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Local immune regulation in human pregnancy

with focus on decidual macrophages

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"There are no facts, only interpretations."

Friedrich Nietzsche

ABSTRACT

During pregnancy, the woman carries a fetus partly foreign to her immune system, because of the expression of paternal antigens. Despite this, the fetus is normally tolerated and not rejected, as is often the case with organs in allogeneic transplantations. Systemic changes in maternal blood occur during pregnancy but, perhaps of greater importance, are changes in tissues locally in the uterus. The pregnant uterine endometrium, the decidua, is infiltrated by large numbers of leukocytes, mainly natural killer (NK) cells but also macrophages and T lymphocytes. Further, various cytokines are known to be secreted at the fetomaternal interface. However, the functions of these cells and the cytokine networks are not fully understood. The aim of this thesis was to investigate the local immune balance in normal human pregnancy decidua, both in the early phase of pregnancy and at parturition.

First trimester decidual mononuclear cells, NK cells and macrophages were all shown to secrete IFN-γ, IL-4 and IL-10, as detected by ELISPOT. The secretion was not mirrored in blood from the same subjects. A significantly larger number of decidual macrophages secreted IL-10 than did their blood counterparts, indicating potential regulatory functions of this cell type.

Further examination of early pregnancy decidual macrophages by microarray revealed 120 genes being differentially regulated at the transcriptional level in decidual compared to blood monocytes/macrophages. Several genes were associated with alternative activation/M2 polarization of macrophages, including CCL-18, CD209, IGF-1, MRC-1 and FN-1. Genes connected to immune regulation and tissue remodelling were common, in line with the potential functions for this cell type *in utero*. In addition, some molecules not previously connected to decidual macrophages, such as TREM-2, A2M and PGDS, were found to be up-regulated, gaining new insights into the regulatory functions of decidual macrophages.

Term decidual mononuclear cells spontaneously secrete IFN-γ, TNF, IL-4, IL-10, and TGF-β. No differences were seen between tissues obtained before and after the onset of labour, indicating that decidual mononuclear cells are not the main cell population responsible for plausible cytokine regulation in the process of labour induction. Placental and fetal membranes as well as cells in the maternal systemic circulation may instead contribute to a possible shift in immune balance prior to pregnancy termination.

In conclusion, decidual leukocytes, including NK cells and macrophages, are potential producers of both Th1-like/pro-inflammatory and Th2-like/anti-inflammatory cytokines in early pregnancy as well as at parturition. Decidual macrophages are of a specialized phenotype with effector functions contributing to a proper invasion of the placenta and to immunological protection of the semi-allogeneic fetus. This thesis adds new knowledge on local immune balance during normal human pregnancy, however, the clinical significance of the presented data needs to be clarified.

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SAMMANFATTNING

Immunförsvaret är programmerat att reagera på och stöta bort allt främmande som kommer in i kroppen. Därmed reagerar kroppen förutom på t.ex. bakterier och virus även på främmande vävnad såsom ett transplanterat organ. Avstötningsreaktionen är riktad mot främmande HLA-typer. Under graviditet bär kvinnan i sin kropp ett foster som är till hälften främmande för hennes immunförsvar, eftersom det bl.a. uttrycker HLA-molekyler från fadern. Trots detta stöts fostret inte bort vid normala graviditeter. Mycket talar för att en av skyddsmekanismerna för fostret är en omstyrning av moderns immunförsvar. Ett immunsvar kan grovt delas in i Th1- eller Th2-typ. Avgörande för vilket svar man får, är vilka cytokiner som utsöndras av celler och vävnader i den lokala miljön. Avstötningsreaktioner vid organtransplantationer är av Th1-typ och man tror att omstyrningen av immunförsvaret under normal graviditet sker från den aggressiva Th1-typen till den i detta sammanhang mer skonsamma Th2-typen. Dessa förändringar sker dels systemiskt, d.v.s. i blodet, men är mest genomgripande lokalt i livmodern, i deciduan (livmoderslemhinnan) och i placentan (moderkakan). Att immunförändringarna är mest lokaliserade till livmodern är bra med tanke på att modern måste ha kvar ett skydd mot infektioner under graviditeten. En alltför kraftig modulering av det systemiska immunförsvaret skulle därför kunna vara skadlig för både moder och foster.

För att undersöka den lokala utsöndringen av cytokiner vid tidig normal graviditet togs i arbete I deciduavävnad från inducerade voluntära aborter i första tredjedelen av graviditeten. Från decidua separerades mononukleära celler fram och cytokinutsöndringen undersöktes med ELISPOT; en känslig metod som upptäcker cytokinsekretion på enskild-cell nivå. Cytokinerna som undersöktes var Th1-cytokinet IFN-γ, Th2-cytokinet IL-4 och det framför allt anti-inflammatoriska cytokinet IL-10. Mononukleära celler från blod analyserades för jämförelse av den lokala och systemiska utsöndringen. Alla tre cytokinerna utsöndrades spontant i både decidua och blod men ingen korrelation mellan utsöndringen från de två vävnaderna kunde ses.

Fördelningen av immunceller i decidua skiljer sig markant från fördelningen i perifert blod. T.ex. står NK-celler under tidig graviditet för så mycket som 70-80% av leukocyterna i decidua, jämfört med bara 3,5% i blod. Även andelen makrofager är högre i decidua än i blod, medan andelen T- och B-celler är lägre. Alla dessa celltyper, liksom celler av icke-hematopoietiskt ursprung (s.k. stromaceller), är tänkbara producenter av immunmodulerande cytokiner. För att undersöka vilka celler som står för utsöndringen av IFN-γ, IL-4 och IL-10, separerades i arbete II olika celltyper fram ur de mononukleära cellerna från decidua. Detta gjordes med hjälp av en immunomagnetisk metod baserad på de olika celltypernas uttryck av ytmarkörer. De ytmarkörer som användes var CD45 (uttrycks på alla leukocyter och kan därmed skilja immunceller från stromaceller), CD56 (markör för NK-celler) och CD14 (markör för monocyter/makrofager). De olika cellpopulationernas cytokinproduktion analyserades med hjälp av ELISPOT. För att jämföra dessa celltypers aktivitet lokalt med den systemiska aktiviteten separerades celler från blod på samma sätt och undersöktes med ELISPOT. IFN-γ, IL-4 och IL-10 utsöndrades mer från NK-celler i decidua än från motsvarande celler i blod. Hos makrofager sågs en högre utsöndring av IL-10 i decidua jämfört med monocyter/makrofager i blod.

Makrofager är celler som normalt sett har kraftigt inflammatoriska effekter vid ett immunsvar. Man har dock på senare år funnit en alternativ typ av denna cell som istället har anti-inflammatoriska och nedreglerande effekter. Ett av kännetecknen för denna alternativa celltyp är en hög produktion av IL-10 jämfört med klassiska makrofager. Detta stämmer med resultaten från arbete II där makrofager från decidua i hög grad utsöndrade IL-10. Man har funnit att alternativa makrofager är vanliga i placenta från sen graviditet. I arbete III undersöktes fenotypen hos deciduala makrofager vid tidig graviditet. Celler från decidua och blod separerades fram som i arbete I och II samt med flödescytometribaserad cellsortering. Med microarray-analys kartlades uttrycket av 14 000 gener och en jämförelse mellan de två vävnaderna gjordes. Resultaten visar att makrofager i decidua i hög grad uttrycker gener som tidigare associerats med alternativ aktivering av makrofager. Uttryck av gener kopplade till immunreglering och vävnadsombildning var vanliga. Av resultaten framkom även ett antal potentiellt intressanta molekyler som inte tidigare har kopplats till makrofager i decidua, såsom TREM-2, A2M och PGDS.

För att undersöka den lokala cytokinmiljön vid slutet av graviditeten togs i arbete IV deciduavävnad från normala graviditeter vid kejsarsnitt. Förhållandena i livmodern innan en spontan förlossning påbörjats kunde därmed mätas. Även förändringarna som sker efter att graviditeten avslutats genom en naturlig förlossning undersöktes genom att analysera deciduavävnad efter vaginal förlossning. Celler preparerades fram som i arbete I och utsöndringen av IFN-γ, IL-4, IL-10, det anti-inflammatoriska cytokinet TGF-β samt det pro-inflammatoriska cytokinet TNF mättes med hjälp av ELISPOT. Alla fem cytokinerna utsöndrades spontant hos mononukleära celler från decidua och ingen skillnad kunde ses mellan vävnader tagna före respektive efter förlossningsarbete.

Sammanfattningsvis visar arbetena i avhandlingen att leukocyter i decidua, innefattande NK celler och makrofager, är potentiella producenter av både Th1/pro-inflammatoriska och Th2/anti-inflammatoriska cytokiner, vid tidig och sen graviditet. Makrofager i decidua har en speciell fenotyp med funktioner som underlättar en optimal invasion av placenta i endometriet samt bidrar med immunologiskt skydd mot avstötning av fostret.

ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by roman numerals I–IV:

I. **Ekerfelt C, Lidström* C, Matthiesen L, Berg G, Sharma S and Ernerudh J:** "Spontaneous secretion of interleukin-4, interleukin-10 and interferon-γ by first trimester decidual mononuclear cells". *American Journal of Reproductive Immunology* 2002;47:159-166.

II. **Lidström* C, Matthiesen L, Berg G, Sharma S, Ernerudh J, och Ekerfelt C:** "Cytokine secretion patterns of NK-cells and macrophages in early human pregnancy decidua and blood: implications for suppressor macrophages in decidua". *American Journal of Reproductive Immunology* 2003;50:444-452.

III. **Gustafsson C, Mjösberg J, Matussek A, Geffers R, Matthiesen L, Berg G, Sharma S, Buer J and Ernerudh J:** "Gene expression profiling of human decidual macrophages: Evidence for immunosuppressive phenotype". *Manuscript.*

IV. **Gustafsson C, Hummerdal P, Matthiesen L, Berg G, Ekerfelt C och Ernerudh J:** "Cytokine secretion in decidual mononuclear cells from term human pregnancy with or without labour: ELISPOT detection of IFN-γ, IL-4, IL-10, TGF-β and TNF-α". *Journal of Reproductive Immunology* 2006:71:41-56.

* The author's maiden name is Lidström

ABBREVIATIONS

A2M	alpha-2-macroglobulin
BSA	bovine serum albumin
C1Q	complement component 1 q subcomponent
CCL	chemokine (C-C motif) ligand
cDNA	complementary DNA
COX	cyclooxygenase
cRNA	complementary RNA
CRP	C reactive protein
CSF	colony stimulating factor
dNK	decidual NK
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FBS	fetal bovine serum
FN	fibronectin
Foxp3	forkhead box p3
GAS	growth arrest specific
GM-CSF	granulocyte-macrophage CSF
HBSS	Hank's balanced salt solution
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HPE	high performance ELISA
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IDO	indoleamine 2,3-dioxygenase
KIR	killer immunoglobulin-like receptors
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LT	lymphotoxin
M1	classically activated macrophages
M2	alternatively activated macrophages
MACS	magnetic cell sorting
mAb	monoclonal antibody
M-CSF	macrophage CSF
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MNC	mononuclear cell
MØ	macrophage
mPGES	microsomal prostaglandin E_2 synthase
MRC	mannose receptor C type
mRNA	messenger RNA
NK	natural killer
NKT	natural killer T
NO	nitric oxide
NRP	neuropilin
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	PBS Tween
PG	prostaglandin
PGDS	prostaglandin D_2 synthase
PGF	platelet growth factor
PHA	phythaemagglutinin
PMA	phorbol myristate acetate
PROS1	protein S alpha 1
PSG	pregnancy specific glycoprotein
RA	rheumatoid arthritis
ROS	reactive oxygen species
RPS9	ribosomal protein S9
RT	reverse transcriptase
SLC2A1	solute carrier family 2 member 1
SLE	systemic lupus erythematosus
SPP	secreted phosphoprotein
TAG	tumour associated glycoprotein
TAM	tumour associated macrophages
Tc	cytotoxic T lymphocyte
TCM	tissue culture medium
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
Treg	regulatory T lymphocyte
TREM	triggering receptor expressed on myeloid cells
VEGF	vascular endothelial growth factor

INTRODUCTION

GENERAL INTRODUCTION

The ability to distinguish between self and non-self is central to the immune system. A proper response against invaders such as viruses and bacteria is as important as a lack of response against one's own tissues, and requires close cooperation between many different cell types and regulatory systems. During pregnancy the woman carries a fetus partly foreign to her immune system because of the expression of paternal antigens. Despite this, the fetus is normally tolerated. Decades of research since Medawar first stated this immunological paradox (Medawar, 1953) have revealed many clues on the restoring functions but a good deal remains to be clarified.

BASIC IMMUNOLOGY

The immune system can be divided into innate and adaptive immunity (Abbas and Lichtman, 2005). The first line of defence against an infectious agent is provided by mucosal barriers, by innate cells such as phagocytic neutrophils and macrophages, natural killer (NK) cells and by complement factors in the blood. The innate immune system recognizes a limited number of evolutionary stable structures shared by many microbes, and the response does not change in case of re-infection by the same microbe. Adaptive immunity, on the other hand, is more specific with an enormous ability to recognize different antigens, and an infection generates immunological memory. This second line of defence consisting of T and B lymphocytes is more fine-tuned but is dependent on the initial response by innate components. When a microbe succeeds in passing an epithelial membrane and enters the tissue or circulation, it is internalized by neutrophils, macrophages and dendritic cells. Neutrophils and macrophages kill the phagocytosed enemy with the contents of toxic granules and by secreting cytokines, which are also important for regulating the proceeding immune response. Complement factors are able to kill microbes but they also opsonize to facilitate phagocytosis as well as B cell activation. NK cells recognize and kill infected cells and, like the other innate cells, secrete cytokines. Macrophages and dendritic cells digest the phagocytosed microbe and present these fragments to lymphocytes. The activated T and B lymphocytes proliferate and develop into effector cells secreting cytokines and antibodies, respectively. This clonal expansion is specific for the presented antigen and alongside, memory cells are created. Adaptive immune cells also regulate the functions of innate cells, for example they increase their phagocytic capacity by secreting cytokines. Hence innate and adaptive immunity work in close contact in both directions during an immune response.

CYTOKINES, TH1/TH2 PARADIGM

Cytokines are important effectors and regulators of immune responses. Cytokines are polypeptides, which regulate cellular function, growth and differentiation. These signal molecules can be divided into different patterns based on their functions, one common division being the T helper (Th) cell type 1/Th2 paradigm. This concept was first described 20 years ago by Mosmann *et al.* (1986). Murine T helper lymphocytes were found to be separable into two subsets, with distinct differences in cytokine production and the effector mechanisms mediated. Murine Th1 cells produced interleukin (IL)-2, interferon (IFN)-γ and IL-3 and deviated the immune system towards a cellular response against intracellular

microbes (Mosmann et al., 1986; Mosmann and Coffman, 1989). Th1 cells also stimulated phagocytosis of infected cells by triggering the production of antibodies effective on complement activation and opsonization. Th2 lymphocytes, in turn, produced IL-3 and IL-4 and mediated a humoral defence against extracellular antigens and inhibited macrophage functions. Human T cells show cytokine and effector patterns comparable to the murine results, although IL-2 is not as restricted to Th1 cells (Romagnani, 1995). Besides IFN-γ, lymphotoxin (LT) is the major human Th1 cytokine and in addition to IL-4, IL-5 and IL-9 also represent Th2 cytokines (Romagnani, 1995; Mosmann and Sad, 1996). The Th1/Th2 concept, used extensively by researchers for many years, has not very surprisingly been shown to be too simplistic, at least in humans. The two major antagonists IFN-γ and IL-4 however represent two distinct types of immune reactions, counterbalancing each other. The pattern can also be applied to other cytokine secreting cell types such as NK cells (Chan et al., 2001) and cytotoxic T lymphocytes (Tc cells (Mosmann and Sad, 1996)), and therefore, the concept is now referred to as Th1- and Th2-like reactions or immunity in this work. Importantly, cytokines have different effects in different contexts and some cytokines fit into several functional groups which might change depending on other factors. One way to divide cytokines is into pro- or anti-inflammatory molecules. Pro-inflammatory cytokines include those leading to the production of, for example, nitric oxide (NO), prostanoids and leukotrienes (reviewed by (Dinarello, 2000)), typically represented by tumour necrosis factor (TNF) and IL-1. IL-8 is a chemokine that leads to the recruitment of inflammatory cells and fits into the same group, as does the peliotropic IL-6 (Dinarello, 2000; Abbas and Lichtman, 2005). Anti-inflammatory cytokines like transforming growth factor (TGF)-β and IL-10 inhibit the functions of aggressive macrophages and other immune cells. IL-4 and IL-13 are generally connected with Th2-like immunity, and thus lead to Th2-mediated inflammation such as allergic inflammation, but in other contexts, such as rejection and tolerance in pregnancy, they could be regarded as anti-inflammatory. All four cytokines suppress the expression of TNF, IL-1 and IL-8 and also down-regulate vascular adhesion molecules.

MACROPHAGE POLARIZATION

Macrophages are important components of innate immunity due to their ability to present digested antigens (although they are far from as effective in activating naïve T cells as dendritic cells), and by secreting cytokines priming the forthcoming immune response. Monocytes, the precursor of macrophages, are able to take different routes of activation in response to different microenvironmental signals. Classically, macrophages are activated by agents such as IFN-γ, TNF or bacterial lipopolysaccharide (LPS), thereby enhancing their antigen presenting capacity as well as effector functions such as the production of toxic intermediates (NO, reactive oxygen species (ROS)) and pro-inflammatory and Th1 inducing cytokines (Abbas and Lichtman, 2005). The Th2-related cytokines IL-4 and IL-13 were considered suppressors of macrophage activation at first. When it was realised that this was not just a deactivation of the cell, but instead an activation program of its own, the term alternatively activated macrophages was suggested (Stein et al., 1992; Goerdt and Orfanos, 1999; Gordon, 2003). Subsequently, monocytes activated in ways other than in the presence of IL-4/IL-13 were found to differ from classically activated macrophages, thus fitting the alternative mode of activation. Mantovani *et al.* (2004) suggested a nomenclature where M1 represents classically activated macrophages and M2 represents macrophages activated by different non-classical stimuli. Typically, M1 macrophages are present in Th1-like immune

responses, inflammation and the killing of intracellular pathogens, whereas M2 macrophages are more connected to Th2-like immune responses, tissue remodelling and are important immune regulators. No clear-cut or lineage-defined subsets of monocytes/macrophages have been identified; rather the different types should be seen as extremes of a continuum, reflecting environmental priming (Mantovani et al., 2004; Martinez et al., 2006). It has also been suggested that the functional differentiation of macrophages is reversible and an old pattern may be restored when the environment changes (Stout and Suttles, 2004; Porcheray et al., 2005; Gratchev et al., 2006).

