsets, inhibiting receptor CD94/NKG2A and the activating receptors CD94/NKG2C and CD69 as well as IFN-γ production. PBMCs from healthy blood

donors were incubated with these cytokines, alone or in combinations. The IFN-γ production was measured in supernatants of cultures stimulated with the same cytokines.

The total number of NK cells from blood donors was significantly increased when stimulated with IL-15. IL-15 is a cytokine important for NK-cell development and proliferation [187, 286]. We observed an increase both in $CD56^{bright}$ and $CD56^{dim}$ subsets. $CD56^{bright}$ cells obtained from purified NK cell are known to proliferate in the presence of IL-15 [187, 286]. Our observation that also $CD56^{dim}$ cells seemed to expand following IL-15 stimulation, can be explained by the fact that the $CD56^{dim}$ subset is reported to need more signals than $CD56^{bright}$ cells to be able to proliferate properly. This need might not be sustained in purified NK cell cultures [287] but in cultures of whole PBMC. We also found an upregulation in percentage and levels of $NKG2A^{+}$ NK cells in contrary to NKG2C-levels that were down regulated after cytokine addition. The activating receptor CD69 was increased when stimulated with both IL-12 and IL-15, in line with previous results from others [166]. As NKG2A can inhibit the intracellular signalling pathway of CD69 and interfere with CD69 initiated cytotoxicity [168], the upregulation of NKG2A could indicate an inhibition of CD69 mediated cytotoxicity. IFN-γ production was significantly enhanced after all cytokine stimuli, except for IL-23. IL-12 is a well known enhancer for IFN-γ production of NK cells, but it has been found that IL-12 in combination with IL-15 give an even higher IFN-γ production [123, 187, 188].

As the investigated monokines had a marked influence on the NK cells in PBMC cultures and their IFN-γ production *in vitro*, we examined if the same alterations were detected *ex vivo.* Unstimulated NK cells from healthy and preeclamptic women were analysed by FACS and IFN-γ was measured in sera. IL-10 and sHLA-G were also measured in sera from these women. These two factors are suggested to have important regulatory roles and to create tolerance during pregnancy. We found that women with preeclampsia had normal numbers of peripheral NK cells, an ordinary NK cell-subset distribution and similar NK-cell receptor expression, with regard to CD69, NKG2A, NKG2C, NKG2D and CD94. Additionally, IFN-γ, IL-10 and sHLA-G measured in sera from the two pregnancy groups did not differ.

Little is reported regarding these NK-cell receptors during third trimester pregnancies. NKG2D is reported to be down regulated on lymphocytes from pregnant women, although no down-regulation was selectively found on NK cells [217]. Apparently, the skewed pro-

inflammatory profile in preeclamptic circulation does not affect the activating/inhibitory balance of NK cells regarding the receptors investigated in this study. However, there are indications of altered NK- cell receptor expression in preeclampsia, as reported by Hiby *et al*, where they showed that mothers lacking several activating KIR receptors exhibit a higher risk of preeclampsia [50].

We found similar serum IFN-γ concentrations in healthy and preeclamptic pregnancies although women with preeclampsia have enhanced IL-12 and IL-15 production. The fact that we only measured IL-12/IL-23 p40 could partially explain this finding, although other studies suggest both enhanced [281, 288] and reduced/unaltered [48, 289] IL-12 production in preeclampsia.

The surprisingly normal NK-cell population and function found in women with preeclampsia, suggests potent regulatory mechanisms, counter-acting the inflammatory response in these women. IL-10 has been proposed to be important during pregnancy, with its immunosuppressive role and ability to provide a favourable milieu during pregnancy [61, 63]. IL-10 is suggested to be reduced in preeclamptic pregnancies [93], but these findings are controversial [289, 290]. We reported similar levels of IL-10 in sera from healthy and preeclamptic women, which implicate a physiological regulatory immune function in these women, maybe controlling the NK cell population. Healthy pregnant women and women with preeclampsia also had similar circulating sHLA-G levels [98]. *In vitro* culture of NK cells with sHLA-G results in inhibition of NK cell mediated lysis [291]. Hence, NK cell activity could be under the control of sHLA-G in both healthy and preeclamptic pregnancies. Apparently, NK cell alterations in preeclampsia are restricted to the intra/uterine compartment.

