ESSAY | MASTER BIOMEDICAL SCIENCES | UNIVERSITY OF GRONINGEN

The role of regulatory T-cells (Tregs) in pregnancy, pre-eclampsia and beyond

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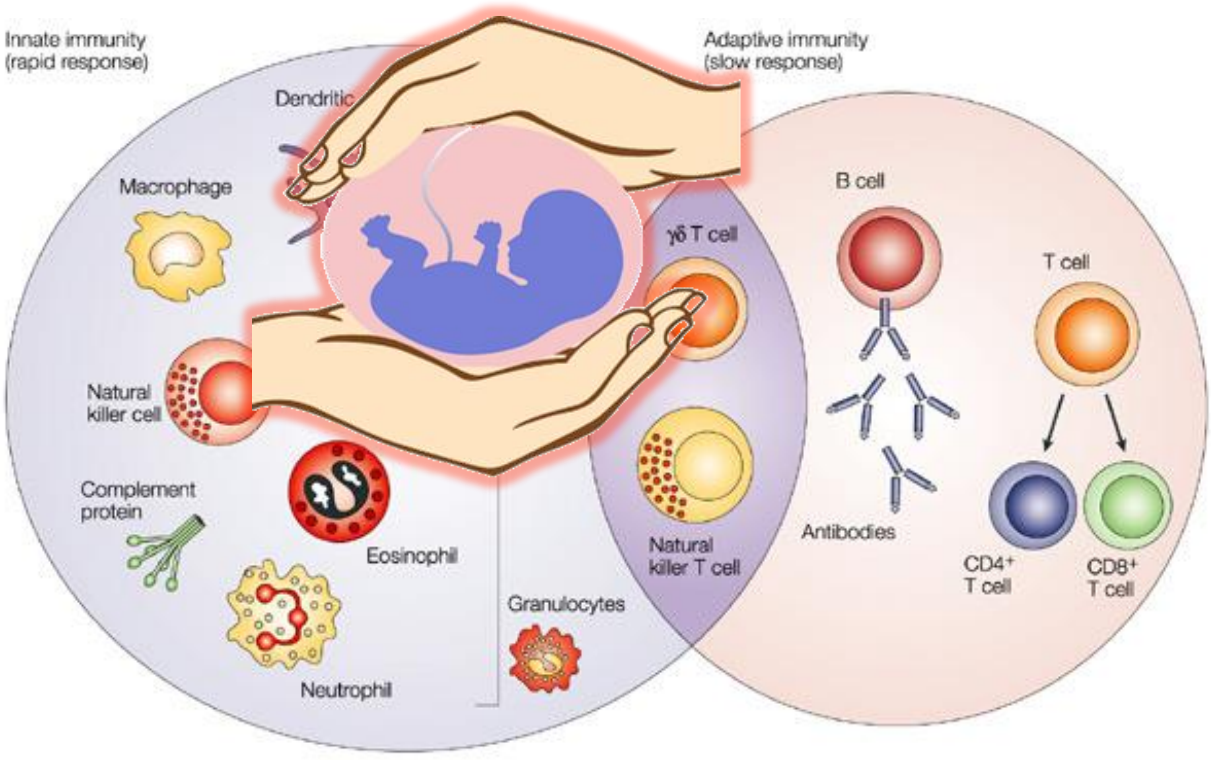