PREGNANCY ANATOMY

Proper development of the placenta is crucial for normal pregnancy. Its complex architecture ensures the nutritional supply for the fetus as well as transport of gases, waste products and hormones (Loke and King, 1995; Moore and Persaud, 2003). About a week after fertilization, the conceptus, at this stage called a blastocyst, is completely embedded into the uterine wall. The exterior of the blastocyst consists of two layers, the inner cytotrophoblast layer consisting of cells that form the outer syncytiotrophoblast cell layer by fusion. At the end of the second week after fertilization, the two trophoblast layers form chorionic villi which are the first stage of placental development (Figure 1). The chorionic villi invade the uterine endometrium, which is already prepared for implantation during the luteal phase of the menstrual cycle and during pregnancy is called the decidua (L. *deciduus*, a falling off). The two trophoblast layers also form the chorionic sac, surrounding the fetus. At the implantation site the villous chorion remains and develops, while it is degenerated around the cavity, forming the smooth chorion. The maternal decidua can be divided into three parts; decidua basalis at the implantation site, decidua capsularis surrounding the smooth chorion and the amniotic sac, and decidua parietalis lining the uterine wall. As the fetus and the amniotic sac enlarge, the decidua capsularis and decidua parietalis eventually fuse and is then called decidua parietalis. The villous chorion consists of stem villi dividing into branch villi and anchor villi that attach the chorionic sac to the decidua (Figure 2). The decidua, in turn, grows as placental septa towards the chorionic plate when the placental development proceeds, forming the placental cotyledons. In between the villi in the cotyledons is the intervillous space where the fetal villi are bathed by maternal blood. Spiral endometrial arteries from the decidua basalis grow through the cytotrophoblastic shell of the cotyledons into the intervillous space. The epithelium of the arteries is replaced by migrating extravillous trophoblasts; this together with the degeneration of vascular smooth muscles enhances the blood flow. Altogether, the placental barrier between the fetal and the maternal circulation is made up of four layers (hemochorial placenta): the endothelium of fetal capillaries, the connective tissue of villus, the cytotrophoblast and the syncytiotrophoblast.

GENERAL PREGNANCY IMMUNOLOGY

Medawar (1953) proposed three mechanisms that might explain the maternal tolerance of the fetus. Two of these explanations, antigenic immaturity of the fetus and physical separation between the fetus and the mother, have proven not to be fully correct. The fetus itself is not in direct contact with the mother's immune system but the fetally derived trophoblasts are. Fetal tissues indeed express and present antigenic molecules to the maternal immune system and fetal cells can be detected in the maternal circulation (reviewed by (Sargent et al., 2003)),

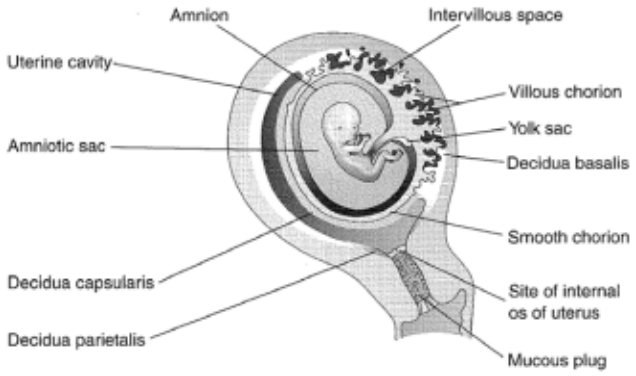

Figure 1. Picture of the pregnant uterus showing decidua, placenta and fetal membranes. Picture from The Developing Human 7th ed, Moore KL and Persaud TVN, Saunders Philadelphia, USA, 2003. © Elsevier, published with permission.

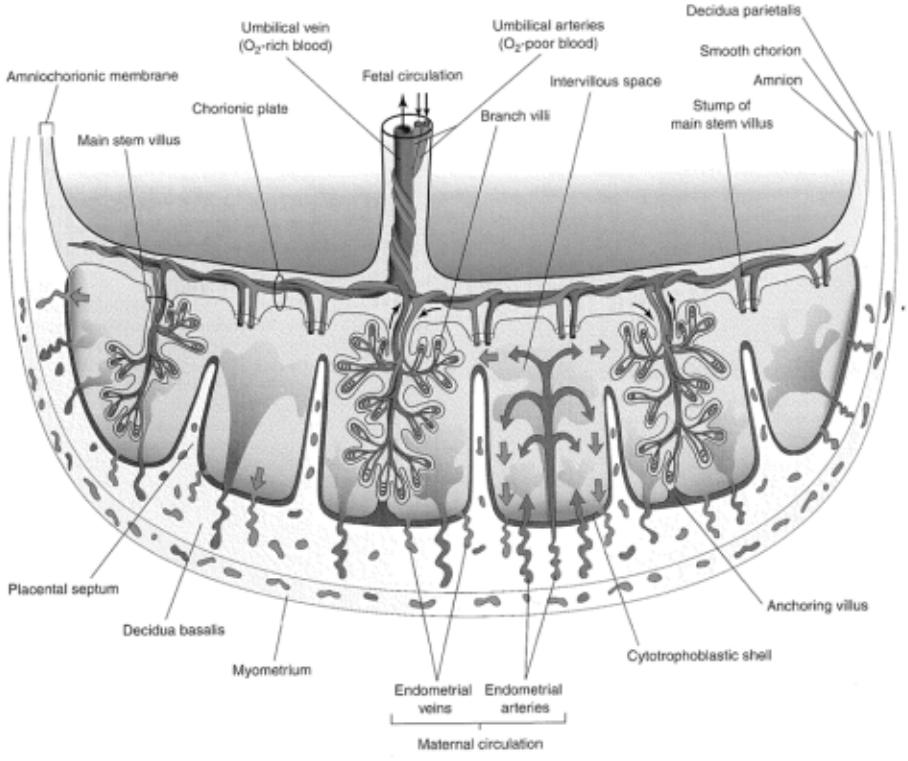

Figure 2. Schematic drawing of a full-term placenta, showing the relation of the villous chorion to the decidua basalis. Picture from The Developing Human 7th ed, Moore KL and Persaud TVN, Saunders Philadelphia, USA, 2003. © Elsevier, published with permission.

indicating a closer contact than was first believed. The focus of most reproductive immunology research has therefore been based on Medawar's third hypothesis, deviation or suppression of the maternal immune system during pregnancy. As well as regarding the placenta as a fetal allograft that ought to be protected from the maternal immune system, it must also be viewed as an invasive tumour-like structure. From the point of view of both rejection and invasion, a proper interaction between the fetal and maternal immune systems rather than just maternal suppression, has recently been shown to be essential for the success of pregnancy (reviewed by (McIntire and Hunt, 2005; Aagaard-Tillery et al., 2006; Hunt, 2006).

During pregnancy, there are two sites where the fetal and maternal immune systems may interact. The first, which is most important in early gestation, is in the decidua (Loke and King, 1995; Moore and Persaud, 2003). Fetal extravillous trophoblasts during implantation invade the spiral arteries and also the mucosa. Extravillous trophoblasts express human leukocyte antigen (HLA) class I molecules, although not of the HLA-A, -B or -D types normally connected with T cell activation, but instead the classical HLA-C, the non-classical HLA-E and in particular HLA-G are expressed (reviewed by (Hunt, 2006)). The selective expression is believed to prevent activation of T lymphocytes but ensure the essential interaction with local NK cells (and thereby avoiding rejection as foreign non-HLA expressing cells). This local interface is accompanied by a second interface, i.e. the systemic interactions in the intervillous space of the placenta. It is first created when the uteroplacental circulation is established at gestation weeks 8–9, and consists of maternal peripheral blood bathing the chorionic villi (Loke and King, 1995; Moore and Persaud, 2003). The size of this systemic interface increases throughout pregnancy, and is dominant in late pregnancy. The syncytiotrophoblasts lining the villi do not express HLA antigens and therefore cannot induce immune reactions in maternal T cells (Hunt, 2006). Yet, many changes caused by pregnancy have been shown in the maternal peripheral circulation, an extension of the interface in the intervillous space (Wegmann et al., 1993).

SYSTEMIC IMMUNOLOGY DURING PREGNANCY

Clinical data on pregnant women show a deviation of the immune system consistent with a weakening of cell-mediated immunity and strengthening of humoral immunity (Wegmann et al., 1993). Cell-mediated disorders such as rheumatoid arthritis (RA) and multiple sclerosis (MS) tend to show fewer or milder symptoms in pregnant women, whereas antibody-mediated diseases such as systemic lupus erythematosis (SLE) seem to flare up during pregnancy. In addition, a number of diseases caused by intracellular pathogens, requiring cellular immunity to be combated, tend to exacerbate during pregnancy. These observations contributed to the hypothesis of pregnancy being a Th2 deviated condition (Wegmann et al., 1993). In rodents, a number of studies supported the hypothesis that Th2-like cytokines were beneficial for pregnancy while Th1-like were detrimental (reviewed in (Raghupathy, 1997). In humans, reports on systemic immunity indicate a decrease in Th1-like cytokines and an increase in Th2-like cytokines during normal as compared to pathological pregnancy (Marzi et al., 1996; Reinhard et al., 1998; Raghupathy et al., 2000). Results from our own group show a specific secretion of IL-4 towards paternal antigens compared to stimulation by unrelated antigens (Ekerfelt et al., 1997c). Besides the hypothesis of a Th2 deviation of adaptive immunity in pregnancy, late human pregnancy has systemically been associated with symptoms similar to those in sepsis, possibly mirroring a strengthening of innate immune

responses (Sacks et al., 1998). C reactive protein (CRP) is elevated in early pregnancy (Sacks et al., 2004) and Th1-like and pro-inflammatory cytokines such as IFN-γ, IL-12, IL-6, TNF (Austgulen et al., 1994; Piccinni and Romagnani, 1995; Matthiesen et al., 1998; Melczer et al., 2003; Sacks et al., 2003), are also elevated in blood from pregnant women compared to non-pregnant controls. Even though some Th2 cytokines (i.e. IL-4) override the functions of some Th1 cytokines (i.e. IFN-γ) when co-expressed (Sadick et al., 1990; Morris et al., 1993; Racke et al., 1994; Röcken et al., 1996), it would be more appropriate to talk about systemic immune balance during pregnancy rather than a systemic, distinct Th2 deviation.

LOCAL IMMUNOLOGY DURING PREGNANCY

At the local interface in the decidua, immune interactions may be even more complex. Around the time point for implantation, the endometrium is infiltrated by a large number of leukocytes, mainly NK cells, as well as macrophages and T lymphocytes. These cells, together with decidual stromal cells and the fetal trophoblast cells, secrete large amounts of various cytokines and other regulatory molecules. In addition, natural killer T (NKT) cells are present in the decidua (Tsuda et al., 2001), as well as low numbers of dendritic cells (Gardner and Moffett, 2003). B cells are barely detectable and there are few granulocytes (Mincheva-Nilsson et al., 1994; Trundley and Moffett, 2004; Milne et al., 2005). Data on the numbers of cells in decidua vary between different studies, but leukocytes account for 15–40% of decidual cells in first trimester human pregnancy (Kurpisz and Fernandez, 1995; Mincheva-Nilsson, 2003; Trundley and Moffett, 2004). The numbers and proportions of immune cells also differ between blood and decidual tissue, as well as between different phases of gestation (see Table I).

Table I. Approximate proportions of some leukocytes in blood and decidua

Cell type	**Blood, non pregnant**	**Blood, early pregnancy**	**Blood, term pregnancy**	**Decidua, early pregnancy**	**Decidua, term pregnancy**
NK cells	3%	1%	1%	50–70%	few
Macrophages	4%	5%	5%	20–30%	50–60%
T cells	27%	20%	20%	10–20%	40–50%
B cells	3%	3%	2%	few	few
Granulocytes	63%	70%	75%	few	few
References	*(Abbas and Lichtman, 2005)*	*(Matthiesen et al., 1995, 1996; Luppi et al., 2002a,b, 2007)*	*(Matthiesen et al., 1995, 1996; Luppi et al., 2002a,b, 2007))*	*(Kurpisz and Fernandez, 1995; Saito, 2000; Mincheva-Nilsson, 2003; Trundley and Moffett, 2004; Milne et al., 2005; Shimada et al., 2006)*	*(Kurpisz and Fernandez, 1995; Trundley and Moffett, 2004)*

Proportions presented as % of leukocytes.

NK cells in decidua

In blood, most NK cells are of the potent lytic $CD56^{low}CD16^{+}$ phenotype with their main function to act cytotoxic against cells not expressing HLA class I molecules, i.e. foreign cells or own cells where infecting viruses have down-regulated HLA molecules to escape recognition by T lymphocytes (Abbas and Lichtman, 2005). In decidua, almost all NK cells are of the $CD56^{bright}CD16^{dim}$ phenotype with less lytic capacity and with great potential to secrete regulatory cytokines (Cooper et al., 2001). The observed differences between decidual and blood NK cells during pregnancy were confirmed in a global microarray analysis on early human pregnancy tissues, where clear differences could be seen between decidual $CD56^{bright}$ cells compared to both blood $CD56^{bright}$ and blood $CD56^{dim}$ cells (Koopman et al., 2003). In fact, blood $CD56^{bright}$ cells were more similar to blood $CD56^{dim}$ cells than to decidual $CD56^{bright}$ cells. This unique population of decidual NK (dNK) cells peaks in number between gestation weeks 6 and 12 but declines from week 20 (Croy et al., 2006) to become very few at term. Consequently, their function is believed to be most prominent in early pregnancy, for example, by promoting trophoblast growth and invasion (Dekker and Sibai, 1998; Saito, 2001), as well as controlling it by killing cells in the case of excessive invasion (King and Loke, 1993; Saito et al., 1993). dNK cells recognize and interact with HLA-G on villous and extravillous cytotrophoblasts, leading to inhibited lytic effects of the NK cell (Rouas-Freiss et al., 1997). Decidual NK cells express potentially immunomodulatory receptors such as CD9 and killer immunoglobulin-like receptors (KIRs), of which CD9 was exclusively shown on dNK cells compared to blood populations (Koopman et al., 2003). Levels of dNK cells have also been associated with pregnancy complications. An increase in decidual $CD56^{dim}$ and a decrease in $CD56^{bright}$ cells could be seen in patients with pregnancy failure compared with those who continued the pregnancy until term (Fukui et al., 1999). On the other hand, high to very high numbers of dNK cells were associated with implantation failure (Ledee-Bataille et al., 2004). These two studies again report on the need for immune balance in pregnancy.

Macrophages in decidua

As the second largest leukocyte population, macrophages account for about 20% of the infiltrating white blood cells in the decidua during early gestation and they persist in numbers throughout pregnancy (Vince et al., 1990; Bulmer, 1994; Kurpisz and Fernandez, 1995; Trundley and Moffett, 2004). While most decidual macrophages are of maternal origin, the placenta consists of a substantial amount of fetal macrophages, so-called Hofbauer cells (Sutton et al., 1983). The fetoplacental macrophages are important in the formation of the placenta by phagocytosis of apoptotic cells that otherwise could have harmful effects on the fetus by causing inflammation (reviewed by (Mor and Abrahams, 2003). Another task for uterine macrophages is of course fighting pathogens in the case of infection. However, studies reveal uterine macrophages are partly of a suppressive or regulatory phenotype, in the placenta (Mues et al., 1989; Hunt and Pollard, 1992) as well as in the decidua (Parhar et al., 1988; Mizuno et al., 1994; Heikkinen et al., 2003; Cupurdija et al., 2004). The fetomaternal interface is known to contain IL-10 (Roth et al., 1996; Chaouat et al., 1999; Hanna et al., 2000; Sacks et al., 2001), IL-4 (Chaouat et al., 1999; Sacks et al., 2001) and IL-13 (Dealtry et al., 1998; Rieger et al., 2002; Brown et al., 2004), suggesting the possibility of alternative activation of macrophages. Another possible way for decidual macrophages to be primed to

an immunosuppressive phenotype is by the interaction with HLA-G on trophoblast cells (reviewed by (McIntire and Hunt, 2005).

T cells in decidua

T lymphocytes comprise 10–20% of leukocytes in the decidua (Kurpisz and Fernandez, 1995; Trundley and Moffett, 2004; Shimada et al., 2006). Both conventional αβ T cell receptor (TCR) bearing T cells and cells with γδ chains are present (Morii et al., 1993; Mincheva-Nilsson et al., 1994; Vassiliadou and Bulmer, 1998; Mincheva-Nilsson, 2003). Many of the decidual αβT cells are $CD8^+$ (Bulmer et al., 1991; Mincheva-Nilsson et al., 1994) and studies on women with unexplained recurrent abortion indicate their numbers are important for normal pregnancy (Piccinni, 2006). Adding to the evidence of immunological activity in the uterus, most T cells in decidua are memory cells (Saito, 2000).

