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Regulatory T cells in human pregnancy

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To me, myself and I

Abstract

During pregnancy, fetal tolerance has to be achieved without compromising the immune integrity of the mother. CD4⁺CD25^{high}Foxp3⁺ regulatory cells (Tregs) have received vast attention as key players in immune regulation. However, the identification of human Tregs is complicated by their similarity to activated non-suppressive T cells. The general aim of this thesis was to determine the antigen specificity, frequency, phenotype and function of Tregs in first to second trimester healthy and severe early-onset preeclamptic human pregnancy. Regarding antigen specificity, we observed that in healthy pregnant women, Tregs suppressed both T_H1 and T_H2 reactions when stimulated with paternal alloantigens but only T_H1, not T_H2 reactions when stimulated with unrelated alloantigens. Hence, circulating paternal-specific Tregs seem to be present during pregnancy. Further, by strictly defining typical Tregs (CD4^{dim}CD25^{high}) using flow cytometry, we could show that as a whole, the Treg population was reduced already during first trimester pregnancy as compared with non-pregnant women. This was in contrast to several previous studies and the discrepancy was most likely due to the presence of activated non-suppressive cells in pregnant women, showing similarities to the suppressive Tregs. Although deserving confirmation in a larger sample, severe early-onset preeclampsia did not seem to be associated with alterations in the circulating Treg population. The circulating Treg population was controlled by hormones which, alike pregnancy, reduced the frequency of Foxp3 expressing cells. Yet, *in vitro*, pregnancy Tregs were highly suppressive of pro-inflammatory cytokine secretion and showed an enhanced capability of secreting immune modulatory cytokines such as IL-4 and IL-10, as well as IL-17, indicating an increased plasticity of pregnancy Tregs. At the fetal-maternal interface during early pregnancy, Tregs, showing an enhanced suppressive and proliferating phenotype, were enriched as compared with blood. Further, CCR6⁻ T_H1 cells, with a presumed moderate T_H1 activity were enhanced, whereas pro-inflammatory T_H17 and CCR6⁺ T_H1 cells were fewer as compared with blood. This thesis adds to and extends the view of Tregs as key players in immune regulation during pregnancy. In decidua, typical Tregs seem to have an important role in immune suppression whereas systemically, Tregs are under hormonal control and are numerically suppressed during pregnancy. Further, circulating pregnancy Tregs show reduced expression of Foxp3 and an increased degree of cytokine secretion and thereby also possibly plasticity. This would ensure systemic defense against infections with simultaneous tolerance at the fetal-maternal interface during pregnancy.

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Sammanfattning

Graviditet är ett immunologiskt utmanande tillstånd. Detta beror på att fostret till hälften genetiskt härstammar från pappan och därmed är delvis främmande (semi-allogent) för mammans immunförsvar. För att fostret ska tolereras under graviditeten måste därför mammans immunförsvar dämpas utan att infektionsförsvaret försämras alltför mycket. Generellt verkar det ske en hämning av det cellmedierade, T hjälparcell typ 1 (T_H1) drivande immunförsvaret, medan T_H2 immunitet samt det förvärvade immunsystemet tycks förstärkas under en graviditet. Regulatoriska T celler är en del av immunförsvaret och finns där för att reglera och dämpa skadliga immunreaktioner. En subgrupp av dessa celler med en särskild fenotyp (utseende) och funktion är fokuset för denna avhandling. Dessa tillhör T hjälparcells-familjen och bär på sin yta CD4, höga nivåer av IL-2 receptorns α -kedja (CD25) samt det intracellulära proteinet forkhead box p3 (Foxp3). Således benämns dessa celler $CD4^+CD25^{high}Foxp3^+$ regulatoriska T celler (Treg) hos människa. Treg har i djurmodeller visat sig ha en avgörande betydelse för fostertolerans, något som inte helt bekräftats hos människa. Tregs tros även ha betydelse för graviditetskomplikationen preeklampsi. Ett stort problem med forskning kring Treg, särskilt hos människa, är att det inte finns några absoluta markörer. Treg liknar i många avseenden aktiverade effektor T celler vilket försvårar identifieringen av typiska Tregs med hämmande funktion.

Det övergripande syftet med de fyra delarbetena var att med hjälp av uppdaterade markörer och tekniker kartlägga förekomsten, fenotypen och funktionen hos Treg i cirkulationen (systemiskt) och i den gravida livmoderslemhinnan (deciduan). Cirkulerande Tregs undersöktes vid första och andra trimester frisk graviditet och vid tidig och svår preeklampsk graviditet. Deciduala Tregs undersöktes i första trimestern. Totalt ingick i studierna 87 friska gravida kvinnor, 95 icke-gravida kvinnor samt 10 kvinnor med tidig och svår preeklampsi.

Syftet med arbete I var att undersöka Tregs förmåga att hämma T_H1 och T_H2 reaktioner mot paternella (från pappan) jämfört med orelaterade alloantigen. Gravida ($n=21$) och icke-gravida ($n=10$) kvinnors perifera mononukleära blodceller (PBMC) stimulerades *in vitro* med fixerade PBMC från papporna/orelaterade män, som ett surrogat för paternella/orelaterade alloantigen, i närvaro/frånvaro av autologa (kvinnans egna) Tregs. I en Enzyme-Linked Immunospot (ELISPOT) assay kunde vi visa att autologa Tregs hämmade både T_H1 och T_H2 immunitet i närvaro av paternella men bara T_H1 , inte T_H2 immunitet mot orelaterade alloantigen under graviditet. Detta indikerar att Tregs specifika för paternella alloantigen förekommer i cirkulationen hos gravida kvinnor.

Genom att utveckla en flödescytometrisk metod (arbete II) för att strikt definiera typiska Treg fann vi att friska gravida kvinnor så tidigt som i första trimestern har färre cirkulerande Tregs jämfört med icke-gravida kvinnor. Detta var i motsats till många tidigare rapporter men vi kunde visa att dessa studier troligen inkluderat en population av celler hos gravida kvinnor som, i vissa avseenden, liknar Tregs men som saknar hämmande funktion. Vidare verkade inte tidig och svår preeklampsi, vilket studerades i arbete III, vara associerat till förändrade andelar cirkulerande Tregs, en slutsats som dock behöver bekräftas i ett större patientmaterial. I arbete II kunde vi visa att den cirkulerande Treg populationen styrs av hormonerna progesteron och estradiol som, i likhet med graviditet, sänker uttrycket av Treg relaterade markörer såsom Foxp3. Typiska Tregs hos friska gravida kvinnor var dock fullt hämmande av pro-inflammatoriska reaktioner *in vitro*. Från dessa celler skedde också en mer uttalad utsöndring av immunmodulerande cytokinerna IL-4, IL-10 samt IL-17. Detta indikerar att Tregs hos gravida kvinnor uppvisar en större möjlighet till plasticitet, något som, tillsammans med den sänkta andelen cirkulerande Tregs, kan vara ett sätt att behålla ett funktionellt infektionsförsvar under graviditeten.

Arbete IV visade dock att i deciduan hos kvinnor med tidig graviditet (n=18) är andelen Tregs förhöjd jämfört med blod. Dessa deciduala Tregs visade en mycket typisk suppressiv fenotyp och genomgick celledelning, vilket kan förklara anrikningen i deciduan. I deciduan förekom dessutom en stor andel potentiellt låg-aggressiva CCR6⁺ T_H1 celler medan andelarna pro-inflammatoriska T_H17 och T_H1 CCR6⁺ celler var lägre i decidua än i blod. Dessa fynd tyder på att hög Treg aktivitet samt moderat T_H1 aktivitet är en normal del av tidig lokal immunreglering vilket tycks bidra till fostertolerans och etablering av graviditeten.

Slutsatsen från denna avhandling är att typiska Tregs verkar spela en större roll för tolerans i deciduan än i cirkulationen och att Tregs därmed regleras olika beroende på lokalisering. Denna kunskap har betydelse för möjligheten att använda Tregs i behandling av graviditetskomplikationer och infertilitet. I cirkulationen kan Tregs specifika för paternella alloantigen förekomma under graviditet. Den totala Treg populationen står dock under hormonell kontroll och under normal graviditet uppvisar de en sänkt förekomst samt en ökad förmåga till cytokin-utsöndring och plasticitet. Totalt kan detta ge ett bibehållet infektionsförsvar systemiskt med samtidig tolerans lokalt i deciduan där det initiala mötet mellan fostret och mamman sker.

Original publications

I. Jenny Mjösberg, Göran Berg, Jan Ernerudh and Christina Ekerfelt

CD4⁺CD25⁺ regulatory T cells in human pregnancy - Development of a Treg-MLC-ELISPOT suppression assay and indications of paternal specific Tregs

Immunology, 120: 456-466, 2007

II. Jenny Mjösberg, Judit Svensson, Emma Johansson, Lotta Hellström, Rosaura Casas, Maria C Jenmalm, Roland Boij, Leif Matthiesen, Jan-Ingvar Jönsson, Göran Berg and Jan Ernerudh

Systemic reduction of functionally suppressive CD4^{dim}CD25^{high}Foxp3⁺ Tregs in human second trimester pregnancy is induced by progesterone and 17 β -estradiol

Journal of Immunology, 183:759-769, 2009.

III. Jenny Mjösberg, Roland Boij, Leif Matthiesen, Maria C Jenmalm, Jan Ernerudh and Göran Berg

Circulating CD4^{dim}CD25^{high}Foxp3⁺ regulatory T cells in severe early-onset preeclampsia.

Manuscript

IV. Jenny Mjösberg, Göran Berg, Maria C Jenmalm and Jan Ernerudh

Foxp3⁺ regulatory T cells, T helper 1, T helper 2 and T helper 17 cells in human early pregnancy decidua.

Accepted for publication in Biology of Reproduction

Abbreviations

AML1	Acute myeloid leukaemia 1
APC	Antigen presenting cell
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
ChIP-chip	Chromatin immunoprecipitation (ChIP) of transcription factor-bound genomic DNA followed by microarray hybridization(chip) of IP-enriched DNA
CRTH2	Chemoattractant receptor-homologous molecule expressed on T helper 2 cells
CTLA	Cytotoxic T lymphocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DMNC	Decidual mononuclear cell
EAE	Experimental autoimmune encephalomyelitis
EBI	Epstein-Barr virus-induced gene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FBS	Fetal bovine serum
Foxp3	Forkhead box p3
FRET	Fluorescence resonance energy transfer
GATA	GATA binding protein
GITR	Glucocorticoid induced tumor necrosis factor receptor
HA	Hemagglutinin
HBSS	Hank's balanced salt solution
hCG	Human chorionic gonadotropin
HELLP	Hemolysis-Elevated Liver enzymes-Low Platelets
HLA	Human leukocyte antigen
HO	Heme oxygenase
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IPEX	Immune dysregulation Polyendocrinopathy, Enteropathy, X-linked syndrome
IUGR	Intrauterine growth restriction
KIR	Killer immunoglobulin-like receptor
LAP	Latency-associated peptide
LFA	Lymphocyte function-associated antigen
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
MAB	Monoclonal antibody
MACS	Magnetic activated cell sorting

MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLC	Mixed leukocyte culture
mRNA	Messenger RNA
MS	Multiple sclerosis
MΦ	CD14 ⁺ macrophages
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
(n)Treg	(natural) Regulatory T cell
NKT	Natural killer T (cell)
Nrp	Neuropilin
PBMC	Peripheral blood mononuclear cells
PFA	Paraformaldehyde
PIBF	Progesterone-induced blocking factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PHA	Phytohemagglutinin
PMT	Photo-multiplier
RA	Rheumatoid Arthritis
RORC	Rar-related orphan receptor C
rRNA	Ribosomal RNA
RT	Reverse transcriptase
SGA	Small for gestational age
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
STBM	Syncytiotrophoblast microparticles
TBX21	T-box 21
TCM	T cell culture medium
TCR	T cell receptor
TF	Transcription factor
TGF	Tumor growth factor
T _H	T helper cell
Tim-3	T cell Ig and mucin domain-3 cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	Regulatory T cell type 1
(u)NK	(uterine) Natural killer
WHO	World health organization

Introduction

This introduction will deal with the role for regulatory T cells in human pregnancy. Much that is known about human immune mechanisms, and particularly regulatory T cells, has originally been discovered, and later on often elegantly examined, in the murine system. However, murine and human physiologies do differ, in ways that are beyond the scope of this thesis. To this end, the focus of this introduction will be on research performed in the human system. First, the anatomy, physiology and immunology of healthy and diseased pregnancy, especially preeclamptic pregnancy, will be presented. Second, regulatory T cells will be introduced, with special emphasis on the role for regulatory T cells in pregnancy.

Pregnancy

Overview of the anatomy and physiology of healthy pregnancy

Establishment of pregnancy

One of the first critical steps in establishing the human pregnancy is implantation of the blastocyst into the decidualized uterine wall (Trundley et al. 2004; Moffett et al. 2006). Decidualization, with swelling of stromal cells, transformation of spiral arteries and enrichment of specialized leukocyte populations, is initiated already during ovulation and continues if pregnancy takes place (King 2000). In implantation, the first contact is achieved by the shell of the blastocyst, the trophectoderm, which consist of an outer syncytiotrophoblast layer and the inner cytotrophoblasts. Cytotrophoblasts are further differentiated into villous and extravillous cytotrophoblasts. During implantation, the trophoblasts invade the endometrium to form the finger-like structures called chorionic villi (Fig 1) which are lined by villous trophoblasts (Trundley et al. 2004). The extravillous trophoblasts penetrate even further and affect the maternal uterine arteries, causing them to erode and to fill the intervillous space, surrounding the chorionic villi, with maternal blood. Hence, the fetal blood stream will be in close proximity to, but not in direct contact with, the maternal blood stream, enabling nutrient exchange without raising immunologic problems. The interstitial extravillous

trophoblasts, on the other hand, migrate into the decidua basalis (simply called decidua throughout this thesis) and come in contact with maternal cells (including maternal leukocytes) (Moffett et al. 2006). These interstitial trophoblasts affect the spiral uterine arteries, causing dissociation of the smooth muscle layers and formation of a fibrinoid layer in which trophoblasts are embedded (Pijnenborg et al. 2006). The endovascular extravillous trophoblasts, migrating against the arterial blood stream, then merge with and replace the endothelial cell layer. Spiral artery remodeling is facilitated and regulated by specialized uterine natural killer cells (uNK) (Hanna et al. 2006) which are discussed more below. By this process, the spiral arteries will reduce their resistance and possibility of responding to vaso-regulatory signals, which secures the fetal blood supply (Trundley et al. 2004; Pijnenborg et al. 2006).

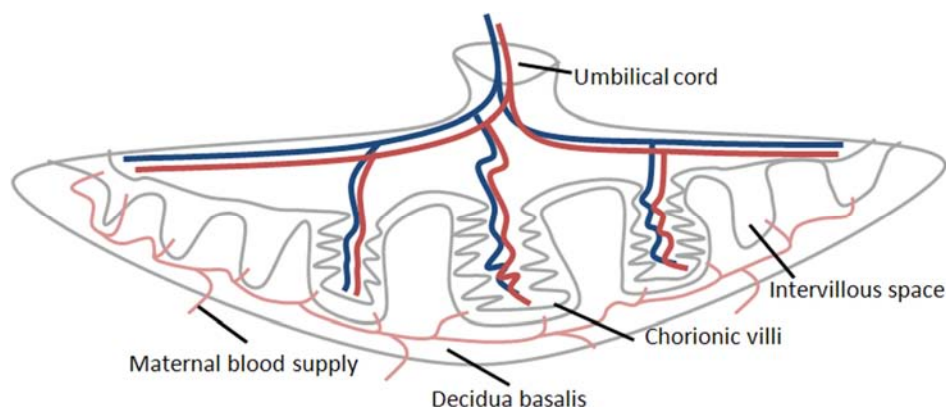


Figure 1

Simplified schematic overview of the structural organization of the fully developed placenta.

Obvious uteroplacental circulatory changes occur during pregnancy and these are echoed to the maternal systemic circulation. During normal pregnancy, there is an increase in the plasma volume with subsequent reduction of hemoglobin concentration. Further, the peripheral vascular resistance is decreased, caused partly by the vasodilatory action of estrogen and progesterone, ensuring high perfusion of uterus as well as kidneys and skin (Nisell 2008b). Hematological changes include slight reduction of thrombocytes and increase in leukocytes,

mainly neutrophils, whereas the lymphocyte numbers, as a whole, remain unchanged (Nisell 2008b).

Steroid hormone levels during pregnancy

Successful pregnancy depends on the action of steroid hormones and blocking of their effects induces abortion in mice (Stites et al. 1983; Szekeres-Bartho et al. 2001). Estradiol (referring to 17 β -estradiol throughout this thesis) and progesterone are powerful immune modulators, released primarily from the fetoplacental unit. Both these hormones increase dramatically during the course of pregnancy. Steroid hormones are normally bound to transport proteins, making them problematic to analyze (Siiteri et al. 1982; Soldin et al. 2005). Further, due to methodological problems, including the process of homogenization of the placenta, information on local hormone concentrations at the fetal-maternal interface is somewhat incongruent and unreliable. Receptors for estradiol can be found in most immune cells (Lang 2004) and receptors for progesterone seem to be up-regulated on peripheral blood cells in pregnant women (Szekeres-Bartho et al. 2001).

During the first trimester of pregnancy, serum estradiol has been estimated to 2-3 nM, increasing to 10-20 nM in the second trimester and 20-50 nM at term (O'Leary et al. 1991; Soldin et al. 2005). Non-pregnant levels are < 0.5 nM, but fluctuate during the menstrual cycle (Soldin et al. 2005; Arruvito et al. 2007). Homogenised decidual tissue in the first trimester of pregnancy show a concentration of estradiol of approximately 20 nM (Wang et al. 1994), likely increasing during the course of pregnancy.

During pregnancy, large amounts of progesterone are produced initially by the corpus luteum and subsequently by the placenta where local concentrations may exceed 10 μ M (Stites et al. 1983; Arck et al. 2007). Serum progesterone increase gradually during the course of pregnancy, starting from 3 nM in the luteal phase in the non-pregnant woman (Soldin et al. 2005), increasing to approximately 50 nM at gestational week 5, 100-200 nM in the second trimester and 200-500 nM at term (O'Leary et al. 1991; Soldin et al. 2005). Most of the immune modulatory effects of progesterone are probably mediated via progesterone-induced blocking factor (PIBF) produced by lymphocytes (Szekeres-Bartho et al. 2001).

Anatomy and physiology of complicated pregnancy – Preeclampsia

Pathogenetic mechanisms in preeclampsia – an overview

Preeclampsia, and its more progressed form eclampsia, affects 4 million women worldwide each year and is a considerable source of morbidity and mortality in both mother and child (Wim Van Lerberghe 2005). Preeclampsia has been called “the disease of theories” since great research efforts during the last decades have resulted in numerous but not very concurrent theories as to the cause of the disease. Many mechanisms are generally accepted, whereas others are not and very little is understood about the likely complex network of mechanisms that initiate and thus cause preeclampsia. In short, the etiology of preeclampsia remains unknown and neither preventive nor curing treatments are available.

It is generally thought that preeclampsia starts with a so called “stage 1” (Noris et al. 2005; Ilekis et al. 2007) in the placenta with insufficient placentation leading to reduced placental blood perfusion. Although the cause for this insufficient placentation remains unknown, immune maladaptation seems highly likely to be involved in this process. As described in the first chapter, normal placentation requires well regulated trophoblast invasion and remodeling of the spiral arteries (Pijnenborg et al. 2006). This process, most likely involving immunological factors, is disturbed in preeclamptic placentas where interstitial trophoblasts seem less invasive (Noris et al. 2005) and spiral arteries with the normal (non-pregnant) thick smooth muscle wall can be found (Pijnenborg et al. 2006). Interestingly, defective spiral artery remodeling also seems to occur in the absence of preeclampsia, e.g. in intrauterine growth restriction (IUGR) (Pijnenborg et al. 2006). Ultimately, the presence of high resistance, un-remodeled, spiral arteries leads to reduced placental perfusion with resulting ischemia/reperfusion damage (Noris et al. 2005). With this follows a maternal hemodynamic response (increased blood pressure) and release of placental factors. This initiates “stage 2”, which is not seen in IUGR, with effects on the endothelial cells, e.g. in kidneys and liver, causing hyper-vaso-reactivity with systemic vasoconstriction to virtually all organs. Preeclampsia can indeed be viewed as a vascular disease as many risk factors and pathophysiological mechanisms are shared with coronary artery disease (Sibai et al. 2005). The released placental factors could be placental debris, such as microparticles and exosomes (Redman et al. 2007). Syncytiotrophoblast microparticles (STBMs), capable of disturbing endothelial cell organization, are increased in preeclampsia but not in IUGR, indicating an actual role in development of preeclampsia but not in isolated placental deficiency (Redman et al. 2007).

In the end, all these changes are more or less involved in the development of the cardinal clinical manifestations of preeclampsia.

Clinical manifestations, diagnosis and management of preeclampsia

There are a number of consensus statements published by various societies to determine the diagnosis criteria of hypertension disorders in pregnancy, including preeclampsia, gestational hypertension, chronic hypertension and preeclampsia superimposed on chronic hypertension (Brown et al. 2001; Noris et al. 2005; Sibai et al. 2005). A summary of these diagnosis criteria for preeclampsia are given in Table I. Preeclampsia is a placenta-dependent disorder only occurring during pregnancy (Noris et al. 2005). Importantly, to be regarded as preeclampsia and not chronic symptoms, all manifestations need to be *de novo* induced after 20 gestational weeks, which is the time of full placental maturation, and return to normal within three months post partum (Brown et al. 2001). As early onset of disease (before week 32) is associated with increased severity of the disease, research focused on this group of patients has been recommended (Ilekis et al. 2007). Further, the blood pressure and proteinuria measurements should be repeated at least twice with 4-6 hours apart in a standardized manner. The diagnosis preeclampsia is set if blood pressure exceeds 140/90 mmHg and proteinuria is detected. Further, if preeclampsia is complicated by even higher blood pressure, proteinuria or one of the other complications listed in table I, the diagnosis is defined as severe preeclampsia.

Table I. Summary of symptoms associated with preeclampsia
(Brown et al. 2001; Noris et al. 2005)

Parameter	Preeclamptic symptoms
Blood pressure (mm/Hg)	≥ 140/90 *(≥ 160/110)
Proteinuria	≥ 1+ (urine dipstick) or 0.3 g/day *(≥ 3+ or 5 g/day)
Subjective symptoms	headache, epigastric pain, vision disturbances
Hematological changes	thrombocytopenia, decreased plasma volume, hemolysis, coagulation disturbances
Renal insufficiency	oliguria, increased plasma creatinine, increased plasma uric acid
Liver damage	elevated liver enzymes

*Severe preeclampsia

Preeclampsia may develop into the highly fatal state of eclampsia which is manifested as convulsions and unconsciousness. The Hemolysis-Elevated Liver enzymes-Low Platelets (HELLP) syndrome is very closely related to preeclampsia and occurs in about 10 % of all severe preeclampsia cases (Nisell 2008a). As indicated by its name, this syndrome is characterized by hemolysis, possibly due to microvessel constriction, elevated liver enzymes and lowered platelets, likely caused by platelet aggregation (Nisell 2008a).

Preeclampsia is symptomatically treated with substances reducing maternal blood pressure (Nisell 2008a). Further, cortisone may be used in preeclamptic women with threatening prematurity to prevent neonatal respiratory distress syndrome (Sibai et al. 2005). However, there are no preventive or curing treatments. Although antioxidants, neutralizing the placental oxidative stress, initially showed promising results, several large randomized studies failed to confirm this beneficial effect (Ilekis et al. 2007).

Pregnancy as an immunological issue

Overview of the adaptive immune system – with focus on T helper immunity

The adaptive immune system involves the action of T, B and NK cells. T cells, expressing CD3 (CD3⁺), are divided into T helper (T_H) cells, expressing CD4 (CD4⁺) and cytotoxic cells, expressing CD8 (CD8⁺). This thesis focuses on the role for Tregs, which are CD4⁺ cells, in suppressing immune responses during pregnancy.

CD4⁺ cells are classically activated by antigens presented on major histocompatibility complex class II (MHC II) molecules on antigen presenting cells (APCs). The most potent APCs seem to be dendritic cells but macrophages and B cells can also present antigens. The MHC II-antigen complex is recognized by the CD4⁺ cell via its T cell receptor (TCR) which act in concert with co-stimulatory molecules such as CD28 (on the T cell) and CD80/CD86 on the APCs (Abbas et al. 2005).

T helper cells are naïve when they leave the thymus and by that time they express the marker CD45RA. Upon antigen encounter, CD45RA is lost and the cells start expressing (“become positive for”) CD45R0 (CD45R0⁺) and also CD25 (CD25⁺) and HLA-DR (HLA-DR⁺), indicating that they are activated (CD25⁺, HLA-DR⁺) or memory (CD45R0) cells (Johannisson et al. 1995). T_H cells can acquire different properties depending on the environment in which they encounter their antigen (Fig 2). At least six different fates of T helper cell activation have been identified. Three of these subsets have established suppressive roles (T_H3, T_r1 and Tregs) and these are described in detail further on in the text.

The T_H1/T_H2 hypothesis, i.e. the balance between T_H1 and T_H2 cells, originally described in mice by Mosmann and Coffman (Mosmann et al. 1989), was used for many years as a basis for understanding various immunological diseases and conditions, including pregnancy (Wegmann et al. 1993). However, it was soon clear that this hypothesis had its limitations, particularly in the human system where T_H1 and T_H2 cells are now seen as extremes of a continuum. Today the T_H1/T_H2 terminology is used with reservation, and should be used as a working model, but applied for the sake of simplicity in this thesis.

Importantly, in humans there are cells that preferentially secrete interferon- γ (IFN- γ) and these might be called T_H1 cells. IFN- γ production is induced via interleukin (IL)-12 and IFN- γ , but also IL-23 and IL-18, produced mainly by

macrophages. These cytokines act via several transcription factors, including signal transducer and activator of transcription (STAT)-4, STAT-1 and T-box 21 (TBX21), ultimately activating IFN- γ gene transcription (Grogan et al. 2002; Murphy et al. 2002; Borish et al. 2003). T_H2 cells, producing IL-4, are induced via the action of the very same cytokine, IL-4 (Swain et al. 1990), which signal through several transcription factors, including STAT-6, to activate GATA binding protein 3 (GATA3), involved in IL-4, IL-5 and IL-13 transcription (Murphy et al. 2002). There is a significant interplay between the subsets, with reciprocal inhibition of each other (Swain et al. 1990; Nakamura et al. 1997). Further, it has been hypothesized, but not fully demonstrated, that when simultaneously present, T_H2-like immunity is dominating the effects of T_H1 responses (Racke et al. 1994).

T_H2-like immunity, potentially inducing humoral responses, significantly contributes to allergy via B cell stimulation and IgE isotype switch induced by IL-4 and IL-13 (Borish et al. 2003). It is also involved in defense against extracellular parasites and microbes. Further, IL-4 has profound anti-inflammatory effects on the innate system, inhibiting reactions against lipopolysaccharide (LPS) (Steinke et al. 2006).

T_H1-like immune responses protect against intracellular (e.g. viral) infections and have been discussed in a vast number of diseases including rheumatoid arthritis (RA), multiple sclerosis (MS) and graft rejection (Murphy et al. 2002; Boniface et al. 2008). These effects are mediated via IFN- γ that stimulates cell-mediated immunity by enhancing macrophage, NK cell and neutrophil cytotoxicity and phagocytosis (Borish et al. 2003).

Interestingly, when mice were depleted of IFN- γ and its receptor, inflammation in a mouse model of experimental autoimmune encephalomyelitis (EAE) was actually enhanced, opening up the possibility of a third T_H subset with proinflammatory actions (Boniface et al. 2008). As for T_H1- and T_H2-like cells, T_H17 cells were first described in mice (Boniface et al. 2008), where forced expression of the TF ROR γ t, but not TBX21, led to secretion of IL-17A, one of the T_H17 signature cytokines (Ivanov et al. 2006). In humans, “naïve” CD4⁺CD45RA⁺CD45RO⁻ cells from peripheral blood may be induced to produce rare-related orphan receptor C (RORC; the human ortholog of murine ROR γ t), IL-17A as well as IL-22, IL-26 and the CCR6 ligand CCL20 upon stimulation with combinations of IL-1 β , IL-23, IL-6, IL-21 and TGF- β (Wilson et al. 2007; Manel et al. 2008; Volpe et al. 2008; Yang et al. 2008b). However, the actual necessity of each of these cytokines, and the optimal combination needed for the

differentiation of T_H17 cells has been a matter of great debate (Boniface et al. 2008; Annunziato et al. 2009). Of particular notion, the role for TGF- β was confused by exogenous addition of this cytokine in serum-supplemented culture media in many studies. However, the role for TGF- β as a facilitator of T_H17 deviation, directly or indirectly, especially at lower concentrations, has been accentuated by others (O'Garra et al. 2008; Das et al. 2009; Santarlaschi et al. 2009).

T_H17 cells, implicated in several autoimmune diseases including RA and MS (Sallusto et al. 2009), allograft rejection (Afzali et al. 2007) and immunity against e.g. fungal pathogens (Acosta-Rodriguez et al. 2007) are phenotypically characterized by expression of CCR6 and CCR4 chemokine receptors as well as the IL-23 receptor (Acosta-Rodriguez et al. 2007; Annunziato et al. 2007). In addition to CCR4, binding to CCL17 and CCL22, T_H17 cells share another receptor with T_H2-like cells, namely the prostaglandin D2 receptor, chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) (Tsuda et al. 2001; Boniface et al. 2008). Further, T_H cells secreting IFN- γ express the chemokine receptor CXCR3, binding to CCL10-11, and might also express CCR6 (Acosta-Rodriguez et al. 2007).

In humans, IL-12 and IL-4, thus T_H1 and T_H2 immunity, inhibit the development of IL-17 secreting cells (Wilson et al. 2007). However, there are T_H cells capable of producing both IL-17 and IFN- γ , termed T_H1/T_H17 cells (Acosta-Rodriguez et al. 2007; Wilson et al. 2007; Annunziato et al. 2009) and this finding, in analogy with the history of the human T_H1/T_H2 concept, is a reminder of the plasticity of T_H subsets. It is possible that T_H17 immunity will emerge as an extreme of a continuum, as was the case for T_H1 and T_H2 immunity.

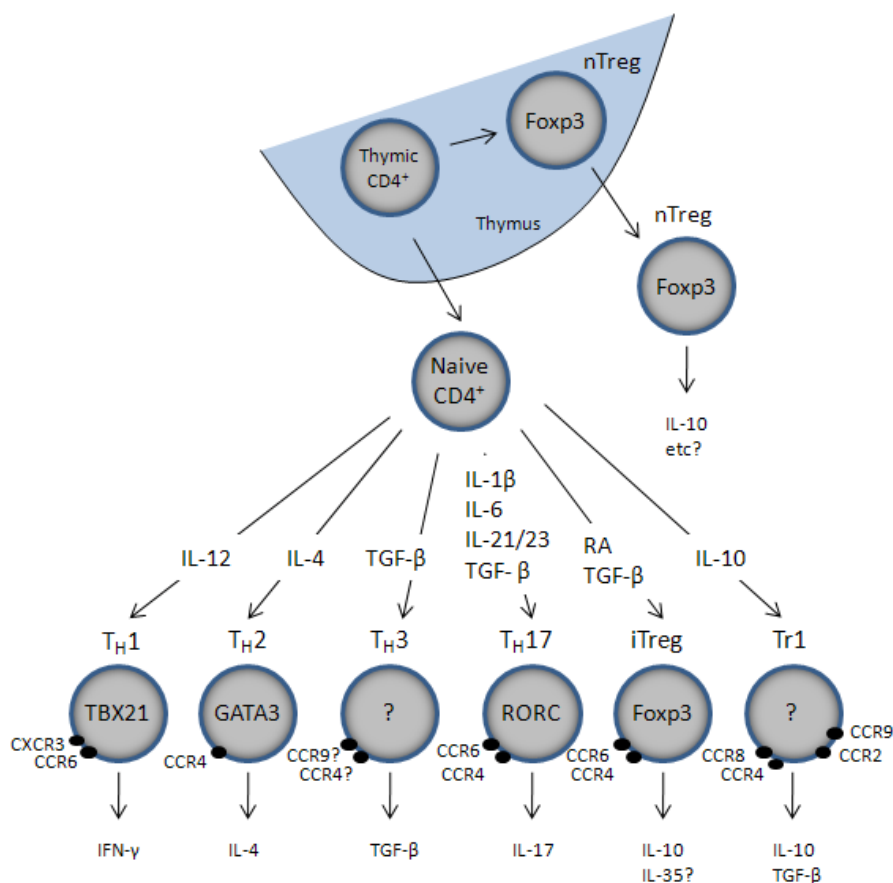


Figure 2

A schematic and simplified view of T_H cell development and differentiation to different cell subsets. Their phenotypes are presented with focus on molecules used for identification in this thesis. For T_H1 , T_H2 , T_H17 and Tregs, this is presented in more detail in the text. Regarding mucosa-associated T_H3 cells, very little seems to be known about their chemokine expression profiles (Weiner 2001; Faria et al. 2005). Tr1 cells express different chemokine receptors depending on activation status, antigen-activated Tr1 cells showing expression of, and responding to ligands for, CCR2, CCR4, CCR8 (Sebastiani et al. 2001) and CCR9 (Papadakis et al. 2003). RA, retinoic acid; iTreg, induced Treg; nTreg, natural Treg.

Alloantigen recognition

Alloantigen recognition is caused by the genetic variation between individuals within a species and can be defined as immune recognition of structures belonging to a different individual. The fetus consists of one maternal and one paternal human leukocyte antigen (HLA or major histocompatibility complex; MHC) haplotype, making the fetus semi-allogenic. As described in the first chapter, there is no major direct contact between the maternal and fetal blood circulation and trophoblasts that are in contact with maternal cells show limited HLA-expression. However, fetal cells do escape to the maternal blood circulation and seem to live on for decades (Barinaga 2002), a phenomena called microchimerism, which raises the issue of alloantigen recognition as a possible immunological problem during, and seemingly even after, pregnancy.

Alloantigen recognition is somewhat of an immunological puzzling phenomenon. Regarding CD4⁺ T cells, during a conventional immune response, for example against an infection, self-T cells recognize the foreign antigen presented by self-MHC II molecules, expressed on self-APCs. The MHC II molecules are highly polymorphic, differing between, and even within individuals, since several different alleles might be expressed in one individual (Felix et al. 2007). However, it is believed that since there are no non-self MHC II molecules present during thymic selection, T cells are able to recognize conserved regions of the MHC II, enabling direct allorecognition (Felix et al. 2007). In line with this, crystal structure studies have revealed that T cells can respond to the same antigen presented on both allo- and self-MHC, with no interaction with the polymorphic regions of the MHC, indicating that allorecognition is in fact a case of cross-reactivity (Felix et al. 2007).