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Abstract

This review is about regulatory T-cells (Tregs) during pregnancy, pregnancy complications and their application against transplant rejection. Tregs can be identified based on the expression of CD4, CD25 and the FOXP3 transcription factor. These cells can be formed within the thymus or within the periphery from naïve T-cells. Tregs use a diverse set of mechanisms to cause immunosuppression and maternal fetal-tolerance. For example inhibition of other T-cell subsets (Th17 and Th1) and the production of anti-inflammatory cytokines (IL-10, TGF-β and IL-35). For a successful pregnancy an increase in Tregs is essential. No increase in Tregs or impaired increase will lead to miscarriage or severe pregnancy complications such as preeclampsia (PE), because Tregs play an important role in creating a tolerant state against the semi-allogenic fetus. Maintenance of maternal fetal tolerance is probably regulated by PDL1 signalling. Many animal and human studies are performed about Tregs in healthy pregnancies and in PE. Sometimes results are controversial. However, most studies show a local/peripheral increase in Tregs during healthy pregnancy and a local/peripheral decrease in Tregs during PE pathology. Mechanisms how a lack of Tregs contributes to PE pathology include impaired trophoblast invasion/incomplete spiral artery remodelling, failure of maternal fetal tolerance and an imbalance in anti- and pro- inflammatory cytokines resulting in inflammation. Chronic inflammation in PE causes endothelial dysfunction contributing to the main symptom of PE: high blood pressure. At the moment there is still no treatment for PE, besides delivery. Therefore, it should be useful to develop Treg based immunotherapy for PE in the future to restore immune homeostasis. In a rat model of PE (RUPP) expansion of Tregs during pregnancy is able to reduce blood pressure. Moreover clinical trials are running at the moment to examine the efficacy of Treg immunotherapy against transplant rejection.

Table of contents

1 Introduction

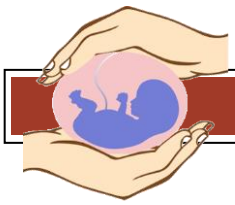
Pregnancy is a complex biological phenomenon. In order to achieve a successful pregnancy the correct development of the placenta and the occurrence of immunological changes during pregnancy are essential. The placenta is required for gas and nutrient exchange. Without a correct functional placenta, the fetus dies (1). The immunological changes are essential in pregnancy to create fetal tolerance. Without fetal tolerance, the fetus will be rejected. A fetus is genetically different from the mother, because half of the genotype is from paternal origin. This feature is called semi-allogenic. The same is true for the placenta. The maternal blood and fetal tissue lay close to each other which forms a challenge for the immune system to tolerate the foreign antigens present on the fetus (2). It is fascinating that a semi-allogenic fetus is not rejected by the immune system, while a transplanted organ is rejected immediately without administration of immune-suppressive drugs.

Organ transplant recipients have to deal with the adverse effects of strong immunosuppressive drugs including increased infection rate and higher incidence of cancer. These are serious adverse effects and even with these drugs immune rejection can occur in a minority or the patients. Take for example kidney transplants, approximately 10% of the transplanted kidneys undergo rejection within three year after transplantation (3). What can we learn from pregnancy immunology for the problem of transplant rejection? A pregnant woman does not have to take daily medications to suppress the immune system to keep the fetus alive, this all occurs naturally.

This can be explained by the temporary local and systemic immunological changes which occur during pregnancy. More than 50 years ago, Medawar was the first researcher who hypothesized that there must exist a suppressive immune regulatory mechanism of the mother to tolerate the semi-allogenic fetus (4). Nowadays, it is known that fetal tolerance during pregnancy involves many immunological changes which can be local at the decidua or peripheral changes of the innate or the adaptive immune system. In general, the innate immune system shows increased activity during pregnancy. In contrast, the adaptive immune system is inhibited during pregnancy and more shifted to a regulatory and immune suppressive state (5) (6).

The importance of immunological changes during pregnancy is even more strengthened by the fact that pregnancy complications are often associated with failure of the immune adaptations during pregnancy (7) (8). This is also the case in women suffering from a severe pregnancy disorder: pre-eclampsia (PE). 5-10% of the pregnant woman suffer from PE. PE is a life-threatening condition for mother and baby which mostly occurs ≥ 20 weeks of gestation. Every year, 50.000 woman die from PE worldwide (9). Currently, the only efficient cure is delivery of the placenta. PE is characterized by proteinuria and new onset hypertension. It is a progressive disease which finally can lead to eclampsia with complications such as: convulsions, liver damage, kidney damage and stroke.

The last couple of years research in pregnancy immunology states the importance of regulatory t-cells (Tregs) in maintenance of maternal fetal-tolerance. This essay will mainly focus on the role that Tregs play in achieving pregnancy success and it will be described how deficiency of Tregs contribute to PE pathology. Furthermore, applications of the knowledge derived from



studies of immunology in pregnancy will be discussed. Examples of such applications can be immune modulatory therapy to prevent PE or transplant rejection. To understand how immunological changes during pregnancy influence PE and how it can be applicable in transplantation research, it is first important to understand the physiology of normal pregnancy and the development and function of Tregs.