Regulatory T cells

Several different subtypes of regulatory T lymphocytes have been characterized, among them the Th3 cells producing mainly TGF-β together with some IL-10 and IL-4, and the Tr1 cells producing mainly IL-10 and some TGF-β (Shevach, 2002; Mincheva-Nilsson, 2003; Abbas and Lichtman, 2005). In contrast to Th3 and Tr1 cells, which are primed in the periphery to become suppressive, there are also natural $CD4^+CD25^{bright}$ regulatory T cells (Treg), which mature in the thymus (reviewed by (Shevach, 2002; Aluvihare et al., 2005). They are selected despite their capacity to recognize self peptides and subsequently down-regulate the function of self reactive cells. Treg cells may suppress the activity of dendritic cells, naïve $CD4^+$ lymphocytes, $CD8^+$ cells, B cells and NK cells. Natural regulatory T cells express the marker forkhead box p3 (Foxp3) and act suppressive mainly via direct cell–cell contact; their capacity to secrete suppressing cytokines is still unknown. Tregs are important in preventing autoimmunity as well as graft rejection (Sakaguchi et al., 1995; Kingsley et al., 2002). Elevated numbers of $CD4^+CD25^{bright}$ cells were seen in early pregnancy decidua (Sasaki et al., 2004) and blood (Somerset et al., 2004) compared with non-pregnant controls. Further, frequencies of Tregs were higher in normal pregnant decidua than in tissues from spontaneous abortions (Sasaki et al., 2004). Tregs account for up to 20% of $CD4^+$ cells in decidua (Heikkinen et al., 2004; Sasaki et al., 2004), meaning a percentage of total leukocytes as low as 1%. Tregs can be activated antigen specifically but mediate suppression also rather generally when activated (Aluvihare et al., 2005). Zenclussen (2006) suggested that paternal antigens shed to the circulation are encountered by Tregs in blood and that the cells then migrate to the local interface in the decidua where immunosuppression may take place.

γδ T cells

While T cells bearing the αβ TCR chains are active in adaptive immunity, γδ T cells are involved in innate reactions (Szekeres-Bartho et al., 2001; Mincheva-Nilsson, 2003; Abbas and Lichtman, 2005). They are not major histocompatibility complex (MHC) restricted and their diversity in antigen recognition is limited compared to αβ T cells. Most γδ T cells are double negative in expression of CD4 and CD8. Due to their secretion of IL-10, TGF-β and IL-4 they functionally fit into the Tr1 group of regulatory T cells. Studies on uterine tissues show decidual γδ T cells are resident cells that divide locally (Mincheva-Nilsson, 2003) and in murine allogeneic pregnancies, decidual γδ T cells are more abundant than in syngeneic pregnancies, suggesting an immunoprotective role (Kimura et al., 1995). Although γδ T cells

act mainly as immunoregulators, they also have cytotoxic potential. In pregnancy their cytotoxic ability may function as protection against pathogens, controlling trophoblast invasion or by killing reactive T cell clones (Mincheva-Nilsson, 2003).

LATE PREGNANCY AND LABOUR

Studies on the immune system in mid pregnancy in particular, but also in term pregnancy, have not been as extensive as in early gestation and the establishment of pregnancy. As mentioned earlier, the main immunologic interface between fetus and mother in late pregnancy is the systemic connection between maternal blood and the fetally derived villi in the intervillous space. At the local level, the chorioamnion membranes, the placenta and the decidua are all important tissues for interactions and secretions of immunoregulatory molecules. The immune system most certainly acts to prevent rejection of the fetus throughout gestation until term but is also believed to be important in the onset of labour. Prostaglandins (PGs) are crucial for labour induction and they and their receptors are, in turn, regulated by hormonal changes, mechanical pressure of the uterine wall and the secretion of cytokines (Keelan et al., 2003; Yellon et al., 2003; Hertelendy and Zakar, 2004). The coordinated actions of these factors lead to cervical ripening and dilatation, contractions of the uterine muscles, rupture of the amnion sac and finally expulsion of the fetus. The exact mechanisms are, however, unknown. Levels and functions of secreted cytokines and prostaglandins vary depending on the different tissues or fluids from the uterine environment studied (Marvin et al., 2002; Young et al., 2002; Alfaidy et al., 2003; Osman et al., 2003; Mitchell et al., 2004). Labour has been associated with an increase in pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 (Winkler et al., 1998; Sennström et al., 2000) and TNF is believed to be important. Data on TNF levels before and after labour are, however, conflicting (Young et al., 2002; Osman et al., 2003), as are results from studies of IFN-γ (Veith and Rice, 1999; Hanna et al., 2000). IL-10 has been shown to prevent LPS-induced labour in mice (Dudley et al., 1996b), which is in line with the observed decline in decidual IL-10 throughout gestation and at labour in humans (Simpson et al., 1998; Hanna et al., 2000). Studies on decidual IL-4 and TGF-β show no differences in expression before and after labour (Marvin et al., 2002; Wilczynski et al., 2002). In late pregnancy decidua, the NK cells have almost disappeared, while macrophages and T cells have remained stable in numbers but consequently increased in proportion of decidual leukocytes (Kurpisz and Fernandez, 1995). The decidua as a whole is a comparatively smaller part of the fetomaternal tissues at term than in early pregnancy, although still with potential regulatory functions due to its location.

As representative for term pregnancy before the onset of labour, tissues from caesarean sections are often studied, while tissues from vaginal delivery are used to represent term pregnancy after labour (Vince et al., 1992; Dudley et al., 1996a; Vives et al., 1999; Hanna et al., 2000). Since levels of prostaglandins are believed to be elevated in labour, measurements of key regulators in the PG pathway would be useful to validate differences between tissues from the respective stages of pregnancy. Prostaglandin synthesis can be both constitutive and inducible; the inducible pathway is of most interest for the present study. Cyclooxygenase (COX)-2 is the most important enzyme in inducible PG synthesis and its messenger RNA (mRNA) and protein levels have been shown to increase in fetal membranes with gestational age and in association with labour (Slater et al., 1999; Hanna et al., 2006; Astle et al., 2007; Choi et al., 2007). In addition, microsomal prostaglandin E_2 synthase (mPGES), the inducible

form of the terminal enzyme in COX-2 dependent PGE_2 synthesis, was increased at the protein level in chorion (Alfaidy et al., 2003) and at the mRNA level in myometrium (Astle et al., 2007) after labour.

SUMMARY OF INTRODUCTION

During pregnancy, a well regulated immune system is essential to avoid rejection of the semi-allogenic fetus but at the same time permitting placental invasion and maternal defence against infections. Many studies have been done on immunological functions during pregnancy but much information is lacking, especially at the local level. The properties of both leukocytes and non-leukocytes in the decidua and placenta need to be further examined, as do the mechanisms and the interplay between those populations, both in promoting tolerance as well as during the onset of parturition.

AIM

GENERAL AIM

The general aim of this thesis was to clarify the mechanisms of local immune balance in normal human pregnancy decidua, both in the early stage of pregnancy and at parturition.

SPECIFIC AIMS

To determine the cytokine secretions from early pregnancy decidua mononuclear cells, natural killer cells and macrophages, and compare them with secretions from the corresponding cells in blood.

To further characterize early pregnancy decidual macrophages by analysing their gene expression profile and compare it with the expression profile of monocytes in blood.

To clarify the possible role of decidual cytokines in labour induction, by measuring their secretion in mononuclear cells from term pregnancy decidual tissues collected before and after labour.

MATERIALS AND METHODS

SUBJECTS

In total, tissues from 47 women at early pregnancy and 44 women at term pregnancy were studied in this thesis. From first trimester pregnancy, decidual tissue was collected after elective surgical abortions. Blood samples were obtained from 37 of the donors. All pregnancies were detected viable and dated by crown–rump length measurement with ultrasound. From women with normal term pregnancy, decidual tissue was collected from 17 subjects undergoing elective caesarean section before the onset of labour and from 15 subjects after normal vaginal delivery. Further, placental biopsies were collected from 12 additional women after caesarean section (n=7) or vaginal delivery (n=5). For details on subjects and tissues used in the different analyses, see Table II. The study was approved by the Local Ethics Committee of Linköping University and tissues were collected after informed consent.

COLLECTION AND HANDLING OF SAMPLES

Decidual specimens (papers I–IV)

Tissues from elective abortions were rinsed with saline to remove blood, and decidual tissue was then macroscopically separated from placental and fetal tissues. In term pregnancy, the placenta was carefully rinsed with saline and decidua basalis was subsequently scraped from the maternal side of the placenta. The decidual tissue was placed in a test tube filled with tissue culture medium (TCM) consisting of Iscoves modification of Dulbecco's medium (Gibco BRL, Paisley, Scotland) supplemented with (given as final concentrations in the medium): L-glutamine (Flow Laboratories, Irvine, Scotland) 292 mg/L; sodium bicarbonate 3.024 g/L; penicillin 50 IE/mL and streptomycin 50 µg/mL (Flow Laboratories) and 100 × non-essential amino acids 10 mL/L (Flow Laboratories). The tube was put on ice and all samples were prepared within 1 h.

Blood samples (papers I–III)

Heparinized blood was collected and handled within 2 h.

Placental specimens (paper IV)

Placental biopsies (5 mm^3) were collected from 5 women after vaginal delivery at term and from 7 women after elective caesarean section prior to labour. The tissue was immediately put in a collection tube containing RNAlater RNA Stabilization Reagent (Qiagen, Valencia, USA) to avoid changes in the gene expression pattern and stored at 4°C or –70°C until use depending on the storage time until analysis.

Table II. Data on subjects and donated tissues used in the different methods in this thesis. If not stated otherwise, samples from each subject were only used in one analysis

	Early pregnancy					**Term pregnancy**				
	ELISPOT MNC (Paper I)	**ELISPOT NK (Paper II)**	**ELISPOT Mø (Paper II)**	**MicroArray Mø (Paper III)**	**Real-time PCR Mø (Paper III)**	**Protein secretion Mø (Paper III)**	**ELISPOT MNC no labour (Paper IV)**	**ELISPOT MNC labour (Paper IV)**	**RT-PCR no labour (Paper IV)**	**RT-PCR labour (Paper IV)**
Decidua (*n*)	16	10	10	7	3	5*	17	15	–	–
Placenta (*n*)	–	–	–	–	–	–	–	–	7	5
Corresponding blood samples (*n*)	9	7	10	7	3	5*	–	–	–	–
Gestational week	10 (8–12)	10 (7–11)	10 (9–11)	9 (7–10)	10 (9–11)	9 (7–9)	38+4 (33+4–41+0)	40+2 (38+6–41+2)	38+4 (37+5–38+6)	40+2 (38+1–40+5)
Age (years)	30 (17–40)	26 (17–39)	29.5 (17–42)	35 (24–41)	22 (18–23)	35 (28–41)	31 (21–41)	28 (24–37)	29 (21–36)	31 (23–34)
Misoprostol treatment (*n*)	No data	(5/10)	(5/10)	(1/7)	(1/3)	(1/5)	–	–	–	–

Values are given as median values and range for gestational week and age. MNC; mononuclear cell, Mø; macrophage, RT-PCR; reverse transcriptase PCR.
*Four of those samples were also included in the microarray analyses.

CELL SEPARATIONS

Separation of decidual mononuclear cells (papers I–IV)

The decidua was placed in a mesh and rinsed carefully with TCM. Blood clots were removed with a scalpel and the remaining tissue was cut into smaller pieces. The tissue pieces were then minced through a fine-meshed strainer and the cell suspension was centrifuged in a 50 mL test tube for 10 min at 400 × *g* at room temperature. The supernatant was discarded and the pellet was resuspended in 280 mL of TCM. The suspension was divided into eight 50 mL test tubes and layered onto 10 mL of Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden). The tubes were centrifuged for 30 min at 400 × *g* at room temperature. The interface was collected, washed twice with TCM by centrifuging for 10 min at 400 × *g* at 4°C, and counted by phase-contrast microscopy. For ELISPOT in paper I, cells were suspended in TCM supplemented with 5% fetal bovine serum (FBS; Sigma Chemicals, St Louis, MO), and the concentration of mononuclear cells was adjusted to 0.5×10^6/mL. In papers II–IV, cells were further separated (see below).

Separation of mononuclear cells from blood (papers I–III)

Blood samples were separated on Lymphoprep (Medinor AB, Stockholm, Sweden) according to Bøyum (1968). After removing from the interface, peripheral blood mononuclear cells (PBMCs) were washed three times with Hank's balanced salt solution (HBSS), pH 7.2 (Life Technologies, Paisley, Scotland) and counted by phase-contrast microscopy. For ELISPOT in paper I, cells were suspended in TCM/FBS and the concentration of mononuclear cells was adjusted to 0.5×10^6/mL. In papers II–IV, cells were further separated.

Immunomagnetic cell-sorting of NK cells or macrophages (papers II, III)

To obtain NK cells or monocytes/macrophages, mononuclear cells from decidua and blood were further separated according to their expression of CD56 or CD14, respectively. Positive selection was used. Mononuclear cells were suspended in 10 mL of separation buffer containing sterile phosphate buffered saline (PBS; EC Diagnostics AB, Uppsala, Sweden) supplemented with 2 mM EDTA (LabKemi, Stockholm, Sweden) and 0.5% bovine serum albumin (BSA; Miltenyi Biotec, Bergish Gladbach, Germany) and centrifuged for 10 min at 400 × *g* at 4°C. The pellet was resuspended in 500 µl of separation buffer and filtered through a magnetic cell sorting (MACS) pre-separation filter (Miltenyi Biotec) with a pore width of 30 µm. The filtered cell suspension was centrifuged for 10 min at 400 × *g* at 4°C. All supernatant except 80 µL was discarded and the suspension was incubated with 20 µL of anti CD56- or anti CD14-antibody coated MACS cell sorting microbeads (Miltenyi Biotec). After 15 min at 4°C, the cell suspension was applied on a MACS MS+ separation column (Miltenyi Biotec) and both positive and negative populations were collected. All cells were washed twice with 10 mL of TCM/FBS and counted under a phase-contrast microscope. For ELISPOT assay (paper II), cells were diluted to a concentration of 0.5×10^6 cells/mL. For MicroArray (paper III), $CD14^+$ cell populations were pelletted, lysed in 350 µL of RNeasy RLT lysing buffer (Qiagen, West Sussex, UK) and stored at –70°C until analysis.

FACSAria cell sorting of macrophages (paper III)

FACSAria cell sorting is based on flow cytometry, i.e. phenotypic analyses of cells based on their size, granularity and emission of light from bound fluorochrome conjugated antibodies (Shapiro, 2003). Essentially, cells are labelled with antibodies against the marker or markers of interest, and loaded into the fluidics system of the FACSAria. Before sorting, the sample is analysed and gates for cell sorting are set according to morphological criteria of the cell or to the expression of desired marker(s). During sorting, the cells first pass the lasers and for each cell a decision is made whether it fits in a sort gate or not. By vibrations, the stream is broken into droplets with a single cell in each. By the application of positive or negative voltage pulses to the droplet stream, single cell droplets that are decided to be sorted are loaded with a charge. All droplets then pass an electrostatic field between a positively and a negatively loaded deflection plate. The route of direction for negatively charged droplets is slightly bent towards the positively charged plate and vice versa. Selected cell populations can thereby be collected in different tubes and can be used for further experiments. Droplets without a charge pass the electrostatic field without influence and are sent to waste.

In paper III, decidual and blood mononuclear cells were resuspended at a final density of 10^7 cells/mL in PBS with 2% FBS and labelled with the following mouse anti-human antibodies: CD4FITC, CD14APC (Miltenyi Biotech), CD45PE-Cy5 and CD3 PE-Cy7 (BD Bioscience, Stockholm, Sweden) for 30 min on ice. Cells were then washed in PBS with 2% FBS by centrifugation at 400 × *g* for 10 min and resuspended at a final density of 4×10^6 cells/mL for sorting. Sorting was performed on the FACSAria cell sorter (BD Bioscience) using the 488 nm and 632 nm lasers for excitation. FACSComp Beads (BD Bioscience) labelled with the above mentioned antibodies were used for automatic compensation in the FACSDiva software (BD Bioscience). Unlabelled cells were used to set PMT voltages. Sorting was performed at low pressure with the 100 µm nozzle. Monocytes/macrophages were sorted as $CD45^+CD3^-CD14^+$ and collected in TCM followed by spinning for 10 min at 400 × *g* before lysing in RNeasy RLT lysing buffer (Qiagen).

CD9 depletion of mononuclear cells from term pregnancy (paper IV)

Mononuclear cells from decidua and blood were suspended in TCM/FBS and filtered through a MACS pre-separation filter (Miltenyi Biotec). To eliminate contaminating decidual stromal cells expressing CD9, a negative selection step using the immunomagnetic technique was done. Goat-anti-mouse IgG-coupled magnetic beads (Dynabeads; Dynal, Norway) were labelled with mouse anti-CD9 antibody (1 µg mAb/10^7 Dynabeads) according to the manufacturer's recommendations. The decidual cell suspension was incubated with anti-CD9-coated Dynabeads (with a minimum of 20×10^6 beads/ml and a minimum of 4 beads per target cell) for 15–30 min under rotation at 4ºC to allow beads to bind to the cells. To remove contaminating CD9 positive cells, the tube was placed in the magnetic device for 2–3 min and the cell suspension containing mononuclear cells was transferred to a new test tube. Finally, after washing twice in TCM supplemented with 2% FBS (TCM/FBS), the cells were counted and diluted in TCM/FBS at a concentration of 0.5×10^6 cells/mL for further ELISPOT analysis.

FLOW CYTOMETRY (PAPERS II–IV)

Flow cytometry was performed to check the purity of the cell populations.

Paper II

For analyses of NK cell enriched cell populations from early pregnancy, a mixture of antibodies containing FITC-conjugated anti-CD3, PE-conjugated anti-$CD16^{+}56^{+}$ and PerCP-conjugated anti- CD45 antibodies (Becton Dickinson, San Jose, USA) were used. For cells separated with CD14 microbeads, FITC-conjugated anti-CD14 was also used (Becton Dickinson). The flow cytometry was performed on a FACSCalibur (Becton Dickinson).

Paper III

MACS separated decidual and blood macrophages were analysed by flow cytometry as described for paper II. For details on the antibodies used for analyses of FACSAria sorted macrophages, see the FACSAria cell sorting section above.