During direct allorecognition, TCRs recognize both allogenic MHC II-peptide complexes (Fig 3) or alternatively, the allogenic MHC II or the presented peptide alone (Felix et al. 2007). Allorecognition is a frequent phenomenon, with many T cells being able to react to any alloantigen, which might be explained by TCR polyspecificity (crossreactivity) (Felix et al. 2007). The presented peptide recognized during alloresponses does not seem to differ in source (extracellular or endogenous) or structure, from those presented during a conventional response.

Indirect allorecognition, being a part of conventional antigen recognition, involves the presentation of self-processed allogenic peptides by self-MHC on autologous APCs (Fig 3) (Felix et al. 2007) and is believed to be responsible for the later issues of transplantation mismatch, chronic rejection (Gokmen et al. 2008). Interestingly, indirect recognition seems to induce regulatory T cells which means that

alloresponses are self-limiting (Gokmen et al. 2008), a mechanism that could be used for therapeutic purposes. From a broader, evolutionary, point of view there is yet no convincing explanation to why allorecognition has developed. However, one intriguing suggestion would be to control non-self cells during microchimerism and even to induce regulatory T cells during a successful model of semi-transplantation, namely pregnancy.

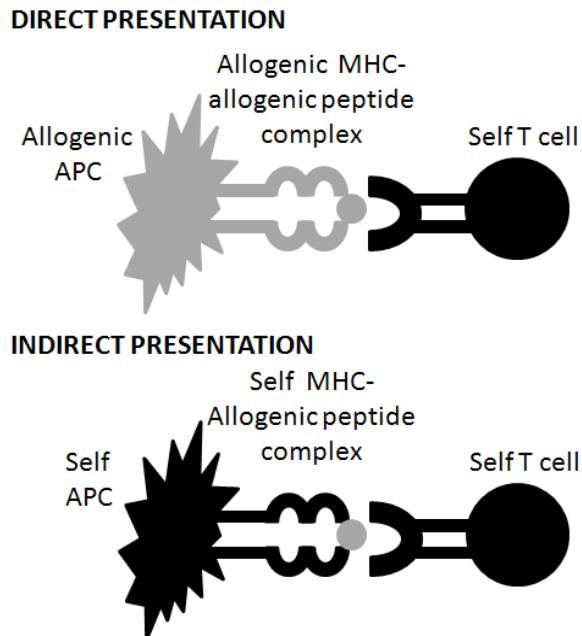


Figure 3

Principal description of direct and indirect recognition of alloantigens. In direct presentation, allogenic (grey) peptides are presented on allogenic MHC by allogenic APCs. In indirect presentation, self (black)-APC take up, process and present allogenic peptides on self-MHC.

Immune regulation during pregnancy – an overview of current understandings

Pregnancy is considered a state of immune tolerance towards fetal alloantigens, as suggested already by Medawar (Medawar 1953). There are two principal ways for anti-fetal sensitization: 1. by presentation of paternal alloantigens by local APCs or 2. by systemic immunization due to escape of fetal cells, such as fetal red blood cells and trophoblasts, to the maternal circulation. Several studies in mice indicate that local immune events are more important than systemic ones (Chaouat 2007). However, systemic changes do occur.

The explanation to fetal tolerance does not lie in a general inability of the mother to mount an alloantigen response. Pregnant rats, immunized with paternal skin grafts, were indeed able to reject a transplanted fetal tissue outside the uterus while leaving the fetus unharmed (Woodruff 1958). However, tissues transplanted to the uterus are rejected if estradiol is not administered (Beer et al. 1970), indicating that the uterus is not inherently immunologically privileged, at least not in rats.

Immune reactions against alloantigens in non-pregnant individuals, as determined by mixed leukocyte cultures, are dominated by IFN- γ (Svenvik et al. 2003). However, it is generally believed that the immune tolerance during an established pregnancy is, at least in part, ascribed a deviation of the adaptive immune system towards a T_H2 -like, humoral-dominated and non-rejecting, non-cell-mediated immunity (Chaouat 2007). Simultaneously, the innate immunity seems to be primed (Sacks et al. 1999). Although debated, pregnant women have been suggested to be more susceptible to, and show more aggressive lapse of, certain bacterial and parasitic infections such as *Toxoplasma gondii*, *Listeria monocytogenes* and influenza (Jamieson et al. 2006), supporting the notion that the cell-mediated immunity is indeed weakened during pregnancy. However, it should be made clear that pregnant women can hardly be viewed as generally immunosuppressed (Chaouat 2007). Further, the immune changes occurring during an established pregnancy are different from those during placental implantation and very early pregnancy since proinflammatory mechanisms are highly involved in these processes (Chaouat 2007; Sharkey et al. 2007; Kwak-Kim et al. 2009). In mice, IFN- γ is both an important mediator of implantation and can simultaneously act as an abortifacient on established pregnancy (Lin et al. 1993; Chaouat 2007). Tumour necrosis factor (TNF) is another proinflammatory cytokine with dual effects on pregnancy. Being widely expressed in trophoblasts during implantation and early pregnancy, the expression declines to undetectable

levels later in pregnancy (Haider et al. 2009). It is believed that in successful pregnancy, TNF and IFN- γ function as breaks on trophoblast invasion and migration. In addition, TNF induces trophoblast apoptosis, thereby ensuring correct spiral artery remodeling. However, increased levels of these cytokines have also been linked to recurrent spontaneous abortion and preeclampsia. Consequently, well regulated presence of TNF, IFN- γ and their receptors is crucial for pregnancy whilst dysregulated expression can lead to disease (Haider et al. 2009; Murphy et al. 2009). Thus, the burden on the immune system to regulate its own processes during pregnancy, to prevent them from jeopardizing the fetus, is huge.

Wegmann was the first to suggest that pregnancy is a T_H2 phenomenon (Wegmann et al. 1993) and they showed that murine feto-placental tissues released T_H2-related cytokines such as IL-4 and IL-5, and especially IL-10, throughout pregnancy, whereas IFN- γ was only transiently produced in early pregnancy (Lin et al. 1993). Interestingly, the levels of all these cytokines were very low in lymphoid tissues, implicating that T_H2 deviation occurs predominantly at the fetal-maternal interface. The reason for this T_H2 deviation has been ascribed paternal alloantigens (Ekerfelt et al. 1997) as well as pregnancy hormones, such as progesterone, estradiol and human chorionic gonadotropin (hCG), which will be discussed in the next section.

Much of the clinical support regarding the T_H1/T_H2 hypothesis in human pregnancy comes from experiences with RA and systemic lupus erythematosus (SLE) patients, generally considered as T_H1 and T_H2 diseases, respectively (Doria et al. 2006; Ostensen et al. 2006). Initially, RA was thought to improve whereas SLE was suggested to deteriorate during pregnancy as a result of the pregnancy induced T_H2 deviation (Doria et al. 2006). Regarding flare frequencies in SLE, studies are not univocal. However, if the SLE disease is active before pregnancy, then the risk of flare seems increased during pregnancy (Doria et al. 2006). Although debated, RA appears to improve during, but worsen after, pregnancy (Doria et al. 2006; Ostensen et al. 2006). Further studies are needed to shed light on the role for pregnancy-induced changes in modulating the T_H17 immunity recently shown to be involved in RA.

Hormonal effects on the immune system during pregnancy

Nuclear progesterone receptors, of which there are three nuclear isoforms (A, B and C) and also less characterized membrane bound forms (Gadkar-Sable et al. 2005), are found in lymphocytes where their expression seems primed during pregnancy (Szekeres-Bartho et al. 2001). Progesterone reduces the cytotoxic activity of decidual lymphocytes (Laskarin et al. 2002) such as NK cells (Szekeres-Bartho et al. 2001). However, since it is unclear if these cells can actually express the progesterone receptor (Szekeres-Bartho et al. 2001), it is likely that decidual trophoblasts respond to progesterone by producing PIBF (Laskarin et al. 2002; Anderle et al. 2008), thereby affecting the decidual lymphocytes. PIBF is produced by trophoblasts but also by peripheral lymphocytes and its excretion increases in urine throughout pregnancy (Szekeres-Bartho et al. 2001; Arck et al. 2007; Anderle et al. 2008). Lack of increasing PIBF levels in pregnancy is associated with prematurity and spontaneous abortion (Arck et al. 2007). Alike IL-4, progesterone and/or PIBF utilizes a receptor that seems to be composed of the IL-4 receptor α -chain and the PIBF receptor (Kozma et al. 2006) to induce a T_H2 -like cytokine shift (Arck et al. 2007) with STAT-6 activation and induction of IL-10 (Kozma et al. 2006) and leukemia inhibitory factor (LIF) (Piccinni et al. 2001) whereas TNF and IFN- γ production in peripheral lymphocytes is abrogated (Kozma et al. 2006).

Supporting the progesterone-induced T_H2 -like deviation, hCG produced by trophoblasts maintains the corpus luteum and its progesterone production. hCG in turn, is sanctioned by cytokines such as IL-4 and LIF, ultimately maintaining a tolerogenic environment during pregnancy (Saito 2000). Further, hCG might have more direct anti-inflammatory effects as it inhibits phytohemagglutinin-induced lymphocyte activation (Siiteri et al. 1982).

Nuclear estrogen receptors α and β are expressed in NK, B and T cells as well as macrophages, the two latter also expressing the membrane-associated receptor (Lang 2004). While low estrogen levels could have immune promoting effects, the levels present during pregnancy inhibit proinflammatory pathways including TNF, IL-1 β and IL-6 while promoting secretion of IL-4, IL-10 and TGF- β (Whitacre et al. 1999; Beagley et al. 2003; Straub 2007). As an example of how potent estradiol is in deviating an already established immune response, in the murine MS model EAE, pregnancy itself and pregnancy levels of estradiol increased the secretion of IL-10 while reducing that of IFN- γ and IL-12 in lymphocyte-APC cocultures (Polanczyk et al. 2006). In general, pregnancy levels of estradiol dampen T cell activity, even causing thymic atrophy (Lang 2004), whereas B cells are promoted by estradiol at all physiological concentrations.

Local immune regulation in healthy and complicated pregnancy

The decidua is the main arena for the immunologic encounter between the mother and fetus. For obvious reasons, most information on local immune regulation in pregnancy is obtained from first (elective abortions) or third trimester (post partum) pregnancy. In first trimester, the decidua is highly enriched by maternal immune cells, actually constituting almost half of all cells present there. Of these, the specialized uterine NK (uNK) cells make up approximately 70%, the macrophages 20% and the T cells 10%. The NK cells gradually decrease with the progress of pregnancy whereas macrophages and T cells persist in relatively stable numbers until term (Trundley et al. 2004).

The local immune response during pregnancy is considered to be dominated by T_H2-like immunity with some proinflammatory features which are thought to be essential for tissue remodeling during early pregnancy and especially during implantation (Kwak-Kim et al. 2009).

Trophoblasts

Several mechanisms involved in overcoming the alloantigen problem of pregnancy have been identified. The villous trophoblasts that are in direct contact with the maternal blood in the intervillous space lack expression of classical MHC I molecules (HLA-A, B and C) whereas extravillous trophoblasts seem to express HLA-E (King et al. 2000a) and HLA-C but not HLA-A or HLA-B (Redman et al. 1984; King et al. 2000b; Koch et al. 2007). HLA-DR (MHC II) does not seem to be expressed on the surface of extravillous or villous trophoblasts, neither in first nor in third trimester of pregnancy (Redman et al. 1984; Sutton et al. 1986). However, intracellular expression of HLA-DR has been reported (Ranella et al. 2005). Hence although both villous and extravillous trophoblasts are poor inducers of classical MHC-II restricted immune responses, they in principal have the capacity to do so, given that their intracellular pool of HLA-DR is activated. Further, trophoblasts do express HLA-C which is able of inducing, not only CD8⁺, but also CD4⁺ immunity at the fetal-maternal interface (Tilburgs et al. 2009).

Trophoblasts, especially extravillous trophoblasts, express the non-classical HLA-G molecule (Kovats et al. 1990; King et al. 2000b), which is thought to ensure immunological acceptance while simultaneously mediating inhibitory effects on cytotoxicity via inhibitory receptors on NK cells and macrophages (Hunt et al. 2000; Hviid 2006). Further, soluble HLA-G (sHLA-G), also presenting anti-inflammatory activities, can be found both at the fetal-maternal interface and

circulating in serum during all trimesters of pregnancy (Hunt et al. 2000; Hviid 2006). In various *in vitro* models, HLA-G stimulation of lymphocytes has been shown to reduce the secretion of TNF and IFN- γ and, although not consistently, increase that of IL-4 and IL-10 (Hviid 2006).

Beside their unique HLA profile, more first and third trimester villous and extravillous trophoblasts spontaneously produce the T_H2 related cytokine IL-4 than proinflammatory cytokines like IL-12, IFN- γ and TNF (Sacks et al. 2001). They also, along with glandular cells, produce the CCR4-ligand CCL17 which seems to attract CCR4-bearing T_H2-like cells to the decidua (Tsuda et al. 2002). Further, the enzyme indoleamine dioxygenase (IDO), starving surrounding T cells of tryptophan, is secreted by villous explants (Kudo et al. 2001).

In preeclampsia, fetuses carrying a certain HLA-G genotype (+14/+14) are over-represented and lowered expression of HLA-G on extravillous trophoblasts has been seen in preeclampsia (Hviid 2006). Further, certain combinations of HLA-C on trophoblasts and NK cell receptors (on NK cells) have been suggested to contribute to the poor trophoblast invasion seen in preeclampsia (Sargent et al. 2007).

NK cells

NK cells in decidua have very unique properties, expressing high levels of CD56 but low levels of CD16 (CD16⁻CD56^{bright}) distinguishing them from their blood counterparts, most of which are CD16⁺CD56^{dim} (Starkey et al. 1988; Poli et al. 2009). CD16⁻CD56^{bright} cells can be found in many secondary lymphoid organs and are generally less cytotoxic and more cytokine producing than CD16⁺CD56^{dim} cells (Poli et al. 2009). Uterine NK cells have been shown to promote trophoblast invasion and angiogenesis via secretion of chemokines, cytokines and growth factors as well as via interactions between NK cell receptors (both activating and inhibitory) and HLA-C/E/G on trophoblasts (Hanna et al. 2006). As mentioned, uNK cells exhibit poor cytotoxicity (Poli et al. 2009), making them good trophoblast and vascularization coordinators, without risk of being harmful to the fetus. Interestingly, it was recently shown that TGF- β could convert blood CD56⁺ NK cells (CD16⁺) to decidua-like CD16⁻ cells (Keskin et al. 2007). During normal early pregnancy, decidual NK cells produce TGF- β , whereas spontaneous abortion is associated with an increased proportion of NK cells producing IFN- γ (Higuma-Myojo et al. 2005; Saito et al. 2008), suggesting that pregnancy failure is

accompanied by skewing of NK cells towards a proinflammatory and cytotoxic nature.

In preeclampsia, the combination of NK cells expressing the inhibitory KIR- (killer-immunoglobulin-like receptor) A receptor, and trophoblasts expressing HLA-C2, seems more prevalent and could be one mechanism behind the poor trophoblast invasion seen in preeclampsia (Sargent et al. 2006; Trowsdale et al. 2008).

Antigen presenting cells

CD14⁺ macrophages (MΦ) are the second largest population of immune cells present in the early pregnancy decidua. A portion of these cells are actually of fetal origin, so called Hofbauer cells (Huppertz 2008). Maternal decidual MΦ express lower levels of co-stimulatory CD86 (Heikkinen et al. 2004), higher levels of HLA-DR (Heikkinen et al. 2004), the immune modulatory enzyme IDO (Kudo et al. 2001; Heikkinen et al. 2004) as well as high levels of IL-10 (Heikkinen et al. 2003; Lidstrom et al. 2003) and have been proposed to be of an alternative, so called M2 type (Cupurdija et al. 2004; Gustafsson et al. 2008). Performing a global gene expression profile analysis of early pregnancy decidual and blood MΦ, we could show that decidual MΦ were characterized by molecules involved in tissue remodeling and immune modulation (Gustafsson et al. 2008) rather than proinflammation, as is the case for classical MΦ, supporting their importance in pregnancy.

The relation between MΦ and dendritic cells (DCs) in decidua is not clear, much owing to their overlapping cell surface phenotypes. Defining DCs as CD45⁺HLA-DR⁺CD14⁻, it was shown that DCs make up around 1% of all leukocytes in decidua, of which most are of the myeloid lineage producing low levels of IFN-γ and inducing a strong IL-4 response in T_H cells (Miyazaki et al. 2003). A third subset of antigen presenting cells in decidua is the immature monocyte-derived APCs (Kammerer 2005).

Preeclampsia is associated with increased oxidative stress initiated in the placenta and later on transmitted, possibly via activated cells, to the systemic circulation. Indeed, activated neutrophils and monocytes are found in the circulation of preeclamptic patients (Roberts et al. 2001). Further, in both spontaneous abortion and preeclampsia, activated dendritic cells (CD83⁺) have been shown to be enriched (Askelund et al. 2004; Huang et al. 2008), indicating that potent stimulatory antigen presenting cells are not desirable in the decidua.

T cells

The T_H1/T_H2 hypothesis was the dominating explanation model for immune regulation during pregnancy for at least a decade, and this model, along with other, more recently added mechanisms, still provides a foundation for explaining fetal tolerance.

The T cells found at the fetal-maternal interface have an activated/memory phenotype (Saito et al. 1992; Saito et al. 1994), indicating that they are in fact primed. Looking at their cytokine secretion profile, decidual T cells from healthy pregnancy, but not spontaneous abortion cases, secrete IL-4 and IL-10 (Piccinni 2002) as well as the pregnancy facilitating LIF and colony stimulating factor 1 (M-CSF) (Piccinni et al. 2001) which goes along with Wegmann's observations in the murine system. Further, as compared with blood, more T cells in decidua produce IL-4 but fewer produce IFN- γ (Saito et al. 1999b). Interestingly, T_H2 -like cells seem to be recruited to the decidua via local CCL17-production (Tsuda et al. 2002). Importantly, in decidua, cells producing IFN- γ were actually more frequent than cells producing IL-4, pointing towards a role for this potentially immune triggering cytokine in early pregnancy (Saito et al. 1999a).

In severe preeclampsia, the number of naive T cells and uterine NK cells were reduced in decidua. Further, decidual lymphocytes from preeclamptic women produced lower levels of IL-6, IL-10 and IL-12, but enhanced levels of IFN- γ as compared with healthy pregnant controls (Wilczynski et al. 2002; Wilczynski et al. 2003). There was however no difference in the secretion or production of IL-4, the major T_H2 polarizing cytokine.

In conclusion, local immune regulation during normal pregnancy seems to be characterized partly by a skewing towards tolerogenic and less aggressive immune mechanisms. This skewing seems dysfunctional in pregnancy complications, including preeclampsia. In addition, proinflammatory mechanisms, including presence of T_H1 cells, are also involved in healthy pregnancy. The recent discoveries of new T helper subsets, including Tregs and T_H17 cells, raises the question about the role for these cells in regulating local immune events during pregnancy.

Systemic immune regulation in healthy and complicated pregnancy

As suggested by Wegmann (Lin et al. 1993; Wegmann et al. 1993), the changes that occur during pregnancy are likely to be most pronounced at the fetal-maternal interface. However, systemic immune changes do occur during pregnancy.

NK cells

The proportion of CD56^{bright} NK cells present in blood is low relative to those found at the fetal-maternal interface. It has been suggested that akin to the T_H1, T_H2, T_H3 and T_R1 cells, NK1 cells produce IFN- γ , NK2 produce IL-4 and NK3 possess TGF- β . NKr1 cells produce IL-10 and these cells are increased in peripheral blood during normal pregnancy, but not in miscarriage patients (Higuma-Myojo et al. 2005; Saito et al. 2008). This is in accordance with Hanna *et al.* implying IL-10 as the main pregnancy facilitator as increased levels of IL-10, but not IL-4, were found in sera during normal early pregnancy as compared with non-pregnant women (Hanna et al. 2000).

Sargent and Redman have proposed a theory that normal pregnancy is a state of controlled systemic immune activation caused by reactivity to debris released from the placenta. They argue that this is mediated mainly via NK cells with T_H1 deviating properties. In preeclampsia, the release of debris is increased, leading to exaggerated systemic immune activation, ultimately causing the systemic symptoms of preeclampsia (Sargent et al. 2007).

Antigen presenting cells

Pregnancy has been suggested to induce changes in the innate immune system actually of the same kind albeit milder than those seen during sepsis, including activation of monocytes and granulocytes (Sacks et al. 1998; Sacks et al. 1999). Supporting this, CD14⁺ monocytes seem more easily primed to produce IL-12 upon LPS stimulation *in vitro* during pregnancy (Sacks et al. 2003). However, monocytes derived from women with preeclampsia spontaneously produce even more proinflammatory IL-1 β , IL-6, IL-8 and TNF than healthy pregnant women (Luppi et al. 2006). Hence, normal pregnancy seems to depend on a well balanced activation and priming responsiveness of monocytes which can be altered during pregnancy complications such as preeclampsia.

Recently, the T cell Ig and mucin domain (Tim)-3 cell receptor was shown to be increasingly expressed by monocytes during the progress of pregnancy. This receptor was induced by IL-4, which was also shown to be increased systemically during pregnancy, and blocking of Tim-3 inhibited both innate and adaptive effector mechanisms, including clearance of *E.coli* bacteria and T cell proliferation. However, the possible ligand for Tim-3 was not investigated (Zhao et al. 2009).

T cells

Polyclonally stimulated peripheral blood mononuclear cells from early normal pregnant women produced higher levels of IL-4 and IL-10, and lower levels of IL-2, IFN- γ and TNF than recurrent aborters (Raghupathy et al. 2000). This was supported by findings showing fewer IL-10 and more TNF producing T cells in recurrent spontaneous abortion (RSA) and pregnancy failure cases than non-pregnant controls (Ng et al. 2002). In that study, normal pregnant women, followed from first to third trimester of pregnancy, showed high numbers of IL-4, IFN- γ and TNF producing T cells in first trimester then declining with the progress of pregnancy (Ng et al. 2002). However, no differences in IL-4 and IFN- γ producing cells were found as compared with non-pregnant women, an observation also done by others (Saito et al. 1999b) but disputed by even others showing higher IL-4 activity in first or third trimester pregnancy as compared with non-pregnant women (Marzi et al. 1996; Saito et al. 1999a). Further, IL-10 was shown to be higher in pregnant as compared with non-pregnant women and women with recurrent spontaneous abortion (Marzi et al. 1996).

Spontaneous secretion of cytokines is perhaps more representative of *in vivo* conditions. Using the sensitive ELISPOT technique, or in situ hybridization on resting circulating cells from pregnant women, pregnancy was shown to be associated with more IL-4 and IFN- γ production in all trimesters of pregnancy as compared to post-partum or non-pregnant controls (Matthiesen et al. 1998; Matthiesen et al. 2003; Persson et al. 2008), indicating that healthy pregnancy involves priming of both IL-4 and IFN- γ secretion. However, since the effects of IL-4 could dominate those of IFN- γ , this was interpreted as a possible T_H2-like deviation during normal pregnancy.

In general, preeclampsia seems to be associated with a T helper cell response that is deviated towards IFN- γ rather than IL-4 secretion (Saito et al. 1999a). PBMC from late-onset preeclamptic women showed increased production of IL-2 and IFN- γ but reduced production of IL-10 and IL-4 upon polyclonal *in vitro*

stimulation (Darmochwal-Kolarz et al. 1999; Saito et al. 1999a; Darmochwal-Kolarz et al. 2002). Hence, the T cell response during preeclampsia is prone to stimulate cytotoxicity and other effects mediated via IFN- γ . Indeed, preeclampsia is associated with increased T cell activation as judged by increased expression of CD45RO on T helper cells (Matthiesen et al. 1995; Chaiworapongsa et al. 2002).

In conclusion, alike local immune regulation, systemic immunity during normal, in contrast to complicated pregnancy, seems to be characterized by a skewing towards tolerogenic and less aggressive mechanisms, in particular with regard to T cell immunity. However, systemic immunity also involves a certain degree of innate immune enhancement that seems exaggerated in preeclampsia. Still, factors regulating systemic immune responses in pregnancy, including regulatory CD4⁺ T cells, are poorly investigated.

Regulatory CD4⁺ T cells

There are many types of T cells that could potentially be defined as regulatory T cells as all cells intrinsically have regulatory properties. This thesis deals with the role for “regulatory T cells” or “Tregs” expressing the transcription factor Foxp3, which since the discovery of naturally occurring regulatory T cells, implicitly refers to CD4⁺ cells with immune suppressive function. However, even defined as CD4 expressing cells, several different subtypes of regulatory T cells exist.

Subsets of regulatory T cells

Type 1 regulatory T (T_r1) cells

Type 1 regulatory (T_r1) cells are believed to be activated in the presence of IL-10 and to secrete both IL-10 and TGF- β , thereby inducing immune tolerance (Roncarolo et al. 2006). IL-10, also produced in large quantities by monocytes and B cells, has a vast array of anti-inflammatory effects including inhibition of both T_H1 and T_H2 responses as well as of phagocytosis (Borish et al. 2003). However, IL-10 also enhances cytotoxicity and IgG4 production, suggesting that IL-10 promotes humoral and cytotoxic immunity while controlling cell-mediated immunity (Borish et al. 2003). TGF- β is also a pluripotent cytokine with pre-dominating anti-inflammatory and tissue healing properties (Borish et al. 2003).

Many studies reveal the existence of T_r1 cells. $CD4^+$ cells activated by alloantigens in the presence of IL-10 fail to respond to the same alloantigens upon restimulation (Groux et al. 1996). Further, stimulating $CD4^+$ cells with antibodies against CD3 and CD46 generates a population of cells suppressing activated $CD4^+$ cells via the action of IL-10 and granzyme B (Kemper et al. 2003; Grossman et al. 2004). Interestingly, these cells also secrete IFN- γ and IL-12, implying that they are closely linked to the T_{H1} subset, a finding supported by others (Papadakis et al. 2003). Whether these CD3/CD46 activated cells are of the same lineage as IL-10 activated cells remains unknown. Interestingly, CD3/CD46 activated T_r1 cells may have reduced suppressive activity in patients with the autoimmune disease MS (Martinez-Forero et al. 2008).

T helper type 3 (T_{H3}) cells

Oral tolerance is a very potent and important way of preventing the immune system from reacting to food allergens and non-pathogenic microorganisms in the mucosal flora. One of the proposed main mechanisms of reaching tolerance in the mucosa is by induction of T helper type 3 (T_{H3}) cells secreting TGF- β (Weiner 2001; Faria et al. 2005). This seems to be mediated via activation of T cells by mucosal dendritic cells in the unique mucosal environment that is intrinsically rich for TGF- β , IL-10 and IL-4 (Weiner 2001; Faria et al. 2005). In humans, it has been shown that oral administration of bovine myelin to MS patients generates circulating antigen-specific T cells secreting TGF- β 1 (Fukaura et al. 1996).

The relation between T_r1 cells, T_{H3} cells and Foxp3 $^+$ Tregs has not been fully established and it is very likely that they co-exist even if one type may be dominating. By cloning human $CD4^+$ cells, type 1 regulatory T cells, differentiated in the presence of IL-10 and IFN- α have been stated to be distinct from Foxp3 (Levings et al. 2002). However, T_r1 cells have the capability of transiently expressing Foxp3 upon activation.

CD4⁺CD25^{high} regulatory T cells (Tregs)

Treg origin

It was the work of Sakaguchi and colleagues that led to the discovery of so called natural CD4⁺CD25⁺ regulatory T cells (nTregs), present even in naïve mice (Sakaguchi et al. 1995). They showed that transfer of spleen and lymph node cell suspensions, depleted of CD4⁺ cells bearing the IL-2 α -chain CD25, caused CD4⁺ cell dependent development of gastritis, oophoritis, thyroiditis and several other organ-specific autoimmune diseases in the athymic recipient mice. Importantly, disease could be prevented by co-transfer of CD4⁺CD25⁺ cells. Later, it was shown that these cells migrate from thymus as a distinct cell subset (Asano et al. 1996).

In humans, the cell type corresponding to nTregs was identified a few years later (Baecher-Allan et al. 2001; Dieckmann et al. 2001; Jonuleit et al. 2001; Levings et al. 2001; Ng et al. 2001; Taams et al. 2001). Since humans are constantly exposed to antigens, and CD25 is an activation marker (Malek 2008), Tregs have not been as easily identified as in mice. However, Tregs showing a naïve phenotype can be found among the naïve cells in neonate cord blood and also in thymus (Annunziato et al. 2002; Wing et al. 2002), indicating that alike the situation in mice, Tregs in humans are generated in the thymus and are present before antigen encounter.

Suppressive cells showing markers associated with natural Tregs can be induced from non-regulatory CD4⁺CD25⁻ cells, called adaptive or induced Tregs. This group also comprises the T_H1 and T_H3 cells which were described above. Although non-regulatory CD4⁺CD25⁻ cells can be induced to express markers of Tregs, it is not completely settled whether these cells acquire suppressive features. CD4⁺CD25⁻ cells, either naïve (CD45RA⁺) or memory (CD45R0⁺) cells, activated via their T cell receptor, induce the expression of the Treg-associated transcription factor Foxp3 (described below). The resulting CD4⁺CD25⁺ cells also show suppressive activity against polyclonal stimuli and alloantigens in humans (Walker et al. 2003; Walker et al. 2005) and in mice (Chen et al. 2003). The mechanism by which suppression was achieved was, similarly to naturally occurring Tregs, contact dependent and cytokine (IL-10 and TGF- β) independent *in vitro*. Treg activity was also seen after retroviral transfer of Foxp3 to naïve CD4⁺ cells (Yagi et al. 2004). However, other groups have shown that T cell receptor stimulation of CD4⁺CD25⁻ cells induces TGF- β dependent Foxp3 expression (Tran et al. 2007), which is transient (Gavin et al. 2006; Allan et al. 2007; Wang et al. 2007) and not linked to suppressive function (Tran et al. 2007). This however, is in sharp contrast to the murine situation, where TGF- β induced Foxp3 expression is

tightly linked to suppressive function (Chen et al. 2003). One study, showing that human CD4⁺CD25⁻ cells, cultured with IL-2, acquire suppressive function and Foxp3 expression, ascribed the suppressive function to cytotoxic T lymphocyte antigen 4 (CTLA-4), and not to Foxp3 (Zheng et al. 2008).

Antigen presenting cells are required for initiation of immune responses and are also important for induction of Tregs. In mice, dendritic cells expressing CD103, present in gut-associated lymphoid tissue, can induce Tregs (Siddiqui et al. 2008). Further, plasmacytoid dendritic cells, stimulated via toll-like receptor (TLR) 9, induce Foxp3 expression and suppressive function in naïve T cells, a mechanism mediated via IDO and tryptophan metabolites (Chen et al. 2008).

As for macrophages, T cells activated by allogenic anti-inflammatory type 2 macrophages have been shown to inhibit the response of activated autologous T cells to the same alloantigens (Savage et al. 2008). Although not all of these M2-activated regulatory T cells expressed high levels of CD25, those that did also expressed Foxp3 and glucocorticoid induced tumor necrosis factor receptor (GITR), signature markers of Tregs.

Self APCs presenting alloantigens (via indirect alloantigen recognition) also play an important role in inducing Tregs, as shown in both murine *in vivo* and human *in vitro* systems (Gokmen et al. 2008). As for the human systems, the Tregs generated were alloantigen-specific and could be expanded without loss of function, introducing the possibility of therapeutic usage (Gokmen et al. 2008).

Treg phenotype and the role for Foxp3

In humans, Tregs are not as easily identified as in naive mice where the IL-2 receptor α -chain CD25 is sufficient to find cells with suppressive function (Sakaguchi et al. 1995). In humans, Tregs have been characterized to express high levels of CD25 (Baecher-Allan et al. 2001), hence called CD25^{high} or CD25^{bright}. However, since the CD25 molecule is a marker of activation, the CD25^{high} population is easily contaminated by activated cells lacking suppressive function, highlighting the need for a better marker of immune suppressive cells.

Scurfy mice show massive autoimmune activation due to a mutation in the gene coding for the scurf protein, Foxp3 (Schubert et al. 2001). Humans show a homologous defect, the unusual disorder Immune dysregulation Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX) (Chatila et al. 2000). IPEX patients, which can display an array of more or less severe mutations in the

Foxp3 locus, are males affected already as newborns with multi-organ autoimmune diseases, often resulting in death before two years of age if left untreated (van der Vliet et al. 2007).

In both mice and humans, it was soon found that CD4⁺CD25⁺ regulatory T cells expressed Foxp3 and that this protein was expressed already in thymus. Furthermore, Foxp3 expression could confer suppressive function to non-Tregs upon retroviral transfer and consequently, was important for lineage commitment to the Treg subset (Fontenot et al. 2003; Hori et al. 2003; Walker et al. 2003; Yagi et al. 2004; Wing et al. 2005).

The Foxp3 protein is a member of the Forkhead family of transcription factors and functions to silence proinflammatory cytokine production and proliferation in the cell, rendering the cell anergic while acquiring the typical Treg phenotype (Fig 4). This is accomplished by Foxp3 interacting with hundreds of factors, including transcription factors such as nuclear factor of activated T cells (NFAT), NF-kappaB (NFκB) and acute myeloid leukaemia 1 (AML1)/Runx. Foxp3 alters the activity of these transcription factors, being crucial for activation of effector cells and transcription of cytokine genes (Schubert et al. 2001; Bettelli et al. 2005; Ono et al. 2007; Sakaguchi et al. 2008). It should be pointed out that despite intense research, the mechanisms behind Foxp3 mediated selective gene silencing are largely unknown. By using mouse models with moderate Foxp3 expression it was suggested, in several reports, that Foxp3 works along a continuum inducing increasing grades of suppressive characteristics upon increasing Foxp3 expression (Gavin et al. 2007; Wan et al. 2007). The role for Foxp3 in Treg phenotype and function is summarized in figure 4.

Using a ChIP-chip assay, it was discovered that Foxp3 binds to the promoter of the IL-7 receptor α -chain CD127, down-regulating its expression, thereby giving the Foxp3-expressing cells a phenotype with low expression of CD127 (CD127^{low}) (Liu et al. 2006). It was shown that while reduced Foxp3 expression was associated with less pronounced suppression (Venken et al. 2008), CD127 was negatively correlated with suppressive function. While the nuclear localization of the Foxp3 protein disables its use as a viable cell sorting marker, viable and suppressive Tregs could be sorted by combining the CD25^{high} and CD127^{low} phenotypes (Liu et al. 2006; Seddiki et al. 2006).