2 The placenta

Important physiological phases during pregnancy are implantation and placentation. Approximately 5 days after fertilization the blastocyst is embedded within the endometrium of the uterus. The blastocyst consists of an inner and outer cell mass. During gestation the inner cell mass (embryoblast) will develop into the fetus and the amnion, while the outer cell mass (trophoblast) gives rise to the placenta and chorion. Different types of trophoblasts can be distinguished. Mononuclear cytotrophoblasts have stem cell properties and can differentiate into multinuclear syncytiotrophoblasts or extrovillus trophoblast cells (EVT). Mature syncytiotrophoblasts are essential for hormone production. EVT cells are involved in anchoring the placenta to the uterus and essential for regulation of placental blood supply (1).

Contact of trophoblasts with the endometrium initiates trophoblast invasion into the endometrium. The endometrium consists of endometrium stromal cells. Due to trophoblast invasion these cells undergo a decidualization process and change into decidual stromal cells(DSC) which contain many nutrients. The syncytiotrophoblasts expand and lacunae develop.Lacunae fuse with maternal blood vessels to form sinusoids: places filled with maternal blood. The cytotrophoblasts develop into the chorionic villi which invade into the syncytiotrophoblasts. The chorionic villi are connected with the circulatory system of the fetus which is in contact with the placenta via the umbilical cord (10).The human placenta is a haemochorial placenta. Meaning that there is direct contact between fetalsyncytiotrophoblasts and the maternal blood. This location is called the fetal maternal interface. Various immune cells within the maternal blood lay in direct connection with fetal tissue at the fetal-maternal interphase. For example macrophages, NK-cells and T-cells (figure 1) (11).

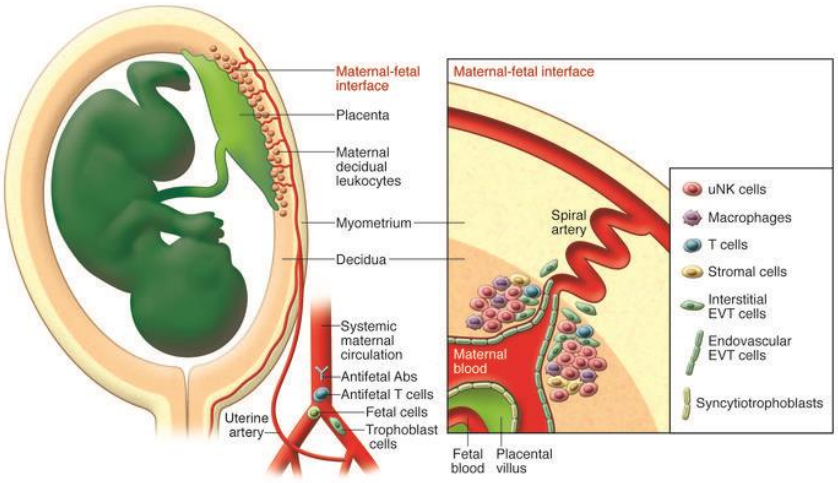
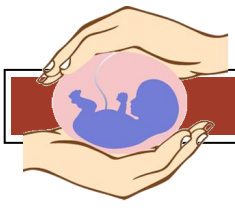


Figure 1: Immunology at the fetal maternal interface. Immune cells including macrophages, T-cells and NK-cells are located in the decidua and are derived from the maternal blood. Direct contact is established between the maternal blood and the chorion. So fetal trophoblast cells are in direct contact with various cells of the maternal immune system (11).