But unfortunately, a very limited amount of serum precluded a complete analysis of IL-12 p40 and IL-15 of the women included in this particular study. Therefore, we cannot exclude that the normal NK cell features and IFN-γ levels in preeclampsia described here are due to normal serum levels of IL-12p40 and IL-15 in these particular women. However, measuring IL-15 in a sub-group of the women included here still indicates elevated levels in the preeclampsia group (data not shown).

Study IV (preliminary data)

Results

Cord blood levels of pro-inflammatory cytokines are influenced by preeclampsia - significantly decreased levels of TNF-α together with an increase in IL-8, in neonates born from preeclamptic mothers

IL-12p70, TNF-α, IL-1β, IL-6, IL-8 and IL-10 were measured in sera from cord blood. There were significantly decreased IL-12p70 ($p=0.003$, fig. 7a) and TNF-α ($p=0.001$, fig. 7b) levels in sera from neonates born from preeclamptic mothers. Further, there were increased levels of IL-8 in sera from neonates born from preeclamptic mothers ($p=0.047$) (fig. 7c). Levels of IL-1β, IL-6, and IL-10 did not differ between the two groups of newborn children.

IL-12p70, TNF-α and IL-8 can be elevated in neonates due to labour, therefore all children, born from healthy and sick mothers, were grouped together and divided into VD and ECS. Significantly higher levels of IL-6 ($p=0.02$) and IL-8 ($p=0.004$) were found in the VD group. There was a significant difference between gestational age, being higher in the VD group ($p=0.0006$, median VD = 40 weeks, median ECS = 38 weeks) and one neonate did not reach full term in the ECS group.

To exclude the influence of labour, neonates from healthy and preeclamptic mothers delivered with ECS were further investigated. TNF-α still showed significantly higher levels in neonates from healthy mothers ($p=0.03$, fig. 8a) and IL-8 was higher in the preeclamptic group ($p=0.03$, fig. 8b). The gestational age was significantly lower in the preeclamptic group ($p=0.02$) (healthy group, median=38 weeks and preeclamptic group, median=37 weeks), one neonate born from a preeclamptic mother was not delivered by full term.

Preeclampsia influences placental morphology

To investigate whether placental morphological changes could be associated with preeclampsia severity, placental morphology was studied in placental biopsies stained with haematoxylin – eosin. The morphology in the preeclamptic placentae, showed an altered and

disturbed SCT layer and malformed villi in both moderate and severe cases of preeclampsia. This was not seen in the healthy pregnancies (fig. 9 a and b).

Is preeclampsia associated with an abnormal maternal-foetal communication? – There is a significant correlation between maternal and cord blood IL-6 serum levels in preeclampsia

To see if we could observe an altered placental barrier function in preeclampsia, we investigated the placental localisation of IgG (neonates from healthy mothers n=8, neonates from preeclamptic mothers n=8). IgG could readily be detected at the trophoblast outer layer, in the villous stroma and in foetal vessel endothelium, with no apparent differences between healthy and preeclamptic placentae.

To further investigate placental function in preeclampsia, correlation studies were performed between children and their mothers regarding all cytokines. A significant correlation for IL-6 levels between preeclamptic mothers and their neonates was observed ($r= 0.83$ $p=0.042$). This was not seen in the healthy control group.

No significant differences in NK-cell numbers, NK-cell subsets or NK-cell receptors in children from preeclamptic mothers

We also analysed the NK-cell population as well as the proportion of $CD56^{bright}$ cells in children with healthy and preeclamptic mothers. Further, comparisons were made between the two groups regarding the $NKG2A^{+}$ and $NKG2C^{+}$ NK cells. No differences were found between the neonates regarding any of these parameters.