In mice, Foxp3 seems to be an absolute marker of Tregs. Yet, in later years it has become increasingly apparent that in humans, Foxp3 is not completely specific for cells with suppressive function. Rather, Foxp3 expression seems to be a self-limiting, natural consequence of activation. Thus, activated CD4⁺ cells start

transiently expressing Foxp3, which is not linked to suppressive function according to most investigations (Allan et al. 2005; Allan et al. 2007; Wang et al. 2007), but not all (Walker et al. 2003). Importantly, stable expression of Foxp3, as under non-inflammatory conditions, is still coupled to suppressive function (Wang et al. 2007). Further, very recent investigations have shown that stable Foxp3 expression and suppressive function is associated with epigenetic DNA modifications, demethylations, of the Foxp3 locus. It was demonstrated that non-Tregs, induced to express Foxp3 via TCR or TGF- β stimulation, did not display Foxp3 demethylations, whereas resting suppressive Tregs did (Baron et al. 2007).

Tregs express several molecules associated with their suppressive function (Table II), such as CTLA-4. In resting Tregs, CTLA-4 is found intracellularly but is rapidly upregulated following activation (Jonuleit et al. 2001; Birebent et al. 2004). About 40 % of the Tregs show surface HLA-DR expression (Baecher-Allan et al. 2001; Baecher-Allan et al. 2006). The issue of membrane-bound TGF- β (mTGF- β) has been debated. Whereas some find mTGF- β on resting Tregs (Jonuleit et al. 2002), most studies have failed to detect mTGF- β and its latency-associated peptide (LAP) (Levings et al. 2002; Birebent et al. 2004). Further, Tregs (Cosmi et al. 2003), particularly those clones actually showing suppressive function (Levings et al. 2002), show high expression of GITR.

CD39 was recently shown to be expressed by Foxp3⁺ Tregs (Borsellino et al. 2007). CD39 is an ectonucleotidase that, alongside other enzymes, convert adenosine triphosphate (ATP), released during e.g. tissue damage and activation, to adenosine (Borsellino et al. 2007).

Table II. Expression of selected markers associated with human Tregs, defined either as Foxp3⁺ or CD4⁺CD25^{high}

Highly expressed markers	Intermediate expression	Low expression
CD25, Foxp3, CTLA-4, CD45R0, CD39, Nrp1? GITR, CD27	HLA-DR, mTGF-β?	CD127

In addition to the Treg-associated factors introduced above, there has been a steady flow of reports suggesting different molecules as markers of Tregs. CD27 is one marker proposed to be useful in inflamed tissue such as synovia (Ruprecht et al. 2005). Based on a global gene array analysis, the surface marker Neuropilin-1 (Nrp1) emerged as a promising tool for identifying Tregs (Bruder et al. 2004) but turned out not to be very useful (Bruder, D; personal communication).

Treg function – mechanisms of suppression and target cells

Much information on Treg mechanisms in humans come from *in vitro* assays developed by numerous groups in the beginning of the 21st century (Baecher-Allan et al. 2001; Dieckmann et al. 2001; Jonuleit et al. 2001; Levings et al. 2001; Ng et al. 2001; Taams et al. 2001). In the typical case, CD4⁺ cells lacking the CD25 molecule, CD4⁺CD25⁻ cells, are used as responders. These responders are then cultured alone or in combination with increasing numbers of Tregs (CD4⁺CD25⁺ or CD4⁺CD25^{high} cells). As expected, the responders will proliferate vigorously and secrete cytokines in response to a polyclonal stimulus such as anti-CD3/CD28 antibodies. However, in the presence of Tregs, both proliferation and secretion of cytokines such as IFN-γ and IL-2 is inhibited. Interestingly, Tregs alone are fairly unresponsive, showing low proliferation unless strongly stimulated via their TCR and CD80/CD86 (Baecher-Allan et al. 2002) or in the presence of high concentrations of IL-2 (Dieckmann et al. 2001; Levings et al. 2001). Tregs have been shown to produce cytokines such as IL-10, but also IL-4 and TGF-β, at least under stimulation (Dieckmann et al. 2001; Levings et al. 2001; Stephens et al. 2001; Baecher-Allan et al. 2002).

The mechanisms for suppression in these assays have been thoroughly investigated and cytokines have obviously been prime candidates. However, separating Tregs and responder cells with a semi-permeable membrane, allowing

passage of soluble agents such as cytokines but preventing direct cell-cell contact, prevents Treg suppression of responder cells (Dieckmann et al. 2001; Jonuleit et al. 2001; Ng et al. 2001; Stephens et al. 2001). Further, activated formalin-fixed Tregs are as suppressive as viable Tregs (Dieckmann et al. 2002; Jonuleit et al. 2002). Hence, Tregs seem to rely on activation and contact with the responder cells for suppression and the action of cytokines seems insufficient, since blocking cytokines such as IL-10, IL-4 and TGF- β cannot cogently prevent suppression (Levings et al. 2001; Azuma et al. 2003; Birebent et al. 2004). However, it should be remembered that in these systems, the cytokines might be strongly diluted, leading to underestimations of their importance. Further, Treg clones, showing actual immune suppressive effect, do seem to rely on TGF- β for suppression (Levings et al. 2002). The action of TGF- β , bound to the Treg surface membrane in its inactive form (TGF- β plus LAP), has also been reported by others (Nakamura et al. 2004). However, in contrast to murine Tregs, human Tregs do not express surface bound active TGF- β (Nakamura et al. 2004). Tregs are able to induce IL-10 (Dieckmann et al. 2002) or TGF- β (Jonuleit et al. 2002) secretion in the CD4⁺ responder cell population, a phenomenon termed infectious tolerance (Jonuleit et al. 2002), and these T_r1- or T_H3-like cells, in turn, are completely dependent on IL-10 or TGF- β , respectively, for suppression of their target cells. Jonuleit *et al* hypothesized that this secondary cytokine dependent suppression was aimed at a systemic immune suppression (Jonuleit et al. 2002), where contact dependence would not be very manageable.

Since Tregs express high levels of the IL-2 receptor α -chain CD25, it has been hypothesized, but also debated, that Tregs act as an IL-2 sink, consuming all available IL-2 at the expense of T effector cells. This mechanism was supported by observations showing that addition of IL-2 to Treg-T effector co-cultures partially restored proliferation (Dieckmann et al. 2001). However, since IL-2 induces Treg proliferation, it is unclear if the restored proliferation in these cultures were caused by actual T effector proliferation. Ruling out the role for IL-2 consumption in Treg suppression, it has recently been shown that Tregs are suppressive of T effector cell IL-2 mRNA and proliferation even when IL-2 is at excess in culture medium (Oberle et al. 2007; Tran et al. 2009).

In mice, cytokines seem to play a more pronounced role in Treg suppression (Vignali et al. 2008). In infections, IL-10 blockade, TGF- β blockade or adoptive transfer of Tregs from IL-10 deficient mice disables suppression of immune responses against *H. hepaticus* (Maloy et al. 2003). Further, Treg suppression of graft rejection largely depends on IL-10, as well as CTLA-4 (Kingsley et al. 2002).

In total, this opens up the possibility that cytokines are important mediators of Treg suppression *in vivo* but not *in vitro* (Vignali et al. 2008).

In summary, the immune suppressive actions of IL-10 and TGF- β are undoubtedly important and closely linked to the function of regulatory T cells. However, whether this, in humans, is mediated via direct secretion of these cytokines from Tregs *in vivo* remains unresolved.

Interestingly, in mice it was recently shown that contact between Tregs and their responder cells upregulates the secretion of the IL-12 family cytokine IL-35, consisting of the Epstein-Barr virus-induced gene 3 (EBI3) and p35 (IL12a) subunits, secreted from Tregs. Tregs from mice lacking the EBI or p35 genes displayed reduced suppressive function and were unable of preventing inflammation in a murine model of inflammatory bowel disease (Collison et al. 2007). Further, ectopic expression of IL-35 in non-Tregs induced suppressive properties in these cells (Collison et al. 2007). Although production of IL-35 from human Tregs (cultured alone, not together with effector cells) was recently dismissed (Bardel et al. 2008), these data not only introduces a new Treg-associated suppressive cytokine, but also a new mode of suppressive action since secretion of IL-35 from Tregs was potentiated by interaction with effector cells (Collison et al. 2009), highlighting the active role of the effector cells in Treg suppression. This principle may possibly explain the lack of suppression in systems where Tregs and effector cells are physically separated.

So what mediates the contact in Treg suppression? The contact itself, acting to stabilize the immunological synapse, seems important since blocking intracellular adhesion molecule 1 (ICAM-1) (Azuma et al. 2003) as well as its ligand lymphocyte function-associated antigen 1 (LFA-1) (Tran et al. 2009) abrogates suppression. CTLA-4 has been a major candidate for many years and is believed to be very important in mice (Kingsley et al. 2002; Sansom et al. 2006) e.g. based on the dramatic T cell activation occurring in CTLA knock-out mice (Bour-Jordan et al. 2009). Importantly, mice specifically lacking CTLA-4 in the Treg compartment show defective Treg suppression and consequently exhibit lymphoproliferation and autoimmunity whereas tumor immunity is enhanced (Wing et al. 2008).

On Tregs, CTLA-4 mediates its suppression via intrinsic pathways, enhancing Treg suppressive characteristics such as TGF- β secretion, and extrinsic pathways, involving APCs (Bour-Jordan et al. 2009), the latter being described in more detail below. Further, by the mere fact that CTLA-4 has a higher affinity for CD80/CD86 than CD28, Tregs could outcompete T effector cells for binding to CD80/CD86 on

APCs (Bour-Jordan et al. 2009). In humans, despite the notion that CTLA-4 is highly expressed intracellularly and can be induced on the surface of activated Tregs (Birebent et al. 2004), CTLA-4 has been dismissed as the responsible molecule in several reports (Baecher-Allan et al. 2001; Jonuleit et al. 2001; Levings et al. 2001). It is however plausible that the nature (Fab fragment or whole antibody), dose and target (Treg *vs.* T effector cells) of the CTLA-4 blocking reagent has confused the role for CTLA-4 in Treg suppression (Bour-Jordan et al. 2009). Indeed, blocking CTLA-4 in suppression-assays performed using only CTLA-4-expressing Tregs did reduce suppression (Birebent et al. 2004). Further, Tregs obtained from patients with rheumatoid arthritis show decreased expression of CTLA-4, contributing to reduced suppressive function in these patients (Flores-Borja et al. 2008).

Tregs may also suppress via lysis of the target cell. Stimulated Tregs express granzyme A and kill autologous CD4⁺, CD8⁺, CD14⁺ and dendritic target cells via a perforin dependent pathway (Grossman et al. 2004).

Another possible way for Treg suppression is via CD39/CD73 mediated generation of adenosine from ATP, acting on the anti-inflammatory adenosine receptor A2a on effector T cells (Vignali et al. 2008). Interestingly, about 60% of Foxp3-expressing Tregs also express CD39 and these cells are suppressive whilst those lacking CD39 are not (Borsellino et al. 2007).

As mentioned above, Tregs have many target cells, including T cells (as described above), antigen presenting cells (Taams et al. 2005; Tiemessen et al. 2007), NK cells (Ralainirina et al. 2007), NKT cells (Azuma et al. 2003) and B cells (Lim et al. 2005), ultimately causing immunosuppression. Tregs are able to induce a phenotype of alternative activation in monocytes/macrophages. Alternatively activated macrophages, in contrast to classical macrophages, are associated with extracellular matrix and tissue remodeling and are less prone to induce inflammation. Treg-affected macrophages show CTLA-4 dependent suppressed expression of MHCII and co-stimulatory molecules (CD80/86) (Oderup et al. 2006), and also inhibited phagocytic activity and proinflammatory response against LPS (Taams et al. 2005; Tiemessen et al. 2007). In mice, Tregs are able to induce expression of the enzyme IDO in dendritic cells via the action of CTLA-4 on Tregs (Fallarino et al. 2003). This enzyme is involved in catabolism of tryptophan to pro-apoptotic products, leading to tryptophan starvation and apoptosis induction in surrounding effector T cells ultimately leading to immune tolerance. Further, in APCs, a particular IDO metabolite induces hemeoxygenase-1 (HO-1), actually expressed also in Tregs, leading to formation of carbon

monoxide (CO), creating a local immune suppressive environment (Bour-Jordan et al. 2009). Regarding NK cells, Tregs reduce NK cell cytotoxicity and IFN- γ production (Ralainirina et al. 2007).

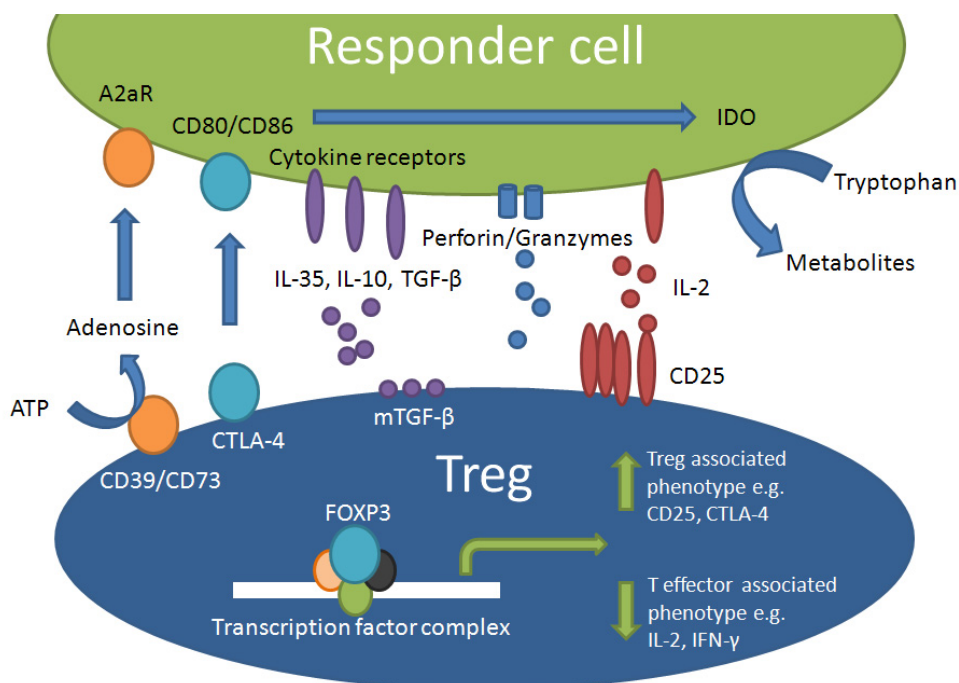


Figure 4

Proposed suppressive mechanisms for Tregs acting on responder cells (such as effector T cells and APCs) and the role for Foxp3. The mechanisms are explained in more detail in the text. Expression of Foxp3 poses the key switch to development of a regulatory phenotype and expression of Treg associated molecules. Simultaneously, Foxp3 downregulates the phenotype associated with non-suppressive effector T cells.

Thus, Tregs suppress T helper cell responses. Whether suppression is more aimed at any certain polarized T helper response is not fully known. IPEX patients display a broad spectrum of immune mediated diseases ranging from T_H2-dominated allergy to T_H1-dominated type 1 diabetes, (Chatila et al. 2000) (van der Vliet et al. 2007), indicating that Tregs control much of the T helper repertoire. Upon polyclonal stimulation, T_H17 cells, defined as CD4⁺ cells secreting IL-17, do not seem to be suppressed by Tregs (Annunziato et al. 2007), which rather

increase the secretion of IL-17 in co-culture *in vitro* (Flores-Borja et al. 2008). However, Tregs do suppress IFN- γ (T_H1-like cytokine) (Baecher-Allan et al. 2005) and patients with rheumatoid arthritis, a T_H1-associated disease, show defective Tregs (Flores-Borja et al. 2008). Regarding T_H2-like cytokines, Tregs seemingly suppress IL-13 (Baecher-Allan et al. 2005) but not IL-4 or IL-10 (Stephens et al. 2001). Interestingly, although later refuted (Annunziato et al. 2007), T_H2, as compared to T_H1, clones are not as easily suppressed by Tregs (Cosmi et al. 2004). Further, T_H2 deviated pollen allergic individuals show even more diminished Treg suppression during pollen season (Grindebacke et al. 2004), indicating that in non-allergic individuals, Tregs may be involved in controlling T_H2 responses.

Treg antigen-specificity

Tregs were originally discovered as mediators of self tolerance. However, Tregs have been shown to possess an equally broad repertoire of $\alpha\beta$ T cell receptors as non-Tregs (CD4⁺CD25⁻) (Fazilleau et al. 2007). Thus, Tregs may have the same capability as non-Tregs of responding to self- as well as non-self antigens. This broad reactivity is demonstrated by the fact that Tregs participate in immune regulation during infections, tumor diseases and most importantly, in the context of pregnancy, allorecognition and transplantation. In mice, Tregs activated against a certain allogenic donor is suppressive only of immune responses towards skin transplant from that particular allogenic donor (Kingsley et al. 2002).

In humans, Tregs need to be activated via their TCR to be suppressive, but once activated, they seem capable of suppressing the response against other antigens as well. Thus, influenza hemagglutinin (HA)-tetramer-binding Tregs suppressed influenza HA-responses whereas tetramer-non-binding Tregs did not. Further, these tetramer-binding Tregs also suppressed immune response against tetanus antigen. Hence, Treg activation is specific, while suppression is not, a phenomenon termed bystander suppression (Walker et al. 2005).

Treg migration and circulation

Natural Tregs originate in the thymus but soon leave this site to patrol the body as a part of the peripheral tolerance to self as well as to non-self antigens. Generally, as naïve cells (CD45A⁺), Tregs express receptors associated with lymphoid homing such as CCR7 and CD62L (Lim et al. 2006). Upon activation and expression of CD45R0, Tregs start expressing receptors coupled to non-

lymphoid homing and inflammation (such as CCR2, CCR4, CCR5, CCR6, CXCR3) while reducing the levels of lymphoid homing receptors (Lim et al. 2006). These activated Tregs are destined to migrate to inflamed tissues and suppress effector cells at that site. This wide expression of receptors ensures that Tregs can suppress both the initiation and effector phase of an immune response in lymphoid and inflamed tissues, respectively.

Table III. Chemokine receptor expression in resting peripheral blood Tregs (shown as proportional expression in the Treg population)

Reference	Expression over 50%	Expression 25-50%	Expression under 25%
(Hirahara et al. 2006)	CD45R0, CD62L, CLA, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CD11a	CD49d	CCR8, CXCR5, CXCR6, $\beta 7$ integrin
(Lim et al. 2006)	CCR4, CCR6, CCR7, CXCR4, CD62L	CCR5, CXCR3, CXCR6, $\alpha 4\beta 7$ integrin, CLA	CCR2, CCR9, CXCR5
(Iellem et al. 2001)	CCR4, CCR8		
(Lim et al. 2008)*	CCR4, CCR5, CCR6	CCR7, CXCR3, CXCR6	CCR2, CXCR5

* Information in this paper relies partly on data reported in (Lim et al. 2006)

Most peripheral Tregs are phenotypically activated/memory cells and thereby express non-lymphoid chemokine receptors such as CCR4, CCR5 and CCR6 and a smaller portion (25-50%) also express CCR7, CXCR3 and CXCR6 (Table III). In tonsils, almost all Tregs express CCR4 and CCR6, indicating that these receptors are not stably down-regulated upon recruitment to secondary lymphoid tissue (Lim et al. 2008).

It is clear that Tregs express CCR4 and CCR6 and also migrate in response to ligands for CCR4 (CCL17 and CCL22) and CCR6 (CCL20) (Iellem et al. 2001; Hirahara et al. 2006). In addition, Tregs have been shown to express CCR8 and migrate preferentially in response to the CCR8 ligand CCL1 (Iellem et al. 2001). The expression of CCR6 indicates that Tregs preferentially migrate to mucosal

tissues such as Peyer's patches where Tregs are indeed found. In mice, CCR4 and its ligand CCL22 have been shown to direct Treg invasion of cardiac allografts (Lee et al. 2005) and in humans, CCR4 was suggested to mediate T_H2-like cell invasion of the decidua during pregnancy (Tsuda et al. 2002).

Treg activation and suppression – regulating the regulators

In addition to the mechanisms that induce regulatory T cells, a number of factors and mechanisms have been shown to regulate Treg capacity. These mechanisms have been thoroughly investigated in various murine models.

Co-stimulatory signals are viewed as an essential part of Treg homeostasis. Supporting this, mice deficient in CD28 show defective Treg numbers (Yang 2008). In thymic Tregs, signaling through CD28, although poorly understood, seems to be able to induce and stabilize Foxp3 expression. However, once established and moved to the periphery, natural, as well as adaptive Tregs, seem to depend on CD28 stimulation for survival but not for suppressive effect (Bour-Jordan et al. 2009). As mentioned before, CTLA-4 in Treg function has been a matter of great debate and its role in Treg suppression is not settled. Importantly, data from CTLA-4 deficient mice show that CTLA-4 may be important when present but is not critical for normal Treg development (Yang 2008).

Several cytokines affect Treg suppression and Foxp3 expression. IL-2 is a T cell growth factor and necessary for Treg development, as shown by a lack of Tregs in IL-2 or IL-2 receptor deficient mice (Yang 2008), but also in Treg maintenance and suppressive activity (de la Rosa et al. 2004; Yates et al. 2007), especially when Tregs are activated under suboptimal conditions (Tran et al. 2009). Since Tregs themselves seemingly produce very little IL-2, they rely on effector cells for their survival. However, high doses of IL-2 abrogate Treg unresponsiveness (Dieckmann et al. 2001) and can render T effector cells insensitive to Treg suppression (de la Rosa et al. 2004). IL-2 shares the common γ receptor chain with IL-4, IL-7 and IL-15, all which seem able of maintaining or even enhancing Treg suppressivity *in vitro*, independent of TCR stimulation (Maerten et al. 2005; Yates et al. 2007). In mice, Tregs are induced by TGF- β , a process that is inhibited by IL-6 and promoted by LIF (Gao et al. 2009) and the vitamin A metabolite retinoic acid (Mucida et al. 2007). The role for retinoic acid in Treg augmentation was recently confirmed in humans where retinoic acid and TGF- β induced stable and suppressive *de novo* Tregs from naïve CD4⁺CD25⁻ cells (Wang et al. 2009). In humans, TNF reduces Foxp3 expression in the Treg population. The Treg defect

seen in asthmatic patients was linked to the TNF dependent airway inflammation seen in these patients (Lin et al. 2008).

In mice, signaling through GITR on Tregs attenuate the suppressive effects of Tregs and cause autoreactivity *in vitro* (Shimizu et al. 2002). Further, Tregs stimulated strongly via their TCR before addition to stimulated T effector cells show reduced suppression (Baecher-Allan et al. 2002).

In total, it has been discussed that regulation of Tregs may result in a shutdown of Treg suppressive function at times when the immune activating signals are very high, during e.g. an infection, to later be restored when the need for immune limiting mechanisms is increased. Further, this would render Tregs capable of suppressing weakly activated cells, during e.g. the onset of autoimmunity (Baecher-Allan et al. 2002).

Very recently, lessons from murine studies have pointed out that the Treg target cells have an active role in the suppression, providing signals, presumably via receptor-ligand interactions, to the Tregs, enhancing their suppression (Collison et al. 2009). Although it is currently unknown precisely what mediates these signals, the above mentioned factors, e.g. CTLA-4, CD28 etc. constitute good candidates.

Treg relation to other T cell subsets – the plasticity of Tregs

Tregs and T_H17 cells have been suggested to be in reciprocal relation to each other (Bettelli et al. 2006). Thus, it was hypothesized that high levels of TGF- β promoted Treg, whereas lower levels of TGF- β in combination with IL-6 enhanced T_H17 development (O'Garra et al. 2008). In mucosal tolerance in mice, retinoic acid co-operates with TGF- β to promote Foxp3 expression. Reciprocally, RA inhibits TGF- β /IL-6 induced T_H17 development (Mucida et al. 2007). EBI3, a subunit of the Treg cytokine IL-35 discovered in mice, forms IL-27 with p28. IL-27 inhibits RORC expression and IL-17 secretion from naive T cells *in vitro*, hence acting with Tregs to suppress T_H17 development (Diveu et al. 2009).

Tregs may also be re-programmed into other T helper phenotypes. Interestingly, in humans, resting double-positive Foxp3⁺RORC⁺ cells have been found in tonsils. Further, purified Tregs from blood, particularly those lacking HLA-DR (Beriou et al. 2009) can develop into IL-17 producers upon *in vitro* stimulation (Koenen et al. 2008). They may also express transcription factors associated with T_H1-(TBX21) and T_H2-(GATA3) like immunity (Voo et al. 2009). However, the reduction in suppressive function accompanying a Treg-T_H17 shift was shown to be transient

(Beriou et al. 2009). It is yet to be shown if T_H17 cells can be reprogrammed to Tregs (Zhou et al. 2009a).

Tregs do not only show flexibility towards T_H17 development. Murine Tregs, expressing moderate levels of Foxp3, could develop into T_H2- or T_H1-like cells, as shown by production of IL-4 or IL-10, respectively (Gavin et al. 2007; Wan et al. 2007).

In conclusion, during the last decade, intensive research on Tregs and their role in immune regulation has resulted in vast knowledge about this subset. However, there are still many questions remaining. Especially, the role for Tregs in specific conditions, including pregnancy, is still unclear. Further, the dynamics of the Treg population, especially in relation to other T helper subsets, has recently received a lot of attention but remains unsettled.

Regulatory CD4⁺CD25^{high} cells in pregnancy

Findings on regulatory T cells in healthy and complicated murine pregnancy

Already in 1982, Chaouat and co-workers demonstrated that splenic cells from multiparous mice lacked cytotoxic activity against paternal cells. This suppression was explained by the presence of antigen-specific regulatory cells, capable of inhibiting cytotoxicity as a third party in a mixed leukocyte culture cytotoxicity assay (Chaouat et al. 1982).

More than twenty years later, the interest for the revived Treg population, now defined as CD4⁺CD25⁺, in pregnancy was raised. Using murine models of normal pregnancy, it was shown that CBA mated C57BL/6 mice and C57BL/6 mated BALB/c mice displayed increased numbers of functionally suppressive Tregs in all tissues investigated (blood, lymph nodes, spleen and uterine tissue) (Aluvihare et al. 2004; Zhao et al. 2007). In adoptive transfer trials, BALB/c nude females transferred with CD25⁺ depleted lymphocytes from BALB/c females were unable to conceive with C57BL/6 (allogenic) males but were successfully mated with BALB/c (syngenic) males (Aluvihare et al. 2004). Tregs can also be depleted *in vivo* by treating mice with the anti-CD25 antibody PC61. Using this approach, Treg depleted mice carrying allogenic fetuses showed strikingly smaller litter sizes, or alternatively, if depleted prior to mating, reduced implantation rates

(Zenclussen et al. 2005b; Darasse-Jeze et al. 2006), corresponding well to the adoptive transfer experiments. These data suggested that Tregs were important for initiation of pregnancy, independent of a prior exposure of the maternal immune system to paternal alloantigens. Further, it also implicated that Tregs were only needed during pregnancies actually involving paternal alloantigens.

The classical murine abortion model CBA/J (female) × CBA/2J (male) is characterized by increased abortion rates and decidual T_H1 reactivity against paternal antigens (Zenclussen et al. 2005b). In this model, Tregs were diminished in spleen, thymus and placenta (Zenclussen et al. 2005a; Zenclussen et al. 2005b). When transferring Tregs from normal pregnant mice (CBA/J × BALB/c) to the abortion model prior to implantation, the abortion rate was dramatically decreased. The reduced abortion rate was explained by infiltration of Tregs in the decidua, causing a rise in Foxp3, IL-10, TGF- β , HO-1, LIF and neuropilin-1 levels. This was not linked to T_H1 abrogation since TNF and IFN- γ levels were unaffected (Zenclussen et al. 2005a). Further, IDO and IL-4, both highly implicated in fetal tolerance, were not a part of the tolerogenic environment created by Treg transfer (Zenclussen et al. 2005a). Of note, although Tregs from both normal pregnant and non-pregnant females could infiltrate the decidua, only pregnancy-primed Tregs were fetal-protective (Zenclussen et al. 2005b).

The question of what exerts the Treg enhancement during normal pregnancy is obviously important. In C57BL/6 female mice, matings with both CBA and C57BL/6 males caused Treg expansion, indicating that pregnancy itself, alternatively minor histocompatibility complexes, was the driving force (Aluvihare et al. 2004). The role for minor histocompatibility complexes in fetal tolerance is not to be neglected. In fact, the CBA/J (H2k) × CBA/2J (H2d) abortion model depends on these allogenic disparities. Thus, CBA/J females mated with BALB/c males (H2d) present with healthy pregnancy, despite the similar major histocompatibility backgrounds of the CBA/2J and BALB/c males. Further, Tregs transferred from CBA/J × BALB/c mated, but not virgin, females were able to rescue CBA/J × CBA/2J mated females from fetal resorption (Schumacher et al. 2007). Interestingly, Tregs from CBA/J × C57BL/6 (H2b) matings, where the males were major histocompatibility complex mismatched, were less protective than those from CBA/J × BALB/c (H2d) matings (MHC-matched, minor histocompatibility complex mismatched males) in preventing fetal rejection in the CBA/J (H2k) × CBA/2J (H2d) abortion model (Schumacher et al. 2007). This suggested that Tregs indeed are MHC-specific and that in the abortion model, the reactions against minor compatibility antigens were prevented by Tregs generated against the same MHC (but different minor histocompatibility

antigens) by so called “linked immune suppression”. Unfortunately, syngeneically mated females were not investigated, leaving the role for pregnancy itself (e.g. hormonal effects) unclear. However, when addressed in the C57BL/6 and BALB/c models (Aluvihare et al. 2004), syngenic pregnancy actually caused a similar Treg expansion as seen upon allogenic matings and it was concluded that hormones were urging the Tregs. It was later shown that the expansion seen in syngenic pregnancy correlated with the number of male fetuses in uteri, i.e. presence of male minor histocompatibility antigens (Zhao et al. 2007).

As to the role of pregnancy hormones, C57BL/6 mice treated with estradiol showed increased systemic Treg numbers, Foxp3 expression and enhanced suppression, comparable to the situation during pregnancy (Polanczyk et al. 2004; Polanczyk et al. 2005). This was also seen in female Kunming mice where E2 enhanced Foxp3 and IL-10 levels (Tai et al. 2008). However, BALB/c mice treated with progesterone and/or estradiol showed similar levels of Tregs (Zhao et al. 2007).

In C57BL/6 females, Foxp3 levels were elevated in uteri even prior to pregnancy, during the estrous phase, very likely as a preparation for pregnancy. The Foxp3 accumulation was associated with an increase in chemokines and their receptors, in particular the CCL4-CCR5 combination, once pregnancy took place (Kallikourdis et al. 2007). Along the same line, Robertson and colleagues have recently elegantly shown that seminal factors, among them TGF- β (Robertson et al. 2002), induce prompt tolerance to paternal alloantigens and that this tolerance is associated with an expansion of the Treg population (Robertson et al. 2009).

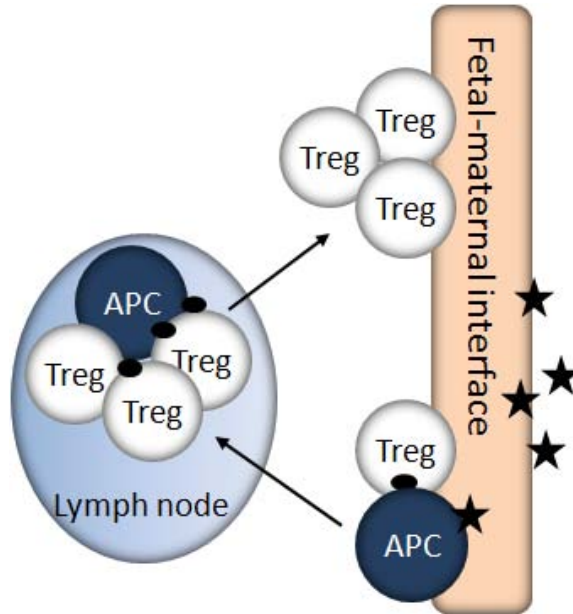


Figure 5

Explanation model for generation and expansion of Tregs in murine pregnancy.

To summarize, a model could be build based on murine data (Fig 5) (Zenclussen 2006; Guerin et al. 2009). Paternal alloantigens and seminal factors (black stars and dots) encountered already in the genital tract (Guerin et al. 2009), could initiate an early local generation of Tregs by help of specialized uterine APCs present in decidua. In addition, once reaching lymph nodes via efferent lymphatics, APCs could cause further peripheral expansion of Tregs. The Tregs would then migrate to decidua, via e.g. CCL4-CCR5 interactions (Kallikourdis et al. 2007), and create an immune privileged environment (Zenclussen 2006). Of note, in this model (Zenclussen 2006; Guerin et al. 2009), the hormonal effects on Tregs during pregnancy are left out.

Findings on regulatory T cells in healthy and complicated human pregnancy

Investigating Tregs in humans, in any condition, is complicated by the presence of activated cells sharing phenotypic properties with the Treg population. This is even more complicated during inflammatory conditions and in tissues, where T cell activation is high. In early reports, Tregs were defined as CD4⁺CD25^{+/high} but with the discovery of more specific Treg markers such as Foxp3, data on Tregs have become more reliable. As an example, when investigating the total CD25 population (holding both activated CD25^{dim} and suppressive CD25^{high} cells) in blood and decidua of early pregnant women, CD25 seemed to be down-regulated in decidua, which was interpreted as reduced activation of decidual T cells in normal pregnancy (Chao et al. 2002). Future specific markers, such as soluble cytokines that are more readily detectable, will provide new insights the role of Tregs in pregnancy. Interestingly, the subunits p35 and EBI3 of the newly discovered murine Treg-specific cytokine IL-35 can be found in placenta throughout pregnancy and more specifically in differentiated trophoblasts such as extravillous trophoblasts (Devergne et al. 2001).

Tregs seem to be enriched at the fetal-maternal interface throughout healthy pregnancy (Heikkinen et al. 2004; Sasaki et al. 2004; Tilburgs et al. 2006; Tilburgs et al. 2008), and Foxp3⁺ cells localize primarily to the decidua basalis (Tilburgs et al. 2008). However, in contrast to the findings in mice, all reports in the human system have not been univocal regarding the systemic Treg changes. Most early studies showed that the number of CD4⁺CD25^{+/high} cells was increased in early pregnancy (Sasaki et al. 2004), peaking in second trimester and declining towards the end of pregnancy and post-partum (Heikkinen et al. 2004; Somers et al. 2004). However, there are also reports showing unaltered circulating CD4⁺CD25^{high} frequencies in second trimester (Tilburgs et al. 2006; Tilburgs et al. 2008).