Paper IV

Mononuclear cell populations from term pregnancies were analysed by using a mixture of antibodies containing FITC-conjugated anti-cytokeratin-7 (Dakopatts, Copenhagen, Denmark) for detection of trophoblasts, PE-conjugated anti-CD9 for detection of stromal cells, and PerCP-conjugated anti-CD45 (BD Biosciences, San José, CA) to enumerate the leukocyte content. Flow cytometry was performed on a FACSCalibur (BD Biosciences).

For results on purity of different cell populations, see Table III.

Table III. Purities of cell populations analysed by flow cytometry. Values shown are medians and/or range.

Cells analysed	**CD56$^+$**	**CD56$^+$CD3$^+$**	**CD14$^+$**	**CD45$^+$**	**CD9$^+$**	**Cytokeratin-7$^+$**
Trimester I decidua MACS sorted CD56$^+$ for ELISPOT (paper II) *n*=2	91–94%	2.7–7.9%	–	–	–	–
Trimester I decidua MACS sorted CD56$^-$ for ELISPOT (paper II) *n*=2	6–13%	25–39%	–	–	–	–
Trimester I decidua MACS sorted CD14$^+$ for ELISPOT (paper II) *n*=2	–	–	62–86%	–	–	–
Trimester I decidua MACS sorted CD14$^-$ for ELISPOT (paper II) *n*=2	–	–	6–20%	–	–	–
Trimester I blood MACS sorted CD14$^+$ for ELISPOT (paper II) *n*=2	–	–	88–95%	–	–	–
Trimester I blood MACS sorted CD14$^-$ for ELISPOT (paper II) *n*=2	–	–	0.3–6%	–	–	–
Trimester I decidua MACS sorted CD14$^+$ for MicroArray (paper III) *n*=3	–	–	72–76%	–	–	–
Trimester I blood MACS sorted CD14$^+$ for MicroArray (paper III) *n*=3	–	–	84–92%	–	–	–
Trimester I decidua FACSAria sorted CD14$^+$ for MicroArray (paper III) *n*=2	–	–	96.2–97.2%	–	–	–
Trimester I blood FACSAria sorted CD14$^+$ for MicroArray (paper III) *n*=2	–	–	98–99.8%	–	–	–
Trimester I decidua FACSAria sorted CD14$^+$ for Real-time PCR (paper III) *n*=3	–	–	86–96%	–	–	–
Trimester I blood FACSAria sorted CD14$^+$ for Real-time PCR (paper III) *n*=3	–	–	93–96%	–	–	–
Term decidua CD9 depleted mononuclear cells for ELISPOT (paper IV) *n*=6	–	–	–	90% (85–93%)	<1%	7% (3.6–10%)

ELISPOT (PAPERS I, II AND IV)

Enzyme-linked-immunospot-assay (ELISPOT) is a very sensitive method for detecting low levels of secreted molecules such as cytokines at a single cell level (Czerkinsky et al., 1984; Ekerfelt et al., 1997a). It is therefore particularly useful for cytokines where similar methods such as enzyme-linked immunosorbent assay (ELISA) fail to detect a secreted molecule. On the other hand, the method gives no information about concentrations but only shows the number of cells secreting a certain cytokine.

Basically, nitrocellulose-bottomed 96-well multiscreen plates are coated with monoclonal antibodies against the molecule of interest (Figure 3). Cells are incubated in the plates overnight and the secreted molecule binds to the antibody. After washing away the cells, a second antibody against the secreted molecule is added. This second antibody is biotinylated and by adding enzyme bound avidin and an enzyme substrate, each cytokine-secreting cell gives rise to one coloured spot at the bottom of the well. These spots are counted to get a value for the number of secreting cells.

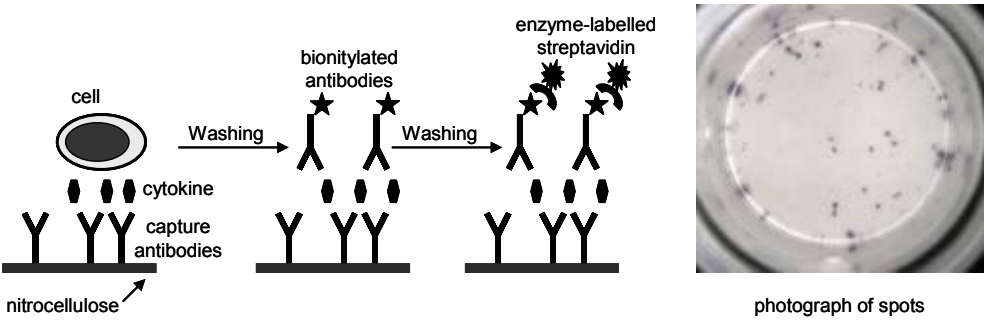

Figure 3. Cytokine secreting cells are incubated in antibody-coated wells. Secreted cytokine binds to the antibody. The cells are washed away and a secondary antibody against the cytokine is added. This second antibody is biotinylated and after adding enzyme labelled streptavidin and a colour substrate, a colour spot can be seen in the bottom of the well. Each spot represents one cytokine secreting cell.

ELISPOT as performed in papers I, II and IV

The ELISPOT was performed essentially as described by Czerkinsky *et al.* (1984), slightly modified for the detection of cytokine secreting cells as described previously (Ekerfelt et al., 1997a). Sterile nitrocellulose-bottomed 96-well multiscreen plates (Multiscreen HA, Millipore, Bedford, MA) were coated overnight with 100 μL/well of mouse anti-human IFN-γ monoclonal antibody (mAb), mouse anti-human IL-4 mAb, rat anti-human IL-10 mAb (papers I, II and IV) or mouse anti-human TNF mAb (all antibodies were purchased from Mabtech, Stockholm, Sweden) diluted to a concentration of 15 μg/mL in sterile PBS, pH 7.4 or with recombinant human TGF-β1 Receptor II (R&D Systems, Abingdon, UK) diluted to a concentration of 10 μg/mL in sterile PBS (paper IV). The plates were incubated at 4°C overnight, whereupon they were emptied by suction using a multiscreen vacuum manifold and washed eight times with 100 μL per well of sterile PBS. Non-specific binding sites on the

nitrocellulose were blocked by incubation with 100 µL per well of TCM (containing 5% FBS) for 30 min at 37°C and 5% CO_2. After emptying, 200 µL aliquots of cell suspension in TCM/FBS, containing 50 000 cells were applied to the wells. Cell populations were trimester one decidual and blood mononuclear cells in paper I, trimester one decidual and blood $CD56^{+/-}$ and $CD14^{+/-}$ cells in paper II and term decidual mononuclear cells in paper IV. Cells were analysed in triplicate for each cytokine in most cases. Because of limited numbers, cells were occasionally analysed in duplicate or in single wells; for details see papers. Additional wells were stimulated with 100 µL of phytohaemagglutinin (PHA; 10 µg/mL; Sigma Chemicals) as a positive control. As a negative control, wells were incubated with TCM/FBS alone, without cells, otherwise treated likewise. The cells were then cultured undisturbed overnight at +37°C in a humidified atmosphere with 5% CO_2. The plates were emptied and washed twice with PBS and twice with PBS containing 0.05% Tween 20 (PBS-T; EC Diagnostics AB, Uppsala, Sweden). 100 µL/well of biotinylated anti-human IFN-γ mAb, biotinylated anti-human IL-4 mAb, biotinylated anti-human IL-10 mAb, biotinylated anti-human TNF mAb (all purchased from Mabtech) diluted to 1 µg/mL in PBS-T or biotinylated anti-human TGF-β1 mAb (R&D Systems) diluted to 10 µg/mL in PBS-T were added. After 2 h of incubation in a dark, moistened chamber at room temperature, the plates were washed four times with PBS-T. 100 µL of streptavidin-alkaline phosphatase (Mabtech) diluted 1:1000 in PBS-T, was added to each well and incubated for another 60 min. The plates were washed four times with PBS and to develop the spots, 100 µL of AP conjugate substrate (BioRad, Hercules, CA) was applied to each well. The reaction proceeded for 15 min, then the plates were rinsed carefully under running water and left to dry overnight at room temperature.

In paper I, the spots were counted in a light microscope with 4× magnification. In paper II and IV, spots were counted using the ELISPOT reader system Transtec 1300 (Autoimmune Diagnostica GmbH, Straßburg, Germany). The median value of the triplicates was used.

ISOLATION OF TOTAL RNA (PAPERS III, IV)

Paper III

Total RNA was isolated according to the RNeasy™ Mini Protocol (Qiagen), for isolation of total RNA from animal tissues. RNA was quantified by UV absorption at 260 nm. The samples were kept at –70°C until use.

Paper IV

Placental biopsies were placed in 600 µL of RLT buffer (Qiagen) and homogenized using a conventional rotor-stator homogenizer. After centrifugation of the lysate, the supernatants were treated as described for paper III above.

MICROARRAY (PAPER III)

Background

Microarray is a method that enables analysis of dozens to millions of molecules in a single sample (Diaz-Mochon et al., 2007). Examples of such molecules are oligonucleotides, cloned DNA, antibodies and peptides. In paper III, microarray was used for relative expression analysis of genes from decidual compared to blood CD14 positive cells. A chip probed with oligonucleotides corresponding to 14 000 genes was used. Basically, complementary RNA

(cRNA) generated from total RNA was hybridized to the chip. If bound to a matching oligonucleotide, a fluorescent signal was detected and mRNA from the gene of interest was considered present in the original sample (Affymetrix, 2007). The relative expression of mRNA in decidual and blood samples was calculated and expressed as expression fold change in decidua compared to blood. In more detail, each gene on the microarray chip was represented by 11 probe pairs, of which one oligonucleotide in the pair had a perfect match to the mRNA sequence of interest, while in the other a middle base was exchanged to create a mismatched probe. By analysing the signal ratio of perfect match/mismatch oligonucleotides, a value for unspecific binding could be created. The mean results of signals in the 11 probe pairs, graded a gene as present or absent call. Using logarithmic signals and setting up stringency criteria for fold change and statistical difference, the amount of present call genes could be limited to a number reasonable for further analysis.

Affymetrix GeneChip Assay as performed in paper III

Samples were amplified for GeneChip analysis according to the manufacturer's recommended protocols (Affymetrix, Santa Clara, USA). In short, total RNA was reversely transcribed to double stranded complementary DNA (cDNA) which, in turn, was transcribed to cRNA. Another round of reverse transcription to cDNA and then transcription back to cRNA was done and in the latter process, biotinylated ribonucleotides were used. Subsequently, 10 µg of each biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre), as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix HG U133A 2.0 GeneChips for 16 h. After hybridization, the GeneChips were washed, stained with streptavidin-PE, and read using an Affymetrix GeneChip fluidic station and scanner.

Data analysis of gene expression

Analysis of microarray data was performed using the Affymetrix GCOS 1.2 software. For normalization, all array experiments were scaled to a target intensity of 150, otherwise using the default values of GCOS 1.2. Further downstream analysis was performed using Array Assist 4.0 (Stratagene, La Jolla, USA). Data was normalized by the PLIER algorithm (Affymetrix) using default parameters. Genes whose signal maximum intensity did not exceed 100 across all samples were excluded from further analysis. Student's *t*-test was applied to identify differences between decidual and blood macrophages. Genes whose *p* values were below or equal to 0.05 and mean relative fold changes more than 2 fold in cells from two of the high purity FACSAria separated subjects were considered differentially regulated in decidual samples compared with blood cells. These genes were used as a gene expression signature for the experiment, resulting in 408 regulated genes. Among these, genes fulfilling the same criteria in cells from the five MACS separated subjects were selected, resulting in the final 120 regulated genes. These genes were grouped according to plausible functions in the context of macrophages in early pregnancy, resulting in the following groups: immune modulation, tissue remodelling, cell cycle-related and cell metabolism/transport.

REAL-TIME RT-PCR (PAPER III)

To confirm regulated genes in the microarray, real-time PCR was performed on flow-cytometrically sorted CD14 positive cells. The two up-regulated genes triggering receptor expressed on myeloid cells (TREM)-2 and CD209 together with the down-regulated

intercellular adhesion molecule (ICAM)-3 were analysed. Indoleamine 2,3-dioxygenase (IDO) and neuropilin (NRP)-1 with two-fold up-regulation in all seven subjects but just below statistical significance, were also analysed. RNA was reversely transcribed with 500 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. For primers used for real-time PCR see paper III. Relative quantification was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) under the following conditions: 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60 s at 60°C. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems) in duplicate. The housekeeping ribosomal protein S9 (RPS9) gene was used for normalization.

ELISA (PAPER III)

The protein level of chemokine (C–C motif) ligand (CCL)-18 in cell culture supernatants was determined by an in-house double-antibody sandwich ELISA. Costar 3690 plates (Costar Inc., Corning, NY) were coated with 0.5 μg/mL monoclonal anti-human CCL18 (clone 64507) in 50 μL of carbonate buffer (pH 9.6) per well (R&D Systems). The plates were incubated on a plate shaker for 1 h and overnight without shaking. The coated plates were washed 4 times with a microplate washer (Anthos microplate washer, Fluido, Salzburg, Austria) with PBS with 0.05% Tween and blocked with 100 μL of prewarmed (37°C) PBS supplemented with 2% low-fat milk per well. After incubation for 1 h on a plate shaker, the plates were washed 4 times. A 7-point standard curve with 2-fold dilutions in PBS with 1% BSA (Sigma-Aldrich, Stockholm, Sweden) was constructed, using recombinant human CCL18 (7.8–500 pg/mL) (R&D Systems). The cell culture supernatants were diluted 1:5 in PBS with 1% BSA. The plates were incubated with 50-μL samples in duplicate on a plate shaker for 1 h. After washing 4 times, 50 μL of biotinylated anti-human CCL18 antibody (polyclonal; R&D Systems), diluted to a concentration of 200 ng/mL in high-performance ELISA (HPE) dilution buffer (CLB, Amsterdam, the Netherlands), were added to the wells. After a second 1-h incubation on a plate shaker, the plates were washed 4 times and 50 μL of streptavidin-poly-horseradish peroxidase (CLB), diluted 1/10 000 in HPE buffer, was added to the wells. After 30 min on a plate shaker, the plates were washed 4 times and 50 μL of 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma-Aldrich) were added to each well, then incubated on a plate shaker for 30 min in the dark. The reaction was terminated with 1.8 M H_2SO_4. The ODs were read at 450 nm with a wavelength correction at 540 nm in a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, USA). Data acquisition was performed using SOFTmaxPRO Version 3.1.2 computer software (Molecular Devices). All steps were performed at room temperature unless otherwise stated. The lower detection limit was 8 pg/mL CCL18.

MULTIPLEX BEAD ARRAY (PAPER III)

The levels of CCL2, matrix metalloproteinase (MMP)-9 and IL-10 in cell culture supernatants were analyzed according to the manufacturer's instructions. Base kits LUH000 and LMP000 together with analyte kits LUH279 and LMP911 were used (all purchased from R&D Systems). Protein concentrations were analyzed using the Luminex 100 instrument (Luminex Corporation, Austin, TX) and the STarStation software (v 2.3, Applied cytometry Systems, Sheffield, UK).

REVERSE TRANSCRIPTASE-PCR (PAPER IV)

To confirm that elective caesarean section and vaginal delivery can be used as representative for the state before and after labour, respectively, COX-2 and mPGES-1 mRNA levels in placental tissues were determined by reverse transcriptase (RT)-PCR.

cDNA was generated in a 20 μL reaction according to the First-Strand cDNA Synthesis Using Superscript™ II RT protocol (Invitrogen). For amplification of the COX-2 and mPGES-1 genes, 2 μM of each primer was used. To control for RNA concentration, each sample was corrected for β-actin. For primers used see paper IV. PCR was performed on a Peltier Thermal Cycler, PTC-200 (SDS Biosciences, Falkenberg, Sweden). Both primers and an appropriate amount of template DNA were added to a mixture consisting of PCR buffer (containing 15 mM $MgCl_2$), Q-solution, dNTP and HotStarTaq DNA polymerase to a total volume of 25 μL. Before the PCR thermal-cycler program was initiated the temperature was raised to 95°C for 15 min to activate HotStarTaq DNA polymerase. The PCR procedure continued with denaturation at 94ºC for 1 min, annealing at 59ºC for 45 s and extension at 72ºC for 1 min. Twenty-four cycles were used for amplification of β-actin, 37 cycles for COX-2 and 33 cycles for mPGES-1. After the last cycle the temperature was raised to 72ºC for 10 min for a final extension. Samples were analysed on a 1% agarose gel containing ethidium bromide.

STATISTICS (PAPERS I–IV)

In papers I–II and IV, non-parametric statistical tests were used. Parametric tests are powerful when data is normally distributed, while non-parametric statistics is preferable in studies where the data is not. Data in papers I-II and IV did not show normal distribution. Friedman test and Wilcoxon's test were used for comparison of the number of cells secreting the different cytokines in paper I. Spearman's rank correlation test was used for test of correlations between cells secreting the different cytokines in papers I, II and IV. Spearman's test was also used for comparisons of cytokine secretion in relation to gestational age in paper II. Wilcoxon's test was used for paired comparisons of spontaneous and PHA-induced secretion of cytokines in paper I, for comparisons between cell populations within decidua and blood, as well as for comparisons between decidual and blood cell populations in paper II and for comparisons of protein secretion in decidual and blood cells in paper III. The Mann–Whitney *U*-test was used for unpaired comparisons between groups treated with or without Misoprostol in paper II, for comparisons of cytokine secretion and to compare ratios of different cytokines between the two groups in paper IV and for comparisons among patient characteristics in paper IV. In addition, Fisher's exact test was used for comparisons among patient characteristics in paper IV.

In paper III, Student's *t*-test was used for analyses of microarray data.