Due to shortage of cells, few children were included and phenotyped for the activating receptors NKG2D, CD69, NKp30 and NKp46 (neonates born from preeclamptic n=5 and healthy n=3 mothers). No significant differences were seen when comparing either numbers or levels of NKG2D expression on NK cells, but there was a trend towards a higher level of $NKG2D^{+}$ NK cells in neonates born from healthy mothers (median=100.5, range 99-142.2) when compared to the preeclamptic group (median=60.2, range 24.8-104.6) ($p= 0.4$) (fig. 10). A continuation of these studies with new material supports these findings.

To study if mode of delivery could influence NK cell numbers and receptor expression all neonates were divided into VD and ECS groups. An increased number of NK cells were

detected in VD (p=0.03). There was a significant difference in gestational age (p=0.012), being shorter in ECS pregnancies.

Soluble HLA-G levels are similar in healthy and preeclamptic children

sHLA-G was measured in sera obtained from cord blood from children with healthy mothers (n=15) and preeclamptic mothers (n=14). Overall there were low levels of sHLA-G in both groups and no differences were found (healthy cord sera, median=12.14 U/mL and preeclamptic cord sera, median=13.89 U/mL).

No detectable levels of intracellular IFN-γ and IL-4 production of NK cells in neither healthy nor preeclamptic children

Intracellular production/expression of IFN-γ and IL-4 was studied in NK cells following stimulation with IL-15 + PGN. There was a very low production of both IFN-γ and IL-4 in both groups of children. Further, IFN-γ production of IL-15 + PGN stimulated CBMCs measured in supernatants after 24 hours showed an IFN-γ production below the levels of detection in most cases.

Discussion

Preeclamptic mothers are known to have a higher inflammatory response in their circulation than healthy pregnant mothers, including elevated serum levels of IL-6, IL-8, IL-12 and TNF-α [48, 281, 292, 293].

In this study we investigated whether the escalated inflammation during preeclamptic pregnancies could influence placental morphology and possibly also function as well as the immunological profile in the neonates. The following parameters were evaluated: serum levels of pro- and anti-inflammatory cytokines and sHLA-G, NK cell numbers and receptor expression and NK cell IFN-γ production. We found a significant correlation between maternal and foetal serum IL-6 levels. Further, there was a significant decrease in IL-12p70 and TNF-α levels in preeclamptic children compared to controls, while IL-8 levels were increased in preeclamptic cord blood.

Oxygen and nutrients can readily cross the placental barrier, while it is still debated if cytokines can pass. We found a strong correlation between IL-6 measured in sera from

preeclamptic mothers and their neonates, indicative of a transfer over the maternal-foetal interface. Data supporting a low-level transfer of IL-6 in placental perfusion models have been reported [15], while others fail to find such a transfer [14]. Despite the contradictory results regarding IL-6 passage in healthy placentae, it is not known how a placenta with altered morphology, as seen in preeclampsia, deals with maternal cytokines. It is known that antibodies, both IgG and IgA, (and perhaps also IgE) can cross the placental barrier during healthy pregnancies [8, 10]. The transfer of IgG and IgA, together with IgM, is further pronounced during inflammatory conditions [12]. We found similar localisations of placental IgG between the two groups. This could be explained by the low number of severe preeclamptic cases included in the study where the inflammatory response often is more exaggerated than in moderate cases of preeclampsia.

IL-8 levels were significantly increased in foetal serum from preeclamptic pregnancies. IL-8 production can be influenced by labour and therefore we subdivided all children into mode of delivery. Not surprisingly, IL-6 and IL-8 levels were elevated in VD children. These cytokines are produced in higher levels during labour [103]. IL-6 induces oxytocin, an important hormone believed to induce labour work [294]. IL-8 can also be further escalated during stressful deliveries like acute ECS and VD with assistance [295]. Also, IL-8 production in cord blood is higher in preterm ECS associated with infections [19].