Supporting the importance for systemic Tregs in pregnancy, patients experiencing RSA show reduced levels of circulating CD4⁺CD25^{high} cells, both during (Sasaki et al. 2004; Yang et al. 2008a; Jin et al. 2009) and after pregnancy (Fraccaroli et al. 2009), the latter finding also confirmed by the more specific marker Foxp3. Interestingly, in fertile women, the pre-ovulatory rise in estradiol level is associated with increased circulating Foxp3⁺ Treg numbers, a phenomenon not seen in RSA patients (Arruvito et al. 2007). Infertility and RSA have also been connected to decreased levels of Foxp3 mRNA in uterine tissue (Jasper et al. 2006)

and reduced numbers of CD4⁺CD25^{+/high} cells in decidua (Sasaki et al. 2004; Yang et al. 2008a).

As for RSA, in preeclampsia, the prevailing hypothesis has been that diminished Treg numbers and/or function are somehow involved in the disease. When defined as CD4⁺CD25^{+/high}, preeclamptic women show reduced circulating Treg numbers (Darmochwal-Kolarz et al. 2007; Sasaki et al. 2007) or levels similar (Paeschke et al. 2005; Hu et al. 2008) to those of healthy pregnant women. More recent studies of Foxp3 protein support the role for reduced Treg numbers (Steinborn et al. 2008; Toldi et al. 2008; Prins et al. 2009) and even suppressive function (Steinborn et al. 2008) in preeclampsia. Further, preeclamptic placentas contain fewer Foxp3-expressing T cells (Sasaki et al. 2007). However, none of these studies stratified their material for onset of disease. Early-onset, as opposed to late-onset, preeclampsia is associated with disease severity and might also represent a more primary placental disease with a different pathophysiology, why research on this subgroup of women has been recommended (Ilekis et al. 2007).

How Tregs are enriched at the fetal-maternal interface in mice seems fairly settled, involving chemokine receptors, hormones, seminal factors and paternal alloantigens. However, in human pregnancy, Treg enrichment remains somewhat of a mystery. Alike the murine model, human Tregs could be attracted to the fetal-maternal interface. hCG and CCL5 are released by trophoblasts and were recently suggested to attract Tregs and to enhance their Foxp3 expression, respectively (Fracaroli et al. 2009; Schumacher et al. 2009). Further, estradiol, present at increased concentrations in pregnancy serum and even more in placenta, enhances the suppressive function of Foxp3-expressing Tregs (Prieto et al. 2006). The role for seminal factors in Treg expansion in humans remains unclear.

Yet another unanswered question is the occurrence of paternal/fetus-specific Tregs. In mice, Tregs seems to protect the fetus in a MHC-specific manner. Since, in the CBAJ x CBA/2J (H2d) abortion model, only Tregs from MHC-matched pregnant (CBAJ x BALB/c (H2d)), not MHC-mismatched pregnancies (CBAJ x C57/BL6 (H2b)) or virgin mice protected against fetal rejection (Zenclussen et al. 2005b; Schumacher et al. 2007). In humans, it was shown that *in vitro* depletion of Tregs from decidual leukocytes, but not from peripheral leukocytes, leads to enhanced immune reactivity against fetal antigens (umbilical cord blood cells) during normal pregnancy (Tilburgs et al. 2008). This, together with the finding of

reduction of peripheral Tregs, was interpreted as selective migration of fetus-specific Tregs from peripheral blood to decidua.

In conclusion, several studies have highlighted the role for Tregs in murine pregnancy. Still, the situation in healthy and preeclamptic human pregnancy remains obscure. The confusion likely owes much to the problems associated with identifying “true” Tregs in humans. Before the issue of correct definition of Tregs has been solved, no reliable information on Treg frequency, phenotype and function can be obtained. Further, the big issue is of course the presence of fetus-specific Tregs during pregnancy.

Aims and hypotheses

General aim

There is a widespread interest in understanding the seemingly complex role of regulatory T cells in immune regulation. During pregnancy, which is a state of tolerance of the fetus, without dramatically altering susceptibility against infections, immune regulation is greatly challenged.

The general aim of this thesis was to determine, using updated methods, the antigen specificity, frequency, phenotype and function of regulatory T cells in first to second trimester human pregnancy. Further, by applying this knowledge, we wanted to enlighten the importance of these cells in preeclampsia.

Specific aims

- In paper I, we aimed at characterizing the ability of circulating regulatory T cells to regulate anti-paternal T_H1 and T_H2 responses in second trimester pregnancy. The purpose was to determine if Tregs in pregnancy differentially regulated 1) responses against paternal alloantigens as compared to unrelated alloantigens 2) T_H1 and T_H2 responses against the different alloantigens
- In paper II, the aim was to assess the frequency, phenotype and function of circulating Tregs in PBMC from healthy second trimester pregnant women, non-pregnant women and *in vitro* 17 β -estradiol/progesterone stimulated PBMC of non-pregnant women
- In paper III, the aim was to determine the frequency and phenotype of circulating Tregs found in women with severe early-onset preeclampsia as compared with healthy pregnant and non-pregnant women
- Paper IV was aimed at characterizing the Tregs found at the maternal-fetal interface as compared with those circulating in blood during first trimester pregnancy. Further, we wanted to quantitatively determine the presence of T_H1, T_H2, T_H17 cells in relation to Tregs

Hypotheses

- In paper I, we hypothesized that Tregs from pregnant women would suppress type 1, but not type 2 immune responses towards paternal alloantigens. Further, we anticipated that Tregs from pregnant, as compared with non-pregnant, women would be more suppressive of both type 1 and 2 immune reactions against unrelated alloantigens
- In paper II, we hypothesized that the frequency of circulating Tregs would be enhanced in healthy pregnant women and that this could be induced *in vitro* by estradiol and/or progesterone. Further, we anticipated Treg suppressive phenotype and function to be more pronounced in pregnant as compared with non-pregnant women
- In paper III, we hypothesized that severe early-onset preeclampsia would be associated with a reduced frequency of circulating Tregs and possibly also a less homogenous Treg phenotype with regard to expression of Treg-associated markers
- In paper IV, it was hypothesized that Tregs and T_H2 cells would be enriched, whereas T_H1 cells would be fewer in first trimester decidua as compared with blood. The presence of T_H17 cells was more impartially studied

Materials and Methods

Subjects

All studies were approved by the Local Ethics Committee at Linköping University. Informed consent was obtained from all subjects. A description with relevant and available information on all the subjects included in paper I-IV is given in table IV. The individual characteristics of the preeclamptic patients included in paper III are shown in table V.

Paper I

For methodological development, blood was obtained from two healthy non-pregnant women (age 23 and 37 years, respectively). Twenty-one second trimester pregnant women with no signs of pregnancy complications and the corresponding fathers to be, visiting the maternity outpatient care unit in Linköping (Kvinnohälsan) were asked to participate in the study. Ten non-pregnant women and ten men, who were all employees/students at Linköping University or blood donors at Linköping University Hospital, served as control subjects.

Paper II

Thirty-eight second trimester pregnant women with no signs of pregnancy complications, visiting the maternity outpatient care unit in Linköping (Kvinnohälsan), were asked to participate in the study. Seventy-one non-pregnant women, employees/students at Linköping University or blood donors at Linköping University Hospital, served as control subjects.

Paper III

Women with *de novo* hypertension appearing after gestational week 20, diagnosed with severe early onset preeclampsia, were included in the study group. These

women were recruited at the delivery wards at Linköping University Hospital and Ryhov Hospital, Jönköping, Sweden.

The diagnosis severe preeclampsia was defined according to WHO criteria as:

- High blood pressure: diastolic ≥ 160 mmHg and/or systolic 110 mmHg on two separate occasions ≥ 4 hours apart.
- and/or:
- Proteinuria: 5 g/day or 3+ (dipstick grading) on two separate occasions ≥ 4 hours apart.

The diagnosis early onset preeclampsia was defined according to WHO criteria as:

- Onset of preeclampsia before gestational week 32
- At least moderate preeclampsia: ≥ 140 mmHg and/or systolic 90 mmHg on two separate occasions ≥ 4 hours apart, proteinuria 300 mg/day or 1+ (dipstick grading) on two separate occasions ≥ 4 hours apart.

The preeclamptic patients were included in the study during 2007-2009. Twenty healthy second trimester pregnant and twenty non-pregnant women (recruited during two periods in 2007 and 2008) served as control subjects. Ten of the healthy second trimester pregnant and ten of the non-pregnant women (recruited in 2007) were also included in paper II.

Paper IV

Eighteen healthy first trimester pregnant women who underwent elective surgical abortion at Linköping University Hospital participated in the study. All pregnancies were detected viable and dated by crown-rump length measurement using ultrasound. All pregnant women were administered misoprostol (Cytotec®) prior to the abortion procedure. Venous blood was obtained immediately prior to the termination of pregnancy. Venous blood from four healthy non-pregnant women was used for methodological validation experiments.

Table IV. Characteristics of the subjects included in papers I-IV. All data are given as medians and range (within parenthesis) or as categorical data.

Paper I			Paper II			Paper III			Paper IV	
Method	Treg-MLC ELISPOT		Six-color flow cytometry		Hormonal stimulation	<i>In vitro</i> suppression assay		Six-color flow cytometry	Six-color flow cytometry	
PE/HP/NP ^g	HP	NP	HP	NP	NP	HP	NP	PE	HP	NP
Subjects (n)	21	10	34	10 ^h	13	14	14	10	20 ^h	4
Characteristics of the subjects at inclusion										
Age (years)	28 (19-36)	29 (25-38)	26 (19-35)	30 (27-38)	25 (21-36)	29 (20-37)	29 (23-36)	30 (17-46)	26.5 (20-36)	27 (26-29)
Gestational week (weeks)	Approx. 25	N/A	N/A	25 (25-26)	N/A	Approx. 25	N/A	29 (25-32)	N/A	N/A
Blood pressure (mmHg)	No info	No info	No info	110/70 (105/60- 120/80)	No info	No info	No info	175/110 (140/95- 190/120)	115/70 (110/60- 130/80)	No info No info
Use of hormonal contraceptives (yes/no)	N/A	No info	No info	N/A	0/13	N/A	0/14	N/A	N/A	5/5 ^e 0/4
Menstrual cycle phase (luteal/follicular)	N/A	No info	No info	N/A	6/3 ^a	N/A	6/5 ^d	N/A	N/A	3/1
Characteristics of the subsequent delivery and the baby										
Delivery week (weeks)	40 (34-42)	N/A	N/A	40 (35-41)	N/A	40 (27-42) ^b	N/A	29.5 (26-33)	41 (35-42)	N/A N/A
Gender of baby (male/female)	6/15	N/A	N/A	4/6	N/A	10/2 ^b	N/A	6/4	11/9	No info N/A
Birth weight (g)	3500 (2200- 4380)	N/A	N/A	3413 (2560- 3700)	N/A	3370 (1104- 4560) ^b	N/A	1095 (508- 1653)	3505 (2560- 4190)	N/A N/A
Birth method (PN/VE/CS) ^c	17/2/2	N/A	N/A	8/1/1	N/A	10/1/1 ^b	N/A	0/0/10	17/2/1	N/A N/A
Obstetrical history of the subjects										
Previous pregnancies (n)	0 (0-2)	No info	No info	0 (0-2)	No info	0 (0-6)	0 (0-0)	1 (0-4)	0 (0-2)	1 (0-1) 1 (0-6)
Previous births (n)	0 (0-1)	No info	No info	0 (0-1)	No info	0 (0-0)	0 (0-0)	0.5 (0-2)	0 (0-1)	0 (0-3) 0 (0-0)

^a Data missing on four women. ^b Two unknown outcomes of pregnancy. ^c PN: Normal delivery, VE: Vacuum extraction, CS: Caesarean section. ^d Three women were on day 15 in their menstrual cycle. ^e Only ten of the women gave information about pregnancy history, use of hormonal contraceptives and menstrual cycle status. ^f Data missing on one patient. ^g PE: Preeclampsia, HP: Healthy pregnant, NP: Non-pregnant. ^h Ten of the healthy pregnant and ten of the non-pregnant women in paper II were also included in paper III. N/A not applicable.

Table V. Individual characteristics of the preeclamptic women included in paper III.

Subject no.	Age (years)	Gestational week (weeks)	Blood pressure (mmHg)	Proteinuria (dipstick grading)	Medical treatment (according to referral)	Delivery week (weeks)	Gender of baby (male/female)	Birth weight (g)	SGA ^e	Comment; maternal health and pregnancy	Previous pregnancies (n)	Previous births (n)
1	27	30	160/105	1+	Betamethasone ^a	30	male	1115	Yes		2	1
2	26	29	180/120	3+	Betamethasone ^a dihydralazin ^b	31	male	1074	Yes		0	0
3	36	32	170/120	3+	Betamethasone ^a	33	male	1653	Yes	Mitral insufficiency	1	1
4	31	27	185/115	3+	Unclear if Betamethasone	29	female	1015	Yes		0	0
5	17	25	190/120	3+	No Betamethasone	29	male	1035	Yes		1	0
6	35	26	140/95	3+	Betamethasone ^a Tinzaparinmatritium ^d	26	male	508	Yes	Previous pulmonary embolism	0	0
7	21	28	180/110	3+	No Betamethasone magnesium diazepam ^c	28	female	836	Yes	BMI 33, eclampsia	0	0
8	46	32	170/105	3+	No Betamethasone	32	female	1366	Yes		4	2
9	37	31	170/105	3+	Betamethasone ^a	32	male	1410	No info.	HELLP ^f	1	1
10	29	29	180/110	3+	Betamethasone ^a dihydralazin ^b	29	female	1120	No		1	1

^a Betapred®, ^b Nepresol®, ^c Stesolid®, ^d Innohep due to previous pulmonary embolism, ^eSGA = Small for Gestational Age, ^fHELLP = Hemolysis - Elevated Liver enzymes - Low Platelets.

Cell separation and sorting

Separation of peripheral blood mononuclear cells (PBMC) (paper I-IV)

Whole blood was obtained in diaminoethanetetraacetic acid (EDTA) (for flow cytometry in papers II and III) or sodium-heparin (for functional assays in papers I, II and for flow cytometry in paper IV) vacutainer tubes. PBMC were separated within two hours on Lymphoprep (Axis-Shield, Oslo, Norway). Lymphoprep is a gradient solution with a density of 1.077 g/mL, consisting of sodium diatrizoate (also known as Hypaque; 9.1 % w/v) and polysaccharide (5.7% w/v). Diluted blood is layered on top of Lymphoprep and during centrifugation, erythrocytes aggregate while lymphocytes and monocytes are trapped in the interphase between Lymphoprep and the diluted blood. Erythrocytes are depleted as they pellet at the tube bottom. Further, granulocytes, which shrink by the high osmolarity of the Lymphoprep solution, thereby acquiring a higher density than mononuclear cells, sediment with the erythrocytes. To discard plasma and Lymphoprep remnants, cells were washed in Hank's balanced salt solution (HBSS; Gibco BRL, Paisley, Scotland; UK) before use in further experiments. Percoll gradient solution has been suggested a better reagent for leukocyte separation due to its iso-osmolarity and pH-neutrality (Nagaeva et al. 2002). However, our own experience, using trypan blue exclusion of dead cells, has shown very little negative effects of Lymphoprep on PBMC viability (generally above 90%).

For culturing in all studies, cells were incubated at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were resuspended in T cell culture medium (TCM) consisting of Iscove's modified Dulbecco's medium (IMDM; Gibco BRL) supplemented with L-glutamine (292 mg/mL; Sigma Aldrich, Stockholm, Sweden), sodium bicarbonate (3,024 g/L; Sigma), penicillin (50 IE/mL), streptomycin (50 µg/mL) (Cambrex, New Jersey, USA), 100x non-essential amino acids (10 mL/L; Gibco BRL) and 5% heat inactivated fetal bovine serum (FBS; Sigma). For flow cytometry, cells were resuspended in PBS pH 7.4 (Medicago AB, Uppsala, Sweden) supplemented with 0.1 % or 2% heat inactivated FBS. For MACS separation, cells were resuspended in PBS with 2 mM EDTA (Sigma) and 2% FBS. Alternatively, PBMC were lysed in RNeasy RLT lysing buffer (Qiagen, West Sussex, UK) and frozen at -80°C until RNA extraction.

Separation of first trimester decidual mononuclear cells (DMNC) (paper IV)

Vacuum aspirated abortion tissue was rinsed in sterile saline solution and decidual tissue, representing the maternal side of the fetal-maternal interface, was macroscopically separated from fetal tissue and placenta. The latter tissue was further handled according to the regulations at the Department of Ob/Gyn, Linköping University Hospital. The decidual tissue was placed in IMDM supplemented as described above. Using a scalpel, the tissue was divided into smaller pieces and minced in a strainer (approximately 0.5 mm pores) to disperse the cells from the connective tissue. The cell mixture was placed on top of Ficoll-Paque plus (GE Healthcare, Uppsala, Sweden) and mononuclear cells (DMNC) were acquired as described for PBMC above. Ficoll-Paque plus has traditionally been used for separation of DMNC at our lab but to the best of our knowledge, Ficoll-Paque plus and Lymphoprep are comparable reagents. DMNC were filtered through a 30 µm nylon mesh filter (Miltenyi Biotech, GmbH, Bergisch Gladbach, Germany) before used in further experiments. The method of mechanic dispersal of mononuclear cells, followed by gradient centrifugation, has been shown to maintain, as compared to *in situ* immunohistochemistry, the expression of markers such as CD3 and CD4 on decidual leukocytes (Rasheed et al. 1992). An alternative way of cell dispersal is to use enzymatic digestion of extracellular matrix components such as collagen. However, although increasing the total cell yield, this method has been shown to reduce the expression of critical leukocyte surface markers such as CD4 and CD8 (White et al. 2000).

Magnetic cell sorting (Dyna technology) (paper I)

Dyna beads are small (4.5 µm) uniform paramagnetic iron particles coupled to antibodies against the cell antigen of interest. When exposed to a magnet situated outside the tube wall, the bead-bound cells (positively selected cells) are attracted to the magnet and accumulate on the tube wall. Other cells (negatively selected cells) are left in solution and can easily be acquired for use in further investigations. Positively selected cells can then be treated with the Detachabead reagent, competing out the antigen from the antibody binding site, thereby leaving the cells free from both antibodies and beads. This may be one advantage of Dyna microbeads versus MACS (see below). However, this advantage might be counteracted by the large size of Dyna beads, potentially affecting the cells more than MACS beads. Another advantage is that Dyna beads are polystyrene coated thereby marketed as completely inert and not biodegradable as MACS beads, thereby, at least theoretically, causing less harm to the cells.

In paper I, CD4⁺CD25⁺ cells were separated using the CD4 Positive Isolation kit followed by the anti-CD25 bead portion (positive selection) of the CD4⁺CD25⁺ Treg kit, both from Dynal Biotech (Oslo, Norway). The number of anti-CD25 beads used was reduced to 3 beads/cell to select for those CD25 cells with the highest expression of CD25. This allowed retrieval of first CD4⁻ and then CD4⁺CD25⁻ cells to be pooled and used as Treg depleted PBMC responder cells. Bead bound cells were separated using the magnetic particle concentrator MPC-50 (Dynal Biotech). The negative fractions were saved in TCM and kept on ice. The positively selected cells were treated with Detachabead reagent (Sheep anti-mouse-Fab antibody) directly after selection, prior to a second round of positive selection or culture. Treg depleted PBMC and CD4⁺CD25⁺ cells were resuspended in TCM at 1 million lymphocytes/mL prior to culture.

Flow cytometry cells sorting (FACSARIA) and MACS pre-selection (paper II)

FACSARIA sorting is based on the general principle of flow cytometry, which is described further in the flow cytometry section. In the FACSARIA, the sample is introduced into a fluidics system and by hydrodynamic focusing in the flow cell (Fig 6, 1), cells are forced into the middle of the stream, one by one in a row. When the stream reaches the cuvette (enclosing 2 and 3), cells are slowed down and at the interrogation point (2), cells are intercepted by laser beams (3) which are led via fibre optic cables and focused at the interrogation point by lenses and prisms. Fluorochromes on the cells are excited and emit light that are transported via glass fibres to the detectors. The detectors (photomultiplier tubes; PMTs) are placed as octagons or trigons and equipped with band pass filters narrowing the wavelength detected and minimizing spectral overlap. After encountering the lasers, the stream passes through the nozzle (4), a 70/100 µm large opening, where energy with a given frequency is applied to the stream. This breaks the stream into uniform droplets.

The software monitors the distance between the intact stream and the top (gap (5)) and middle (drop 1 (6)) of the first broken droplet and uses this drop-delay information to identify which cell is in which drop. Depending on which requirements is set, the subsequent sorting of the drops will be based either on purity or yield. The gated cell (droplet) is then charged and when it reaches the deflection plates (7) it is deflected, forming a side-stream, into a pre-set collection tube (9). The non-gated droplets are sent into the waste unit (8).

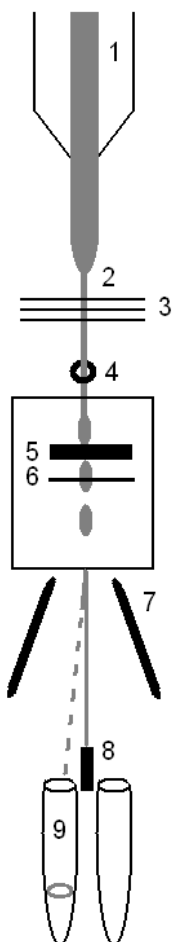


Figure 6

Schematic explanation of FACS Aria sorting. 1. Flow cell. 2. Cuvette. 3. Laser interrogation point. 4. Nozzle. 5. Droplet break gap. 6. Drop 1 (distance to first broken droplet). 7. Deflection plate. 8. Waste. 9. Collection tube.

To increase the purity and time effectiveness, a pre-enrichment step is often used prior to the FACS Aria sorting. In paper II, the CD4⁺ T cell isolation kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for negative selection of untouched CD4⁺ cells from PBMC separated from pregnant (n=14) and non-pregnant women (n=14). This kit uses the MACS (magnetic activated cell sorting) technology, originally described by Miltenyi *et al.* (Miltenyi *et al.* 1990), consisting of small (approx. 50nm) superparamagnetic biodegradable ferri-dextran beads. The beads are anti-biotin labelled and bind biotinylated antibodies attached to the cells. Beads can also be directly conjugated to the antibody. In negative selection, unwanted bead-bound cells are trapped when the sample is applied to the steel sphere matrix in the MACS column, whilst free cells (in this case CD4⁺ cells) pass through the column and can be used further. Dynal microbeads are alternative to MACS nanobeads, but as mentioned above, the former beads are larger and may cause activation of the cells. On

the other hand, the degradable MACS beads may have, at least in theory, toxic effects on cells due to release of iron during bio-decomposition. However, in our study, MACS beads were used for depletion and were thus highly unlikely of affecting the cells of interest.

In paper II, CD4⁺ selection (depletion of CD8⁺ T cells, γ/δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells) was performed according to the manufacturer's description using MS columns and a miniMACS separator (Miltenyi Biotec). The MACS sorted CD4⁺ cells were then labelled with mouse anti-human CD4-FITC (clone MT466; Miltenyi Biotec) and mouse anti-human CD25-APC (clone 2A3, BD Biosciences). For analysis, a portion of cells was also labelled with mouse anti-human CD127-PE (clone hIL-7R-M21, BD

Biosciences). Sorting of CD4⁺CD25⁻ responders and CD4^{dim}CD25^{high} T_{regs} was performed on a FACSARIA cell sorter (BD Biosciences) equipped with a 100µm nozzle. Sorting was set on the purity precision with a purity and yield mask of 32. Sorted populations were collected in TCM and typically showed purities above 99% upon reanalysis. Before culturing, CD4⁺CD25⁻ responders were allowed to rest for 2 hours, and CD4^{dim}CD25^{high} Tregs for 10-30 min, at 37°C.

***In vitro* functional assays**

Treg-MLC-ELISPOT assay (Dynal bead sorted Tregs) (paper I)

This assay was based on the one-way MLC-ELISPOT method originally described by Ekerfelt *et al* (Ekerfelt et al. 1997). Autologous PBMC (pregnant women/non-pregnant control), allogeneic PBMC (father/male control) and a freeze-thawed alloantigen-pool, consisting of PBMC collected from 20 healthy donors, were treated with 4% paraformaldehyde (PFA; Sigma). PFA treatment of PBMC has been shown to totally inhibit cytokine secretion, yet rendering cells capable of stimulating responder cells in the MLC (Ekerfelt et al. 1997). Cells were resuspended at 1 million PBMC/mL in TCM. The alloantigen-pool was used because it, due to the many alloantigens present, increases the probability of eliciting an alloresponse. It has been suggested that alloresponses theoretically reaches a plateau level when the pool consists of ten to fifteen randomly selected blood donors (Dubey DP 1986).

Initially, the minimum number of allogeneic stimulator cells needed for PBMC stimulation was investigated. PBMCs were separated from two healthy blood donors and responder PBMC from both donors were cultured alone or cross-wise with PFA-treated auto- or allogeneic stimulator PBMC in varying numbers (ratios 1:1, 1:2, 1:4, 1:8, 1:16). Cells were incubated over night in TCM, at 37°C, in coated ELISPOT wells for direct analysis of IFN-γ secretion from responder cells. The ELISPOT method is described below. All cultures were analyzed in triplicates at the ratio 1:2 (stimulator:responder) which was found to be the lowest number of stimulator cells needed for allogeneic stimulation to exceed the autologous response. The ability of Tregs to suppress the allogeneic response in MLC was then investigated using the same blood donors as in the previous MLC-experiment. IFN-γ secretion was measured from PBMC or Treg depleted responders cultured alone or with PFA-treated allogeneic stimulator cells in TCM.

In addition, allogeneically stimulated Treg depleted responders (ratio stimulator: responder; 1:2) were cultured with Treg cells (ratio responder:Treg; 1:1 and 2:1). An incubation period of approximately 42 hours was found to be optimal. The incubations were performed at 37°C. All cultures were analyzed in triplicates.

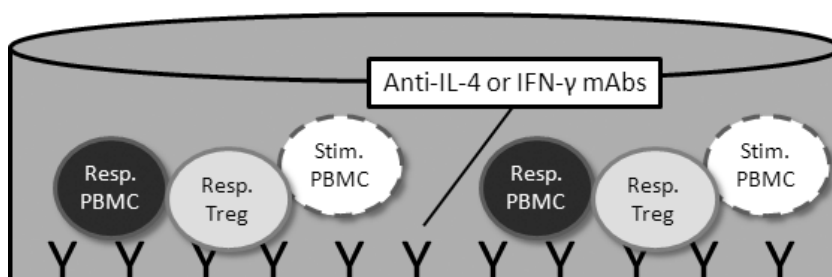


Figure 7

Schematic explanation of the Treg MLC-ELISPOT assay. Maternal/control PBMC responder cells (black) were cultured with PFA-treated paternal/unrelated PBMC (white with dotted borders) in ratio 2:1 (responder: PFA-treated PBMC) and autologous Tregs (light grey) in ratio 1:1 or 2:1 (responder: Treg) in ELISPOT plates coated with anti-IL-4 or IFN- γ monoclonal antibodies.

PBMC and Treg depleted cells from twenty-one pregnant women and ten non-pregnant women were stimulated with PFA treated PBMC from the corresponding fathers to be or control males, respectively. In addition, responder PBMC and Treg depleted cells were stimulated with autoantigens or pooled alloantigens. The stimulator: responder cell ratio was set to 1:2 based on the initial experiments. Stimulated PBMC and Treg depleted responder cells were then cultured with Tregs in responder:Treg ratio 1:1 or 2:1, depending on cell yield. All cultures were analyzed in triplicates and occasionally in duplicates.

MLC-Treg cultures were analyzed for responder cell secretion of IFN- γ and IL-4 by ELISPOT. Ten pregnancy samples were analyzed for secretion of IL-4 and eleven for secretion of IFN- γ . For the non-pregnant controls, IFN- γ secretion was investigated in ten samples, since the normal allogeneic response is predominantly a T_H1 situation with no or very low IL-4 secretion (Danzer et al. 1994; Senvik et al. 2003). To confirm this, IL-4 was analyzed in six of the ten control samples. However, Tregs were not reconstituted to these cultures. Autologously stimulated PBMC were used as a methodological control for the

addition of PFA-treated cells to the wells. As cytokine secretion from these cells did not significantly differ from the unstimulated secretion, unstimulated secretion was chosen as a measure of the spontaneous secretion.

Treg functional assay (MACS-FACSria sorted Tregs) (paper II)

In paper II, 96-well plates (BD Biosciences, Le Pont De Claix, France) were coated by incubation for 24 hours at 4°C with 1 or 5 µg/mL anti-CD3 antibody (clone UCHT1; AbD Serotec) and 5 µg/mL rat anti-human CD28 (clone YTH913.12, AbD Serotec) followed by washing in PBS. The coating antibody concentrations were based on initial titration experiments. CD4⁺CD25⁻ responders were plated alone or in co-culture with CD4^{dim}CD25^{high} Tregs at ratios of 1:1, 2:1 or 4:1 in singlet or duplicate cultures and cultured for 91-93 hours at 37°C before analyzing the supernatants. The ability of CD4^{dim}CD25^{high} cells to suppress cytokine secretion by CD4⁺CD25⁻ responders (at ratios 1:1, 1:2 and 4:1) was calculated as a suppressive index (SI) according to: $(1 - (\text{secretion in co-culture} / \text{secretion from CD4}^+\text{CD25}^- \text{ cells alone})) \times 100$.

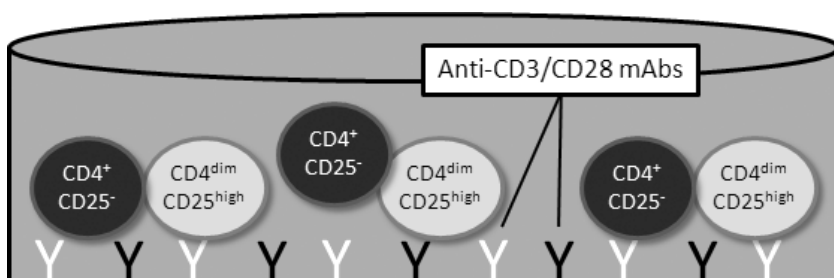


Figure 8

Treg suppression assay. CD4⁺CD25⁻ cells (black) were cultured with CD4^{dim}CD25^{high} Tregs (grey) in 96-well plates coated with α-CD3/α-CD28 antibodies (5 µg/mL). After 3 days, supernatants were analysed for presence of cytokines using Luminex.

Hormonal stimulation of PBMC (paper II)

In paper II, 6-well plates (Costar, Cambridge, MA, USA) were coated with 0.005 µg/mL mouse anti-human CD3 antibody (clone UCHT1; AbD Serotec, Oxford, UK) for 2 hours at 37°C followed by washing the wells with PBS. The anti-CD3

antibody concentration was chosen based on titration experiments where 0.005 μ g/mL anti-CD3 antibody caused a low grade activation of the CD4⁺ cells with slight elevation of CD69 and CD25 expression. PBMC, isolated from non-pregnant women (n=7), were cultured in uncoated or anti-CD3 antibody coated wells with 10 nM-10 μ M 17 β -estradiol (water soluble E; Sigma Aldrich) and/or 200 nM - 200 μ M progesterone (water soluble P; Sigma Aldrich) in TCM for 3 days at 37°C. The starting concentrations of 17 β -estradiol and progesterone were chosen to be similar to the levels found in serum during second trimester pregnancy (O'Leary et al. 1991; Soldin et al. 2005). After incubation, cells were stained for six-color flow cytometry on FACSCanto II as described below.

Flow cytometry analysis of surface and intracellular protein expression

Flow cytometry – general principle

In flow cytometry, cells labeled with fluorochrome conjugated antibodies are introduced into a fluidics system consisting of a carrier liquid (sheath fluid) and the sample stream. By hydrodynamic focusing of the sample stream, the cells are forced to line up and pass laser beams as single cells. Fluorochromes are thereby excited and emit light of a wavelength specific for that particular fluorochrome. The photons are captured by detectors and transferred via filters to the photomultipliers (PMTs) which transmit the signal to a computer. Data are registered as pulse-peaks, with a given height, width and area. Besides providing information about specific antigen expression, cell granularity and size can be determined by flow cytometry, using the light scattering properties of the cells.

Four-color flow cytometry on FACSCalibur (paper I and II)

In paper I, the immunomagnetic selection of T_{regs} from control subjects was evaluated on four occasions by flow cytometry. Briefly, PBMC, CD4⁺CD25⁺ depleted PBMC and CD4⁺CD25⁺ cells were incubated with isotype controls or mouse anti-human CD25-FITC, CD4-PE and CD3-PerCP (paper I). This was followed by lyzing, fixating, washing and analyzing on FACS Calibur using CellQuest Pro version 4.0.2 software (BD Biosciences).

In paper II, PBMC from pregnant (n=14) and non-pregnant (n=34) women were analyzed using four-color flow cytometry to determine the frequency of CD4⁺CD25^{high} cells and the expression of Foxp3, CTLA-4 and CD27. PBMC were stained with antibodies against extracellular markers or isotype controls. Intracellular staining was thereafter performed using the eBioscience protocol for Foxp3 staining (paper II).

The absence of CTLA-4 surface expression on resting cells was confirmed on two separate occasions and this is in agreement with others (Jonuleit et al. 2001). One hundred thousand lymphocytes were collected and analyzed using FACSCalibur and the CellquestPro software (BD Biosciences). The Foxp3 antibody clone PCH101 binds the amino-terminus of the Foxp3 protein and has been shown to recognise both isoforms of the Foxp3 protein (Allan et al. 2007). The Foxp3 antibody clone PCH101 and 206D, as well as 236A/E7, was recently shown to identify the same Foxp3⁺ cell population, strengthening their credibility as being specific (Pillai et al. 2008; Zheng et al. 2008).

Six-color flow cytometry on FACSCANTO II (paper II-IV)

In paper II, PBMC from healthy second trimester pregnant (n=10) and non-pregnant (n=10) women were analyzed by six-color flow cytometry to obtain a more detailed phenotype analysis. In addition, 17 β -estradiol and progesterone stimulated PBMC (see below) were analyzed to evaluate the effects of these hormones on Treg frequency and phenotype. Paper III included PBMC from 10 preeclamptic, 20 second trimester pregnant and 20 non-pregnant women. Ten of these second trimester pregnant and non-pregnant women were also included in paper II. In paper IV, PBMC and DMNC from 18 first trimester pregnant women were used.