Blood supply towards the placenta is provided by spiral arteries located into the decidua, the maternal part of the placenta. The spiral arteries undergo an extensive remodelling process. Spiral artery remodelling is initiated by EVT cell invasion from the placenta into the uterus (endometrium and myometrium). The trophoblast cells replace the normal vessel wall with endovascular trophoblasts (ENVT). The goal of spiral artery remodelling is to obtain increased blood flow to the placenta by vasodilation which promotes growth of the foetus. Moreover the villous tree structures of the fetal part of the placenta optimize diffusion efficiency of gas and nutrients between maternal and fetal blood. During spiral artery remodelling the trophoblasts change in endothelial like cells which replace the muscle layer of the vessel wall (12). Besides the exchange of nutrients, oxygen and waste products are exchanged between mother and fetus via the placenta. Moreover an important function of the placenta is the production of progesterone to maintain pregnancy. Furthermore, the placenta is considered as the main organ which regulates the changes in the immune system during pregnancy due to the secretion of hormones, cytokines, micro-particles and other placental specific factors (13).

3 Development and function of regulatory T cells (Tregs)

3.1 T-cell subsets

Our immune system is equipped with a diverse subset of CD4+ T-lymphocytes, also called T helper (Th) cells. Four major classes of Th cells can be distinguished: Th1, Th17, Th2 and regulatory T cells (Tregs). More recent identified T-cell subsets include Th25, Th22 and Th9 cells. All Th cells are functionally dependent on other cell types. Immature naïve T cells are activated by recognizing aforeign antigen with their CD3 or T-cell receptor (TCR). This antigen is presented with an MHC-II molecule present on antigen presenting cells (APC) such as dendritic cells. For Th activation a secondary signal is required wherein CD28 expressed on the Th cell and CD80 expressed on the APC bind with each other. Th cell activation is followed by differentiation and proliferation of the T-cell into an antigen specific memory or effector T cell of a particular subtype. The type of Th cell which arises depends on the antigen and on the presence of cytokines in the microenvironment (14).

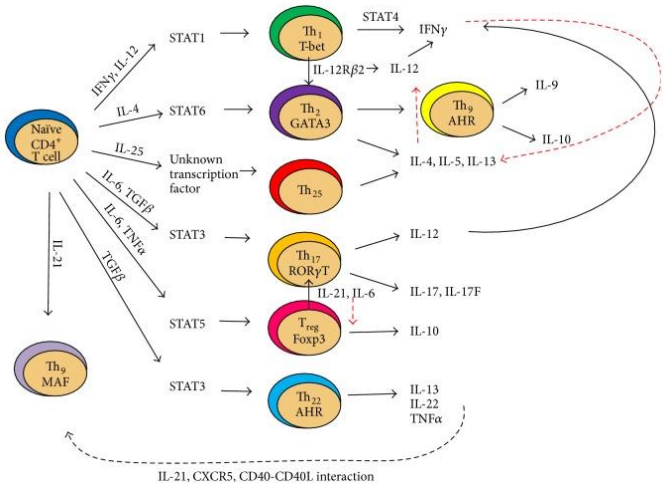
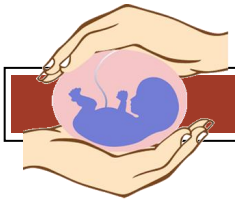


Figure 2:Development of a naïve T cell in different T cell subsets Th1, Th17, Th2, Tregs, Th25, Th22 and Th9. Different effector cells produce different effector cytokines. Tregs are immunosuppressive while the other effector cells induce immunity (14).

Each Th effector subtype has its own specific function, transcription factors and secretion of effector cytokines (Figure 2) (14). Th1, Th17, Th2,Th25, Th22 and Th9 cells are all involved in immunity against invading pathogens. Th1 is directed against intracellular pathogens, Th2 is involved in defence against extracellular parasites and Th17 is mainly active against infections with extracellular bacteria and fungi. Th25 is active against extracellular pathogens. Th22 is



mainly recruited to the skin and mucosal tissue for microbial defence (15). Th9 is involves in defence against infections with parasitic worms (nematodes) (16). In contrast, Tregs have the opposite function compared to Th1, Th2, Th17 and Th25 cells. Tregs are known to have immunosuppressive capacities via inhibition of other t-cell subsets. They prevent the occurrence of an excessive inflammatory response and also play a role in the regulation of immunological tolerance and the prevention of autoimmune diseases. So, Tregs are important regulatory cells of the immune system providing immune homeostasis. However, they only compromise a small subset of cells: 5-15% of the T-cell subsets are Tregs in mice and humans (2).