Preeclamptic pregnancies could be associated with a higher stress during delivery. Also, early bacterial and viral infections are suggested to be risk factors for preeclampsia. To investigate if the increased IL-8 levels in preeclamptic cord sera were influenced not only by mode of delivery, we further divided healthy neonates and preeclamptic neonates delivered by ECS, which still showed increased IL-8 in the preeclamptic group. The higher IL-8 levels in preeclamptic foetal serum could be due to an overall higher load of maternal stress in preeclamptic pregnancies. However, it could also be due to an infection but no such data was available from the women included in our study.

According to the literature, the foetal/neonatal immune system is skewed towards a type 2-profile in neonates from healthy pregnancies, together with an impaired APC production of pro-inflammatory cytokines [17, 18, 21, 26, 27]. Defective intracellular events have been suggested for the low IL-12p70 and TNF-α production found in neonates [17]. These defects appear to be restored within the first days of life [27].

However, we found levels of IL-12p70 and TNF-α in foetal blood from healthy pregnancies that compare to adult levels, while children born from preeclamptic mothers had significantly lower levels of these cytokines compared to neonates with healthy mothers. It is important to remember that most studies describing an impairment of pro-inflammatory activities of APCs, are based on *in vitro* experiments with stimulated cells, while we have measured cytokine levels directly in the cord serum at delivery. Although the mode of delivery can influence levels of IL-12p70 and TNF-α in cord blood with higher levels in VD than in un-laboured ECS, this did not explain the differences between the two groups observed here [28, 103].

While impaired production of cytokines in APCs are found in early life, the TNF-α levels also seem to depend on the chosen stimuli. CBMC have been treated with various bacterial, viral components and mitogens, resulting in levels of IL-12p70 and TNF-α that are either lower or equal to adult levels [17, 19, 21, 23, 26]. Increased maternal antibody levels against bacteria and virus are found in preeclampsia [40]. How or if these pathogens/maternal responses affect foetal production of cytokines is not known.

Maternal preeclampsia had no effect on cord blood NK-cell numbers and NK-cell subset distribution, but as reported previously [29] the mode of delivery influenced NK-cell numbers, with a significant increase in cord blood NK cells in VD. Preliminary data, showed a trend towards reduced levels of NKG2D on NK cells from children with preeclamptic mothers. NKG2D expression is influenced by cytokines [159, 160]. Purified NK cells treated with IL-12 upregulates the expression of NKG2D [296]. The reduced NKG2D expression on NK cells from neonates born from preeclamptic mothers could depend on the lower IL-12 levels found in their cord sera.

sHLA-G is suggested to inhibit NK cell mediated lysis [291] and to induce an allografic tolerance by T_{reg} cells [297]. Levels of sHLA-G secreted from the embryo are reported to correlate with successful *in vitro* fertilizations (IVF) compared to unsuccessful IVF [97]. Similar levels of sHLA-G measured in foetal serum from healthy and preeclamptic pregnancies were found. The same was true for sHLA-G in maternal serum from the two groups, and could reflect a mechanism in which tolerance and NK cell regulation is preserved in both healthy and preeclamptic pregnancies.

The production of IFN-γ and IL-4 in CBMCs following *in vitro* stimulation of PBMCs with PGN + IL-15, was very low, which is in line with earlier studies [21, 27, 30, 298]. No differences between the two groups could be observed. The low IFN-γ production is partly explained by impaired intracellular events in newborns [17], but also on different stimulation requirements for adult and neonatal PBMCs *in vitro*. However, our results are preliminary and need to be confirmed before any firm conclusions can be drawn.