Cells were labelled as described for four-color flow cytometry. One hundred thousand lymphocytes were collected and analyzed using FACSCanto II (BD Biosciences) and the FACSDiva software (version 5.0.1; BD Biosciences). Absolute leukocyte (CD45), T lymphocyte (CD3) and T helper cell counts (CD4) in 50 μ L of EDTA whole blood was determined by using TruCount tubes (BD Biosciences) as described by the manufacturer (paper II and III). Briefly, the determination of lymphocyte density in a whole blood sample is based on the known number of beads in a given volume in the TruCount tubes.

Analysis of flow cytometry data (paper I-IV)

In paper II-III, all gating analysis was performed in a blinded manner, i.e. the evaluator did not know the origin of the sample. In paper I and IV, only cells from pregnant women were analyzed, disabling blinding of the analysis.

In paper I, cells were gated for analysis of lymphocytes by side/forward scatter and gating for analysis of T cells was based on CD3 expression. Gates for co-expression of the CD4 and CD25 molecules were set according to isotype controls. The CD25^{bright} gate was adjusted to contain CD4⁺ cells that expressed CD25 more brightly than CD4⁺CD25⁺ cells.

In paper II-IV, cells were gated for analysis of small lymphocytes by side/forward scatter, and when possible, gating for analysis of T cells was also based on CD3 expression (paper II-IV). Gates for expression of the CD4 and CD25 molecules (CD4⁺CD25⁺ or CD4⁺CD25⁻) were set according to isotype controls. The “classical” CD25^{high} gate was adjusted to contain CD4⁺ cells that expressed higher levels of CD25 than the discrete population of CD4⁺ cells that expressed CD25 (paper II and III). CD4^{dim}CD25^{high} cells were gated as CD25^{high} cells with a slightly lower expression of CD4 than the remaining CD4 population (paper I-IV). To avoid the possible errors introduced when subjectively setting the gate for CD25^{high} expression, the 0.5% of CD4⁺ cells expressing the highest levels of CD25 were also evaluated (paper II). These cells were referred to as “0.5% CD4⁺CD25^{highest}” cells. Mean fluorescence intensity (MFI) was evaluated by dividing the geometric MFI (gMFI) for Foxp3⁺ cells with the gMFI for Foxp3⁻ isotype controls, to correct for the instrumental day-to-day variations in fluorescence intensity measurements (paper II).

Analysis of cytokine production

Enzyme Linked Immuno SPOT assay (ELISPOT) (paper I)

ELISPOT is a highly sensitive technique for *in vitro* detection of cell-secreted cytokines at the single cell level, originally developed by Czerkinsky and colleagues (Czerkinsky et al. 1988). In principle, cells are cultured on anti-cytokine-antibody coated membranes, allowing immediate capture of cell-secreted cytokines on the membrane. Following cell removal, the cytokines are detected using a biotinylated anti-cytokine-antibody followed by adding enzyme-

conjugated streptavidin. The enzyme substrate forms a colored precipitate, under the microscope identified as a “spot”. Each spot corresponds to one cytokine producing cell and the results are expressed as a frequency of cytokine producing cells within a cell population. However, ELISPOT does not provide reliable quantification of cytokine secretion which can be a limitation of this method. Theoretically, a large number of cells, secreting a small amount of cytokine yet adding to a high bulk concentration, might fail to be detected with ELISPOT whilst providing a signal using for example the Luminex technology (see below) or ELISA. Still, when analyzing modest secretion, such as observed spontaneously from resting cells, ELISPOT provides the sensitivity needed for detection of certain cytokines such as IL-4 (Tanguay et al. 1994; Ewen et al. 2001; Ekerfelt et al. 2002). The sensitivity has been ascribed the close proximity between cells and capture antibody preventing *in vitro* consumption of cytokines due to cell surface receptor binding (Ewen et al. 2001).

In paper I, nitrocellulose bottomed 96-well MAHAN 4550 microtiter plates (Millipore, Bedford, MA, USA) were coated with mouse anti-human IFN- γ mAb or mouse anti-human IL-4 mAb (both from Mabtech, Stockholm, Sweden). Cultures were set in ELISPOT plates for direct analysis of cytokine secretion from responder cells. As negative controls, TCM alone, without cells, were added to some wells. No spots were detected in these wells. As positive controls, responder cells were stimulated with phytohemagglutinin (PHA; Sigma Aldrich, St. Louis, MO, USA), which always generated a strong response with several hundred spots. PFA-treated cells did not respond to PHA stimulation, confirming their inability to secrete cytokines. After incubation with cells, plates were emptied and incubated with biotinylated mouse anti-human IL-4 or mouse anti-human IFN- γ detection antibodies (both from Mabtech) followed by incubation with streptavidin-alkaline phosphatase (AP) conjugate (Mabtech). Plates were then washed and wells were incubated with alkaline phosphatase substrate BCIP-NBT (Bio-Rad, Solna, Sweden). Spots were manually counted under a dissection microscope.

Multiplexed microsphere-based flow cytometric assays (Luminex) (paper II)

In multiplexed microsphere-based flow cytometric assays, up to one hundred analytes (using the Luminex 100 system) can theoretically be detected in a single sample (Vignali 2000). Beads filled with a pre-defined mixture of red and infrared fluorescent dyes (1, Fig 9) are coupled to antibodies (2) against a specific analyte

(3). The mixture of red and infrared fluorescence in a bead forms the fingerprint, and the coupled antibody the specificity, for the particular analyte to be analyzed. Beads with different fingerprints and specificities may then be mixed to enable detection of several analytes in a single sample. Beads with bound analytes are then incubated with detection antibodies (4) conjugated with biotin (5) – forming a complex with streptavidin (6) bound to a green light emitting fluorochrome (7). The green fluorescence intensity will be proportional to the amount of bound analyte, thereby providing information about the concentration of analyte in the original sample. This obviously requires the use of a standard curve with known concentrations of the analytes.

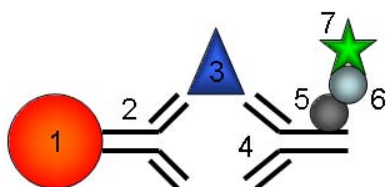


Figure 9

Schematic explanation to the multiplexed microsphere-based flow cytometric assay used in paper II. 1. Fluorescent bead, 2. Anti-cytokine capture antibody, 3. Cytokine, 4. Anti-cytokine detection antibody, 5. Biotin, 6. Streptavidin, 7. Fluorochrome used for quantification of the cytokine.

A great advantage with the multiplexed microsphere-based flow cytometric assays is that the actual secreted cytokine is detected. An alternative method would otherwise be flow cytometric detection of intracellular cytokines. However, this would limit the number of cytokines analyzed simultaneously due to availability of fluorochromes.

In paper II, cell culture supernatants were analyzed by LINCOplex kit 96 well plate assay according to the manufacturer's instructions using the Luminex 100 instrument (Luminex Corporation, Austin, Texas, USA). STarStation software (v 2.3, Applied Cytometry Systems, Sheffield, UK) was used for acquisition and analysis of data. The range of the standard curves was 0.13-10 000 pg/ml with a dilution factor of 5. The lowest (detection limit) and highest standard concentrations used for each cytokine was adjusted according to the standard curve fitting of the standard concentrations after mathematical interpolation. Values below the detection limit were given half the value of the detection limit.

Analysis of mRNA expression – real time RT PCR (paper I-II)

In real time RT-PCR, as used in paper I and II, expression of messenger RNA (mRNA) is measured by reverse transcription to complementary DNA (cDNA) which is subsequently amplified and the products detected in real time. Detection is possible due to the gene-specific probe, which carries a fluorochrome at the 5' and a quencher at the 3' end. As the sample is hit by a laser, the quencher absorbs energy from the laser-excited fluorochrome and this silences the fluorochrome by a process called FRET (fluorescence resonance energy transfer). When the primers are extended during the cyclic amplification process, the polymerase, exhibiting 5' nuclease activity, will cleave off the fluorochrome. As the distance between the quencher and fluorochrome increases, FRET is disrupted and the fluorochrome starts to emit light and this generates the signal for a successful round of amplification. In contrast to end point analysis on agarose gels, real-time PCR provides more reliable quantification (Bustin 2000) since the products are measured during the exponential, not at the plateau, phase of the amplification.

When analysing mRNA expression as a measurement of a protein to be produced, it is important to keep in mind that these processes do not necessarily correlate. In studies comparing several methods for analysis of cytokines, correlation for levels of mRNA and secreted protein largely depended on the protein analyzed (Favre et al. 1997).

In paper I, expression of Foxp3 messenger RNA (mRNA) was analyzed in PBMC, CD4⁺CD25⁺ depleted PBMC and CD4⁺CD25⁺ cells isolated from two pregnant women. In paper II, Foxp3 mRNA was analyzed in PBMC from pregnant (n=13) and non-pregnant women (n=25).

Total RNA was extracted using the RNeasy minikit (Qiagen, West Sussex, UK) and converted to complementary DNA (cDNA) using the cDNA high-capacity archive kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR, 1 µl of cDNA was mixed with 1xTaqMan Universal Mastermix (Applied Biosystems) together with primers and probe for Foxp3 or 18S rRNA. In paper I, commercially available primers were used: Foxp3 Assay ID: Hs00203958_m1 or 18S ribosomal RNA Assay ID: Hs99999901_s1 (both from Applied Biosystems). In paper II, sequences for primers and probes were kindly provided by Jenmalm MC (Linköping University, Sweden) and purchased from Eurogentec S.A. (Seraing, Belgium) (paper II, table II). PCR reactions were performed on the ABI Prism 7700

or 7500 Sequence Detection System (Applied Biosystems). Expression of the 18S subunit of ribosomal RNA was used for normalization of RNA content in all samples. The absence of genomic DNA amplification was controlled by amplifying one sample of RNA. Data was analyzed with the ABI Prism Sequence detector v 1.7a software or the 7300 system SDS software v 1.3.1 (Applied Biosystems). Quantification was determined using the standard curve method. All samples were analyzed in duplicates and the variation limit between duplicates was set to <15%.

Statistics

The statistical guidance resource at Linköping University was consulted for the statistical analyzes in paper I and II.

In paper I, differences within the groups were analyzed by Friedman's test followed by Bonferroni-corrected Wilcoxon signed rank test as a post hoc test to adjust for the number of pairwise comparisons within each group. Differences between groups were analyzed by Mann-Whitney U test corrected for the number of pairwise comparisons between groups by Bonferroni-correction. The significance level was set to 5% i.e. p-values below 0.05 were considered statistically significant.

In paper II, III and IV, due to multiple comparisons and the risk of mass significances, the significance level was set to 1%, i.e. $p \leq 0.01$ was considered statistically significant and $p \leq 0.05$ (paper II) or $p = 0.07$ (paper III) was regarded as a statistical tendency. Results from the flow cytometric analyzes were analyzed using Student's unpaired t-test (paper II and III) or paired t-test (paper IV) and presented as mean \pm SD. Data on cytokines (paper II) did not follow Gaussian distribution and were therefore analyzed using Wilcoxon signed rank test or Mann-Whitney U test and presented as medians and interquartile range (25th and 75th percentile values). In paper II, data on cytokines were also logarithmically transformed and analyzed using parametrical statistical methods. As this did not affect the statistical results, data was kept in linear mode. The coefficient of variation (CV) was expressed as percentage by calculating $(SD/\text{mean}) \times 100$.

All statistical analyzes were performed using the GraphPad Prism version 4 (paper I-II) or 5 (paper III-IV) software (GraphPad Software Inc., San Diego, CA,

USA). Throughout this thesis and papers II-IV, p-values are given as e.g. $p \leq 0.05$ or depicted with stars as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Results & Discussion

Circulating Tregs in healthy pregnancy and preeclampsia

Fetus-specific T_H1- and T_H2-like responses and the role for Tregs in healthy second trimester pregnancy (paper I)

In paper I, the role for Tregs was investigated by the use of the MLC-ELISPOT assay described by Ekerfelt and colleagues (Ekerfelt et al. 1997). In that study, second trimester pregnant women responded significantly more with both IL-4 and IFN- γ secretion upon stimulation with paternal alloantigens (paraformaldehyde fixed paternal PBMC) as compared with a pool of unrelated alloantigens. Further, pregnant women responded with significantly more IL-4 secreting cells than non-pregnant controls when stimulated with paternal alloantigens. It was suggested that this could be interpreted as a paternal-specific T_H2-like response, supported by a paper discussing that the effects of IL-4 could pre-dominate over those of IFN- γ (Rocken et al. 1996). In paper I, we could not detect a differential IL-4 or IFN- γ response against paternal, as compared with unrelated, alloantigens in total PBMC from pregnant women (paper I, Fig 3A and 3B). Of note, the alloantigen response was generally low for both IL-4 and IFN- γ in both pregnant and non-pregnant women. The rise in the number of IFN- γ secreting cells from pregnant women against pooled unrelated alloantigens was the only response that reached statistical significance (paper I, Fig 3B). This suboptimal stimulation, based on titration experiments (1:2, responder:stimulator ratio, see methods section in paper I), was deliberately chosen for the Treg-MLC ELISPOT assay since Tregs were reported to be inhibited by strong antigen stimulation (Baecher-Allan et al. 2002; George et al. 2003). On the other hand, suboptimal stimulation might have hampered not only the quantity but also the quality of the alloantigen responses, explaining the lack of agreement between our study and that of Ekerfelt (Ekerfelt et al. 1997). Pregnant women did however show a significantly higher frequency of IL-4 secreting cells than non-pregnant women (paper I, Fig 3A), regardless of stimulation, speaking for pregnancy as a situation of more general and unspecific spontaneous T_H2 deviation, perhaps driven by the pregnancy itself, including hormones, cytokines, etc. In fact, the maximum IL-4 response yielded in the non-pregnant group was 12 spots/100 000 lymphocytes (paper I, Fig 3A), showing that the systemic T_H2 activity is generally kept low in non-pregnant women.

The purpose of paper I was to develop an MLC-ELISPOT assay for studying the ability of CD4⁺CD25⁺ Tregs to regulate alloantigen responses. The final objective was to apply this method in the study of anti-paternal IL-4 and IFN- γ reactions during second trimester pregnancy. The methodological development was performed using cells from non-pregnant healthy blood donors and a responder: Treg ratio of 2:1 was found to be the minimum number of Tregs needed to observe suppression of the number of IFN- γ secreting cells (data not shown).

For both pregnant and non-pregnant women, depleting the PBMC population of CD4⁺CD25⁺ Tregs did not significantly affect the number of cells secreting IL-4 or IFN- γ (Fig 10-11). Interestingly, although the Treg depleted population was successfully depleted for CD25^{high} cells (paper I, Fig 1B), we did not observe any reduction of Foxp3 mRNA expression as compared with the total PBMC population (Fig 12). This could be explained by the very low expression of Foxp3 in the total PBMC population, making it hard to detect any further reduction of Foxp3 expression. Alternatively, some Foxp3⁺CD25^{dim} cells with suppressive activity were contaminating the Treg depleted cultures, explaining why no effect on cytokine secretion was seen. The consequences of this possible contamination are discussed further on in this section.

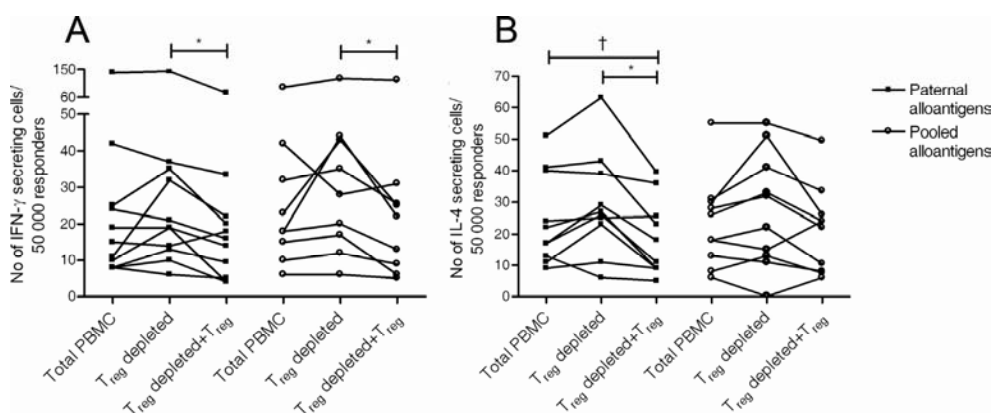


Figure 10. Paper I.

Secretion of IFN- γ (A) and IL-4 (B) from total PBMC, Treg depleted PBMC and Treg depleted PBMC+Tregs from second trimester healthy pregnant women (n=10) stimulated with paraformaldehyde fixed PBMC from the corresponding father (paternal alloantigens) or multiple unrelated donors (pooled alloantigens). * $p \leq 0.05$, † $p = 0.06$.

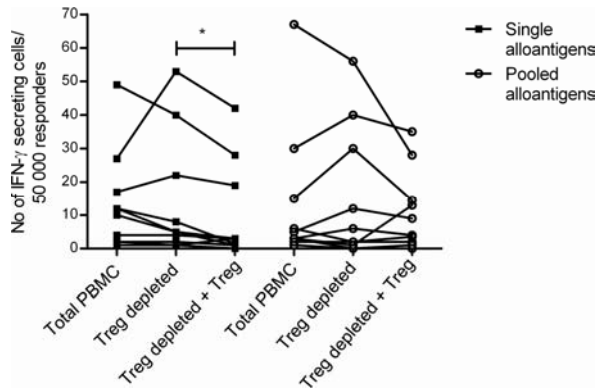


Figure 11. Paper I.

Secretion of IFN- γ from total PBMC, Treg depleted PBMC and Treg depleted PBMC+Tregs from non-pregnant women (n=10) stimulated with paraformaldehyde fixed PBMC from a single donor (single alloantigens) or multiple donors (pooled alloantigens). * $p \leq 0.05$. This is a corrected version of Fig 4 in paper I.

In pregnant women, re-adding Tregs to the depleted population reduced the number of cells secreting IL-4 and IFN- γ in the presence of paternal alloantigens (Fig 10). In the presence of unrelated alloantigens however, only the number of IFN- γ (Fig 10A), not IL-4 (Fig 10B), secreting cells was reduced. Although based on a limited sample, this finding is intriguing, suggesting that maternal Tregs are more potent suppressors of T_H2-like immunity in the presence of paternal than unrelated alloantigens. However, in accordance with models constructed from murine data (Zenclussen 2006; Guerin et al. 2009), it is possible that in pregnant women, fetus-specific Tregs are generated in response to the fetal semi-allograft at the fetal-maternal interface. These cells are then able to home to peripheral lymphoid organs, eventually appearing in blood. Once antigen-specifically re-stimulated in our *in vitro* assay, these cells could be generally more suppressive of anti-paternal than unrelated allogenic responses (of both T_H1 and T_H2 kind). Categorically, pregnancy has been viewed as a state in which IL-4 (T_H2-like immunity) is “good” and IFN- γ (T_H1-like immunity) is “bad” for pregnancy maintenance. For obvious reasons, including the well documented proinflammatory effects of IL-4 in atopy, this is a great oversimplification. Our data suggest that during pregnancy, Tregs suppress both T_H1- and T_H2-like anti-paternal/fetal immunity, protecting the fetus from immunological rejection.

It is interesting that Tregs from pregnant women did not significantly suppress IL-4 secretion in the presence of unrelated alloantigens. Several reports have

suggested that ex vivo isolated T_H2 cells from allergic individuals (Grindebacke et al. 2004) as well as T_H2 clones (Cosmi et al. 2004) are less susceptible to the suppressive effects of Tregs. Keeping this in mind, we argue that in the presence of unrelated alloantigens, maternal Tregs were not re-activated in our *in vitro* assay. This allowed for the responder cells to escape with IL-4 secretion while being preferentially suppressed for the more pregnancy detrimental IFN- γ secretion. As a consequence, we speculate that during pregnancy, Tregs “allow” for responses against unrelated alloantigens to be T_H2-, rather than T_H1-like (Svenvik et al. 2003). The lack of maternal Treg suppression of IL-4 secretion in the presence of unrelated alloantigens should however be interpreted with caution due to the limited number of samples analyzed. Further, there was no statistical difference (in odds ratio) when analyzing suppression as the IL-4 secretion from Treg depleted cells relative to that in the presence of Tregs.

Tregs from pregnant women (Fig 10A), in contrast to those from non-pregnant women (Fig 11, this is a corrected version of Fig 4 in paper I), reduced the number of IFN- γ secreting autologous cells in response to pooled alloantigens. This correction of the results (Fig 11) presented in Figure 4, paper I, leads to a modified interpretation, namely that Tregs from pregnant women are better than Tregs from non-pregnant women in suppressing allogenic IFN- γ responses being unrelated to the pregnancy. This finding was in accordance with our original hypothesis. In line with this, IL-4, which we showed to be spontaneously secreted by a higher number of cells in pregnancy, has beneficial effects on Treg suppressivity (Yates et al. 2007). Further, estradiol, abundantly present during pregnancy, enhances the suppressive function of Tregs in mice (Polanczyk et al. 2005). Hence, pregnancy might enhance Treg suppression of allogenic responses. Importantly, weakening this theory, non-pregnant women did show Treg suppression of IFN- γ secretion in the presence of single alloantigens (from one unrelated donor, Fig 11). Hence, no certain conclusion about differences in Treg suppression of T_H1 responses between pregnant and non-pregnant women could be drawn. Thus, the interpretation of the findings was not seminally altered by the correction of the results (Fig 11) presented in figure 4, paper I.

From a methodological aspect, these data overall confirmed the successful development of a Treg MLC-ELISPOT assay. In our assay, more Tregs were re-added than depleted from the PBMC population, probably explaining why differences were seen between Treg depleted and reconstituted cultures, but not between the total PBMC and Treg depleted cultures. In a recent study addressing the issue of fetus-specific Tregs in pregnancy (Tilburgs et al. 2008), depleting the maternal PBMC population of Tregs did not significantly enhance the

proliferation or IFN- γ secretion in response to TCR stimulation. Further, Treg depletion did not affect the proliferation upon stimulation with umbilical cord blood cells (fetal or unrelated). This was interpreted as a lack of fetus-specific Tregs (or in fact Tregs of any specificity) in the blood of pregnant women. Based on the lack of effect from Treg depletion in our study, we could agree with this. However, speaking against this statement, we showed that re-adding Tregs to Treg depleted cultures caused effects on cytokine secretion. In the physiological situation, it is likely that the ratio of Tregs:responders e.g. in lymph nodes would exceed that seen in circulating PBMC (roughly 1:50 as compared with 1:2 or 1:1 used in our assay). Further, speaking for the methods used in our study, Wing *et al.* stated that analyzing cytokine secretion rather than proliferation increased the sensitivity in detecting Treg suppression (Wing et al. 2005).

The Tregs used in paper I were separated using magnetic beads, an approach that yielded a median purity of 91% with regard to CD25 expression (Fig 12A). However, the median purity regarding CD25^{high} expression was only 53% (Fig 12B). It has been shown by us (paper II) and others (Baecher-Allan et al. 2001), that the Tregs are confined to the population of CD4⁺ cells expressing the highest levels of CD25. Hence, it is likely that the CD25⁺ cells used in this study were contaminated by activated non-suppressive CD4⁺ cells expressing intermediate levels of CD25. By analyzing Foxp3 mRNA expression, we could confirm that the CD4⁺CD25⁺ population was enriched for Foxp3-expressing cells (Fig 12C). However, since Foxp3 is only expressed in CD25⁺ cells (Walker et al. 2003), the CD25⁺ population did not have to be pure, with regard to Foxp3, to obtain these data.

Overall, although it is likely that Tregs dominated the CD4⁺CD25⁺ population and were highly reduced in the Treg depleted population, sorting cells using flow cytometry should be preferred to improve the purity, thereby possibly simplifying the interpretation of data.

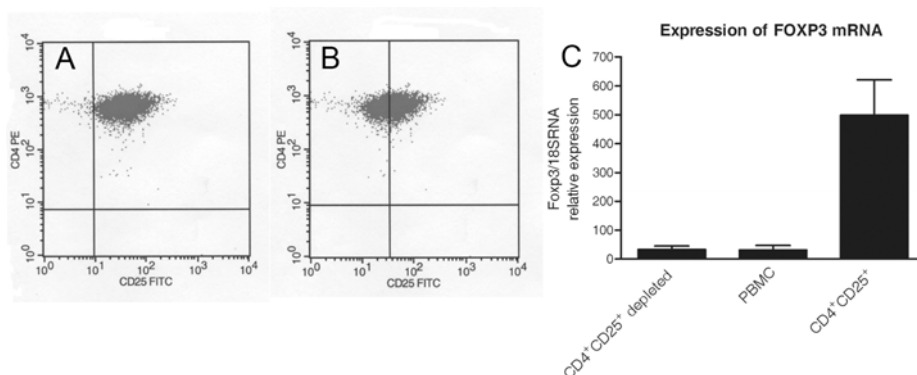


Figure 12. Paper I.

Representative flow cytometric analysis (n=4, non-pregnant women) of immunomagnetically selected CD4⁺CD25⁺ cells defined according to

A. CD25⁺ gate (based on the isotype control).

B. CD25^{high} gate (based on the absence of CD25 expression on CD4⁺ cells).

C. Expression of Foxp3 mRNA in CD4⁺CD25⁺ depleted PBMC, PBMC and CD4⁺CD25⁺ cells as determined using real-time PCR (n=2, non-pregnant women). Bars are medians and error bars represent the highest value.

In conclusion, the results in paper I showed that T_H2-like responses are enhanced during pregnancy and that maternal Tregs are capable of suppressing both T_H1 and T_H2-like immunity against paternal/foetal alloantigens. Since these antigens are present as long as pregnancy is sustained, immune responses against them need to be strictly regulated. In contrast, when encountering unrelated alloantigens, T_H2-like cells seem to escape Treg suppression, which would allow for IL-4, together with Tregs, to maintain immunological integrity while simultaneously controlling potentially detrimental immune reactions against the fetus.

Defining Tregs in healthy second trimester pregnancy using flow cytometry (Paper II)

Regulatory T cells are, to date, best defined as CD4-expressing cells with abundant CD25 and Foxp3 expression, referred to as CD4⁺CD25^{high}Foxp3⁺ cells. However, the intracellular location of Foxp3 rules out its usefulness as a marker for sorting viable Tregs. Up until the discovery of Tregs expressing low levels of CD127, this left the CD4⁺CD25^{high} as the best available sorting phenotype. The

term “CD25^{high}” simply refers to CD4⁺ cells with high expression of CD25, a description leaving huge space for the scientists’ own interpretation. There have been attempts at standardizing the gating of CD4⁺CD25^{high} cells (Hoffmann et al. 2007). Still, it is remarkable that the problems and risks with subjectively gating regulatory T cells using flow cytometry are so seldom discussed in the literature. Especially considering the consequences that incorrect gating of CD4⁺CD25^{high} could lead to, both regarding frequency and function, not to mention reproducibility. Since expression of CD25 is not restricted to Tregs, but can also be found on activated CD4⁺ cells (Malek 2008), the CD4⁺CD25^{high} population is easily contaminated by activated cells lacking suppressive function. This potential problem is worth considering, especially when investigating Tregs in immune challenging conditions, such as pregnancy.

In paper II, we used flow cytometric analysis of markers associated with suppressive function (Foxp3, HLA-DR and CTLA-4) to determine the optimal gating strategy for CD4⁺CD25^{high} cells. The CD4⁺CD25^{high} cells were first gated according to a previously described strategy (Cao et al. 2004; Lundgren et al. 2005) in which the CD25^{high} gate was adjusted to contain CD4⁺ cells that expressed higher levels of CD25 than the discrete population of CD4⁺ T cells expressing CD25. This gate was termed “classical CD4⁺CD25^{high} gate” (paper II, Fig 1A). According to this strategy, pregnant women showed an increased proportion of CD4⁺CD25^{high} cells as compared with non-pregnant women (paper II, Fig 1A). This finding was in accordance with our hypothesis and also the majority (Heikkinen et al. 2004; Sasaki et al. 2004; Somerset et al. 2004; Sasaki et al. 2007), but not all (Tilburgs et al. 2006), of previous papers investigating Treg frequency in pregnancy. However, we noted that within the classical CD4⁺CD25^{high} gate (paper II, Fig 1A), there was a population of cells expressing high levels of CD4 (CD4^{high}CD25^{high}) (paper II, Fig 1B). Few of these CD4^{high}CD25^{high} cells expressed Treg markers Foxp3, HLA-DR and CTLA-4, similarly to activated CD4⁺CD25^{dim} cells (paper II, Fig 1E), and the CD4^{high}CD25^{high} population was larger in pregnant as compared with non-pregnant women (paper II, Fig 1B). To avoid these cells, that we found to be non-suppressive (Fig 22 and paper II, Fig 7), a gate was set to include those CD25^{high} cells with a slightly lower expression of CD4 (CD4^{dim}CD25^{high}, paper II, Fig 1C and Fig 13). This population, in contrast to the CD4^{high}CD25^{high} population, was enriched for cells expressing Foxp3, HLA-DR and CTLA-4 (paper II, Fig 1C), similarly with the 0.5% CD4⁺CD25^{highest} cells (paper II, Fig 1E) that we chose as a reference for cells with a really pronounced Treg phenotype.

To sum up, the CD4^{dim}CD25^{high} phenotype (Fig 13) was considered to be the optimal definition of Tregs in pregnant women, minimizing contamination by CD4^{high}CD25^{high} cells lacking suppressive function as well as Foxp3, HLA-DR and CD127^{low} expression. Hence this definition, which has also been suggested by others (Hoffmann et al. 2007; Luhn et al. 2007) was used throughout papers II-IV as one way of describing Tregs.

Applying the CD4^{dim}CD25^{high} gating strategy - Frequency of circulating Tregs in first-second trimester healthy pregnancy and preeclampsia (Paper II-IV)

Circulating Treg frequency in first and second trimester healthy pregnancy (paper II and IV)

Going against our original hypothesis, when defining Tregs as CD4^{dim}CD25^{high} according to the thorough gating investigation (paper II, Fig 1), these cells were in fact fewer within the CD4⁺ population in second trimester healthy pregnant than in non-pregnant women (Fig 13). This was seen both when using four- and six-color flow cytometry (Four-color: $1.2 \pm 0.35\%$ vs. $1.9 \pm 0.6\%$; $p=0.0005$, Six-color: $1.5 \pm 0.3\%$ vs. $2.8 \pm 1.1\%$; $p=0.002$). In addition, TruCount analysis of absolute cell count/ μ L blood in second trimester healthy pregnant as compared with non-pregnant women (14 ± 4 cells / μ L vs. 21 ± 8 cells/ μ L; $p=0.01$).

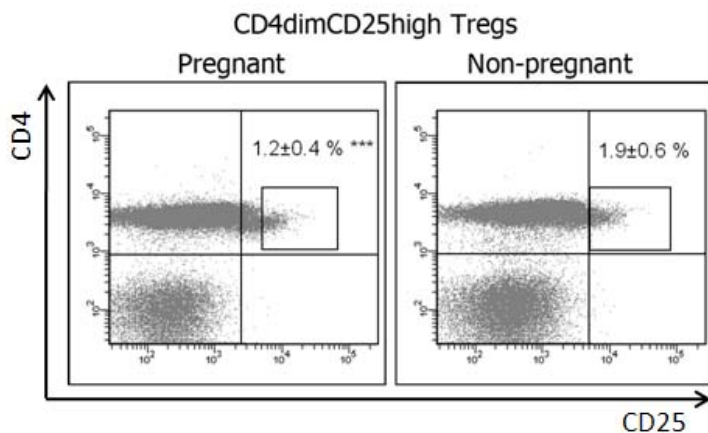


Figure 13. Paper II.

Representative dot plots (four-color flow cytometry) showing the expression of CD4 and CD25 on peripheral lymphocytes isolated from second trimester healthy pregnant (left, n=14) and non-pregnant (right, n=34) women. The frequency of CD4^{dim}CD25^{high} cells in the CD4⁺ population are given as mean ± SD.

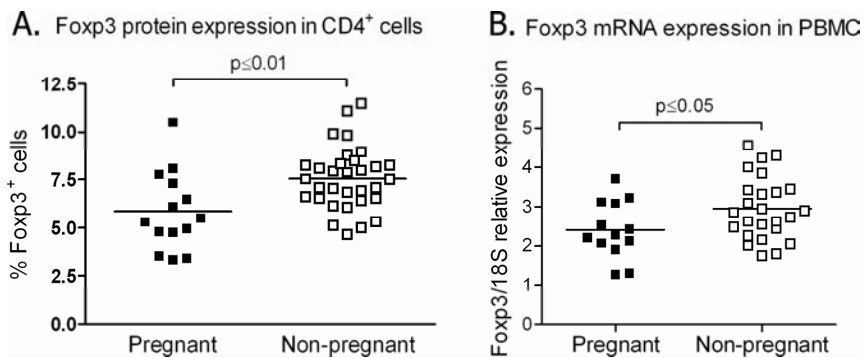


Figure 14. Paper II.

A. Frequency of Foxp3⁺ cells in the CD4⁺ population from second trimester healthy pregnant (n=14) and non-pregnant women (n=32) according to four-color flow cytometry.

B. Expression of Foxp3 mRNA in total PBMC population from second trimester healthy pregnant (n=13) and non-pregnant (n=25) women.

Lines indicate means.

The reduction of CD4^{dim}CD25^{high} Tregs in healthy pregnant women was supported by data from the four-color flow cytometric analysis showing that the number of CD4⁺ cells expressing Foxp3 was reduced in second trimester healthy pregnant women (Fig 14A). Of note, this finding could not be confirmed by data from the six-color flow cytometric analysis where similar proportions of Foxp3⁺ cells were seen in healthy pregnant (n=10) and non-pregnant (n=10) women (paper III, Fig 2C). Due to the smaller sample sizes used in these latter experiments, this was regarded as a statistical power issue. At the transcriptional level, PBMC from second trimester healthy pregnant women tended to express relatively lower levels of Foxp3 mRNA than non-pregnant women (Fig 14B).

In paper III, addressing the role for Tregs in severe early-onset preeclampsia using six-colour flow cytometry, we expanded the non-pregnancy (NP) and second trimester healthy pregnancy (2nd HP) groups used in paper II (from n=10 to n=20 in each group) to gain more power to our statistical analyzes. In paper III, the finding of lower levels of CD4^{dim}CD25^{high} Tregs in healthy second trimester pregnant women was confirmed (Fig 15A). However, as in paper II, CD4⁺ cells from non-pregnant and healthy second trimester pregnant women showed similar expression of Foxp3 (Fig 15B). This did not strengthen the finding of lower frequency of Foxp3⁺ cells in healthy second trimester pregnant women seen using four-color flow cytometry in paper II. Besides the methodological differences in paper II and III (four- *vs.* six-color flow cytometry), a smaller cohort of non-pregnant women was used for six-color flow cytometry in paper III than in paper II (n=20 *vs.* n=32), possibly explaining these discrepancies. Of note, when defining Tregs as CD4^{dim}CD25^{high}Foxp3⁺, healthy second trimester pregnant women did show lower levels of Tregs as compared to non-pregnant women (paper III, Fig 2B). Interestingly, first trimester pregnant women (from paper IV) also showed reduced frequencies of CD4^{dim}CD25^{high} Tregs as compared with non-pregnant women (Fig 15A). Hence, the reduction of systemic Tregs noted during second trimester pregnancy in paper II was a phenomenon that occurred already in the first trimester of pregnancy. The latter finding should however be considered with a certain degree of reservation due to a different antibody staining protocol used in paper IV as compared with paper III.