3.2 Treg identification based on phenotypic markers

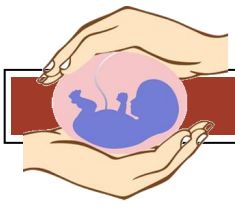
The immunosuppressive functions of Tregs were already recognized a long time ago in 1970 by Gerson and Kondo (17). However, at that time there was a lack of techniques and knowledge about how to separate Tregs from other T-cell subsets which delayed further research for a period of more than 20 years. Currently, it is known which surface markers are expressed on Tregsand can be used for its identification via fluorescent activated cell sorting (FACS).In 1995 it was discovered that CD25 correlated with immunosuppressive activity of T cells (18). CD25 is the α-chain of the interleukin-2 (IL-2) receptor. CD25 is expressed on Tregs although, this marker is also present on other activated CD4+ T-cell subsets. Another marker widely used for Treg identification is cytotoxic T lymphocyte antigen 4 (CTLA4). CTLA4 is a co-inhibitory receptor expressed on the surface and in the cytoplasm of Tregs. However, CTLA4 is just as CD25 not specific for Tregs. Furthermore, in humans CD127 is expressed on the surface of recent activated Tcells which can be used for distinguishing Tregs from those cells wherein Tregs have low CD127 expression (19).

More recently, in 2003 the transcription factor forkhead box P3 (Foxp3) was identified as a master regulator of Treg development and function (20). Expression of Foxp3 is specific for Treg cells and therefore, very useful as an intracellular marker in distinguishing Tregs from other T-cell subsets. Moreover, Foxp3 is essential for Treg cell functionality determined in mice and humans lacking functional Tregs due to mutations in Foxp3. Scurfy mice have no functional Tregs and develop severe autoimmune disease. Humans without functional Tregs also develop a severe autoimmune disease called immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). In both scurfy mice and IPEX patients there is a failure in immune tolerance and development of autoantibodies against keratin in the skin (21).

FOXP3 specificity for Tregs is also shown on the epigenetic level. A region in the Foxp3 gene had specific demethylated CpG islands, known as conserved non-coding sequence 2 (CNS2) or Treg specific demethylated region (22). The methylation status of CNS2 is associated with Foxp3 expression and stability (23). This demethylated region is specific for Tregs and not present in other activated T-cell subsets. Making FOXP3 or epigenetic demethylation of CNS2 specific marks for Treg identification. Taken together, a combination of phenotypic markers is required to identify Tregs. Most studies use the CD4+ CD25+ phenotypic characteristics for Treg identification with or without the combination of the FOXP3+ intracellular marker.

3.3 Development of Tregs

The development of Treg cells occurs either within the thymus (tTregs), also referred as natural Tregs and within the periphery (pTregs) or called induced Tregs. Within the thymus, thymocytes are a source of effector T-cells. In order to produce functional effector T-cells from thymocytes,



the cells undergo positive and negative selection procedures. Various APC in the thymus present self-antigens. Normally, T-cells within the thymus which recognize self-antigens are removed via apoptosis in a process called negative selection. However, thymocytes which develop in tTregswill escape negative selection. Therefore, the reactivity of the TCR with self-antigens and its avidity plays a major role in the development of tTregs (24).

It is thought that moderate levels of TCR self-reactivity induces the expression of the NF-kB component c-Rel which makes FOXP3 transcription possible. Additionally, signals within the microenvironment including IL-2 and IL-15 promote the expression of FOXP3 which results in CD4+ FOXP3+ tTregs. In contrast, low TCR self-reactivity results in the formation of CD4+ FOXP3- naïve T-cells which migrate to the periphery. The mature tTregs also migrate to the periphery (24). In the periphery the earlier generated tTregs are maintained and can be expanded (25). Furthermore, new pTregs can be formed from naïve T cells as described in 3.1.