RESULTS AND DISCUSSION

CYTOKINE SECRETION FROM FIRST TRIMESTER PREGNANCY DECIDUA AND BLOOD (PAPERS I, II)

Decidual mononuclear cells, CD14 enriched monocytes/macrophages and CD56 enriched NK cells, were assayed for secretion of IFN-γ, IL-4 and IL-10 by ELISPOT and the secretion was compared to that from corresponding cells in blood from the same individual. All three cytokines were detected in all cell populations (Figure 4).

Mononuclear cells (paper I)

The number of cytokine secreting decidual mononuclear cells did not differ significantly from the number in blood. There were, however, no significant correlations between decidua and blood when comparing numbers of secreting cells for each cytokine. Instead, significant correlations were found when comparing the number of cells secreting different cytokines within decidua or blood, i.e. a high number of cells secreting one cytokine was accompanied by a high number of cells secreting the other cytokines (Table IV).

Being the major Th2 type cytokine, expression of IL-4 at the maternofetal interface fits well into the original paradigm of pregnancy being a Th2 deviated condition (Wegmann et al., 1993). IL-4 was detected previously in human trimester one decidual and placental tissue using immunohistochemistry (Chaouat et al., 1999) and in decidual mononuclear cell supernatants using ELISA (Ho et al., 2001). Further, IL-4 was detected by ELISA in a phorbol myristate acetate (PMA) stimulated decidual T cell line (Piccinni et al., 1998) as well as by intracellular flow cytometry in PMA stimulated decidual T lymphocytes (Saito et al., 1999; Shimada et al., 2006). Intracellular flow cytometry also showed IL-4 expression in unstimulated decidual NKT cells (Tsuda et al., 2001). Early pregnancy trophoblasts show expression of IL-4 mRNA (Haynes et al., 1993; Vives et al., 1999) and intracellular IL-4 protein (Sacks et al., 2001). Hence, reports on spontaneous secretion of IL-4 in human fetomaternal tissues are few. Immunohistochemistry may locate a cytokine present in a certain tissue but it can never prove the actual secretion of the protein from a specific cell, since the staining does not reveal whether the cytokine is intra- or extracellular or bound to receptors on the cell surface. In addition, measured mRNA levels may not represent secretion of protein. A comparison of methods for detecting IL-4 secretion showed ELISPOT to be outstanding compared to ELISA and real-time PCR in detecting spontaneous secretion of the cytokine (Ekerfelt et al., 2002). Although detected in many of the subjects, IL-4 mRNA levels did not correlate with the number of IL-4 secreting cells as measured with ELISPOT. The difficulties in detecting spontaneous secretion of IL-4 can be explained by the relatively low levels of this cytokine secreted, and the fact that it is highly consumed *in vitro* due to the abundance of IL-4 receptors on different cells (Bullens et al., 1998). Cytokine detection after PMA stimulation of cells might represent a boost of what was going on in the cell before stimulation but since the protein kinase C activating mitogen, PMA (Marquardt et al., 1994) stimulates for example inflammatory COX-2 (Chun et al., 2003) and MMP-9 (Arai et al., 2003) in murine cells, a deviation of the cell is also possible.

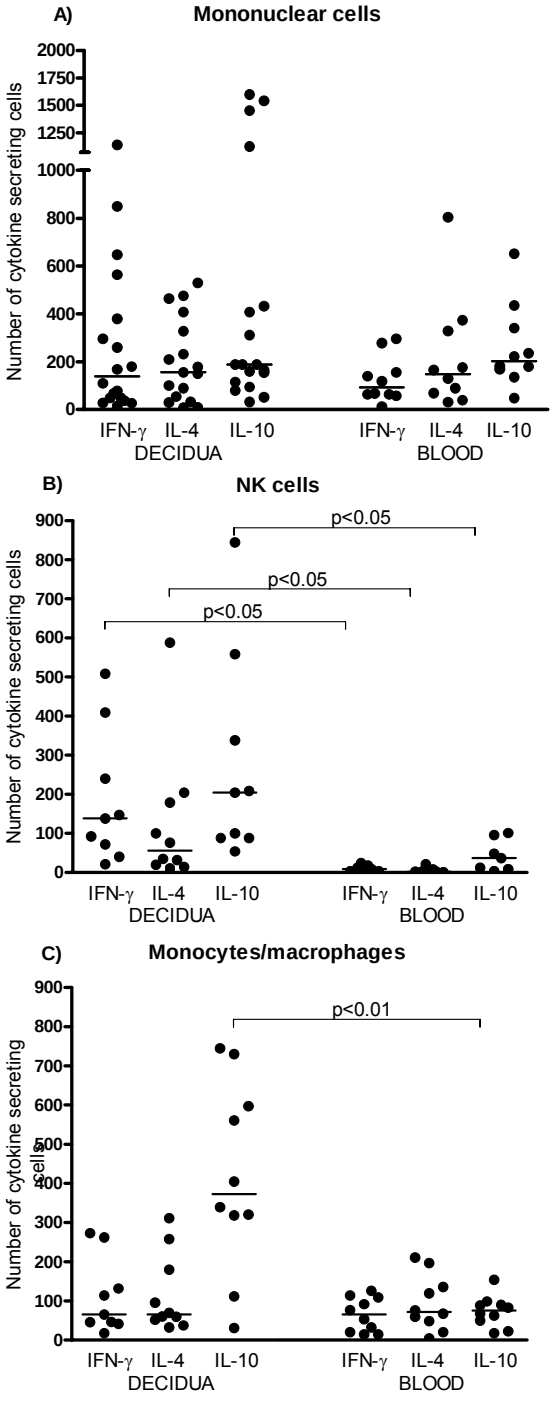

Figure 4. Cytokine secretion from first trimester decidual and blood mononuclear cells (A), NK cells (B) and monocytes/macrophages (C). Values presented are the number of cytokine secreting cells per 50 000 cells as measured with ELISPOT. Bars show median values; *p*-values are from Wilcoxon's paired non-parametric test.

TABLE IV. Correlations (r) between the numbers of cells secreting different cytokines in decidua and blood. *p <0.05, **p <0.01 from Spearman's Rank Correlation Test.

	MNC		CD14+		CD56+	
Correlation	Decidua	Blood	Decidua	Blood	Decidua	Blood
IFN-γ vs IL-4	r=0.92**	r=0.85*	r=0.83**	r=0.88**	r=0.68*	r=0.28
IFN-γ vs IL-10	r=0.80**	r=0.80*	r=0.68*	r=0.56	r=0.452	r=0.11
IL-4 vs IL-10	r=0.67**	r=0.93**	r=0.61	r=0.6	r=0.89**	r=0.55

Unstimulated secretion of IFN-γ has been detected in human first trimester decidual mononuclear cells using ELISA (Ho et al., 2001), at protein (Lash et al., 2006) and mRNA (Saito et al., 1993; Lash et al., 2006) levels in decidual NK cells, by intracellular flow cytometry in NKT cells (Tsuda et al., 2001) and by immunohistochemistry in decidual stromal and NK cells (Plevyak et al., 2002). IFN-γ has also been detected in PMA or PHA stimulated decidual T cells using intracellular flow cytometry (Saito et al., 1999; Shimada et al., 2006) and analyses of cell supernatant protein levels (Piccinni et al., 1998; Scaife et al., 2006). Trophoblast tissues from early pregnancy contain IFN-γ as detected at mRNA levels (Haynes et al., 1993) and by immunohistochemistry (Paulesu et al., 1994).
Despite the first reports on IFN-γ being detrimental to pregnancy (reviewed by (Raghupathy, 1997)), appropriate levels of IFN-γ have been shown to have essential functions in murine spiral artery modulation, maintenance of decidua and development of decidual NK cells (Ashkar et al., 2000; Ashkar and Croy, 2001). This is in line with the findings of IFN-γ secretion in human gestational tissues. However, as indicated by our own results, IFN-γ probably needs to be balanced by, for instance, the co-expressed IL-4 and IL-10, keeping the potential harmful effects of IFN-γ in check. The significant correlations in the numbers of secreting cells between the different cytokines seen in decidua, together with the large interindividual differences, suggest that balance within an individual or within a tissue is more important than the exact amount of cytokines secreted or, as in this case, the amount of cells secreting cytokines. The interindividual differences could also be partly explained by laboratory variations since the ELISPOT method shows significant interassay variations (Czerkinsky et al., 1988; Ekerfelt et al., 1997b).

IL-10 is a cytokine with mainly anti-inflammatory functions but it also promotes inflammation within the innate arm of immunity, especially by activating NK cells (reviewed by (Mocellin et al., 2003)). Adaptive immune responses are dampened by IL-10 via down-regulation of APC maturation, expression of co-stimulatory molecules and pro-inflammatory cytokines (de Waal Malefyt et al., 1991; Ding and Shevach, 1992; Mocellin et al., 2003; Abbas and Lichtman, 2005). For T cells this may cause long-term and antigen-specific anergy (Mocellin et al., 2003) or induction of suppressive cells. As for cytokines in general, the effects of IL-10 are dependent on cytokine concentrations, the type of tissue or cell investigated and the timing in the immune process, as discussed by (Cavaillon, 2001). In pregnancy, IL-10 may be important not only for its anti-inflammatory functions but also due to its anti-thrombotic effects (Downing et al., 1998), given that studies suggest both spontaneous and cytokine induced abortions in mice were dependent on thrombotic processes in blood vessels (Clark et al., 1998). IL-10 may also influence decidual NK cells and

macrophages, as discussed below. The main source of IL-10 in human uteroplacental tissues has been proposed to be trophoblasts as detected by ELISA (Roth et al., 1996; Hanna et al., 2000), immunohistochemistry (Chaouat et al., 1999) and at the mRNA level (Bennett et al., 1999). Immunohistochemistry also shows staining for IL-10 in first trimester decidual tissue (Krasnow et al., 1996; Chaouat et al., 1999; Plevyak et al., 2002) with localization mainly to stromal cells (Plevyak et al., 2002). Unstimulated decidual mononuclear cells (Ho et al., 2001) and macrophages (Heikkinen et al., 2003) secrete IL-10 as measured by ELISA, as do NK cells (Deniz et al., 1996). Decidual NK cells (Vigano et al., 2001) and γδ T cells (Nagaeva et al., 2002) both express mRNA for IL-10 and secreted protein can be measured in PHA stimulated decidual $CD8^+$ T lymphocytes (Scaife et al., 2006) and in a PMA stimulated decidual T cell line (Piccinni et al., 1998).

As for PMA, PHA stimulation might drive the immune response away from the situation found *in vivo*. Using partly the same signalling pathways as PMA, the lectin PHA induces mitosis but is toxic in high doses (Kesherwani and Sodhi, 2007). It acts via binding to the CD2 and T cell receptors and has mainly been considered a T cell mitogen stimulating production of several cytokines such as IFN-γ, IL-2, IL-10 (Fan et al., 1998; Stanilova et al., 2005). However, CD2 is also expressed on NK cells and PHA has recently been shown to stimulate macrophages to produce TNF and IL-1β (Kesherwani and Sodhi, 2007). In paper I, the PHA stimulation of decidual mononuclear cells did not affect the secretion of IL-4 and IL-10 to the same extent as in blood cells. This may reflect that the cells are already activated *in vivo*, as shown for decidual T cells by expression of the activation markers CD69, IL-2 receptors α and β and HLA-DR (Saito et al., 1992) and the phenotype of memory cells (Saito et al., 1994). Keeping the picture of PHA as a T cell mitogen, the difference between decidua and blood may also reflect the differences in cell composition between the two tissues, with far more abundant T cells in blood than in decidua. Alternatively, T cells were not the main IL-4 and IL-10 secreting cell type among the mononuclear cells investigated. Yet, based on the knowledge that both NK cells and macrophages may respond to PHA stimulation, the most probable explanation may be the inability to further stimulate the already activated decidual cells.

The decidual secretion of cytokines was not reflected in blood cells, as no significant correlation between the number of cytokine secreting cells in decidua and blood was found for any of the cytokines. Because the fetomaternal circulation is not fully established until the end of the first trimester, it would be interesting to investigate if any significant correlation could be found in late pregnancy. Unfortunately, blood was not analysed in paper IV for comparison of the local cytokine secretion.

NK cells (paper II)

The number of cells secreting IFN-γ, IL-4 and IL-10 was significantly higher in decidual CD56 positive cells than in blood CD56 positive cells (Figure 4). Within decidua, the number of IL-4 and IL-10 secreting cells correlated but no significant correlations between cytokines were found in blood. Neither there were any significant correlations found between the numbers of cytokine secreting cells in decidua compared to blood. In addition to CD56 enriched cells, CD56 depleted populations were assayed. CD56 negative decidual cells were more frequent in numbers secreting IL-10 than CD56 positive cells (Figure 5). In blood, non-NK cells were also higher in numbers secreting IFN-γ than blood NK cells.

The clear differences seen in decidual CD56 positive cells compared to blood cells reflect and confirm the known typical characteristics of decidual NK cells. Decidual NK cells expressing high amounts of CD56 but low amounts of CD16 ($CD56^{bright}CD16^{dim}$) are known to differ from regular NK cells in blood ($CD56^{low}CD16^{+}$). $CD56^{bright}CD16^{dim}$ cells are also present in low numbers in blood but gene expression analyses showed the two blood populations were more similar than $CD56^{bright}$ cells in decidua and blood (Koopman et al., 2003). Except for the cytokines assayed in paper II, dNK cells secrete, for example TNF, TGF-β, leukaemia inhibitory factor (LIF), colony stimulating factors (CSFs), angiogenic factors such as platelet growth factor (PGF) and vascular endothelial growth factor (VEGF)-C (reviewed by (Croy et al., 2006)), illustrating their diverse but most certainly very important functions. The main source of IFN-γ in murine pregnancy is uterine NK cells (Ashkar et al., 2000). In humans, IFN-γ has also been detected in decidual NK cells, at mRNA (Saito et al., 1993; Lash et al., 2006) and protein levels (Plevyak et al., 2002; Lash et al., 2006). IFN-γ derived from human decidual NK cells reduced trophoblast invasion *in vitro* through increased apoptosis (Lash et al., 2006) and decreased levels of the protease MMP-2 (Hu et al., 2006; Lash et al., 2006). These studies further underline the multiple roles for IFN-γ in the uterus.

IL-10 secreting cells were detected among human decidual NK cells, but in significantly lower numbers than in the CD56 negative population, emphasizing that NK cells are not the major source of IL-10 in decidual leukocytes. IL-10 mRNA (Vigano et al., 2001) and secreted protein (Deniz et al., 1996) have been previously detected in decidual NK cells. In association with NK cells, the otherwise anti-inflammatory cytokine IL-10 has proven pro-inflammatory functions. IL-10 augments IL-2 induced proliferation of blood $CD56^{bright}$ NK cells, and increases their secretion of IFN-γ, TNF, granulocyte-macrophage colony stimulating factor (GM-CSF) (Carson et al., 1995). Even more interesting for pregnancy, since IL-2 is not believed to be present in the uterus, is that IL-10 alone increases NK cytotoxicity against tumour cells, in $CD56^{bright}$ cells more than in $CD56^{low}$. In line with this, IL-10 indeed induces dNK cytotoxicity, with or without IL-2 (Vigano et al., 2001), implying functions in regulating trophoblast invasion.

IL-4 has to our knowledge not been previously detected in decidual NK cells, although studies using intracellular flow cytometry localised the cytokine to decidual NKT cells (Tsuda et al., 2001) and blood NK cells from non-pregnant subjects (Ntrivalas et al., 2006). The absence of reports on IL-4 secretion in decidual NK cells may be due to the difficulties in detecting this cytokine as discussed earlier. The number of cells secreting IL-4 did not differ between $CD56^{+}$ and $CD56^{-}$ cells, showing NK cells are not the only source of IL-4 in decidual mononuclear cells.

When separating $CD56^{+}$ and $CD14^{+}$ cells in papers II and III, negative selection perhaps would have been preferable due to the risk of unwanted activation of the cells when using positive selection. However, positive selection leads to higher purity, which is of particular importance for cell suspensions of decidual mononuclear cells since they contain cell debris and probably fragments of both trophoblasts and stromal cells. CD56 is an adhesion molecule originally described in the nervous system and although the function of the molecule is still unclear (Lanier et al., 1989; Cooper et al., 2001) an effect of binding cannot be excluded. Since blood and decidual tissues were handled in the same way, the differences seen should not be due to receptor activation.

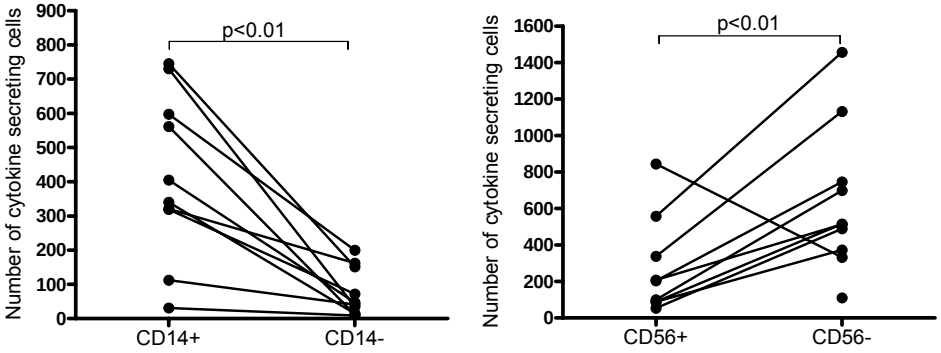

Figure 5. IL-10 secretion from first trimester decidual CD14/CD56 positive and negative cells. Values are the number of cytokine secreting cells per 50 000 cells as measured with ELISPOT. *p*-values are from Wilcoxon's paired non-parametric test.

Macrophages (paper II)

No differences were found in the number of decidual macrophages secreting IFN-γ or IL-4 compared to blood CD14 positive cells, while a significantly larger number of decidual macrophages secreted IL-10 than did their blood counterparts (Figure 4). Within both decidua and blood, numbers of cells secreting IFN-γ and IL-4 correlated but there was no significant correlation of the number of secreting cells between decidua and blood for any cytokine. When comparing CD14 positive cells with CD14 depleted cells, decidual macrophages had significantly higher frequencies of IL-10 secreting cells than decidual non-monocytic cells (Figure 5). In blood, the number of IL-10 secreting cells was higher in CD14 positive than in CD14 negative cell populations, as was the number of cells secreting IL-4.