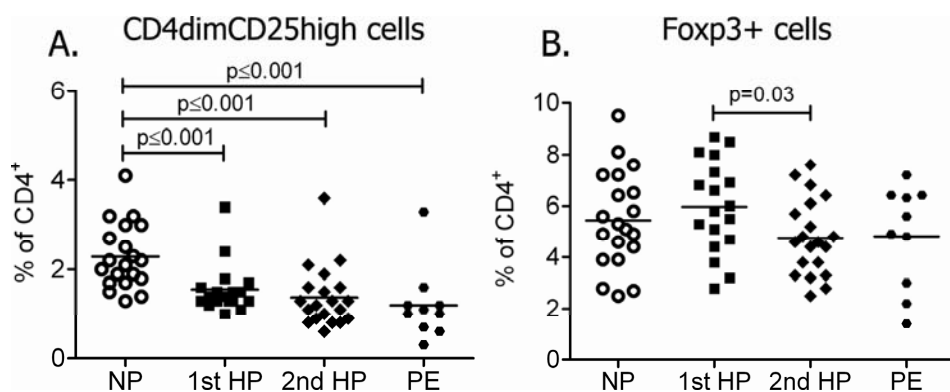


Figure 15. Papers III and IV.

Frequency of CD4^{dim}CD25^{high} (A) and Foxp3⁺ (B) cells in the CD4⁺ population from non-pregnant (NP, n=20), 1st trimester healthy pregnant (1st HP, n=18), second trimester healthy pregnant (2nd HP, n=20) and preeclamptic (PE, n=10) women. Data were obtained using six-color flow cytometry and were merged from paper IV (1st HP) and paper III (NP, 2nd HP and PE). Lines indicate means.

To summarize, the findings on Tregs in healthy pregnancy showed that reduction of the number of circulating Tregs seem to be a healthy physiological response to pregnancy, already in the first trimester. These data are in contrast to our own original anticipations and the majority of previous findings (Heikkinen et al. 2004; Sasaki et al. 2004; Somerset et al. 2004; Sasaki et al. 2007) where circulating Treg frequencies ranged from 8-17.5% Tregs/CD4⁺ cells in second trimester pregnant women. The increase of Tregs was apparent already in first trimester pregnancy (Heikkinen et al. 2004; Sasaki et al. 2004; Somerset et al. 2004). In our study, when inaccurately defining Tregs as CD4⁺CD25^{high} according to the classical gating strategy, we too found an increase of this population in pregnancy. We argue that Treg frequencies of that magnitude would most likely include the population of non-suppressive CD4^{high}CD25^{high} cells showing an atypical Treg phenotype (lacking Foxp3, HLA-DR and CD127^{low} expression). This would lead to misinterpretations, not only of Treg frequency and phenotype, but also Treg function in pregnancy. On the basis of our findings in paper II, these studies need to be re-evaluated. Further, our findings indicate that during pregnancy (early-mid gestation), Tregs are down-regulated systemically, possibly as a measure to ensure maintained defense against infections.

Circulating Treg frequency in severe early-onset preeclampsia (paper III)

In paper III, healthy second trimester pregnant women and women with severe early-onset preeclampsia showed similar levels of CD4^{dim}CD25^{high} Tregs (Fig 15A), gated according to the flow cytometric strategy developed in paper II. Further, preeclamptic women did not show alterations in the frequency of Foxp3⁺ (Fig 15B), Foxp3^{high} or CD4^{dim}CD25^{high}Foxp3⁺ cells (paper III, Fig 2B-C) as compared to healthy second trimester pregnant women. These findings did not support our original hypothesis.

There are now several studies that have addressed the question about the role for Tregs in preeclampsia (Paeschke et al. 2005; Darmochwal-Kolarz et al. 2007; Sasaki et al. 2007; Hu et al. 2008; Steinborn et al. 2008; Toldi et al. 2008; Prins et al. 2009). The majority of these studies, but not all (Paeschke et al. 2005; Hu et al. 2008; Prins et al. 2009), found that Tregs comprise a reduced part of the T helper population in preeclamptic as compared with healthy pregnant women. We showed, in paper II and the present paper, that in preeclamptic, as well as in healthy pregnant women, the population of CD4^{high}CD25^{high} non-Tregs is expanded, thereby contaminating the “classical” CD4⁺CD25^{high} Treg population (paper II, Fig 1B-C). As judged from the frequency of CD4⁺CD25^{high} Tregs found in previous studies (Darmochwal-Kolarz et al. 2007; Sasaki et al. 2007), it is probable that they used a gating strategy that included CD4^{high}CD25^{high} non-Tregs. However, since we observed similar frequencies of both the CD4^{dim}CD25^{high} and CD4⁺CD25^{high} populations in preeclamptic and healthy pregnant women, the different gating strategies used does not provide the full explanation to the lack of agreement between our study and those previously conducted.

Regarding Foxp3, some have shown a reduction in preeclampsia of the CD4⁺Foxp3⁺ (Toldi et al. 2008; Prins et al. 2009) and CD4⁺CD25⁺Foxp3⁺ (Steinborn et al. 2008) population, but not the CD4⁺CD25^{high}Foxp3⁺ population (Prins et al. 2009). Hence, even when analyzing the most reliable Treg marker Foxp3, the changes seen in circulatory Treg frequencies during preeclampsia are not univocal.

In paper III, we chose to study the group of women presenting with severe preeclampsia early in pregnancy (before gestational week 32), hypothesizing that this would provide us with a more homogenous group of diseased women, simultaneously minimizing the effects of prolonged disease. Further, it has been suggested that early-onset preeclampsia might be a more true placental disorder,

why research on this subgroup has been recommended (Ilekis et al. 2007). No study has previously focused on this subgroup or analyzed the Treg changes in relation to gestational age at onset of disease. Hence, it is possible that the Treg changes seen in previous studies were mostly confined to the group of women with late onset of preeclampsia, including those having had a subclinical disease for a long time. The noted changes could then, to a higher degree, reflect the effect of, rather than the cause of preeclampsia. Even though early onset preeclampsia is, in our opinion, one step closer to investigating preeclampsia onset mechanisms, prospective studies identifying the women destined to develop preeclampsia would really solve this issue.

Phenotype of circulating Tregs in second trimester healthy pregnancy and preeclampsia (Paper II-III)

Expression of markers associated with suppressive function (Paper II-III)

In paper II, we used a strict gate for selection of CD4^{dim}CD25^{high} Tregs, minimizing the potential contamination by activated Foxp3⁻ cells. We then found, using four-color flow cytometry, that the frequency of Foxp3⁺ cells as well as the Foxp3 expression intensity were reduced in the CD4^{dim}CD25^{high} cell population in second trimester healthy pregnant as compared with non-pregnant women (Fig 16). This finding was however not confirmed in paper III, where the expression of Foxp3 within the CD4^{dim}CD25^{high} population was similar in preeclamptic, second trimester healthy pregnant and non-pregnant women (paper III, Fig 2D). It is possible that the smaller number of non-pregnant women used in paper III (n=20) as compared with paper II (n=32) could contribute to why no significant changes were observed in paper III.

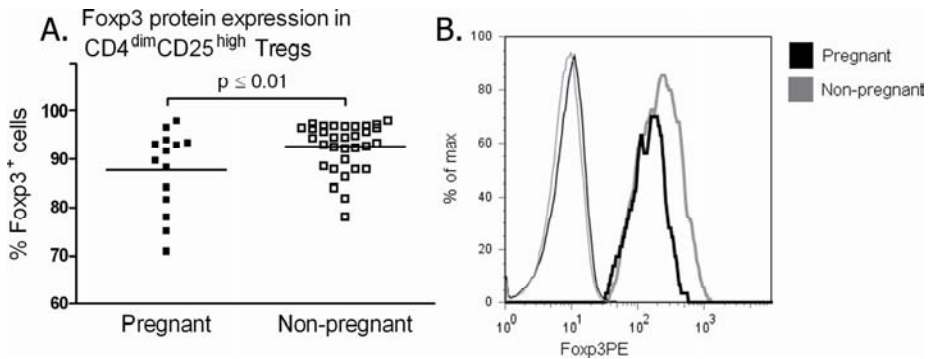


Figure 16. Paper II.

A. Expression of Fxp3 protein in the CD4^{dim}CD25^{high} population, B. Representative Fxp3 fluorescence intensity histograms of Fxp3⁺CD4^{dim}CD25^{high} cells (right peaks) and Fxp3⁻ lymphocytes stained with isotype control (left peaks). The difference between the Fxp3⁺CD4^{dim}CD25^{high}/ Fxp3⁻ lymphocyte gMFI ratios for pregnant and non-pregnant was $p \leq 0.01$.

Cells were isolated from second trimester healthy pregnant (n=14) and non-pregnant (n=32-33) women and analyzed using four-color flow cytometry. Lines in A indicate means.

The findings of reduced Fxp3 expression in healthy second trimester pregnancy (paper II), a notion also made by others (Tilburgs et al. 2008), indicated important, yet unknown, implications in healthy pregnancy. Interestingly, murine models of Tregs expressing intermediate levels of Fxp3 have taught us that Fxp3 works along a continuum, inducing increasing grades of suppressive phenotypes. Diminished Fxp3 expression not only reduced the suppressive function and phenotype, but turned the cells into IL-4, IL-10 or IL-17 producers (Gavin et al. 2007; Wan et al. 2007). With this background, our data suggest altered Treg function in healthy pregnancy which was indeed investigated in paper II (see below, Fig 20). However, since the expression of Fxp3 was similar in preeclamptic and healthy pregnant women, disturbed Fxp3 expression did not seem to be linked to severe early-onset preeclampsia.

The presence of HLA-DR⁺ cells within the CD4^{dim}CD25^{high} population was similar in preeclamptic, second trimester healthy pregnant and non-pregnant women (paper II, Fig 3B and paper III, Fig 6B). Within the Fxp3⁺ population, however, healthy second trimester pregnant women showed a reduced frequency of HLA-DR⁺ cells as compared with non-pregnant women (paper III, Fig 6E). Most importantly, preeclamptic women did not show this reduction of HLA-DR expression and tended to have higher numbers of HLA-DR⁺ cells within the

Foxp3⁺ population than healthy pregnant women (paper III, Fig 6E). HLA-DR is expressed on Tregs and linked to their suppressive function (Baecher-Allan et al. 2006; Peiser et al. 2007). The Tregs lacking HLA-DR expression secreted cytokines such as IL-10 and IL-4 and suppressed in a late cell-cell contact and IL-10 dependent manner (Baecher-Allan et al. 2006). These HLA-DR⁻ Tregs seem closely linked to adaptive/induced Tregs (Roncarolo et al. 2006), which suppression depends on cytokines. In healthy pregnancy, the findings of reduced HLA-DR expression within the Treg population indicate that these cells are more abundant, perhaps contributing to systemic and long-distance suppression of immune reactions, as suggested by others (Jonuleit et al. 2002). Hence, one contributing factor to the exaggerated systemic inflammation seen in preeclampsia could be maladaptations in the HLA-DR⁻ Treg fraction.

CTLA-4 is a marker associated with Treg function (Sansom et al. 2006). Altered expression of this molecule would indicate altered function, which was indeed seen in a study of patients with rheumatoid arthritis (Flores-Borja et al. 2008). The presence of CTLA-4⁺ cells within the CD4^{dim}CD25^{high} and Foxp3⁺ populations was similar in second trimester healthy pregnant and non-pregnant women (paper II, data not shown and paper III, Fig 6A). However, the CD4^{dim}CD25^{high} and Foxp3⁺ populations in preeclamptic women tended to contain more cells expressing CTLA-4 than non-pregnant and healthy pregnant women (paper III, Fig 6A and D). Since CTLA-4 participates in contact-dependent suppression, this could be a compensation for the relative lack of supposedly cytokine-secreting HLA-DR⁻ Tregs seen in preeclampsia (paper III, Fig 6E). CD4⁺CD25⁺ MACS-sorted Tregs from preeclamptic women have been reported to show reduced suppressive capacity (Steinborn et al. 2008). Disregarding the poor purity of such a MACS-sorted Treg population, the dysregulated expression of CTLA-4 and HLA-DR in Tregs from severe early-onset preeclamptic women could participate in the reduced suppressive activity of Tregs and thereby the pathogenesis of preeclampsia.

Expression of markers associated with activation (Paper II-III)

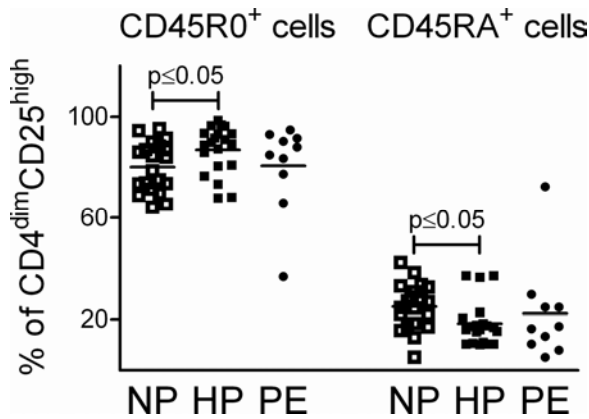


Figure 17. Paper III.

Expression of CD45R0 and CD45RA in the CD4^{dim}CD25^{high} population from non-pregnant (n=20), second trimester healthy pregnant (n=20) and preeclamptic (n=10) women. Lines indicate means.

In paper II, we observed that CD4^{dim}CD25^{high} Tregs in healthy pregnant women showed an activated phenotype with increased expression of CD45R0 and decreased expression of CD45RA as compared with non-pregnant women (paper II, Fig 3C-D). Although not reaching statistical significance ($p \leq 0.05$, significance level set to 99%), we confirmed this finding in paper III (Fig 17). The precise cause of this maternal Treg activation remains unknown but is likely influenced by paternal/fetal alloantigens. Importantly, Tregs from preeclamptic women did not show an activated Treg phenotype. Hence, it would be intriguing to investigate the influence of paternal/fetal alloantigens on functional and phenotypical Treg characteristics in preeclamptic and healthy pregnant women.

Regarding the activation status of Tregs, Sakaguchi and colleagues recently suggested a novel way of discriminating between three subtypes of Foxp3-expressing cells; resting Foxp3^{dim}CD45RA⁺ Tregs (rTreg), activated Foxp3^{high}CD45RA⁻ Tregs (aTreg) and non-suppressive Foxp3^{dim}CD45RA⁻ cells (Miyara et al. 2009). By comparing the strategies for flow cytometric gating (data not shown), we observed that the aTregs corresponded well to the CD4^{dim}CD25^{high} population (paper II, Fig 1C). However, when evaluating the

frequency of aTregs in paper III, we observed similar frequencies in non-pregnant, healthy second trimester pregnant and preeclamptic women (paper III, Fig 5B). The same was observed for rTregs, best corresponding to the least CD25-expressing part of the population termed CD4⁺CD25^{+/high} in paper II (paper II, Fig 1D). The non-suppressive Foxp3^{dim}CD45RA⁻ population corresponded well to the previously described activation induced Foxp3-expressing cells (Gavin et al. 2006; Allan et al. 2007; Tran et al. 2007; Wang et al. 2007), as well as the CD4^{high}CD25^{high} population (paper II, Fig 1B), the former tending to be reduced in women with preeclampsia (paper III, Fig 5C). Hence, the activated non-suppressive Foxp3^{dim}CD45RA⁻ population could be important in maintaining normal pregnancy and its systemic reduction, possibly by recruitment to the placenta, could contribute to development of preeclampsia.

Expression of CCR4, a marker associated with cell recirculation and migration (Paper II-III)

In paper III, we investigated the frequency of CD4^{dim}CD25^{high} and Foxp3^{+/high} Tregs expressing the chemokine receptor CCR4 in non-pregnant, healthy second trimester pregnant and preeclamptic women (paper III, Fig 6C and 6F). The proportion of cells expressing this marker was similar in Tregs from healthy pregnant and preeclamptic women. However, more CD4^{dim}CD25^{high} Tregs from healthy pregnant women expressed CCR4 as compared with non-pregnant women (paper III, Fig 6C).

The ligand for CCR4, CCL17, is found in trophoblasts and endometrial epithelial cells and has the potential to attract CCR4-bearing cells, such as T_H2 cells, to the fetal-maternal interface (Tsuda et al. 2002). However, as shown in paper III, CCR4 is highly expressed on Tregs (paper III, Fig 6C and 6F), making Tregs yet another target for CCR4-CCL17 mediated attraction to the fetal-maternal interface where Tregs are indeed enriched as shown in paper IV and by others (Tilburgs et al. 2006; Tilburgs et al. 2008). Our findings of increased expression of CCR4 on the diminished population of CD4^{dim}CD25^{high} Tregs seen in the circulation of healthy pregnant as compared with non-pregnant women support this idea. However, this adaptation of CCR4 expression was not seen in preeclampsia, possibly explaining the lower occurrence of Tregs in the placentas of preeclamptic women (Sasaki et al. 2007). It would indeed be interesting to investigate the migratory properties of Tregs isolated from preeclamptic women. It was recently shown that Tregs are attracted to the fetal-maternal interface by hCG which is decreased in

placental tissue from patients with RSA (Schumacher et al. 2009), giving a clue to the explanation of reduced decidual Treg numbers seen in these patients.

Hormonal effects on Tregs (Paper II)

In paper II and III, we observed reduced frequencies of CD4^{dim}CD25^{high} cells in the blood of both healthy pregnant and preeclamptic, as compared with non-pregnant, women (Fig 15A). In paper II, the lower occurrence of Tregs could be confirmed by reduced expression of Foxp3 both within the CD4⁺ (Fig 14A) and the CD4^{dim}CD25^{high} population (Fig 16).

There are several suggestions as to the causes of the Treg changes previously observed in pregnancy. In mice, syngenic pregnancy causes a rise in Treg levels (Aluvihare et al. 2004), indicating that pregnancy itself, in the absence of major histocompatibility antigens, is sufficient for induction of Tregs to occur. Based on compelling studies, this leaves minor histocompatibility antigenic dissimilarities (Zenclussen et al. 2005b; Zhao et al. 2007) and pregnancy levels of estradiol (Polanczyk et al. 2004; Polanczyk et al. 2005; Tai et al. 2008) as prime candidates for spurring Treg expansion. In humans, Treg numbers correlated with estradiol levels during the menstrual cycle (Arruvito et al. 2007) and estradiol induced Treg proliferation (Prieto et al. 2006). The effect of progesterone on Tregs had, to our knowledge, not been previously investigated.

With this in mind, we wanted to examine the effects of 17 β -estradiol and progesterone on Treg frequency and phenotype in PBMC from non-pregnant women. These hormones are present at greatly elevated levels in the circulation, and even more so in placenta, during pregnancy. In contrast to our anticipation, we found that 17 β -estradiol and in particular progesterone reduced the number of Foxp3⁺ and CD4^{dim}CD25^{high} Tregs, as well as the the frequency of cells expressing Foxp3, CTLA-4 and HLA-DR within the Treg population (Fig 18). These effects were associated with the hormone doses, where the highest concentrations led to very pronounced effects (Fig 18). At the lowest doses of 17 β -estradiol and progesterone, corresponding to pregnancy serum levels, similar effects, i.e. reduction, were seen on Foxp3 expression (Fig 19). However, these effects were less pronounced and not always statistically significant.

Regarding estradiol, our findings were in disagreement with most previous murine studies. Since the non-suppressive CD4^{high}CD25^{high} population was not expanded by the hormone treatment (paper II, data not shown), the discrepancies

could not be explained solely by the different gating strategies used. Further, our data did not support the generally accepted view that estradiol and progesterone are of anti-inflammatory nature, and therefore should support Tregs. Interestingly, estradiol might have more complex regulatory functions than previously thought since estradiol induces NF- κ B, a key inflammatory switch (Hirano et al. 2007).

However, there are also reports from murine models where Tregs were unaffected by estradiol and progesterone (Zhao et al. 2007). Interestingly, the abortion prone mice (CBA/J x CBA/2J), which show defective Treg levels, actually have enhanced progesterone levels, supporting the idea that progesterone inhibit Tregs (Thuere et al. 2007).

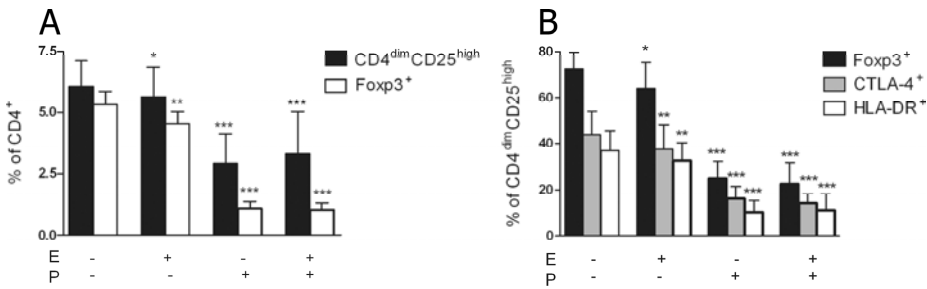


Figure 18. Paper II.

PBMC from non-pregnant women (n=7) were stimulated with 10 μ M 17 β -estradiol (E) and 200 μ M progesterone (P), alone or in combination.

A. Proportion of Foxp3⁺ cells and CD4^{dim}CD25^{high} cells in the CD4⁺ population.

B. Proportion of Foxp3⁺, CTLA-4⁺ and HLA-DR⁺ cells within the CD4^{dim}CD25^{high} population.

Bars represent means with standard deviations. Significance markers (*) indicate differences between unstimulated and hormone stimulated cells. * p \leq 0.05, ** p \leq 0.01, *** p \leq 0.001.

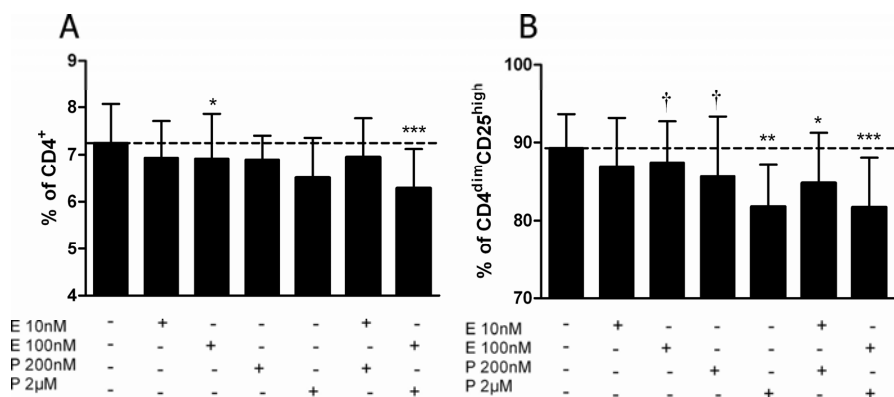


Figure 19. Paper II.

Proportion of Foxp3⁺ cells in the CD4⁺ (A) and CD4^{dim}CD25^{high} (B) population from non-pregnant women (n=6) cultured with two different concentrations of 17β-estradiol (E) and progesterone (P), alone or in combination.

Bars represent means with standard deviations. Dotted lines represent the value of the unstimulated (no hormones) control. Significance markers (*) indicate differences between unstimulated and hormone stimulated cells. † p≤ 0.07, * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001.

In humans, Prieto and colleagues showed that 17β-estradiol, particularly in combination with strong TCR stimulation, induced proliferation of functionally suppressive Tregs (Prieto et al. 2006). Although our results and those of Prieto (Prieto et al. 2006) do not seem to agree, Foxp3 was not investigated in that study, making our data difficult to compare. However, if done, the discrepancies between our studies could depend on the purity of the Tregs analyzed, the amplitude of the TCR stimulation as well as the formulation of the estradiol used. By flow cytometry, we could analyze precisely defined and pure Tregs that had been stimulated by a low grade physiologic TCR stimulation. Further, we used water-soluble 17β-estradiol, thereby avoiding the background cell activating effects associated with ethanol-soluble 17β-estradiol.

In conclusion, the reduction of Tregs seen in second trimester healthy pregnant (and preeclamptic) women could be mimicked *in vitro* by estradiol and in particular progesterone. Hence, as mentioned previously, hormonal control of Tregs could be one mechanism by which immune integrity is maintained in case of infections during pregnancy.

Suppressive function of Tregs in healthy second trimester pregnancy (Paper II)

Due to our newly developed flow cytometric gating strategy (paper II, Fig 1) and the phenotypical changes seen in Tregs from healthy pregnant women, with decreased expression of Foxp3 (paper II, Fig 2) and HLA-DR (paper III, Fig 6E), as well as increased expression of activation marker CD45R0 (paper II, Fig 3C), we obviously wanted to examine the suppressive function of these Tregs.

To this end, we developed an *in vitro* suppressive assay for studying the effects of CD4^{dim}CD25^{high} Tregs from healthy second trimester pregnant and non-pregnant women on cytokine secretion from autologous CD4⁺CD25⁻ responder cells. Due to the relatively weak response that was generated against alloantigens in paper I, we chose to use anti-CD3/CD28 antibodies as a polyclonal and unspecific stimulus. Since Tregs seem to have a lower threshold for activation and their suppressive function is abrogated by very strong TCR stimulation (Baecher-Allan et al. 2002), we carefully evaluated the optimal strength of the anti-CD3/CD28 stimulation used. The concentrations of these antibodies finally used generated a measurable cytokine response from CD4⁺CD25⁻ responder cells while maintaining the suppressive function and limited cytokine production of CD4^{dim}CD25^{high} Tregs (Fig 20). It should however be kept in mind that *in vitro* systems are greatly manipulated and might generate effects that are not physiologically relevant.

In human and murine pregnancy, Tregs suppress T cell proliferation *in vitro* (Aluvihare et al. 2004; Sasaki et al. 2004; Somerset et al. 2004; Zenclussen et al. 2005b; Zhao et al. 2007), a suppression suggested to be more pronounced during pregnancy (Polanczyk et al. 2005; Steinborn et al. 2008). However, the Treg effects on cytokine responses during pregnancy are poorly understood.

In our *in vitro* setting, Tregs from both healthy pregnant and non-pregnant women showed very potent suppression of IL-2, IFN- γ and TNF secretion from the responder cells already at Treg:responder ratio 1:4 (paper II, Fig 5). In the case of IL-2 and IFN- γ , this has previously been shown in non-pregnant individuals (Baecher-Allan et al. 2001; Ng et al. 2001), whereas the suppressive effects of Tregs on TNF secretion is less explored. However, it has been shown that murine Tregs shed TNF receptors, neutralizing the effects of this cytokine (van Mierlo et al. 2008).

The suppressive capacity, expressed as a suppressive index, was similar for healthy pregnant and non-pregnant women (paper II, Fig 5). Hence, although showing an activated but Foxp3- and HLA-DR-diminished phenotype, Tregs

from pregnant women maintained their suppressive function. This was somewhat surprising since reduced Foxp3 expression has been coupled to diminished suppressivity in autoimmune disease (Venken et al. 2008). However, it is possible that an even more pronounced inflammatory environment than that seen in pregnancy (Sacks et al. 1999) is needed for such a functional deficit to break through.

Interestingly, Foxp3 expression is not as stable as first thought, but can be induced transiently at lower levels in activated non-Tregs (Gavin et al. 2006; Allan et al. 2007; Tran et al. 2007; Wang et al. 2007). These activated cells have a Foxp3^{dim}CD45RA⁻ phenotype (Miyara et al. 2009) in line with the Foxp3^{dim}CD45R0⁺ phenotype occurring more frequently in pregnant women (paper II, Fig 2 and 3). However, stable but not transient Foxp3 expression leads to suppressed CD127 expression (Allan et al. 2007). Since Tregs from healthy pregnant women maintained their CD127^{low} phenotype (similarly to non-pregnant women), the Foxp3 expression in these women seems to be stable, thereby making maintained suppression feasible.

Tregs from pregnant and non-pregnant women did not suppress IL-4, IL-10 or IL-17 production from responder cells. Instead, the secretion of these cytokines was enhanced in co-cultures of Tregs and responders (Fig 20), especially from cells isolated from pregnant women. This was most likely due to production of these cytokines from Tregs themselves (Fig 20) which, for IL-17, was a surprise and is further discussed in the next section. It is likely that the secretion of IL-4 and IL-10 contributed to the maintained suppressive function of the Tregs isolated from pregnant women.

Regarding proliferation, it has been shown that Tregs do suppress proliferation of T_H1, T_H2 and T_H17 clones, with T_H2 clones being slightly less susceptible to suppression (Cosmi et al. 2004; Annunziato et al. 2007). However, it is possible, in our system, that proliferation of effector cells secreting IL-17 and IL-4 was suppressed whilst secretion of these cytokines was not.

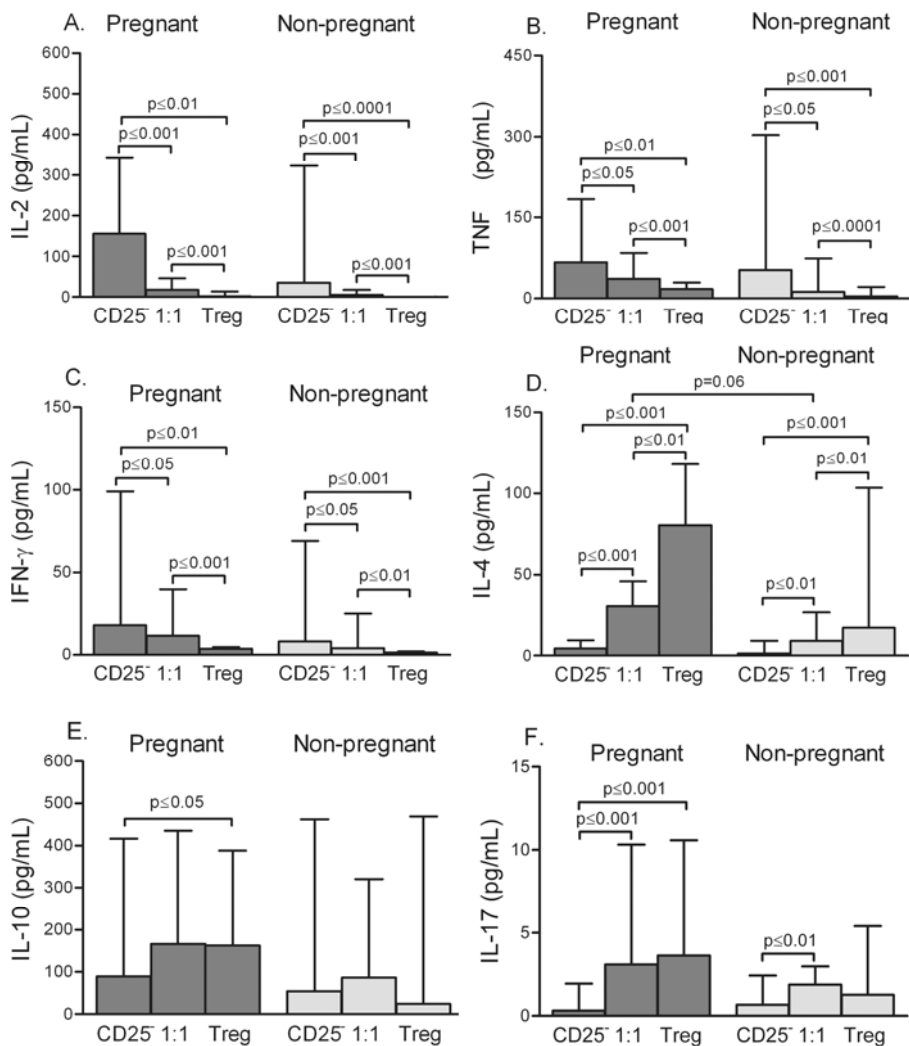


Figure 20. Paper II.

Secretion of IL-2 (A), TNF (B), IFN-γ (C), IL-4 (D), IL-10 (E) and IL-17 (F) from CD4⁺CD25⁻ cells, CD4^{dim}CD25^{high} Tregs and 1:1 co-cultures of CD4⁺CD25⁻ cells and CD4^{dim}CD25^{high} Tregs isolated from second trimester healthy pregnant (n=13) and non-pregnant (n=14) women. Bars represent medians with interquartile range.

Treg cytokine production in second trimester healthy pregnancy (Paper II)

In second trimester pregnant women, the Treg expression of HLA-DR was reduced, indicating that more of these Tregs secrete IL-4 and IL-10 and suppress in a late contact and IL-10 dependent manner (Baecher-Allan et al. 2006). When indeed investigating Treg cytokine production in paper II, we did observe high secretion of these cytokines from Tregs isolated from both pregnant and non-pregnant women (Fig 20).

Although the median secretion of IL-10 and in particular IL-4 from Tregs seemed to be higher in pregnant women, there was no statistically significant difference (Fig 20D-E). This was also the case when IL-4 and IL-10 secretion was considered as a ratio between that from Tregs and effector cells (CD4⁺CD25⁻ cells) (Fig 21). However, co-cultures from pregnant women tended to contain more IL-4, and Tregs from pregnant women tended to produce more IL-10 than the effector cells, something which was not seen in non-pregnant women (Fig 20D-E). Hence, it could be suggested with caution that the IL-4 and IL-10 secretion from pregnancy Tregs was more pronounced. Supporting this, GATA3 mRNA expression was significantly higher in anti-CD3/CD28 stimulated Tregs from pregnant as compared with non-pregnant women ($p \leq 0.01$, data not shown).

The cytokine-producing capability of Tregs is a matter of great debate, likely much due to the different conditions *in vitro* vs. *in vivo*. *In vitro*, human Tregs have been shown to secrete IL-4, IL-10 and TGF- β (Dieckmann et al. 2001; Jonuleit et al. 2001; Levings et al. 2001; Stephens et al. 2001; Dieckmann et al. 2002). In paper II, detection of TGF- β in supernatants was compromised by the existence of TGF- β in fetal calf serum, disabling analysis of this cytokine.

At the time, to our great surprise, Tregs secreted relatively high levels of IL-17 that were significantly enhanced in pregnant women (Fig 21). At the transcriptional level, Tregs from pregnant women tended to express more RORC mRNA than non-pregnant women ($p=0.06$, data not shown). It is now well established that Tregs, under some circumstances, do secrete IL-17 and cells co-expressing the lineage-associated transcription factors Foxp3 and RORC can be found in tonsils (Koenen et al. 2008; Beriou et al. 2009; Voo et al. 2009).