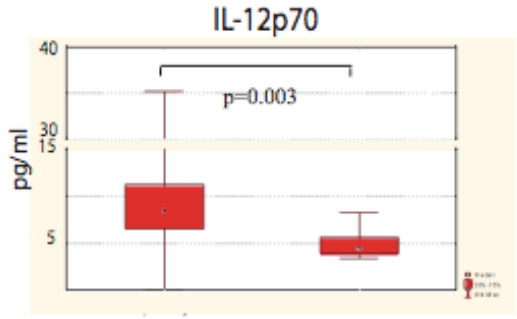

Figure 7 a

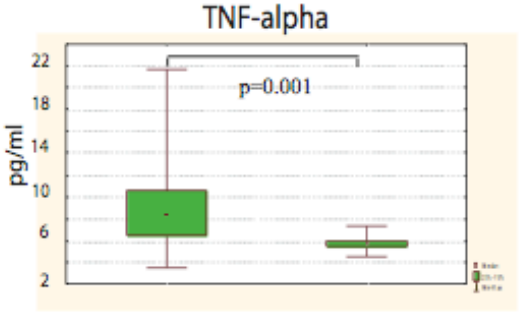

Figure 7 b

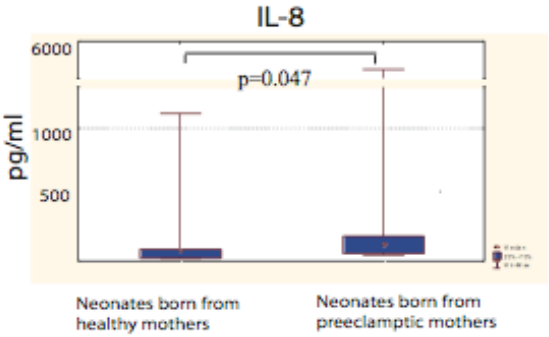

Figure 7 c

Figure 7. Levels of IL-12p70 (a), TNF-alpha (b) and IL-8 (c) in cord sera from neonates born from healthy (n=16) and preeclamptic (n=10) mothers.

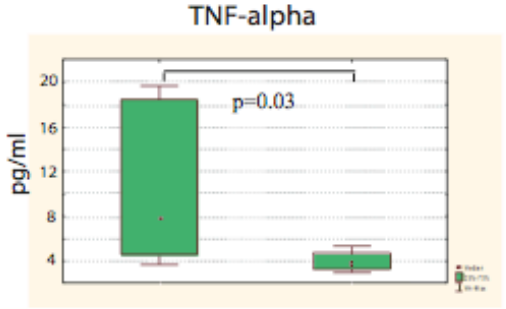

Figure. 8 a

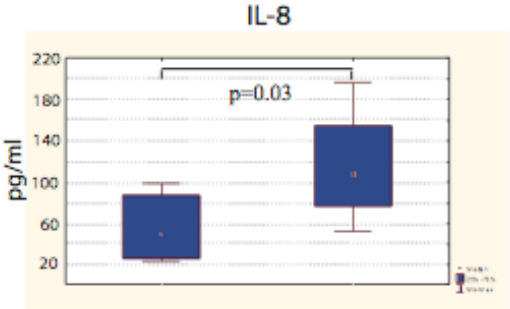

Figure. 8 b

Figure 8. Levels of TNF-alpha (a) and IL-8 (b) measured in cord sera from neonates delivered by ECS born from healthy mothers (n=11) and from preeclamptic mothers (n=4).