Macrophages were not originally described as a source of IFN-γ and IL-4 but recent data shows expression of IFN-γ in mouse macrophages (Frucht et al., 2001) and in human alveolar macrophages (Prieto et al., 2000), the latter a source also for IL-4 (Pouliot et al., 2005). Here, we show that both decidual and blood CD14 positive cells from pregnant women produce IFN-γ and IL-4, with similar frequencies of cytokine producing cells between the two tissues. Studies on cytokine secretion in decidual macrophages are few, but the result on IL-10 secretion is in line with ELISA analyses of decidual macrophages (Heikkinen et al., 2003). The purity of CD14 positive enriched cells in decidua was not optimal (62–86%), why CD14 negative cells might contribute to the number of IL-10 secreting cells detected. However, if CD14 negative cells were the most frequent cells producing IL-10, a decrease in these cells in concert with enrichment of CD14 positive cells, would not cause the increase in IL-10 secreting cells seen in CD14 enriched cells compared to CD14 depleted cells. Further, the difference in IL-10 secreting cells between CD14 positive and negative cell populations match the difference seen in CD56 positive and negative cells, where the pattern was the opposite. IL-10 secreting cells are more frequent in CD56 negative cell populations, i.e. in the populations where, among others, macrophages are found. The number of IL-10 secreting cells in decidua was far higher than the number in blood, indicating potential differences between the two populations. A high secretion of IL-10 is a hallmark of alternatively activated or M2 macrophages (Goerdt and Orfanos, 1999; Gordon, 2003), but it cannot be

ruled out that differences between decidual macrophages and blood monocytes in part mirror discrepancies in differentiation stages. Further studies are needed to gain more insight into this feature of decidual versus blood monocytes/macrophages.

As discussed for NK cell separation in the previous section, negative selection of cells would have been preferable but was not possible. CD14 is a co-receptor for toll-like receptor (TLR)-4 but so far no effector function of binding to CD14 alone has been found (Dobrovolskaia and Vogel, 2002).

GENE EXPRESSION PROFILE OF DECIDUAL MACROPHAGES (PAPER III)

The finding of high numbers of IL-10 secreting cells in decidual CD14 enriched cells led us to further investigate the characteristics of this cell type. Fetally derived macrophages in the placenta (Hofbauer cells) have shown phenotypes alternative to classically activated macrophages (Mues et al., 1989; Hunt and Pollard, 1992). In previous studies, maternal decidual macrophages have shown regulatory functions associated with macrophages of alternatively activated phenotype (Parhar et al., 1988; Mizuno et al., 1994; Heikkinen et al., 2003; Cupurdija et al., 2004), although only a limited number of markers have been analyzed. In paper III, gene expression analysis revealed 120 genes being differentially regulated in decidual compared to blood CD14 positive cells. A selection of genes is shown in Table V. Some genes associated with classically activated macrophages (Hashimoto et al., 2003; Martinez et al., 2006; Volpe et al., 2006) were up-regulated in decidual macrophages but they were accompanied by a larger number of up-regulated markers for alternatively activated macrophages, including CCL18 (Kodelja et al., 1998), CD209 (Puig-Kröger et al., 2004), mannose receptor C type (MRC)-1 (Gordon, 2003), fibronectin (FN)-1 (Gordon, 2003) and insulin-like growth factor (IGF)-I (Kodelja et al., 1998). The up-regulated genes were classified into functional groups based on possible functions in pregnancy. Large groups included genes connected to immune regulation and tissue remodelling. Cell proliferation-related genes and genes functioning in cell metabolism and transport were commonly up-regulated in decidual macrophages compared to cells in blood. Differential regulation of TREM-2, CD209, ICAM-3 and the near-significance regulated genes IDO and NRP-1 was confirmed by real-time RT-PCR data (Figure 6); CCL-18, CCL-2 and MMP-9 were confirmed by ELISA or Multiplex bead array analyses (Figure 7). IL-10 was not differentially regulated in the microarray but analysed by Multiplex bead array and found to be secreted at higher levels in decidual macrophages than in blood monocytes/macrophages.

In the group of up-regulated immune modulating genes, several were receptors or secreted molecules such as chemokines. The often co-expressed lectins MRC-1 and CD209 (dendritic cell specific intercellular adhesion molecule-grabbing nonintegrin; DC-SIGN) are generally linked to alternative activation of macrophages and have been detected previously in human early pregnancy uterine cells (Burk et al., 2001; Soilleux et al., 2002; Kämmerer et al., 2003; Laskarin et al., 2005). Mannose receptors may have a regulatory role via the binding of tumour associated glycoprotein (TAG)-72, which would prevent macrophage uptake and processing of paternal trophoblast antigens and thereby avoid immune reactions against the fetus (Laskarin et al., 2005). The up-regulated tetraspanin CD9 has been suggested to bind pregnancy specific glycoproteins (PSGs) and induce anti-inflammatory cytokines in pregnant mice (Ha et al., 2005) and in human isolated blood monocytes (Snyder et al., 2001). NRP-1 was significantly up-regulated in decidual macrophages as measured by real-time

PCR. The receptor, which is involved in the immunological synapse, was previously linked to regulatory T cells (Bruder et al., 2004) but has recently been shown to be up-regulated in M2 macrophages (Martinez et al., 2006).

A receptor that has not been previously linked to pregnancy but has potential functions of interest is TREM-2. In mouse macrophages, TREM-2 is induced by IL-4 as well as by ovalbumin (OVA)-allergen stimulation but is suppressed by IFN-γ or LPS (Turnbull et al., 2006). In concert with this, high expression of TREM-2 on mouse microglial cells correlates with their ability to phagocytose apoptotic neurons, hence TREM-2 might positively regulate phagocytosis but negatively regulate inflammatory responses (Takahashi et al., 2005). This proposed dual function of TREM-2 would fit well into the pregnant uterus, where the need for both immune regulation and apoptotic clearance is high. In humans, the role of TREM-2 is unclear but it is associated with the Nasu–Hakula syndrome including defective osteoclast and microglial function (Cella et al., 2003). In line with the murine results, TREM-2 has also been found to be down-regulated in LPS-stimulated human monocyte derived dendritic cells (Begum et al., 2004). It is thus plausible that TREM-2 is involved in suppressing production of inflammatory factors and providing immune balance in pregnancy.

Chemokines CCL-2 and CCL-18 were among the soluble molecules up-regulated. Due to its role in recruiting monocytes, CCL-2 (monocyte chemoattractant protein-1, MCP-1) is classically linked to inflammatory responses (Daly and Rollins, 2003). On the other hand, a dual function depending on environmental factors is suggested, in line with the finding that CCL-2 could drive Th2 polarization in mice (Daly and Rollins, 2003). In human lung epithelial cells, IL-4/IL-13 stimulation increased their secretion of CCL-2 (Ip et al., 2006). We suggest that the role of decidual CCL-2 is to recruit monocytes/macrophages to the tissue, where other factors will be decisive for polarization. CCL-18 is constitutively expressed in monocytes, macrophages and dendritic cells, and is strongly up-regulated by Th2-related cytokines and dampened by stimulation with IFN-γ (Kodelja et al., 1997). No agonistic receptor has been found for the chemokine, but CCL18 recruits naïve T cells, possibly directing them towards tolerance (van Lieshout et al., 2006). CCL18 is highly present in lung tissue and in RA in the synovial lining in the joints. These locations have in common that self- and non-pathogenic antigens are constantly present and must be judged by the immune system. During pregnancy, CCL-18 is expressed in human decidual and amnion tissues, interestingly with reduced expression after the onset of labour (Marvin et al., 2002).

The real-time PCR detected up-regulation of IDO in decidual compared to blood monocytes/macrophages; this is in line with previous results on the same cell type (Heikkinen et al., 2003). IDO is a tryptophan depleting enzyme, suggested to be important for preventing T cell activation in murine pregnancy (Munn et al., 1998). Prostaglandin D2 synthase (PGDS) is associated with decidual Th2 cell recruitment and antigen presentation (Saito et al., 2002), functions that may explain the up-regulation of the gene in decidual macrophages.

IL-10, which was detected by ELISPOT in MACS sorted decidual macrophages in paper II, was also detected in the microarray, although no differences were seen between decidua and blood. This could be due to the fact that the *in vivo* induction of cytokine and thereby mRNA expression was lost during the time-consuming preparation of the cells before lysing. This was supported by the measured difference of IL-10 protein secretions in cell culture supernatants between decidual and blood macrophages. A previous study on IFN-γ expression from dNK cells shows similar kinetic results with lost mRNA but not protein expression after culture for 24 h (Lash et al., 2006).

Table V. Selection of genes differentially expressed in decidual compared to blood $CD14^+$ cells in early pregnancy

Gene name	GenBank accession number	Fold change[a]
Immune modulating		
Suppressive/anti-inflammatory functions		
Soluble form		
Alpha-2-macroglobulin; A2M	NM_000014	92
V-set and immunoglobulin domain containing 4; VSIG4	NM_007268	23
Prostaglandin D2 synthase, hematopoietic; PGDS	NM_014485	13
Chemokine (C–C motif) ligand 18; CCL18, AMAC1	Y13710	3.8
Membrane bound form		
Mannose receptor, C type 1; MRC1	NM_002438	36
CD209 antigen; DC-SIGN	AF290886	16
Triggering receptor expressed on myeloid cells 2; TREM2	NM_018965	9.0
Tumor necrosis factor receptor superfamily, member 21; TNFRSF21	NM_016629	7.3
CD9 antigen (p24); CD9	NM_001769	5.3
Dipeptidase 2; DPEP2	NM_022355	–2.8
C-type lectin domain family 4, member A; CLEC4A (LLIR)	AF200738	–3.0
Intracellular protein		
Disabled homolog 2, mitogen-responsive phosphoprotein (*Drosophila*); DAB2	AF188298	30
V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian); MAF	NM_005360	12
Interleukin-1 receptor-associated kinase 3; IRAK3	NM_007199	–2.7
Activating/pro-inflammatory functions		
Soluble form		
Secreted phosphoprotein; SPP1	M83248	101
Complement component 3;C3	NM_000064	48
Chemokine (C motif) ligand 2; XCL2	U23772	7.6
Chemokine (C-C motif) ligand 2; CCL2, MCP1	S69738	5.8
Chemokine (C motif) ligand 1; XCL1 (lymphotactin)	NM_003175	5.7
Chemokine (C-C motif) ligand 8; CCL8, MCP2	AI984980	5.0
Proteolipid protein 2 (colonic epithelium-enriched); PLP2	NM_002668	–4.0
S100 calcium binding protein A8 (calgranulin A); S100A8	NM_002964	–5.8
Properdin P factor, complement; PFC	NM_002621	–6.6
Membrane bound form		
SLAM family member 8; SLAMF8	NM_020125	4.7
Bone marrow stromal cell antigen 1; BST1 (CD157)	NM_004334	–3.1
Asialoglycoprotein receptor 2; ASGR2, CLEC4H2	NM_001181	–4.1
Intercellular adhesion molecule 3; ICAM3	NM_002162	–4.8
EGF-like-domain, multiple 5; EGFL5	W68084	–5.9
Selectin L (lymphocyte adhesion molecule 1); SELL	NM_000655	–7.0
Vanin 2; VNN2	NM_004665	–7.5
Tissue remodelling		
Fibronectin 1; FN1	AF130095	68
Complement component 1, q subcomponent, beta polypeptide; C1QB	NM_000491	58
Collagen, type III, alpha 1; COL3A1	AU144167	27
Heat shock 27 kDa protein 1; HSPB1	NM_001540	21
Complement component 1, q subcomponent, alpha polypeptide; C1QA	NM_015991	16
Endothelial PAS domain protein 1; EPAS1	AF052094	15
Growth arrest-specific 6; GAS6	L13720	11
Collagen, type I, alpha 2; COL1A2	AA788711	10
Collagen, type VI, alpha 3; COL6A3	NM_004369	9.9
Collagen, type IV, alpha 2; COL4A2	X05610	9.3
Serpin peptidase inhibitor, clade F, member 1; SERPINF1	NM_002615	8.2
Nerve growth factor receptor (TNFRSF16) associated protein 1; NGFRAP1	NM_014380	7.9
Matrix metalloproteinase 9;MMP9, plasminogen	NM_004994	6.5
Protein S (alpha); PROS1	NM_000313	5.3
Insulin-like growth factor 1 (somatomedin C); IGF1	AI972496	5.1
Syndecan 2 (heparan sulfate proteoglycan 1); SDC2	AL577322	4.4
Integrin, beta 5; ITGB5	BE138575	3.7
S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog); S100A4	NM_002961	–2.8
Chondroitin sulfate proteoglycan 2 (versican); CSPG2	BF590263	–3.2
Peptidyl arginine deiminase, type IV; PADI4	NM_012387	–5.9

[a]Fold change is the factor of regulation of mRNA from $CD14^+$ cells in decidua versus $CD14^+$ cells in blood. Positive values denote up-regulation and negative values mean down-regulation. Genes included in this table were up- or down-regulated by a factor of at least 2 in all 7 subjects.

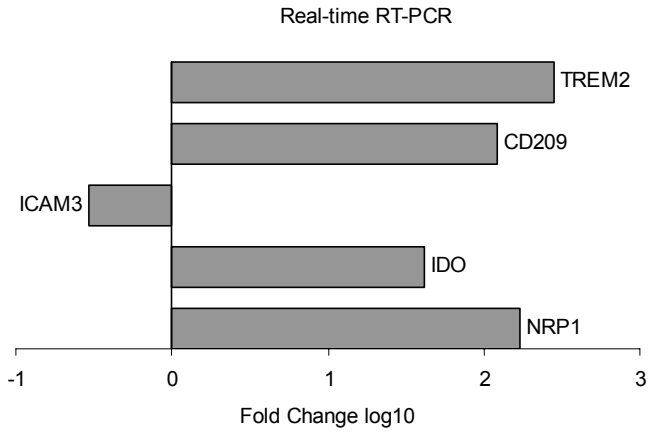

Figure 6. Results from real-time PCR analyses for TREM2, CD209, ICAM3, IDO and NRP-1 showing fold change of mRNA expression in decidual compared to blood CD14 positive cells.

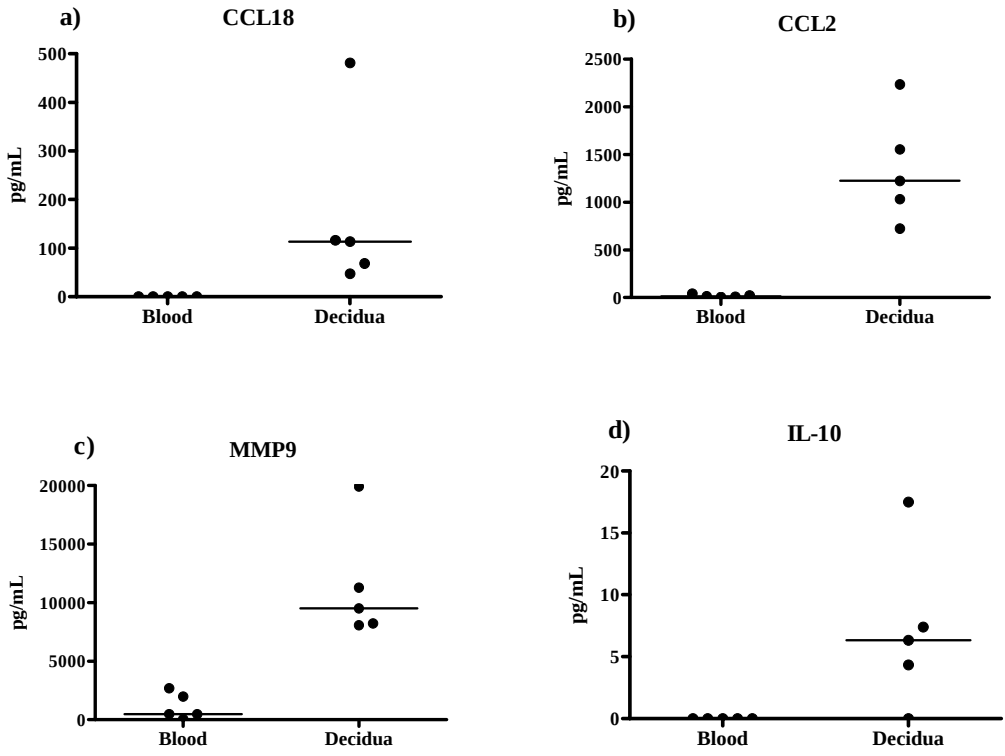

Figure 7. Protein concentrations of (a) CCL18 as measured with ELISA, (b) CCL2, (c) MMP9 and (d) IL-10 as measured by multiplex bead array in 24-h culture supernatants of CD14 positive monocytes/macrophages isolated from 5 subjects. Error bars show median values and $p<0.01$ for differences between decidual and blood CD14 positive cells for all proteins, using Wilcoxon's signed ranked test.