In mice, diminished Foxp3 expression resulted in loss of suppressivity and IL-17, as well as IL-4 and IL-10 production (Gavin et al. 2007; Wan et al. 2007). Although the Tregs from healthy pregnant women in our study did not show reduced suppressive function, they did secrete increased levels of IL-17, linking them to the Foxp3-attenuated Tregs seen in mice. Further, the IL-17 production previously

found in human Tregs (Koenen et al. 2008; Beriou et al. 2009; Voo et al. 2009) was recently shown to originate in those cells expressing the lowest levels of Foxp3 and CD45RA (Miyara et al. 2009), as Tregs in pregnancy preferentially do (Fig 16 and 17).

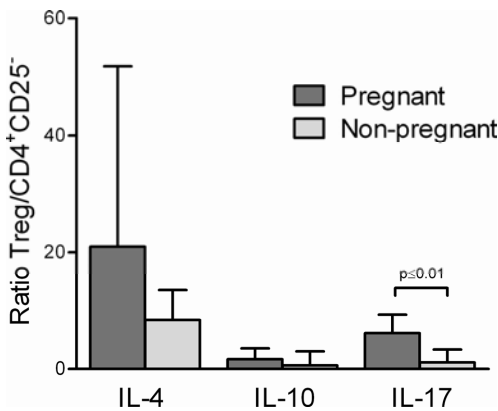


Figure 21. Paper II.

Secretion of IL-4, IL-10 and IL-17 from Tregs in relation to the secretion from CD4⁺CD25⁻ cells (Treg/CD4⁺CD25⁻ ratio) isolated from second trimester healthy pregnant (n=13) and non-pregnant (n=14) women and stimulated with anti-CD3/CD28 antibodies for three days. Bars represent medians with interquartile range.

In conclusion, the cytokine production and phenotypical characteristics of CD4^{dim}CD25^{high} Tregs from healthy pregnant women indicate that their stability as Tregs have been challenged, providing them with properties unlike typical Tregs. However, it should be emphasized that although the pregnancy Tregs did show an increased degree of plasticity, they preserved their suppressive capacity, unlike the CD4^{high}CD25^{high} population that will be discussed in the next section. It is intriguing to speculate that these changes are a normal response to pregnancy which is a situation of increased immune regulatory burden. Studies unraveling this by investigating cytokine production (e.g. IL-35) and expression of lineage-specific transcription factors are ongoing.

Functional characteristics of CD4^{high}CD25^{high} cells found in healthy second trimester pregnancy (Paper II)

It is worth emphasizing that it is becoming increasingly clear that Tregs is not a stable or permanent subset of cells. Besides the very recent reports of Foxp3⁺ cells producing IL-17 (Koenen et al. 2008; Beriou et al. 2009; Voo et al. 2009), murine cells that had lost their Foxp3 expression, hence termed exFoxp3 cells, showed an autoaggressive behavior with production of IFN- γ (Zhou et al. 2009b). It was hypothesized that during autoimmunity, Tregs can not only lose their suppressive function but also acquire proinflammatory features, contributing to pathogenesis. We found that a population of CD4^{high}CD25^{high} cells in healthy pregnant, as well as preeclamptic, women was expanded (paper II, Fig 1B and paper III, Fig 3A). The CD4^{high}CD25^{high} population had an activated phenotype (CD45R0⁺) similar to the CD45A-Foxp3^{dim} non-suppressive cells described by Miyara and colleagues (Miyara et al. 2009), the exFoxp3 cells (Zhou et al. 2009b) as well as the activation induced Foxp3-expressing cells presented by several groups (Gavin et al. 2006; Allan et al. 2007; Tran et al. 2007; Wang et al. 2007). In healthy pregnant women, we could show that, in contrast to CD4^{dim}CD25^{high} Tregs, the CD4^{high}CD25^{high} cells were non-suppressive of IL-2, IFN- γ , TNF (Fig 22) as well as IL-17 (Fig 23) secretion, but rather produced these cytokines themselves (Fig 20-23). Further, the CD4^{high}CD25^{high} cells produced high levels of IL-10 and IL-4 (paper II, page 766).

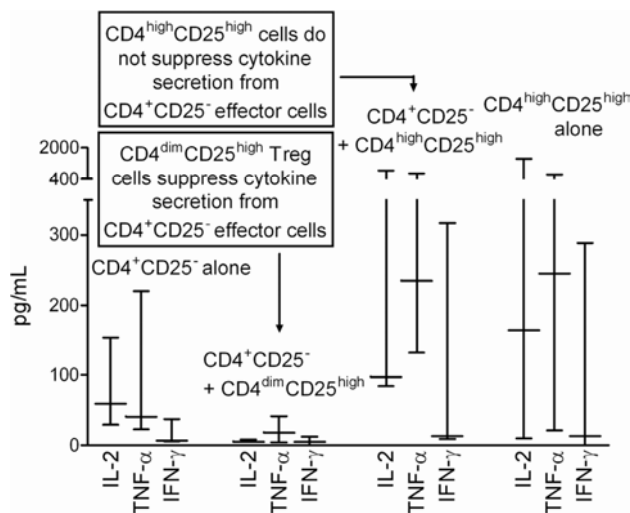


Figure 22. Paper II.

Secretion of IL-2, TNF-α, IFN-γ from CD4⁺CD25⁻ cells alone (far left) or in combination with CD4^{dim}CD25^{high} Tregs (second from left) or CD4^{high}CD25^{high} cells (second from right) in 2:1 (responder:suppressor) ratio. The secretion of the cytokines from CD4^{high}CD25^{high} cells alone is shown to the far right. Data are from cells isolated from three second trimester healthy pregnant women and shown as minimum, maximum and median values.

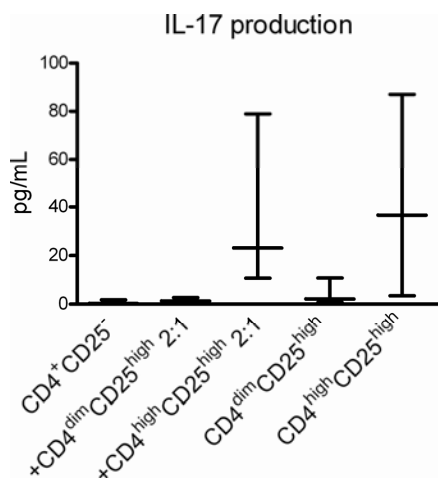


Figure 23. Paper II.

Secretion of IL-17 from CD4⁺CD25⁻ cells alone (far left) or in combination with CD4^{dim}CD25^{high} Tregs (second from left) or CD4^{high}CD25^{high} cells (third from left) in 2:1 (responder:suppressor) ratio. The secretion of IL-17 from CD4^{dim}CD25^{high} and CD4^{high}CD25^{high} cells alone is shown to the far right. Data are from cells isolated from three second trimester healthy pregnant women and shown as minimum, maximum and median values.

It is not completely unfounded to associate these CD4^{high}CD25^{high} cells with exFoxp3 cells or activation-induced Foxp3-expressing cells (Gavin et al. 2006; Allan et al. 2007; Tran et al. 2007; Wang et al. 2007; Miyara et al. 2009; Zhou et al. 2009b). Indeed, both the CD4^{dim}CD25^{high} and CD4^{high}CD25^{high} populations from pregnant women carried features of the Foxp3^{low} non-Tregs but with CD4^{high}CD25^{high} cells being at the extreme non-suppressive end of this population. Perhaps, during pregnancy, these potentially harmful CD4^{high}CD25^{high} cells are generated and, for some yet unknown reason, necessary but kept in check by the unique immune (cytokines etc.) and hormonal environment. A clinical observation that could be linked to this theory is that many women with MS and RA experience aggravated disease after delivery (Ostensen et al. 2006; Argyriou et al. 2008). It is intriguing to speculate that this could be due to the pregnancy-generated CD4^{high}CD25^{high} population that loses its hormonal control post partum and becomes pathogenic in already “autoimmunity-primed” women. Obviously, these are mere speculations. However, in the healthy pregnant woman it is likely that the CD4^{high}CD25^{high} population has an important function in the systemic defense against infections.

Tregs in first trimester pregnancy decidua and blood (Paper IV)

Frequency and phenotype of Tregs in first trimester decidua and blood

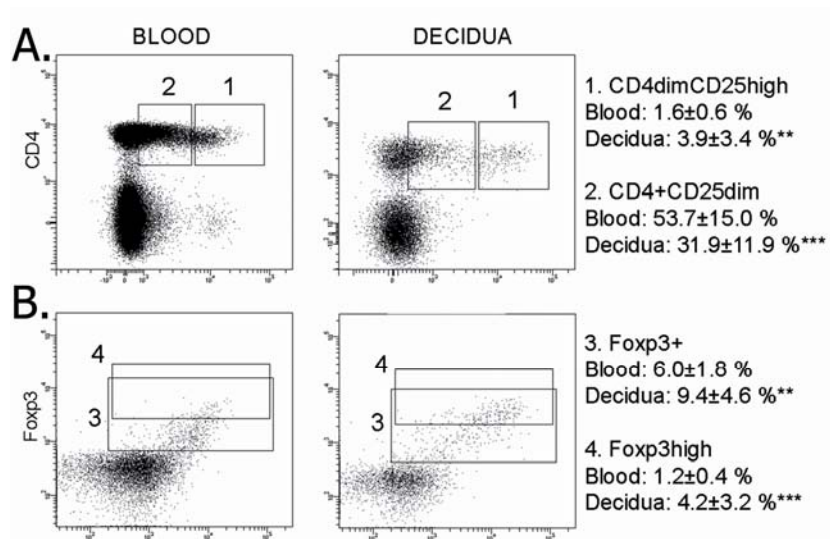


Figure 24. Paper IV.

Presence of CD4^{dim}CD25^{high}, CD4⁺CD25^{dim}, Foxp3⁺ and Foxp3^{high} cells in the CD4⁺ population in first trimester healthy pregnant women.

A. Proportion of CD4^{dim}CD25^{high} cells (gate 1) and CD4⁺CD25^{dim} cells (gate 2) within the CD4⁺ population isolated from blood (left panel) and decidua (right panel).

B. Proportion of Foxp3⁺ cells (gate 3) and Foxp3^{high} cells (gate 4) within the CD4⁺ population isolated from blood (left panel) and decidua (right panel).

Data are given as mean ± SD. ** p ≤ 0.01, *** p ≤ 0.001.

In paper IV, we applied our gating strategy, CD4^{dim}CD25^{high}, developed in paper II, to investigate the frequency and phenotype of decidual and circulating Tregs isolated from first trimester pregnant women. By combining data from paper III and IV, we established that similarly to the situation in second trimester pregnancy, circulating CD4^{dim}CD25^{high} Tregs in first trimester pregnant women were significantly fewer than in non-pregnant women (Fig 15A). This finding is compatible with the view that the Tregs were somehow drained from the

circulation, migrating to the place where they would undoubtedly be most useful, in the decidua at the fetal-maternal interface.

Indeed, in accordance with our hypothesis, regardless if defined as CD4^{dim}CD25^{high}, Foxp3⁺, Foxp3^{high} (Fig 24) or CD25^{high}CD127^{low} (paper IV, Fig 1), Tregs were consistently more frequent in the decidua as compared with blood. This was an important finding since decidual tissue holds fetal antigens and is expected to contain immune cells with an activated phenotype that could complicate the interpretation of Treg markers, most of which are also markers of activation. Supporting the idea that the expanded decidual population of Tregs was “true” Tregs, the majority of these CD4^{dim}CD25^{high} and Foxp3⁺ cells also expressed the Treg markers CTLA-4 and HLA-DR (paper IV, Fig 3B and 3C). Expression of these markers, as well as the activation/memory indicator CD45R0, was consistently more frequent in/on Tregs in decidua as compared with blood (paper IV, Fig 3). Further, Foxp3 expression intensity was higher in the decidua than in blood, as shown by a higher frequency of Foxp3^{high} cells in decidua (Fig 24). Activation-induced Foxp3 expression in non-Tregs, which could be a potential problem in activated tissues such as decidua, results in lower expression of not only Foxp3, but also CTLA-4 as compared to that seen in Tregs (Allan et al. 2007). Hence, activated non-Tregs mimicking the phenotype of true Tregs did not seem to be a major problem in decidua.

Our findings are in line with early previous studies showing that the number of CD4⁺CD25^{+/high} (Heikkinen et al. 2004; Sasaki et al. 2004; Tilburgs et al. 2006) and Foxp3⁺ cells (Tilburgs et al. 2008) was higher in first trimester (Sasaki et al. 2004), second trimester (Tilburgs et al. 2006; Tilburgs et al. 2008) and term decidua (Heikkinen et al. 2004; Tilburgs et al. 2006; Tilburgs et al. 2008) as compared with corresponding blood. However, our study (paper IV) is the first to make use of an extended panel of Treg markers, including Foxp3, in first trimester decidua. Our findings have implications for understanding fetal tolerance mechanisms, as well as implantation mechanisms, since placentation continues throughout first trimester.

Origin of Tregs in first trimester decidua

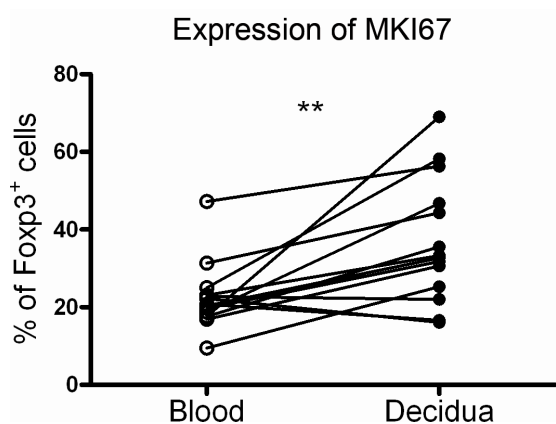


Figure 25. Paper IV.

Frequency of MKI67⁺ cells within the blood and decidual Foxp3⁺ population of first trimester healthy pregnant women (n=12). ** p< 0.01.

Tregs were enriched in first trimester decidua. However, the mechanisms behind this remain unknown. Based on the increased presence of Tregs expressing the proliferation-associated antigen identified by monoclonal antibody Ki-67, MKI67, in decidua (Fig 25), this could be caused by proliferation of Tregs at the fetal-maternal interface. Of note, since MKI67 was also highly expressed in circulating Tregs in first trimester pregnant women, it is possible that the Tregs were also expanded peripherally. However, these cells would have to be rapidly recruited to the decidua, or some other extra-circulatory compartment, since CD4^{dim}CD25^{high} Tregs were reduced in the circulation already in first trimester (Fig 15A). It would be intriguing to investigate the systemic expression of MKI67 in Tregs from non-pregnant women, trying to find out if circulating Tregs expand more during pregnancy. Supporting the idea of Tregs being recruited to the decidua during pregnancy, 30% of the decidual CD4⁺ population bearing the chemokine receptor CCR4 were Foxp3⁺ Tregs as compared with 16% in blood (Fig 26). A ligand for CCR4, CCL17, is produced by trophoblasts and stromal cell in first trimester pregnancy (Tsuda et al. 2002). Hence, the CCR4-CCL17 interaction could be one mechanism behind Treg recruitment to the decidua. Another likely

mechanism is hCG, secreted from the early blastocyst, attracting Tregs to the implantation site very early in pregnancy (Schumacher et al. 2009).

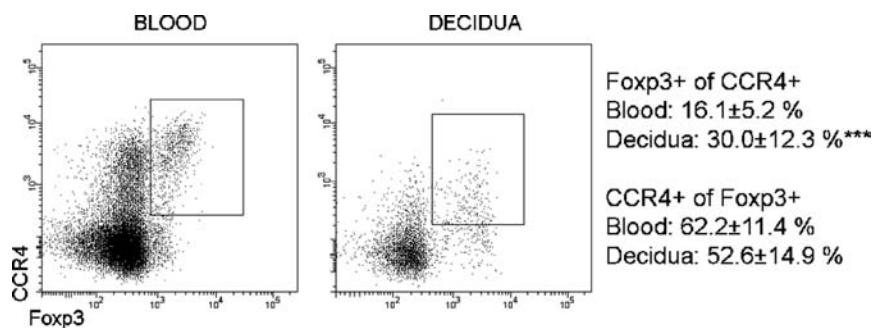


Figure 26. Paper IV.

Representative dotplots showing the expression of CCR4 (y-axis) and Foxp3 (x-axis) within the CD4⁺ population isolated from blood (left plot) and decidua (right plot) of first trimester pregnant women (n=14). Data to the far right in the figure show mean \pm SD. *** $p \leq 0.001$.

We established that Tregs were enriched, at least partly by proliferation, in decidua (paper IV). However, the signals required for such expansion remain unknown. There are two main candidates, presence of fetal antigens and hormonal/cytokine environment. As to the first, based on a functional *in vitro* study, it was recently suggested that Tregs are only present in the decidua parietalis when the mother and fetus are HLA-C mismatched (Tilburgs et al. 2009). We argue that this is because only then was there an activation of leukocytes, preferentially CD4⁺CD25^{dim} cells, to be suppressed by the Tregs. Further, there were no correlations between HLA-A/B/C/DR/DQ disparities and Treg numbers. Hence, Tregs are likely expanded in both decidua parietalis and basalis in all pregnancies, as shown previously by this group (Tilburgs et al. 2006), but only required in decidua parietalis when there is a MHC mismatch, such as HLA-C. Supporting this, Aluvihare et al. noted that Tregs were expanded in both syngenic and allogenic pregnancies in mice. However, pregnancy only failed when the Tregs were depleted from allogenicly, not syngenicly, mated mice (Aluvihare et al. 2004). Also, the expansion seen in syngenic pregnancy correlated with the number of male fetuses in uteri, i.e. presence of male minor histocompatibility antigens (Zhao et al. 2007). Hence, pregnancy environmental factors (hormones, cytokines etc.) but also minor histocompatibility complex

mismatches contribute to Treg expansion. We observed, in paper II, that Tregs were not expanded by estradiol or progesterone at concentrations that can be expected locally in the decidua (Fig 18 and 19). However, it is likely that these and other hormones, such as hCG, and cytokines do promote Tregs in the unique setting in the decidua. Several cytokines known to support Tregs, such as TGF- β , IL-4 and IL-10 (Zheng et al. 2004; Maerten et al. 2005; Horwitz et al. 2008) are present at high levels in decidua (Piccinni 2002; Jones et al. 2006). Further, the role for semen, rich for TGF- β , in local Treg expansion has also been discussed and could be the key switch for induction of tolerance even before implantation (Robertson et al. 2009). In accordance, Zenclussen et al. showed that Tregs could only rescue abortion prone mice from fetal rejection if Tregs were adoptively transferred prior to conception (Zenclussen et al. 2005b).

Expression of CD127 on Tregs in first trimester blood and decidua

Sorting of pure and viable Tregs to be used for *in vitro* and *in vivo* functional experiments is crucial to obtain reliable and reproducible results. The intracellular location of Foxp3 however rules out this molecule. In paper IV, we evaluated a new marker for Tregs, CD127, which is down-regulated in cells stably expressing Foxp3, providing suppressive Foxp3⁺ cells with a CD127^{low} phenotype (Liu et al. 2006; Seddiki et al. 2006). Although CD127^{low} cells were mostly confined to the CD25^{high} population in both blood and decidua, combining the CD127^{low} and CD4^{dim}CD25^{high} phenotypes did not convincingly increase the homogeneity of Foxp3 expression (paper IV, Table 2). Hence, we concluded that the CD127^{low} phenotype was redundant when applying the CD4^{dim}CD25^{high} phenotype for sorting of viable suppressive Foxp3⁺ Tregs in blood as well as decidua.

T helper subsets T_H1, T_H2 and T_H17 in relation to Tregs in first trimester pregnancy decidua and blood (Paper IV)

Expression of chemokine receptors associated with T_H1, T_H2 and T_H17 immunity in first trimester decidua and blood

T_H1, T_H2 and T_H17 cells have been defined by production of their signature cytokines (IFN- γ , IL-4 and IL-17, respectively) or transcription factors (TBX21, GATA3 and RORC, respectively). Transcription factors are analyzed as mRNA by real-time PCR while cytokines can be analyzed by flow cytometry on polyclonally stimulated cells. In paper IV, it was our wish to investigate the balance between Tregs and T_H1, T_H2 and T_H17 cells in freshly isolated unstimulated peripheral and decidual leukocytes. This was done to give an authentic picture of the *in vivo* conditions at the fetal-maternal interface. To our knowledge, there are no previous reports on T_H17 cells in decidua. Considering that this subset is associated with mucosal immunity (Acosta-Rodriguez et al. 2007), angiogenesis and tumor growth (Numasaki et al. 2003), T_H17 could have an important role at the fetal-maternal interface. On the other hand, murine data indicate that there is a reciprocal relationship between Tregs and T_H17 cells (Bettelli et al. 2006). We anticipated Tregs to be enriched which would then, at least in theory, reduce the presence of T_H17 cells.

Tregs can be analyzed by flow cytometry using the vast array of markers available for this subset. However, the T_H1, T_H2 and T_H17 subsets were more challenging since flow cytometry reagents for analysis of transcription factors were not available. To this end, we applied a method described by Acosta-Rodriguez et al. (Acosta-Rodriguez et al. 2007), utilizing chemokine receptors for identification of T_H1, T_H2 and T_H17 cells. According to this strategy (described in table VI), CXCR3-expressing cells could be divided into two populations. One with a more pure T_H1 phenotype, producing IFN- γ and TBX21, as well as one population with a mixed T_H1/T_H17 phenotype, producing IFN- γ and IL-17, as well as TBX21 and RORC. The latter population, termed CCR6⁺T_H1, seemed to produce less IFN- γ and TBX21. T_H2 and T_H17 cells could be identified by the expression of CCR4 on T_H2 cells and the additional expression of CCR6 on T_H17 cells (table VI).

Table VI. T_H1, T_H2 and T_H17 cells can be characterized by expression of chemokine receptors CXCR3, CCR4 and CCR6 (Acosta-Rodriguez et al. 2007)

Subset	Chemokine receptor expression	Secretion of cytokines	Expression of transcription factors
T _H 2	CCR6-CCR4+CXCR3-	IL-4	GATA3
T _H 17	CCR6+CCR4+CXCR3-	IL-17	RORC
CCR6 ⁺ T _H 1	CCR4-CXCR3 ⁺ CCR6 ⁺	IFN- γ and IL-17	TBX21 and RORC
CCR6 ⁻ T _H 1	CCR4-CXCR3 ⁺ CCR6 ⁻	IFN- γ	TBX21

The subsequent commercial availability of antibodies directed against human lineage-associated transcription factors RORC (T_H17), TBX21 (T_H1) and GATA3 (T_H2) protein made it possible to confirm the chemokine receptor profiling strategy for identifying T_H1, T_H2 and T_H17 cells. This was done using non-stimulated PBMC from non-pregnant women (Fig 27). The T_H2 population was dominated by cells expressing GATA3, T_H17 by RORC and T_H1 by TBX21. In accordance with previous observations, CCR6⁺ T_H1 cells also expressed RORC, supporting the notion that these cells have a mixed T_H1/T_H17 phenotype. Hence, we confirmed that the expression of chemokine receptors CCR4, CCR6 and CXCR3 could be used for identifying T_H1, T_H2 and T_H17 cells. Although we did not verify this using decidual cells, due to scarcity of such material, it was shown that the strategy is stable and valid even under inflammatory conditions as in inflamed synovial fluid (Acosta-Rodriguez et al. 2007).

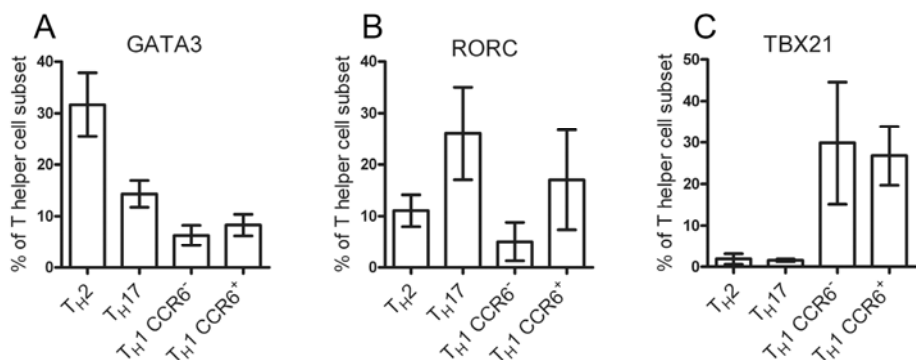


Figure 27. Paper IV.

Expression of lineage-associated transcription factors GATA3 (T_H2), RORC (T_H17) and TBX21 (T_H1) in the T_H2, T_H17 and T_H1 CCR6^{+/−} populations as defined by expression of chemokine receptors CCR4, CCR6 and CXCR3. Data are from four non-pregnant women and shown as mean ± SD.

When utilizing the chemokine receptor profiling for identifying T_H1, T_H2 and T_H17 cells in first trimester pregnancy blood and decidua, T_H17 and CCR6⁺T_H1 cells were significantly downregulated in decidua as compared with blood (Fig 28). T_H2 cells were present at similar frequency in blood and decidua whereas CCR6⁺T_H1 cells were significantly enriched in decidua, a pattern also observed for Tregs (Fig 28).

Regarding T_H1 cells in decidua, relaxin, which is a hormone produced by the corpus luteum and decidua, has been shown to induce IFN-γ secretion from CD4⁺ cells (Piccinni et al. 2001). It was proposed that this was a counterweight to progesterone, preferably inducing IL-4 secretion. Further, IFN-γ has very important functions in establishing pregnancy and is abundantly expressed during implantation and early pregnancy (Wegmann et al. 1993; Bazer et al. 2009). Simultaneously, IFN-γ is regarded as an abortifacient (Chaouat 2007). Hence, the levels and timing for IFN-γ secretion, as well as the co-existence of other cytokines are likely decisive for the outcome of the pregnancy. Interestingly, both murine and human T_H1 cells can produce IL-10, tentatively as a mechanism of avoiding self-damage during chronic infections (Trinchieri 2007). The results from paper IV show that moderately IFN-γ secreting CCR6⁺T_H1 cells are enriched whereas the possibly more aggressive CCR6⁺T_H1 cells are diminished in decidua as compared with blood. This would ensure but also limit the access of IFN-γ, possibly contributing with an appropriate amount of IFN-γ. The role for IL-10 in this limitation would undoubtedly be interesting to investigate.

Within the T_H1/T_H2 paradigm, pregnancy is considered as a T_H2 phenomenon (Wegmann et al. 1993). In paper IV, in contrast to a previous study (Tsuda et al. 2001), the frequency of T_H2 cells was similar in blood and decidua. This does not however rule out the role for T_H2 cells in decidua as it could rather suggest that T_H2 cells are frequent also in blood. Indeed, in paper I, we observed an increased T_H2 activity in PBMC from healthy second trimester pregnancy.

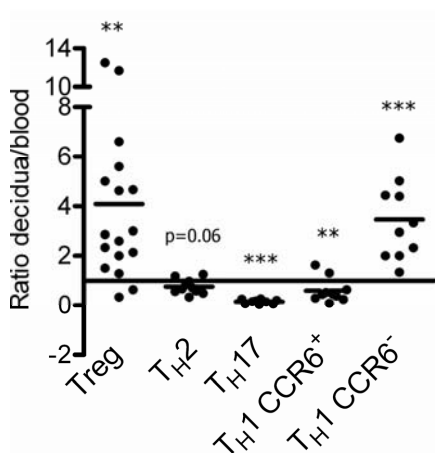


Figure 28. Paper IV.

Ratios of the presence of Tregs ($CD4^{dim}CD25^{high}Foxp3^{high}$, $n=17$), T_H2 ($CCR6^- CCR4^+CXCR3^-$, $n=10$), T_H17 ($CCR6^+CCR4^+CXCR3^-$), $CCR6^+T_H1$ ($CCR4^-CXCR3^+CCR6^+$) and $CCR6^-T_H1$ ($CCR4^-CXCR3^+CCR6^-$) cells in decidua vs blood from first trimester healthy pregnant women.

Lines indicate mean values. Significance markers (*) indicate the statistical difference between blood and decidua. ** $p \leq 0.01$, *** $p \leq 0.001$.

The recently discovered T_H17 subset has yet been poorly investigated in pregnancy and particularly in decidua. IL-17, the T_H17 signature cytokine, promotes vascularisation and tumour growth (Numasaki et al. 2003), events closely linked to establishment of pregnancy. Indeed, IL-17 protein could be found in human term trophoblasts and macrophages (Pongcharoen et al. 2007), indicating a role for this protein in pregnancy. However, regarding T cells, we found, in paper IV, that T_H17 cells were barely detectable in decidua. An attractive mechanism behind this is that Tregs and T_H17 are reciprocally regulated (Bettelli et al. 2006) at the fetal-maternal interface, Tregs being promoted by TGF-

β at the expense of the T_H17 subset which, besides lower levels of TGF- β seems to demand additional factors such as IL-6 for its differentiation (O'Garra et al. 2008). Further, IL-27, a negative regulator of T_H17 immunity (Diveu et al. 2009), is widely expressed in trophoblasts in all trimesters of pregnancy (Coulomb-L'Hermine et al. 2007).

Expression of transcription factors associated with T_H1 , T_H2 , T_H17 and Treg immunity in first trimester decidua and blood (preliminary data)

The T helper cells present in decidua are important. However, the total expression of factors regulating immune processes, such as transcription factors associated with cytokine production, could give a more complete picture of the immune status at the fetal-maternal interface. In first trimester decidua, NK cells represent approximately 70% of the mononuclear leukocytes, macrophages 10-20% and T cells 10-20% (paper IV) (Trundley et al. 2004). The corresponding approximate figures in blood are 5%, 20% and 75%, respectively (paper IV) (Matthiesen et al. 1996; Matthiesen et al. 1999; Luppi et al. 2002). Consequently, a factor expressed in decidual NK cells would completely mask all differences between smaller cell populations such as T cells and macrophages. Hence, analyzing T cell factors in decidua as compared with blood as a bulk could not be done if the factors are expressed by uNK cells. Nevertheless, we considered it important to investigate the total mRNA expression of transcription factors associated with T_H1 , T_H2 , T_H17 and Treg immunity in first trimester decidua as compared with blood to get a more complete picture of these factors at the fetal-maternal interface.

In accordance with the traditional view of pregnancy as a T_H2 phenomenon, GATA3, enhancing the expression of IL-4 was strongly upregulated, whereas TBX21, promoting the expression of IFN- γ was strongly downregulated in decidua (Fig 29B-C). Foxp3 was on the other hand similarly expressed in blood and decidua (Fig 29A). Approximately 2.5% of the mononuclear cells in blood represent CD3⁺CD4⁺Foxp3⁺ cells while the corresponding frequency in decidua is only 1% (calculated from data in paper IV, Fig 1 and 2), explaining the lack of total Foxp3 mRNA enrichment in decidua. In contrast to Foxp3, which is only expressed in T cells (our own unpublished observation), IFN- γ and IL-4, thereby possibly also TBX21 and GATA3, are expressed by NK cells in decidua (Lidstrom et al. 2003; Higuma-Myojo et al. 2005). Using flow cytometry, decidual CD56^{bright} NK cells expressed significantly less TBX21 protein but similar levels of GATA-3 protein as compared with blood CD56^{bright} NK cells (Fig 30). Hence, since the CD56^{bright} NK cells make up the vast majority of the mononuclear cells in decidua,

these data (Fig 30) support the findings of decreased expression of TBX21 and increased expression of GATA3 mRNA in the total decidual, as compared with the peripheral, mononuclear cell population (Fig 29).

Interestingly, RORC transcripts, governing IL-17 expression, were upregulated on the transcriptional level in decidua (Fig 29D) which, given the tumor-promoting and angiogenic effects of IL-17, is very intriguing. Further, since trophoblasts show IL-17 protein expression, it is not unlikely that these cells produce RORC, contributing (by contamination) to the increase in decidual RORC mRNA expression. To our knowledge, there is no information about the possibility for decidual NK cells to produce RORC/IL-17. We found a tendency towards higher expression of RORC protein in decidual than in blood CD56^{bright} NK cells (Fig 30). Further, in mouse gut mucosa, NK cells expressing ROR γ t (mouse homologue of human RORC) and IL-22 have been found, in contrast to conventional NK cells which are negative for both these factors (Malmberg et al. 2009). Further, human fetal lymphoid tissue inducer (LTi) cells, involved in lymph node organogenesis, were recently identified as NK cell precursors expressing RORC, IL-17 and IL-22 (Cupedo et al. 2009), both cytokines involved in controlled chronic inflammation. Since decidua can be viewed as an inducible tertiary lymphoid organ (Kalkunte et al. 2008), it is interesting to speculate about the similarities between LTi and decidual NK cells and their progenitors. This will however have to be the subject of future investigations.

In total, it is highly likely that the NK cells and to some extent also contaminating trophoblasts and stromal cells were the main sources of the discrepancies in expression of TBX21, GATA3 and RORC between blood and decidual mononuclear cells. Nevertheless, collectively these preliminary data indicate that local immunity, as compared with that seen in blood, is skewed towards higher expression of GATA3 and RORC, but not TBX21. This could ascertain a correct and controlled establishment and progression of pregnancy.

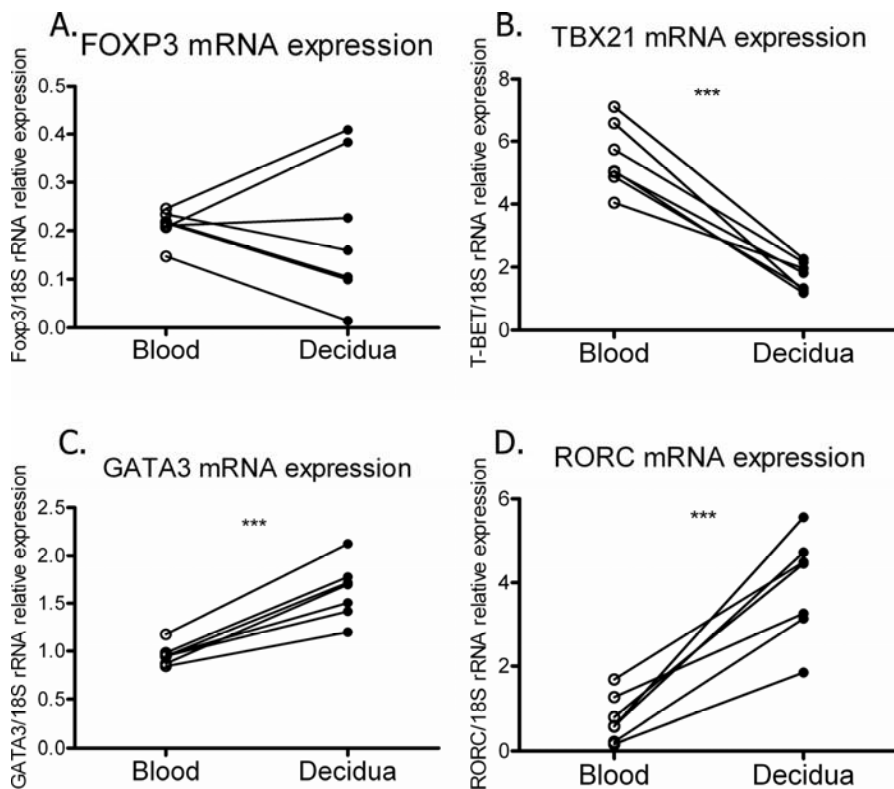


Figure 29. Preliminary data

Expression of Foxp3 (A), TBX21 (B), GATA3 (C) and RORC (D) mRNA of transcription factors associated with Treg (A), T_H1 (B), T_H2 (C) and T_H17 (D) immunity in first trimester decidua mononuclear cells as compared with PBMC from the same woman (n=7).