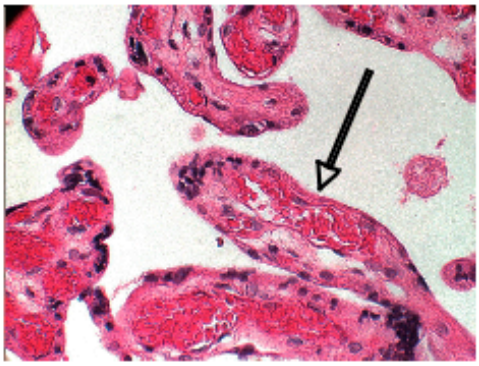

Figure 9 a

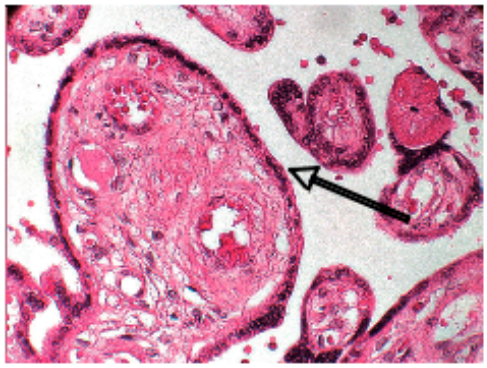

Figure 9 b

Figure 9. Disturbed morphology in third trimester preeclamptic placenta with altered SCT layer (arrow) (a) and abnormal shaped villi (a) compared to the even SCT layer (arrow) and round-shaped villi in healthy placenta (b).

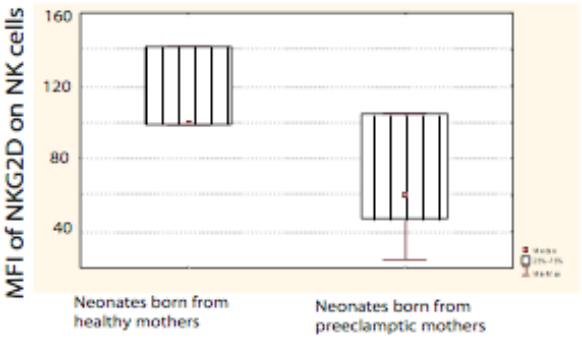

Figure 10

Figure 10. Levels of NKG2D on NK cells analyzed *ex vivo* in cord blood collected from healthy pregnancies (n=3) compared to NK cells in cord blood from preeclamptic pregnancies (n=5).

CONCLUDING REMARKS

Preeclampsia is a complicated pregnancy related disorder with many immunological features. In my studies, I have focused on NK cells and cytokines produced by or activating these cells. Apart from immune cells producing cytokines that are present at the maternal-foetal interface, there are also stromal cells, fibroblasts and trophoblast cells contributing to the cytokine production. A correct interaction between all the cytokine producing cells is a delicate balance and it is important that each cytokine is produced at the right concentrations at the moment when they are needed. Based on my findings, I speculate that the diminished IL-12 expression, perhaps together with an increased HMGB1 expression in preeclamptic placenta, could result in an altered dNK cell cytokine production, receptor expression and function.

HMGB1 is known to cause cell damage in rat liver, when released during ischemic conditions [299]. Ischemia is a hallmark of preeclampsia, and a higher degree of altered morphology could be noted in preeclampsia placentae with damaged villous trophoblast cells and enlarged foetal blood vessels (fig. 1 c, d in paper I and fig. 9 a, b in study IV in this thesis). This could partly be explained by the effects of a higher expression of HMGB1 in preeclamptic decidua. The receptors for HMGB1 are expressed in third trimester placenta and the engagement of HMGB1 to its receptors can result in IFN-γ [246], IL-8 [249, 256, 257] and IL-6 production [249] (fig. 11). If overproduced, these cytokines could be involved in placental damage, premature birth and adverse pregnancy outcomes.

How the immunological changes in preeclampsia influence the foetal immune system is not known. We found a correlation of IL-6 concentrations in maternal and cord sera from preeclamptic pregnancies. The increased HMGB1 could influence the production of IL-6, which partly could be the source of the transfer of IL-6 across the placental barrier and further influence the foetal cells (fig. 11).

We found higher expression of CD94 in preeclamptic placentae together with low IL-12 expression. Peripheral NK cell CD94/NKG2 receptor expression can be altered in disease [161, 162] and when treated with cytokines [159]. Little is known how/if the dNK-cell receptor levels are affected by the altered cytokine milieu in preeclamptic placentae. Maybe the lower IL-12

expression in placentae from preeclamptic pregnancies could result in altered CD94 expression (fig. 11). The dNK cells could play a central role during angiogenesis as these cells express receptors like CD94/NKG2, able to bind to HLA molecules on the invading trophoblasts in decidua during early pregnancy [194, 214]. If the altered receptor expression on dNK cells also exist during early pregnancy this might contribute to the poor placentation.