Decidual macrophages are widely but not evenly distributed throughout the tissue. They are sparse in decidua parietalis but localized in high numbers in the decidua basalis, just underlying the site of trophoblast invasion (Bulmer and Sunderland, 1984). As they are the predominant leukocyte close to the cytotrophoblast, functions in regulating placental formation and invasion would be appropriate, as suggested before (Mor and Abrahams, 2003; Kämmerer, 2005). A large group of genes up-regulated in the microarray was associated with tissue remodelling. This group included different collagens and genes related to angiogenesis and depletion of apoptotic cells, such as growth arrest-specific (GAS)-6 and protein S alpha (PROS1) (Hafizi and Dahlback, 2006). Fibronectin increases apoptotic clearance of cells coated with complement component 1 q subcomponent (C1Q) (Bing et al., 1982), and both were simultaneously regulated in this array. Although the up-regulated MMP-9 is considered a pro-inflammatory chemokine, it also cleaves denatured collagens and type IV collagens in basement membranes, thereby contributing to remodelling of the extracellular matrix and migration of immune cells (Opdenakker et al., 2001). MMP-9 promotes trophoblast invasion via these functions (reviewed by (Staun-Ram and Shalev, 2005)). In tissue fluids, MMPs are known to be regulated by alpha-2-macroglobulin (A2M), which was also up-regulated in decidual macrophages. A2M seems to be involved in trophoblast invasion (Larin et al., 2002; Tayade et al., 2005) and in mice, the placental over-invasiveness and subsequent intrauterine fetal death seen in IL-11 knock-outs, was suggested to be due to defective production of A2M (Bao et al., 2006). IGF-I and other members of the IGF family are important during pregnancy, affecting fetal nutrition and size (reviewed by (Nayak and Giudice, 2003). IGF-I is also an angiogenic factor and is induced in macrophages primed to develop into an alternatively activated phenotype (Kodelja et al., 1997; Martinez et al., 2006), in agreement with the angiogenetic characteristics of this cell type.

Although differentiated macrophages in general are considered to be non-proliferating, the ability of macrophages to proliferate locally in tissues has been suggested before (Cheung and Hamilton, 1992; Bischof et al., 2000). Our results show many regulated genes related to cell cycle functions and when analysed from the perspective of cell proliferation, the net effect of up- and down-regulated genes was in favour of proliferation. An up-regulation of cell proliferation associated genes was also shown in M1 primed as well as M2 primed macrophages compared to macrophage colony stimulating factor (M-CSF) cultured cells (Martinez et al., 2006).

Metabolism and transport associated genes were commonly up- or down- regulated in decidual macrophages. This is in line with results from differentiated and primed human blood macrophages (Martinez et al., 2006) and might simply mirror the functional activity of the cells. Further, solute carrier family 2 member 1 (SLC2A1;Glut-1) which was up-regulated in decidual macrophages has, together with other glucose transporters, been suggested to have invasive and proliferative functions in the establishment of the placenta in early pregnancy (Korgun et al., 2005).

There has been some discussion whether antigen presenting cells in the decidua are mostly dendritic cells or macrophages (reviewed by (Kämmerer, 2005). In early pregnancy decidua, Kämmerer *et al.* (2003) found a large population of cells expressing CD209 which phenotypically resembled immature dendritic cells and were highly proliferative, in line with our findings. Immunohistological studies in decidua showed a $CD14^+$ cell type being HLA-DR^+, $CD68^+$ and $CD209^+$ but negative in CD1a, CD83 and CD86 (Soilleux et al., 2002). These results were confirmed by flow cytometry on isolated term decidual CD14 positive

cells (Heikkinen et al., 2003), showing expression of HLA-DR but low expression of the co-stimulatory molecules CD80 and CD86, suggesting the potential to induce anergy rather than activate naïve T lymphocytes. The CD14 positive cells were not able to differentiate into dendritic cells when cultured in GM-CSF and IL-4. Gardner and Moffett (2003) conclude from flow cytometry studies that CD14 positive cells in early human decidua can be divided into two fractions, dendritic cells being substantially less frequent than macrophages. Hence, these studies suggest cells related to dendritic cells are present in decidua but the main part of CD14 positive cells most likely resemble macrophages.

Mantovani *et al.* (2004) suggests a macrophage nomenclature where M1 represents classically activated macrophages and M2 a, b and c, represent macrophages activated to other phenotypes by defined stimuli: IL-4/IL-13 (M2a), immune complexes in concert with LPS (M2b) and IL-10 (M2c). However, there are many examples of macrophage populations coupled to certain diseases or conditions, exhibiting polarized M2-like features with their own distinct profiles. For example, tumour associated macrophages (TAMs) are macrophages with key properties of M2, or alternatively activated macrophages that enhance tumour infiltration, proliferation and angiogenesis (reviewed by (Lewis and Pollard, 2006)), thus resembling the fetomaternal situation where trophoblast invasion and vascularization need to be permitted. In tumour progression, invasion is enhanced by the expression of hepatocyte growth factor (HGF) which is abundantly produced, especially in tumours with a poor prognosis (Matsumoto and Nakamura, 2006). In line with this, HGF is produced in placenta in murine and human pregnancy, with a clear association between low levels and pregnancy complications (Schmidt et al., 1995; Uehara et al., 1995; Furugori et al., 1997; Aoki et al., 1998; Baykal et al., 2005). Interestingly, the gene expression profile of human blood monocytes cultured with HGF (Rutella et al., 2006) shows many similarities with regulated genes in our decidual CD14 positive cells, e.g. genes involved in immunity as well as in tissue remodelling and cell proliferation. HGF is detected in the CD14 positive cells in the present study but with no significant difference between decidual and blood cells, in line with previous results where placental cells (Clark et al., 1996; Kauma et al., 1997), not macrophages, were shown to be the major producers *in utero* for this cytokine. Thus, macrophage polarization can take several routes within the M2 phenotype. The precise requirements for decidual macrophage polarization remains to be settled, but local factors such as IL-4, IL-10, IL-13, HGF and HLA-G are likely to have important roles, as well as hormonal influence by, e.g. progesterone and oestrogen. In addition, interactions with trophoblast cells may control macrophage polarization. Phagocytosis of human trophoblasts by macrophage cell lines increased their secretion of IDO and IL-10 but decreased their secretion of IL-1β, indicating possible anti-inflammatory interactions (Abumaree et al., 2006). Similarly, in a co-culture system, trophoblasts decreased low-dose LPS-induced inflammatory reactions in monocytes (Fest et al., 2007). In contrast, culture with trophoblasts also induced monocyte migration together with the secretion of inflammatory cytokines from both monocytes and trophoblasts.

The combination of up-regulated genes in decidual macrophages, with both pro-inflammatory or M1 associated genes as well as genes related to immune suppression or M2 macrophages, mirror the complexity of macrophage polarization. Since mainly mRNA was analysed in this study, proteins are not necessarily expressed for all genes. The gene expression pattern may therefore reflect the macrophages' proposed ability to rapidly re-polarize upon changes in the microenvironment (Stout and Suttles, 2004; Takabayshi et al.,

2006). In the context of pregnancy, the different roles for decidual macrophages probably include both suppressive/alternative and inflammatory/classical functions. Some of the differences seen between blood monocytes and decidual macrophages could be caused merely by a differentiation of the cells when leaving the circulation and entering the uterine tissue. However, Martinez *et al.* (2006) showed that macrophage differentiation is mostly associated with the regulation of nuclear factors; macrophage polarization, on the other hand, mostly affects the expression of membrane receptors and extracellular proteins. Our regulated genes include a number of nuclear factors but the major part represents receptors and secreted proteins, indicating a distinct polarization of decidual macrophages. Further, even if the differences seen were connected mainly to differentiation, the pattern of up-regulated genes would still give a picture of the characteristics of the macrophage population. One could speculate on using other types of macrophages as a control population, preferably cells of tissue origin. Alveolar macrophages would be interesting because of their somewhat similar characteristics (Prieto et al., 2000; Soilleux et al., 2002; Pouliot et al., 2005), but the benefit from using cells from the same individual would undoubtedly be lost. Another possibility is of course to culture blood mononuclear cells from the same donor to grow into macrophages, or to stimulate the cells with LPS before cell lysing. However, any of these procedures would introduce methodological differences between the two cell types, since decidual macrophages are preferentially investigated unstimulated immediately after isolation. It is possible to analyse our gene expression data compared to studies done by groups using the same Affymetrix method, for example studies on LPS- or IL-10 stimulated monocytes (Antoniv et al., 2005; Park-Min et al., 2005). Such comparisons were only roughly done in the present study.

For microarray analyses, two different methods were used for separation of CD14 positive cells. Separation with magnetic beads has a lower physical impact on the cells; FACSAria sorting results in a higher purity of cell populations. To handle these differences, the statistical criteria for regulated genes were set firstly according to the two subjects with high-purity flow cytometry sorted cells. Genes showing a two-fold up- or down-regulation and statistical significance within these two subjects (Figure 8) were considered further and classified as differentially regulated if they met the same criteria within cells from the five magnetic bead sorted subjects. These highly stringent criteria revealed a lower number of regulated genes than if all seven subjects were considered as one group and statistical requirements consequently became lower. For a broader view, the cluster picture in Figure 8 shows similarities between the seven patients for a larger number of genes.

Due to the multiple and diverse functions of genes, it is difficult to indisputably place a molecule in a certain group of functions. Localization and microenvironment must be taken into consideration for a correct classification. MMP9, for example, would in another context be classified as pro-inflammatory, but was considered important here for tissue remodelling due to its potential role in trophoblast invasion. CCL2 is another example of a gene with possible dual effects; it was classified here as pro-inflammatory because cell recruitment is generally connected to inflammation, however the macrophages can probably be polarized into either M1 or M2, depending on environmental factors. Furthermore, some genes previously connected to macrophages, such as benzodiazepine receptor, could not, due to very sparse information, be reliably placed into a certain group of functions and were therefore not further considered.

A mapping of expressed genes in decidual macrophages is of great interest, not only for understanding of the normal physiology of pregnancy, but also for understanding the mechanisms behind pathological conditions and for finding possible targets for therapy or disease monitoring. A number of genes differentially regulated in this array were previously found to be dys-regulated in or connected to pregnancy complications such as preeclampsia (MR (Burk et al., 2001), CCL2 (Burk et al., 2001), IGF-1 (Nayak and Giudice, 2003), secreted phosphoprotein (SPP)-1 (Gabinskaya et al., 1998), MMP-9 (Coolman et al., 2007)), preterm labour (CCL18 (Marvin et al., 2002)) and intrauterine growth restriction (IGF-1 (Nayak and Giudice, 2003)). In addition, outside the field of pregnancy, knowledge on the pluripotent roles of non-classical macrophages should have implications for future understanding of several diseases as well as in the search for candidate targets of immune modulation.

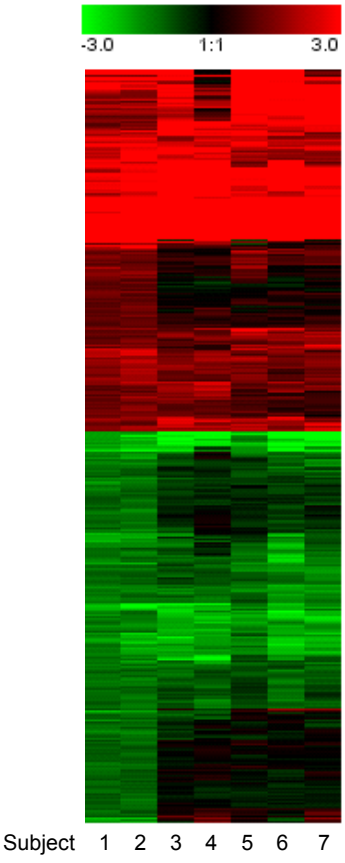

Figure 8. Cluster figure of the gene signature of 408 genes regulated in decidual compared to blood macrophages, showing all seven subjects. Requirements for regulation was two-fold up-/down-regulation and a *p*-value <0.05 in the two subjects with highly pure cell populations (subjects 1 and 2).

CYTOKINE SECRETION FROM TERM PREGNANCY DECIDUA BEFORE AND AFTER LABOUR (PAPER IV)

Secretion of IFN-γ, IL-4, IL-10, TNF and TGF-β

Decidual mononuclear cells were found to secrete IFN-γ, IL-4, IL-10, TNF and TGF-β in tissues taken before the onset of labour and in tissues collected after vaginal delivery (Figure 9). No significant differences in the number of cytokine secreting cells were seen between decidual cells before and after labour. Our results fully corroborate a previous gene expression study on choriodecidual tissues before and after labour, for all five cytokines (Marvin et al., 2002). Further, IFN-γ, IL-10 and TGF-β, but not IL-4, were detected in cell supernatants from unstimulated decidual mononuclear cells in tissues collected before the onset of labour as measured with ELISA (Wilczynski et al., 2002). No tissues after the onset of labour were analysed. The finding that TNF does not increase with labour is surprising since this cytokine induces prostaglandin secretion, which is known to be important in labour induction. mRNA levels in term decidua show up-regulation of TNF in association with labour (Dudley et al., 1996a) or unchanged levels (Vives et al., 1999; Osman et al., 2003). Decidual tissues after the onset of labour stain more intensely for TNF than tissues not in labour using immunohistochemistry (Dudley et al., 1996a; Young et al., 2002). IFN-γ was expressed at higher mRNA levels in decidua collected before labour than after (Jones et al., 1997; Vives et al., 1999); protein levels were higher (Jones et al., 1997) or lower (Veith and Rice, 1999) in the same tissues before the onset of labour. An increase in IFN-γ with labour would be appropriate in cytokine-mediated pregnancy termination, but the results above, by us and others, question its role. Further, IFN-γ has been shown to suppress the secretion of PGE_2 in uterine tissues (Hertelendy et al., 2002; Hanna et al., 2004). IL-4 was detected by immunohistochemistry and at mRNA levels in term decidua but with no differences associated with labour (de Moraes-Pinto et al., 1997). Its hypothetical role in deviating the immune system towards a Th1-like situation at parturition (Wegmann et al., 1993), by reduced expression, is therefore uncertain. On the other hand, IL-4 decreased the LPS-induced PGE_2 increase in cultured term decidual cells (Simhan et al., 2004) suggesting a possible role in regulating parturition.

Protein levels of IL-10 showed no difference in decidua before and after labour (Jones et al., 1997) while mRNA levels were unchanged (Jones et al., 1997) or decreased with labour induction (Vives et al., 1999). Protein levels in trophoblasts have been shown to decrease with gestational age but with no further decline at labour (Hanna et al., 2000), suggesting that the logical loss of the suppressive effects of IL-10 at the onset of labour may be gradual during pregnancy instead of radically changing just before pregnancy termination. Similar mechanisms may be shared by TGF-β. Immunohistochemistry showed less staining for TGF-β signalling molecules in chorion tissue throughout pregnancy (Xuan et al., 2007). As TGF-β reduced the production of prostaglandins in gestational tissues (Berchuck et al., 1989; Bry and Hallman, 1992), a term withdrawal would suit well with labour functions

Some significant correlations between the numbers of cells secreting the different cytokines were found within the groups in our study. Interestingly, correlations were weaker, in tissues after labour compared to tissues collected before the onset of labour (Table VI). Further, 3 of 4 combinations analysed where significant correlations were not found, include

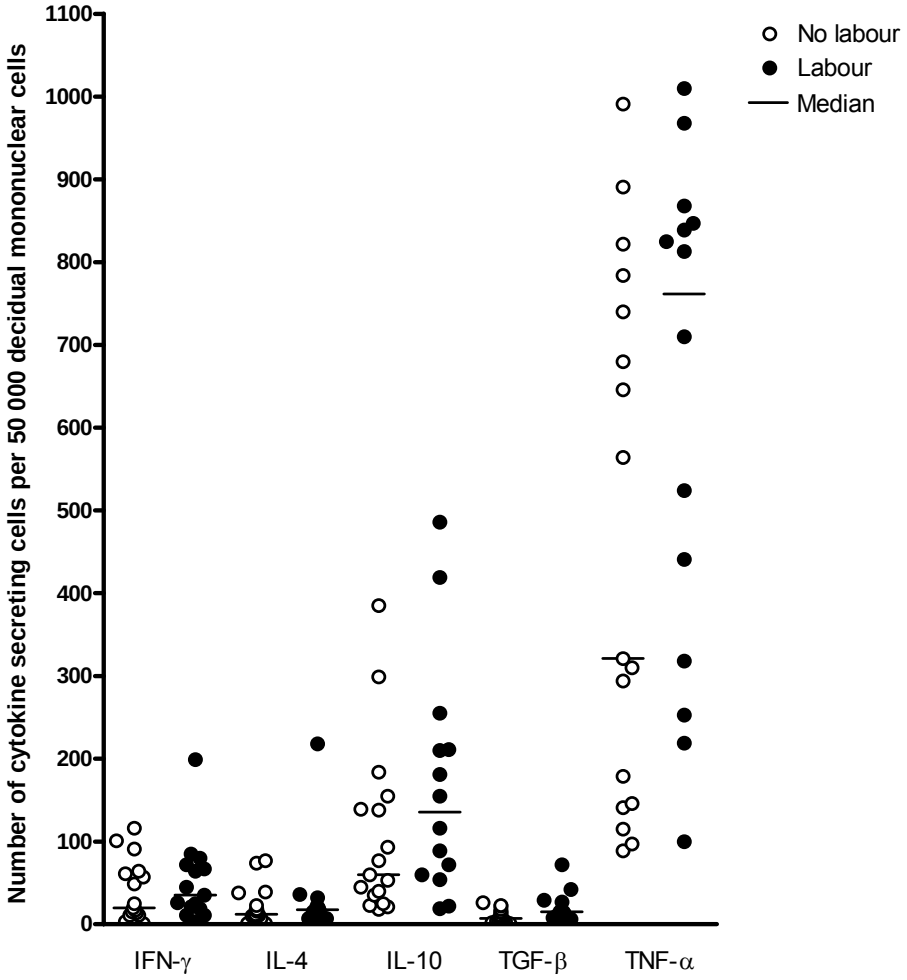

Figure 9. Number of cytokine secreting mononuclear cells in term pregnancy decidual tissues collected at caesarean sections before the onset of labour (open dots) and after spontaneous vaginal deliveries (filled dots). No significant differences in cytokine secretion with or without labour were found using the Mann–Whitney *U*-test.

TNF, indicating a possible regulatory role for this cytokine even in the absence of changes in numbers of other cytokine secreting cells. The significant correlations found between almost all cytokines in decidual mononuclear cells before labour, suggest an immune homeostasis that is disrupted at the onset of labour when correlations decline. A less synchronized pattern of cytokine secretion would allow single cytokines, such as TNF, to have effects without actual changes in secreted levels. The possible change in balance is not due to changes in Th1/Th2-like balance, as indicated by data on ratios where no differences were seen with or without labour.