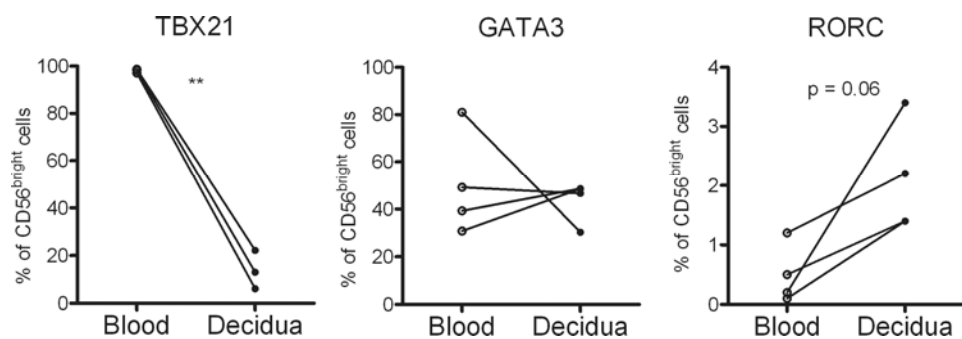


Figure 30. Preliminary data

Expression of TBX21, GATA3 and RORC protein in CD56^{bright} lymphocytes isolated from blood and decidual tissue of first trimester pregnant women (n=3-4).

Methodological aspects and general discussion

This thesis was based on samples isolated from women at one time point during pregnancy. Hence, the results were snapshots of the reality and it is not likely that all the cells analyzed were functionally synchronized at that particular time point. Therefore, repeated samplings from the same woman could provide a more stable and reliable picture. Further, in paper I-III, PBMC were analyzed and this raises the issue about the importance of these cells. A criticism could be that PBMC represent all that is irrelevant to the immune situation, since it is expected that important cells have migrated to tissues, in this case the fetal-maternal interface. Indeed, we do believe that immune tolerance, including enhancement of Tregs, is most pronounced at this interface. Yet, systemic changes do occur, sometimes, but not always, as mirror images of what goes on locally. Since lymphocytes recirculate, both naïve and activated cells could be captured in a PBMC sample. In addition, systemic immunity during pregnancy can be viewed as a situation where tolerance is kept without dramatically altering immune competence. Hence, the underlying mechanisms are intriguing and important to unravel.

T_H2-like immunity (papers I-II and IV)

In paper I, PBMC from pregnant women showed a higher number of cells spontaneously secreting IL-4 as compared with non-pregnant women. In paper II, CD4⁺CD25⁻ polyclonally stimulated cells from pregnant women secreted very low levels of this cytokine, similarly to the situation in non-pregnant women, whereas Tregs themselves secreted higher levels of IL-4. This could imply that in PBMC, most of the IL-4 secretion originates from the Treg population. However, IL-4 can also be secreted from eosinophils, basophils and mast cells (Borish et al. 2003) and although these cells should not be present in PBMC, their relative increase in pregnant women (Matthiesen et al. 1995; Nisell 2008b) might have contributed to a higher degree of contamination, thereby explaining the findings in paper I.

It is interesting to note that in paper I, Tregs were able to reduce the number of cells (Treg depleted PBMC) secreting IL-4. This was not seen in paper II, where Tregs themselves secreted considerable amounts of this cytokine. There are multiple plausible explanations for this. Firstly, in paper I, maternal Tregs were only able to significantly suppress IL-4 secretion in response to paternal, not unrelated alloantigens. In paper II, the antigenic stimulation was polyclonal.

Hence, Treg suppression of IL-4 secretion could be a paternal/fetus-specific phenomenon, as thoroughly discussed in the results and discussion section (paper I). Secondly, the amplitude of the stimulation was much higher in paper II than in paper I, most likely setting a different ground for Treg suppression. It is obvious that the “inertness” originally described for Tregs is somewhat overridden by strong polyclonal stimulation (paper II), possibly explaining the lack of IL-4 suppression in these situations. Thirdly, the impact of the different methods used to investigate Treg suppression should not be neglected. ELISPOT measures, with high sensitivity (Ekerfelt et al. 2002), the frequency of cytokine-secreting cells whereas the multiplexed microsphere-based flow cytometric assays (Luminex) measures the concentration of cytokines in supernatant. These data are not comparable, as shown by a lack of decent correlation between ELISPOT and ELISA for detection of IL-4 and IFN- γ (Ekerfelt et al. 2002).

At the fetal-maternal interface, GATA3 mRNA was strongly upregulated as compared with blood. Since the frequency of T_H2 cells was similar in decidua and blood (paper IV), this signal could originate from the numerous NK cells present in first trimester decidua. In total, this implies that IL-4 is an important part of local immune regulation in normal early pregnancy. Further, IL-4 is produced by circulating Tregs (paper II) and enhanced systemically during pregnancy (paper I).

T_H1-like immunity (papers I-II and IV)

In paper I, pregnant women responded with increased numbers of PBMC secreting IFN- γ in response to unrelated alloantigens. This response was not seen in non-pregnant women. In combination with the IL-4 data, this suggests that systemically, there is an increased baseline IL-4 activity and an increased “alertness” of IFN- γ responses in the pregnant woman. This would ensure maintained immune defense against intracellular bacteria and viruses. Simultaneously, these reactions would be controlled by IL-4 (among others from Tregs) thereby ensuring the safety of the fetal allograft.

As described previously, IFN- γ has intriguing pluripotent roles in pregnancy. While acting as an abortifacient (Chaouat 2007), it is simultaneously crucial for implantation and the very early phases of pregnancy (Wegmann et al. 1993; Bazer et al. 2009). At the fetal-maternal interface in first trimester pregnancy (paper IV), we found a population of CCR6⁺ T_H1 cells that was by far the most prominent T helper population that we could detect in decidua. Although they did not

statistically evaluate their finding, Acosta Rodriguez showed that CXCR3⁺CCR6⁻ T_H1 cells seemed to produce lower levels of IFN- γ and TBX21 mRNA than CXCR3⁺CCR6⁺ T_H1 cells. In total, at the mRNA and transcription factor level, T_H1 activity was lower in decidua than in blood (preliminary data). Hence, in accordance with previous opinions, moderate T_H1 activity seems to be a physiological part of early pregnancy.

Flow cytometric analysis of Treg-associated markers (paper II-IV)

In this thesis, flow cytometry was the single most used and important method. Although providing many benefits, such as quantitative and qualitative measurement of multiple parameters on single cells, it potentially suffers from some drawbacks and pitfalls. On a general level, analyzing isolated markers such as CTLA-4 does not really provide the whole story since e.g. information on down-stream signaling mechanisms and ligands on target cells is missing. Further, to enable detection of intracellular Foxp3 protein expression, fixation and permeabilization had to be performed, possibly introducing cellular alterations. However, to minimize this effect, extracellular markers were labeled prior to fixation and permeabilization. In our hands, the average intra- and inter-assay variation (% CV) was 3.2 % and 3.9%, respectively, when looking at CD4⁺CD25^{high} of CD4⁺ cells isolated from two healthy donors and analyzed at two time points (data not shown). This is in accordance with others, showing a variation (% CV, including methodological, biological and longitudinal variation) ranging from 5-10 % for CD4⁺ cells (Backteman et al. 2007) making it more robust than e.g. ELISPOT where the intra- and inter-assay variation is approximately 30%, depending on the cytokine investigated (Ekerfelt 1999).

Treg suppressive function (papers I-II)

In papers I and II, Treg suppression was evaluated by analyzing the ability of Tregs to suppress cytokine secretion from responder cells. An alternative method to study Treg function would be to analyze the capacity of the Tregs to suppress proliferation. This has indeed been done in MS patients, where Tregs showed reduced suppressive function (Venken et al. 2008). However, this yields a rough quantitative rather than qualitative measure of Treg function and it also disables detection of Treg-mediated immune skewing. Further, others have claimed that measuring Treg suppression as the capacity to suppress cytokine secretion rather

than proliferation is a more sensitive approach (Grindebacke et al. 2004). Still, it would undoubtedly be useful to assess Treg suppression of cell proliferation. A useful method would be the 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay (Lyons 2000) which enables simultaneous analysis of many other fluorescence labeled markers. The interplay between Tregs and their target cells shapes the function of both cell types (Collison et al. 2009). Hence, using the CFSE dilution assay to assess, not only suppressive capacity, but also Treg effects of target cells, would be a natural next step forward in unraveling Treg function in pregnant (both healthy pregnant and preeclamptic) as compared with non-pregnant women.

In papers I and II, depending on the methodological availability at the time, Tregs were sorted by immunomagnetic beads (Dynabeads) or FACSARIA flow cytometric cell sorting, respectively. Whereas Dynabead selection yielded a CD4⁺CD25^{high} cell purity of 53% (median), the purity of FACSARIA sorted CD4^{dim}CD25^{high} cells was typically above 99%. In paper I, we were unable to assess the expression of Foxp3 protein within the selected Treg population, which was however enriched for Foxp3 mRNA expression. In paper II, Foxp3 protein expression purity was expected to be approximately 90%, based on the four-color flow cytometry data. Considering also the high viability of FACSARIA-sorted cells, it is beyond doubt the method of choice when sorting Tregs. This was also the conclusion from a study where FACS-sorted CD4⁺CD25^{high} cells showed markedly higher suppressive capacity as compared with MACS-sorted CD4⁺CD25⁺ cells (Baecher-Allan et al. 2005). It should be emphasized that using a highly defined sorting gate for Tregs, such as the CD4^{dim}CD25^{high} gate used in paper II, may however result in loss of cells with suppressive function since expression of Foxp3 and other Treg-associated markers can be found outside the CD4^{dim}CD25^{high} gate. The different methods used for isolating Tregs are likely considerable sources of the discrepancies seen in the literature regarding Treg phenotype and function.

Studying Treg suppression *in vitro* has its obvious pitfalls and the results have to be viewed with caution. One hurdle is the strength of stimulation needed to obtain responder cell activation and simultaneously allow suppression by the Tregs. For this purpose, in paper I, we choose a suboptimal degree of allogenic stimulation. However, since we did not consistently obtain statistically detectable responses from the responder cells, the stimulation might have been too low. In paper II, the polyclonal stimulation chosen fulfilled the criteria, i.e. measurable responder cell activation that could be suppressed by Tregs. Further, the Tregs

remained unable of producing considerable levels of most of the proinflammatory cytokines measured. However, the Tregs did produce IL-17. Although many reports now support this (Koenen et al. 2008; Beriou et al. 2009; Voo et al. 2009), the actual importance of IL-17 production from Tregs, not just as an *in vitro* artifact due to exaggerated stimulation, will have to be further evaluated.

The impact of hormones on the Treg population (paper II)

In paper II, we observed a dramatic reduction of Treg frequency when stimulating PBMC with 17β -estradiol and progesterone concentrations that were a thousand times higher than those observed in mid-gestational serum and ten-fifty times higher than those seen at the fetal-maternal interface (Stites et al. 1983; O'Leary et al. 1991; Wang et al. 1994; Soldin et al. 2005; Arck et al. 2007). However, lower levels of 17β -estradiol and progesterone (10 times that seen in mid-gestational serum but completely corresponding to placental levels) also reduced the frequency of Tregs. We also observed this at even lower hormone concentrations, although then not consistently statistically significant. Hence, although surprisingly high hormone concentrations were needed to provoke pronounced Treg effects in paper II, we do believe that the results are physiologically transferable and reliable, not the least because we could observe a dose-dependent effect.

Many of the effects of progesterone might be mediated via PIBF that can be produced by lymphocytes (Szekeres-Bartho et al. 2001). However, since we did not analyze this factor, or use it to stimulate the PBMC, the role for PIBF in regulating Tregs cannot be evaluated.

Treg-associated molecules (paper II)

As a part of the methodological considerations for paper II, we investigated several markers of Tregs that were excluded from further consideration. One of these was neuropilin-1 (Bruder et al. 2004). Although we could confirm the expression on $CD4^+$ cells (about 15% of $CD4^+$ cells were positive for neuropilin-1 expression), all expression was situated outside the $CD4^+CD25^{\text{high}}$ compartment. Further, CD27 was used to discriminate between activated and regulatory T cells in inflamed conditions (Ruprecht et al. 2005). We did not consider this marker

useful since it was expressed throughout the entire CD4⁺ population, showing no specificity for Foxp3⁺ cells, and was not differently expressed in pregnant women.

Simultaneously, several interesting Treg-associated markers, involved in suppressive function, such as CD39/CD73 and membrane bound TGF- β /LAP were not investigated in this thesis. We have, yet without success due to lack of proper reagents, indeed attempted to assess the secretion of IL-35, mediating suppression of murine Tregs, from the Tregs isolated in paper II. Future studies will determine the role for these and markers yet to come, in Treg suppression during healthy and diseased pregnancies.

Separation and analysis of decidual cells (paper IV)

In paper IV, we used expression profiles of chemokine receptors CCR4, CCR6 and CXCR3 as markers of T_H1, T_H2 and T_H17 cells. This raises the issue of the chemokine receptor overlap between these subsets and Tregs. Human Tregs have been shown to express a panel of chemokine receptors, including CCR4 and CCR6, but migrate *in vitro* selectively in response to preferentially CCL17 and CCL22 (Iellem et al. 2001; Hirahara et al. 2006; Lim et al. 2006), ligands for CCR8 and CCR4, and to a lesser extent CCL1 and CCL20, ligands for CCR8 and CCR6 (Iellem et al. 2001; Hirahara et al. 2006). Since CCR4⁺ and CCR6⁺ cells (T_H2 and T_H17 cells) were fewer, and Foxp3⁺ cells were more frequent in decidua, it is unlikely that the Foxp3⁺ cells were contaminating the T_H2 and T_H17 populations.

Data regarding presence of CCL1, CCL20 and CCL22 at the fetal-maternal interface are scarce making CCL17-CCR4 interaction (Tsuda et al. 2002) the prime candidate for Treg migration to this site. Our data in paper IV showed that fewer decidual Foxp3⁺ Tregs expressed CCR4, which could be a result of down-regulation due to a high local presence of the CCL17 ligand.

Indeed, many chemokine receptors are rapidly downregulated following exposure to their ligands, owing to receptor internalization and subsequent degradation or recycling to the cell surface (Neel et al. 2005). In paper IV, CCR4 and CCR6 expressing cells were fewer whereas CXCR3⁺ cells were enriched in decidua as compared with blood. Regarding CCR4, it was shown that CCL17, highly expressed at the fetal-maternal interface, did not induce receptor internalization whereas CCL22 did (Mariani et al. 2004). Although, to our knowledge, little is known about decidual CCL22 levels, this indicates that the reduced frequency of decidual CCR4⁺ cells seen in paper IV was not

caused by ligand induced receptor down-regulation. Regarding CCR6, less seems to be known about the dynamics of this receptor. However, the CCR6 ligand CCL20 increases in amniotic fluid of patients with preterm labor and intrauterine infections (Hamill et al. 2008), making it unlikely that high levels of CCL20 are present, and could cause receptor down-regulation, in healthy early pregnancy decidua.

Selection and treatment of patients (papers III-IV)

In papers I-III, healthy second trimester pregnant women were recruited at the maternity outpatient care unit (Kvinnohälsan) in Linköping with a total recruitment ground of approximately 35 000 women (age 18-44 years) and 1 900 pregnant women per year. As the patients were enrolled at a pre-scheduled routine (unlinked from the research participation) midwife appointment in gestational week 25, the recruitment was not biased towards women who themselves sought participation.

The preeclamptic women were recruited at the delivery wards at Linköping University Hospital and Ryhov Hospital, Jönköping. These clinics have a joint recruitment ground of approximately 4 700 deliveries per year and covers a large part of the south east health region of Sweden including a total of 115 000 women (age 18-44 years). Blood samples were taken as soon as possible following admittance but confined to day-time, Monday-Friday, which limited the recruitment ground.

The non-pregnant women were recruited from students/staff at the Faculty of Health Sciences at Linköping University, or from blood donors at Linköping University Hospital. This might have reduced the recruitment area as compared to that of the pregnant women. Many of these women had not previously been pregnant and it is justified to ask why, since these women were all in fertile age and many did not use contraceptives. The median ages for the non-pregnant women were 25-29 (paper I-III). Since most healthy pregnant women in papers I-III were pregnant for the first time and their median ages were 28-30, it is unlikely that more than expected of the non-pregnant women were non-pregnant because of infertility or some other, e.g. social, reasons. It is more likely that the reason was that they were somewhat (although not statistically significantly) younger than the pregnant women.

In papers I-III, all women included fulfilled the inclusion criteria “healthy pregnant women with no signs of pregnancy complications at inclusion”. However, one woman in paper I delivered prematurely in gestational week 34. In paper II, two women delivered prematurely in gestational week 35 and 27, respectively. The data from the women delivering in gestational week 34 and 35, respectively, were not extreme and did not deviate from the overall pattern. Hence, we did not consider it correct to exclude them since they fulfilled the inclusion criteria. In contrast to all the other pregnant women, Tregs from the woman who delivered in week 27 did not secrete higher levels of IL-4 than did CD25⁺ T effector cells. We find this observation intriguing and with reservation to the fact that this is an isolated observation from a single patient, this could indicate a possible role for Treg-associated IL-4 production in healthy pregnancy.

In paper III, seven of the preeclamptic women received betamethasone treatment prior to blood sampling. Glucocorticoids are generally immunosuppressive and have indeed been shown to promote human Tregs (Karagiannidis et al. 2004; Navarro et al. 2006). In the preeclamptic women, this could mask a Treg reduction, possibly restoring the Treg numbers to that seen in healthy pregnant women. Hence, it is of uttermost importance for future studies to include, not only a larger number of samples, but also non-glucocorticoid-treated patients. However, we are proceeding with serum determination of betamethasone levels in these women, thereby hoping to unravel the impact of this treatment on Treg frequency.

In paper IV, patients were given oral prostaglandin (PG) E1 agonist misoprostol (Cytotec) as a cervical priming agent prior to the surgical abortion. PGE is a key player in initiation of labor and given as an abortifacient it expands the decidual neutrophil and macrophage populations (Milne et al. 2005). However, the effects of PGE1-E3 on PBMC were inhibitory, reducing the polyclonal IL-2, TNF, IFN- γ and IL-10 response in *in vitro* cultures (Dooper et al. 2002). Given orally, PGE1 reduced the secretion of IL-2 and IFN- γ from PBMC whereas secretion of IL-4 and IL-10 was unchanged or slightly enhanced (Waiser et al. 2003). PGE2 enhanced IL-17 production, as well as CCR4 and CCR6 expression, in PBMC (Chizzolini et al. 2008). To our knowledge, the effects of PGE1 on Tregs have not been investigated whereas PGE2 seem to enhance Foxp3 expression (Baratelli et al. 2005). In total, PGE1 administered to the women in paper IV could possibly have affected the cell subsets investigated e.g. inhibited the T_H1 population and expanded the Foxp3 and T_H17 populations. However, since PGE1 was orally administered, both peripheral and local immune cells would be affected, thereby limiting the impact of this treatment on the results.

Statistical considerations (papers I-IV)

Since we performed these studies for the first time, and many of them were based on novel methods, it is hard, if not impossible, to formally calculate the statistical power in our studies. Power can be calculated based on the measured means and standard deviations. However, this probably does not equal those of the whole population from where the subjects were sampled. With these issues in mind, we can still carry a discussion regarding statistical power.

In paper I, a larger sample size would undoubtedly secure the findings. Several interpretations were made based on lack of statistically significant changes and this is hazardous when it is suspected that the study has low statistical power. The same issue was evident in paper III. Paper III was based on sample sizes ranging from 10-20 patients in each group and we strongly believe that the sample sizes should be increased to draw certain conclusions. The preeclamptic women in paper III are however a small subpopulation (severe early-onset) of all preeclamptic women which reduces the total number of patients that can be recruited. Importantly, in contrast to previous studies analyzing Tregs in preeclamptic women, our material is very homogenous with regard to onset and severity of disease.

Papers II-IV were based on a large amount of flow cytometric data on subpopulations of cells. Hence, we chose to use a different level of significance (99%) than traditionally used (95%). This method was found to be the most suitable way of correcting for the number of comparisons. However, this method obviously increased the risk of type II errors but we solved this by considering $p \leq 0.05$ - $p \leq 0.07$ as tendencies. Further, throughout papers II-IV, we did not put statistical or biological weight to isolated low p-values that were not supported by other findings in the same direction.

Summary and conclusions

The feto-placental unit is somewhat of a parasite or tumor. Invading the uterine mucosa, the villi and trophoblasts anchor the placenta to the uterine wall and remodulate and connect to the local blood supply to support the fetus. These processes need to be highly regulated and this is achieved, at least in part, by the maternal immune system. If incorrectly so, this could cause pregnancy complications such as preeclampsia. Fetal alloantigens and maternal immune factors are crucial in determining immune balance. In this thesis, we found that the frequency of CD4^{dim}CD25^{high}Foxp3⁺ Tregs was higher at the fetal-maternal interface than in autologous blood. Decidual Tregs could indeed function as breaks on harmful immune reactions and thereby also facilitate trophoblast invasion. We also found an abundant number of CCR6⁻ T_H1 cells, presumably secreting moderate levels of IFN- γ , indicating, in line with previous ideas, that IFN- γ is not simply harmful but also necessary for early pregnancy. Decidual Tregs showed a pronounced and homogenous Treg phenotype. Given the well documented immune suppressive effects of Tregs, these cells could be central players in keeping local fetal tolerance, as suggested in numerous murine studies. Many previous studies have shown that Tregs are promoted by factors present at the fetal-maternal interface, including alloantigens, TGF- β and hormones, providing an explanation to the findings of local proliferation of Tregs. Further, although no direct evidence of this was found in this thesis, recruitment of Tregs from the circulation could be an alternative mechanism of local enrichment. A model for Treg enrichment at the fetal-maternal interface is presented in fig 31.

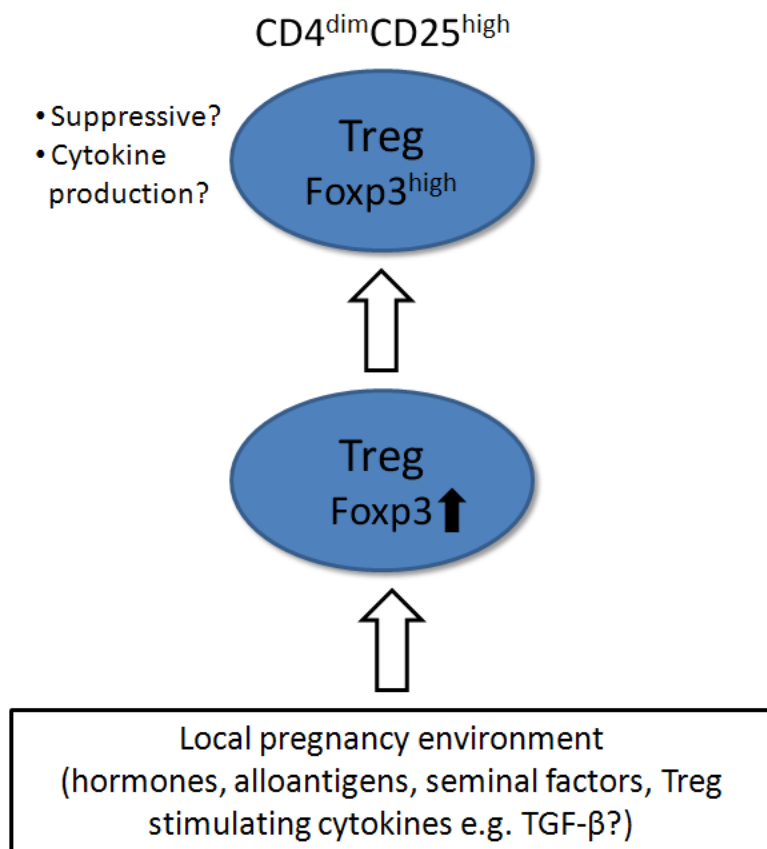


Figure 31

Explanation model for the findings on local, decidual Tregs in this thesis. Local Tregs could be promoted by hormones (e.g. hCG), paternal/fetal alloantigens, factors present in semen (e.g. TGF- β) and factors present at the fetal-maternal interface e.g. TGF- β secreted by trophoblasts and uNK cells. This would enrich Tregs with a very typical and homogenous phenotype. The suppressive function and cytokine secretion capability of these cells remains to be established.

Indeed, circulating $CD4^{dim}CD25^{high}$ Tregs were numerically reduced already in first trimester pregnancy as compared with non-pregnant women.

Although not confirmed in paper III, Tregs from second trimester healthy pregnant women showed reduced expression of Foxp3 protein. We could show that this was controlled by estradiol and in particular progesterone, which, alike

the pregnancy situation, reduced the number of cells expressing Foxp3 protein. Although not evaluated in this thesis, another plausible candidate is TNF which suppresses Tregs and is enhanced systemically during pregnancy. The circulating pregnancy Tregs were more prone to produce cytokines like IL-4, IL-10 and IL-17. This links the circulating pregnancy Tregs to the recently described Tregs with a challenged Foxp3 stability showing plasticity towards the T_H2 and T_H17 lineages. Importantly, although numerically challenged and deficient of Foxp3 protein, the pregnancy Tregs maintained their suppressive capacity. Further, we found indications of that they are indeed paternal-specific since they conveyed a stricter regulation of T_H2 immune responses in the presence of paternal than unrelated alloantigens. A model for the generation of circulating Tregs in pregnancy is given in fig 32.

In the blood of pregnant women, an expanded population of mainly Foxp3⁻ non-suppressive CD4^{high}CD25^{high} cells was present. These cells secreted considerable amounts of all cytokines investigated (IL-2, IL-4, IL-10, IL-17, TNF and IFN- γ). These cells could originate from exFoxp3 cells, as suggested in murine studies, or from activation-induced non-Tregs acquiring a certain degree of Foxp3 expression unlinked from suppressive function, as described by others. Both these processes could be due to presence of alloantigens and/or to the increased innate immune activity, with enhanced TNF levels, that seems to be a part of healthy pregnancy. Further, in the case of exFoxp3 cells, these could be induced by hormones. A model for the generation of circulating CD4^{high}CD25^{high} cells in healthy pregnancy is given in fig 32. In summary, we hypothesize that in healthy pregnancy, circulating CD4^{dim}CD25^{high} Tregs and CD4^{high}CD25^{high} non-Tregs convey a limited degree of immune suppression while simultaneously enabling maintained immune integrity in case of infections.

In patients with severe early-onset preeclampsia similar frequencies of circulating CD4^{dim}CD25^{high} Tregs and CD4^{high}CD25^{high} non-Tregs were found. Although needing to be confirmed in a larger number of non-corticosteroid-treated patients, this indicates that Tregs are not involved in the pathogenesis of severe early-onset preeclampsia. This does not however rule out functional Treg alterations, which we found indications of, or reduction of Tregs at the fetal-maternal interface in preeclamptic women.

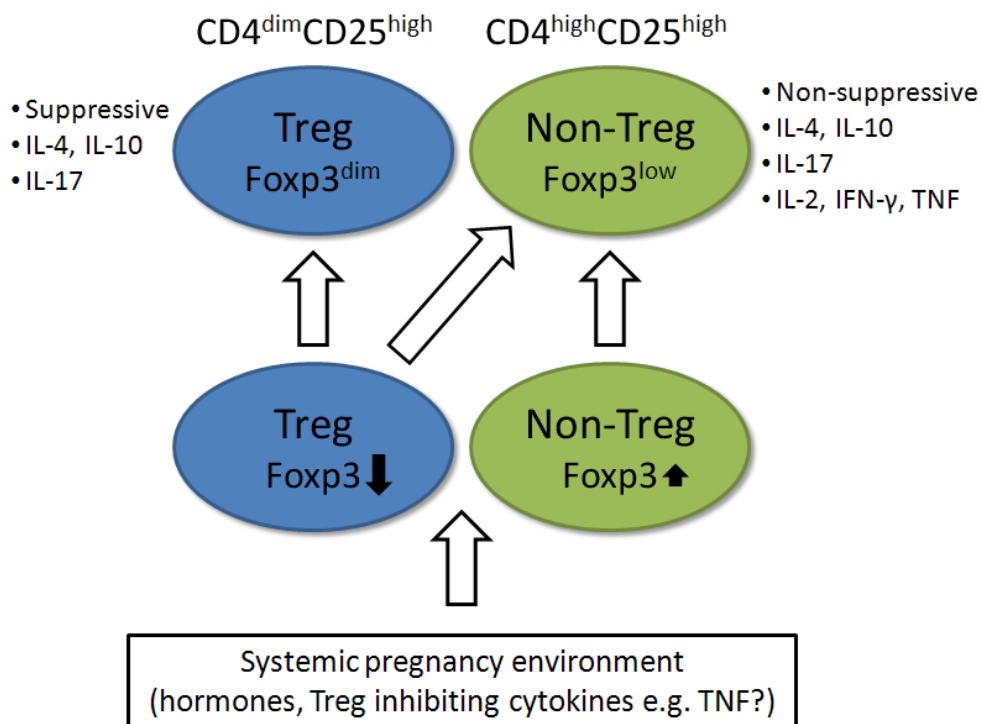


Figure 32

Explanation model for the findings on systemic Tregs in this thesis. Systemic Tregs could be influenced by the systemic pregnancy environment with enhanced levels of hormones (e.g. progesterone) and cytokines like TNF. This could reduce and destabilize the Foxp3 expression, causing secretion of cytokines IL-4, IL-10 and IL-17 while, importantly, maintaining suppressive function. In pregnant women, there was an expanded population of $CD4^{high}CD25^{high}$ cells present, that could originate from exFoxp3 cells, having lost Foxp3 expression due to e.g. hormones. They could also be non-Tregs, having gained some Foxp3 expression due to the presence of e.g. alloantigens.

This thesis adds to and extends the view of Tregs as key players in immune regulation during pregnancy. In decidua, typical Tregs seemed to have a great role in immune suppression whereas systemically, Tregs were under hormonal control and were numerically suppressed during pregnancy. Further, circulating pregnancy Tregs showed reduced expression of Foxp3 and an increased degree of cytokine secretion and thereby also possibly plasticity. This would ensure systemic defense against infections with simultaneous tolerance at the fetal-maternal interface. These results indicate that Tregs are differently regulated systemically and in decidua, findings that have implications when considering Tregs as targets for treating pregnancy complications and infertility.

Future perspectives

The issue of paternal/fetus-specific Tregs is very important. Although we found indications of paternal-specific Tregs, this deserves further examination. An idea would be to use irradiated paternal PBMC, possibly retaining their allogenic structures better than paraformaldehyde fixed PBMC. Since irradiated PBMC keep their cytokine-secreting capacity, maternal cells (with and without Tregs) could be labeled with CFSE, stimulated with irradiated paternal PBMC, sorted and then analyzed for intracellular cytokine production and proliferation. Using this strategy, the impact of Tregs on immune deviation of the maternal immune system upon encountering paternal alloantigens could be assessed.

Since we observed reduced Foxp3 protein levels in CD4^{dim}CD25^{high} Tregs from pregnant women it would be extremely interesting to analyze methylation status of the Foxp3 gene in these women. Demethylations of certain Foxp3 gene locuses are associated with stable Foxp3 expression and suppressive function. Hence, this would reveal if the Foxp3 expression in pregnancy Tregs are indeed destabilized and if so, factors regulating this (e.g. hormones, innate immune factors etc.) would have to be revealed, as would the importance of this in pregnancy complications. Also, the enriched population of non-suppressive CD4^{high}CD25^{high} cells found in pregnant (both healthy and preeclamptic) women deserves to be characterized in more detail.

We failed to detect alterations in the frequencies of circulating Tregs in preeclamptic women, which could be due to statistical power and corticosteroid treatment issues. Also, it would be very interesting to investigate Tregs at the

fetal-maternal interface where the initiation of preeclampsia likely occurs. As we detected differences pointing towards functional alterations in circulating Tregs isolated from preeclamptic women, the suppressive function and migratory properties of these cells would also be intriguing to evaluate.

Since there was an inverse relationship between circulating and decidual Treg frequencies, it is tempting to speculate that Tregs migrate from the circulation to decidua during pregnancy. If so, factors regulating the migration, as well as its timing, are undoubtedly interesting to assess, in both healthy and diseased pregnancies. An approach would be to study *in vitro* migration of peripheral Tregs, in response to decidual cells e.g. trophoblasts, macrophages, NK cells, in transwell systems. Further, since they seem to be differentially regulated, decidual and circulating Tregs should be functionally compared. This could be done in an antigen-specific setting, using paternal or umbilical cord blood cells as stimulation.

It has previously been reported that alternatively activated macrophages, sharing phenotypical similarities with decidual macrophages, induce Tregs. Hence, the (bidirectional) interaction between decidual Tregs and macrophages, as well as uterine NK cells and trophoblasts, could provide clues to how these cells acquire their specialized phenotypes and functions.

It has for long been an, albeit poorly investigated, idea that IFN- γ is essential for placental implantation and the early phases of pregnancy (Lin et al. 1993; Kwak-Kim et al. 2009). However, since IFN- γ is also associated with pregnancy loss (Kwak-Kim et al. 2009), the access of IFN- γ would have to be tightly regulated. The numerous CCR6-T_H1 cells found in the decidua are intriguing since they may contribute with just the right amount of IFN- γ . An idea could be to sort these cells, utilizing surface chemokine receptors, and then study cytokine production (e.g. IL-10) and effects on trophoblast migration.

At the fetal-maternal interface, we found high levels of RORC mRNA that can possibly be attributed to NK cells. In gut mucosa, NK cells expressing RORC seem involved in chronic inflammation and this deserves to be examined in the context of pregnancy.

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