Interestingly, the cytokine pattern in the circulation was the opposite of that in the placental situation, with higher serum levels of IL-12/IL-23p40 and IL-15 in women with preeclampsia. This could reflect an overall escalated inflammatory condition, or be due to an inflammatory response towards the higher load of foetal cell debris found in the circulation from preeclamptic women [47]. Sargent *et al* have proposed an interesting theory about altered activation of APCs in circulation due to the excess of foetal debris in preeclampsia [46]. When monocytes and DCs encounter the debris, they are thought to influence the NK cells incorrectly and produce more pro-inflammatory cytokines [46]. Although increased levels of IL-12/IL-23p40 and IL-15 are seen in preeclampsia and the fact that these cytokines clearly could influence NK-cell number, receptor expression and IFN-γ production *in vitro*, the *ex vivo* peripheral NK-cell pool was remarkably unaffected in women with preeclampsia. There appears to be several mechanisms to keep the circulating NK cells dampened during preeclampsia.

From my studies, it seems as if preeclampsia primarily influences placental immunity with regard to NK-cell phenotype and function, while the maternal peripheral NK-cell pool is strikingly normal. However, the APC population seems affected both in the local placental environment as well as in the periphery, as monokine production was altered at both sites in preeclampsia, although in different ways. To further study the APC population, as well as the NK-APC collaboration in these patients would be interesting in the future.

From my preliminary data, it also seems as if the cord blood peripheral NK cell receptor NKG2D is influenced by preeclampsia. How the immune system of the newborn and of the infant is influenced by preeclampsia is largely unknown and studies are currently being performed in our laboratory to investigate this in more detail.

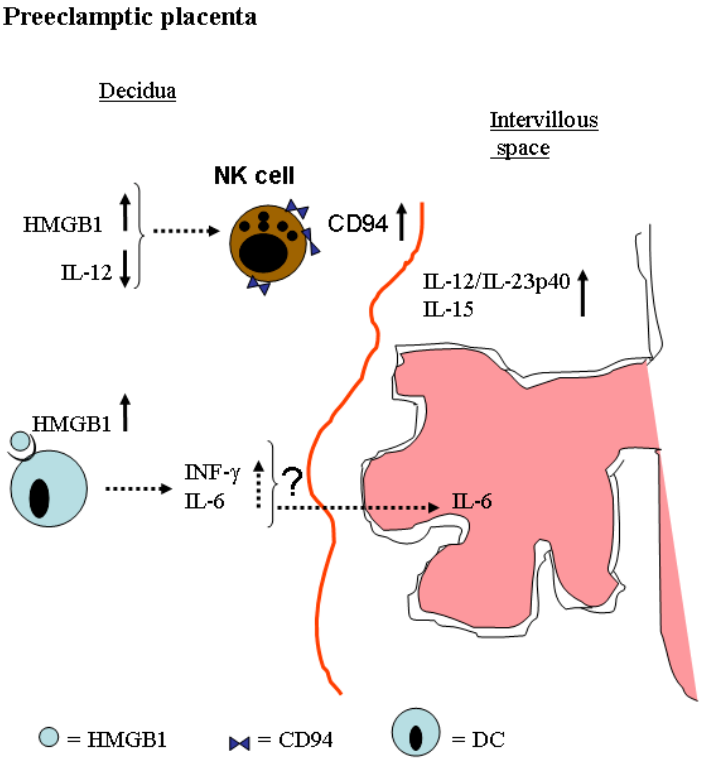

Figure 11. Preeclamptic placenta. The disturbed cytokine balance in decidua from preeclamptic pregnancies could have an effect on NK cell receptor expression (CD94) and cytokine production.

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