Table VI. Cytokine correlations

Correlation	No labour (*n*=17)	Labour (*n*=15)
IFN-γ vs IL-4	0.82**	0.65*
IFN-γ vs IL-10	0.62**	0.15
IFN-γ vs TGF-β	0.65**	0.60*
IFN-γ vs TNF-α	0.56*	–0.26
IL-4 vs IL-10	0.56*	0.62*
IL-4 vs TGF-β	0.76**	0.71**
IL-4 vs TNF-α	0.47	0.16
IL-10 vs TGF-β	0.52*	0.67*
IL-10 vs TNF-α	0.76**	0.64*
TGF-β vs TNF-α	0.68**	0.28

Correlations between the number of decidual mononuclear cells secreting different cytokines with or without labour. *p<0.05, **p<0.01 from Spearman's rank correlation test.

The ELISPOT method does not give any information on concentrations of secreted cytokines, and therefore changes in cytokine secretion might be present even in the absence of differences in numbers of cytokine secreting cells before and after labour. From our own previous experience (unpublished observations) and other studies (Kabilan et al., 1990; Tanguay and Killion, 1994), prominent differences in cytokine secretion are also mirrored by the number of cytokine secreting cells. The effect of cytokines is also regulated by other mechanisms than the amounts secreted, i.e. by regulating degrading enzymes or the expression of agonistic, antagonistic or decoy receptors. In pregnancy, prostaglandins are regulated by such mechanisms throughout gestation (reviewed by (Olson, 2003) and IFN-γ receptors were shown to be reduced in term placental tissues after the onset of labour, in line with the simultaneous demonstration of IFN-γ as an inhibitor of the PG-regulating enzyme COX-2 (Hanna et al., 2004). Differences in receptor expression may explain the differences in function seen for different cytokines in different tissues. IL-10 had opposite effects in term chorionic compared to amnion tissues (Mitchell et al., 2004) and IL-4 stimulated prostaglandin synthesis in term choriodecidual cells (Adamson et al., 1993) is quite the opposite of its normally anti-inflammatory functions in cell-mediated immunity and also in contrast to other studies (Simhan et al., 2004).

ELISPOT data indicate leukocytes in decidua basalis are not the main regulators of possible labour-associated changes in the cytokine milieu of the uterus. Placental trophoblasts, decidual stromal cells and fetal membranes contribute to the production of cytokines and other regulatory proteins (reviewed by (Bowen et al., 2002) and changes in these tissues may regulate labour induction. Otherwise one could speculate that the lack of differences seen in term decidua reflects the minor importance of local immunological effects in term compared to early pregnancy. The systemic interface with maternal blood bathing placental villi is large at the end of gestation and changes in cytokine expression in blood may also influence labour induction.

To eliminate contaminating decidual stromal cells, CD9 depletion of the decidual mononuclear cells was carried out. After designing the study, CD9 was reported to be

expressed on subpopulations of T cells (Kobayashi et al., 2004) and on decidual NK cells (Koopman et al., 2003; Eriksson et al., 2004). Therefore, the negative selection of CD9 expressing stromal cells could also have eliminated CD9-expressing leukocytes. Flow cytometry analyses performed on decidual mononuclear cells before and after CD9-depletion revealed that most CD9 expressing cells in our preparations were CD45 negative and CD9 was expressed only on 0.3–1.2% of all decidual leukocytes. The measured low expression of CD9 in decidual leukocytes and the fact that tissues obtained before and after labour were treated likewise, makes it unlikely that the elimination of CD9 could have influenced the results in a substantial way.

Measuring of COX-2 and mPGES-1 for confirmation of the study design

To investigate the method of using tissues from caesarean sections and tissues obtained after vaginal delivery as representatives of term pregnancy before and after the onset of labour, respectively, expression of enzymes crucial for prostaglandin synthesis were analysed in placental biopsies by RT-PCR. In line with the reported increase of COX-2 (Slater et al., 1999; Hanna et al., 2006; Astle et al., 2007; Choi et al., 2007) and mPGES-1 (Alfaidy et al., 2003; Astle et al., 2007) in gestational tissues in relation to labour, COX-2 expression was higher in the five placenta biopsies obtained after vaginal delivery than in the seven biopsies obtained at caesarean section (Figure 10). mPGES-1 mRNA was also higher in samples obtained after labour (Figure 11), although not to the same extent as for COX-2. This is in line with a previous study where PGE_2 protein secretion was shown to not necessarily be preceded by a co-ordinated up-regulation of mPGES and COX-2 (Premyslova et al., 2006). This could also explain the presence of more studies showing up-regulation of COX-2 than of mPGES in labour. Our results support the use of tissues from caesarean and vaginal deliveries for research on mechanisms of labour induction.

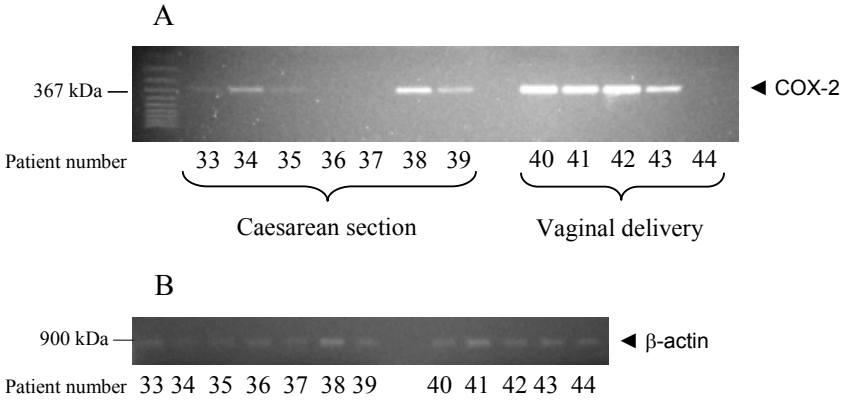

Figure 10. (A) COX-2 mRNA expression in term pregnancy trophoblast cells collected at elective caesarean sections before the onset of labour (left group) and after spontaneous vaginal deliveries (right group). (B) shows the level of β-actin expressed constitutively.

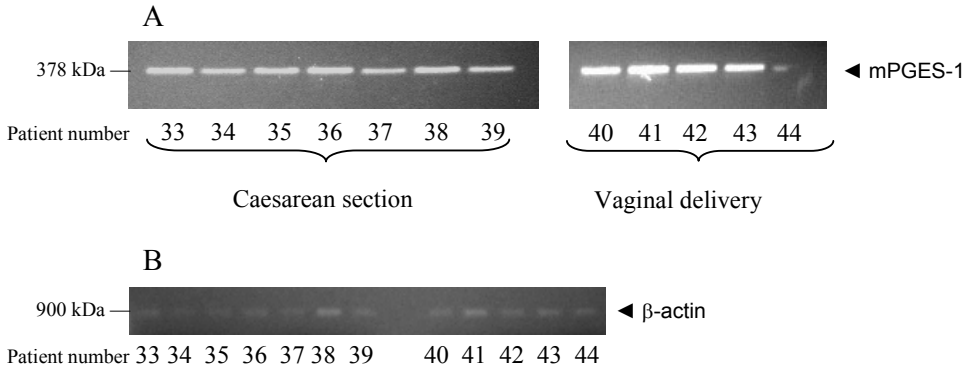

Figure 11. (A) mPGES-1 mRNA expression in term pregnancy trophoblast cells collected at elective caesarean sections before the onset of labour (left group) and after spontaneous vaginal deliveries (right group). (B) shows the level of β-actin expressed constitutively.

GENERAL METHODOLOGICAL ASPECTS

Working with human material, the collection and handling of tissues, as well as the separation of cells, are central to enable high-quality research. Purity of cells is an important factor to consider. In papers I–III, decidual tissue was macroscopically separated from placental and fetal tissues. First trimester placenta is quite distinct in its appearance and only minor fragments should contaminate the decidual tissue collected. Fetal tissues are also mainly distinct but minor contaminations cannot be excluded. In paper IV, decidual tissue was scraped from the maternal side of the placenta and the risk of going too deep and including trophoblast tissue is apparent. However, trophoblasts are hard to collect from solid tissue by mechanical separation as used in papers I–IV; instead enzymatic disaggregation is needed to obtain single cell trophoblasts. Hence, trophoblast fragments would be mainly discarded at the Ficoll separation. Decidual stromal cells could also contaminate the preparations but their fragile characteristics increase the chance that they are discarded during the separation steps. In line with this, flow cytometry results show that stromal or trophoblast cell contamination in our preparations was fairly low (see Table III). The risk of tissue contamination by blood cells should also be noted, although this was avoided by removing clots and by careful rinsing of the tissues. In papers I–III, the comparison of decidual cells and blood cells reduced the possible impact of blood cell contamination on the results. In paper IV, blood contamination would be similar in the decidual tissues from both groups, thus potential differences would not be due to blood contamination. The risk of contaminating cells in CD14 and CD56 separations of decidua using the MACS technique was partly discussed in previous sections. It is important to remember that cells other than those expected could contribute to the results, although the enriched cell type in each cell suspension should be the most probable source of cytokine secretions. FACSAria separated cells used for the microarray showed satisfactory purity.

It is impossible to say whether the cytokine secretions or mRNA expressions detected in decidual cells represent the *in vivo* situation during gestation or if changes were caused by the termination of pregnancy. Mechanical stress during early pregnancy terminations or caesarean sections may indeed affect the cells; changes due to dissociation of the placenta from the uterine wall after vaginal delivery were at least induced naturally. During the separation procedure, cells might have been affected by the mechanical disaggregation, which also adds a significant, but unavoidable, difference in treatment between decidual and blood cells. In trimester one terminations, the prostaglandin analogue misoprostol, was given to some of the patients in these studies before vacuum aspiration. Studies on human early pregnancy decidua showed no increase in the numbers of macrophages, neutrophils, NK cells or expression of steroid receptors after administration of PGE_2 analogue for 3 h (Milne et al., 2005). Cytokine or chemokine expression was not analysed but might have been affected. In paper I, data on misoprostol administration is unfortunately not available but in paper II, when comparing data from subjects receiving misoprostol with those who did not, no significant differences in cytokine secretion were found (data not shown). In paper IV, possible differences due to misoprostol administration would automatically be excluded by the statistical criteria requiring the same expression pattern in all subjects. Nevertheless, the optimal study design would be to include only subjects with no misoprostol administration before surgery.

The ELISPOT method used in papers I, II and IV is very sensitive but cannot be used for quantification of amounts of cytokines secreted, as discussed earlier. Another drawback is that data analysis is also subjective when defining spots versus artefacts. Using light

microscopy for spot counting, as in paper I, requires blinded analyses of the different wells and all plates counted by the same person, which was also done. Both these problems are circumvented by the usage of an ELISPOT reader system as in papers II and IV; the criteria for counting were set up before analysis and used for all plates.

The enormous generation of data using microarray technology is somewhat hard to handle, but looked upon as the beginning of a story, the possibilities and different points of view are close to endless. Mechanisms and molecules of potential interest for further investigation can be found and, as described earlier, comparison of results from totally different studies can be made by analysis of raw data. However, conclusions drawn from microarray data are never the only truth. It is indeed biased not only by the statistical criteria set up, but also by the interests and prior knowledge of the researcher.

GENERAL DISCUSSION AND CONCLUSIONS

In early gestation, proper interactions between the invading fetal trophoblasts and the maternal decidual cells are without doubt crucial for the establishment of pregnancy. Several functions, such as a well co-ordinated expression of paternal antigens, regulate these interactions. Trophoblast invasion must be permitted but at the same time prevented from going too deep into the uterine mucosa. This requires the expression of signals promoting tissue degrading, but also of factors suppressing these signals and promoting tissue repair and angiogenesis. Further, the fetus must be protected from rejection by the maternal immune system, requiring immunosuppressive signals. Decidual mononuclear cells spontaneously secrete IFN-γ, IL-4 and IL-10 (Figure 12), secretions derived from NK cells, macrophages and perhaps other sources. IFN-γ is important for spiral artery modulation and possibly other aspects of tissue remodelling and invasion but may be detrimental to the fetus if stimulating Th1-like cell mediated immunity. The co-expression of IL-4 may suppress such harmful functions. IL-10 may also act as an immunosuppressor and thereby protect the fetus but may also act as an immunostimulator by, for example, activating NK cells. NK cells, in turn, could be cytotoxic against trophoblasts to avoid over-invasion. Both IL-4 and IL-10 may, together with other local factors such as different hormones, growth factors and expressed antigens, deviate macrophages to adopt specialized characteristics within the M2 or alternatively activated polarization pathway. Decidual macrophages express genes for regulating trophoblast invasion (MMP-9, A2M), angiogenesis important for placental development (IGF-1, GAS-6, PROS1) and clearance of apoptotic cells (FN1, C1Q). Fragments of phagocytosed cells are presented on the macrophage MHC class II molecules but may, due to the low expression of co-stimulatory molecules and a suitable microenvironment, induce anergy or suppressive deviation of present T lymphocytes instead of activation. The macrophage expression of immunoregulatory receptors (MRC-1, CD9, TREM-2, NRP-1, CD209) may also stimulate macrophages to a balanced antigen presentation, production of anti-inflammatory factors or proper interactions with other immune cells. Macrophage secreted soluble molecules (CCL18, IDO, PGDS, CCL-2) could also affect recruitment and deviation of T lymphocytes and recruitment of more macrophages.

Term decidual mononuclear cells spontaneously secrete IFN-γ, TNF, IL-4, IL-10, and TGF-β. Hypothetically, IFN-γ and TNF would be increased *in utero* at the induction of labour, driving immune responses towards a Th1-like situation and causing inflammatory reactions such as prostaglandin production. Accordingly, IL-4, IL-10 and TGF-β would be withdrawn. Even if such a cytokine deviation exists in labour induction, decidual mononuclear cells are not the main source of these changes since no differences were seen before and after labour. Placental and fetal membranes, as well as cells in the maternal systemic circulation, may instead contribute to a possible shift in immune balance prior to pregnancy termination.

In conclusion, decidual leukocytes, including NK cells and macrophages, are potential producers of both Th1-like/pro-inflammatory and Th2-like/anti-inflammatory cytokines, in early pregnancy as well as at parturition. First trimester decidual macrophages are of a specialized phenotype with effector functions contributing to a proper invasion of the placenta and to immunological protection of the semi-allogeneic fetus. This thesis adds new knowledge on local immune balance during normal human pregnancy, however, the clinical implications of the data needs to be clarified.

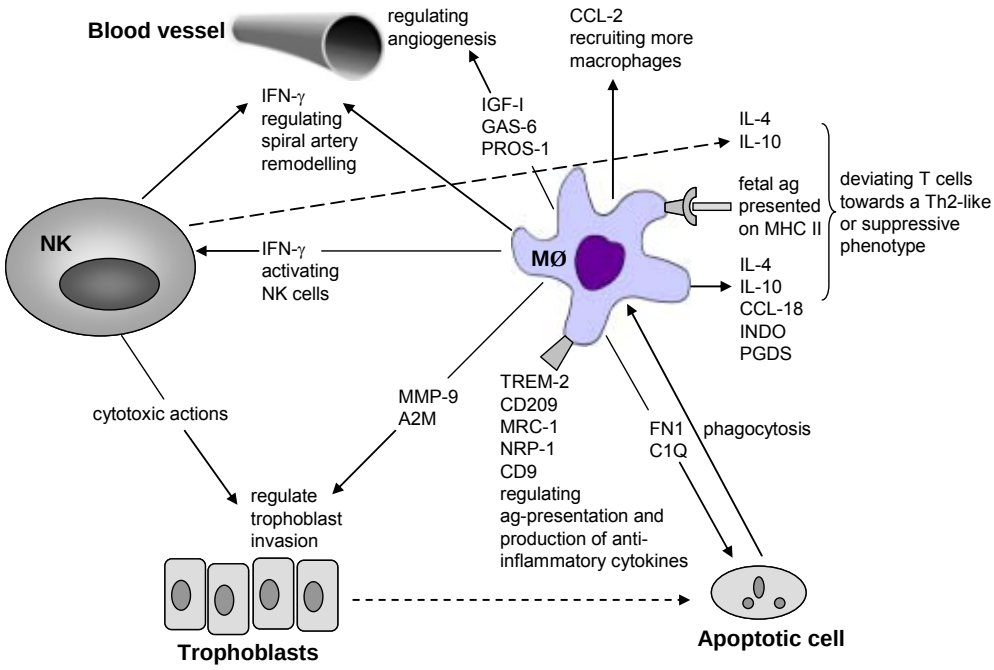

Figure 12. Possible functions of decidual NK cells and macrophages in early human pregnancy, based on the findings in this thesis.

FUTURE PERSPECTIVES

In early pregnancy, further studies on local tissues would be of interest, not necessarily with comparisons with blood cells because of the increasing data on the specialized local milieu in the uterus and the absence of correlation to systemic changes. Rather, it would be interesting to focus on the interplay between different local cell types; leukocytes, trophoblasts and perhaps stromal cells. In late pregnancy, on the other hand, the systemic aspects would be of more interest, for comparisons with local changes and to study the influence of blood cells on local tissues and vice versa.

The gene expression analysis of decidual macrophages in paper III presents many ideas for future studies. In particular, the functional properties of TREM-2 expression on macrophages would be of interest since this molecule was not previously connected with pregnancy but indeed has potential regulatory functions. It would also be interesting to investigate the factors responsible for macrophage polarization, for example the possible role of HGF, based on the similarities between HGF-cultured and decidual macrophages. For TREM-2 and several other molecules regulated in decidual macrophages, such as A2M and PGDS, it would be interesting to compare their expression in normal pregnancy with pathological pregnancies. In early pregnancy, decidua from missed abortions or repeated spontaneous abortions is of great interest. For investigations of conditions late in gestation, such as preterm labour and preeclampsia, characterization of term decidual macrophages would first be needed.

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