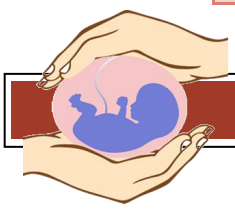
pTregs are antigen specific. Antigens are distinguished in foreign and self-antigens. pTregs are able to tolerate foreign antigens. Peripheral immune tolerance means that there is no immune response initiated against the antigens which are tolerated. Both types of Tregs inhibit the formation of autoreactive T-cells. It is like an unresponsive state of the immune system. During the formation of pTregs the microenvironment plays the main regulatory role. TGF-β is mentioned as the main factor inducing pTreg formation. In vitro experiments showed that TGF-β induces the expression of FOXP3 which is essential for differentiation into Treg cells (26).

Natural and induced Tregs show similarities and differences. The main similarity is their immunosuppressive and immune tolerant function. The main difference is their origin. Moreover, TCR of natural Tregs are directed towards thymus self-antigens, while induced Tregs can also display TCR specificity towards foreign antigens (non-self) (table 1). These differences indicate that both forms of Tregs play different roles with different mechanisms of function. However, this is not proven yet, because distinct identification of natural and induced Tregs seems to be difficult. Studies used the expression of helios to distinguish Tregs (27) (28). However the use of this marker is controversial.

Although, it is hard to distinguish them in vivo, it is possible to induce the formation of peripheral Tregs by administration of antigens (29). Moreover, a mice model exists which completely lacks natural Tregs: the RAG-deficient 1B3 mice. These mice are born without FOXP3+ cells. FOXP3+ Tregs emerge approximately at an age of 3 weeks in the periphery.

Table 1: Differences between natural and induced Tregs

	Natural Tregs	Induced Tregs
Origin	Thymus	Periphery, lymph nodes, spleen
Specificity for	Self-antigens	Self –antigens or foreign antigens
Marker expression (in mice)	CD4, CD25, FOXP3 High Neuropilin-1 Helios CD73 PD-1	CD4, CD25, FOXP3 Lack of Neuropilin-1



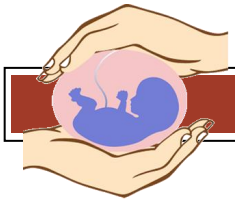
Researchers used this model for transcriptome analysis. They compared peripheral Tregs derived from RAG-deficient 1B3 mice with Tregs derived from wild-type IB3 mice. They found many differences, including 700 differentially expressed transcripts between the Tregs from the two types of mice. Helios, CD73 and PD-1 (programmed cell death-1) transcripts were significantly higher expressed in blood from wild type mice (natural Tregs + induced Tregs). Unfortunately, these markers are also expressed in induced Tregs and therefore not specific enough to distinguish natural from induced. An important finding was the transcript neuropilin-1 (Nrp-1). Nrp-1 is a selective marker for natural Tregs (table 1). RAG-deficient 1B3 mice lack Nrp-1 expression. This marker is not applicable to use in human studies because human lymphoid derived Tregs also express Nrp1. Functional tests with mouse Nrp-1 low and Nrp-1 high expressing cells shows that peripheral (Nrp-1 low) Tregs were not able to prevent autoimmune disease, while they were better in immunosuppression via inhibiting other T-cell subsets. Natural (Nrp-1 high) Tregs were good at preventing autoimmunity (30).

3.4 Mechanisms of Treg induced immunosuppression

The immunosuppressive and regulatory functions of Tregs to maintain immune homeostasis is mediated by a diverse range of molecular mechanisms which are still not exactly understood. The main components involved in immunosuppression are based on cell-cell contact and cytokine production and reach a broad set of other immunological cell types. The activity of many cell populations can be inhibited by Treg including APC (DC, macrophages, B-cells), CD4+ T cells, CD8+ cytotoxic T cells and natural killer (NK) cells. Key players in this process are the secretion of anti-inflammatory cytokines by Tregs and their expression of CTLA-4. Furthermore, the FOXP3 transcription factor upregulates the expression of CD25 and CTLA-4 towards a constitutive level which is thought to maintain immunosuppressive functions of Tregs (figure 3) (31).

CTLA-4 mediates immunosuppression by decreasing the expression of CD80/CD86 on antigen presenting cells and the inhibition of CD80/CD86 co-stimulatory signals on other T-cell subsets. CTLA-4 can eliminate CD80/CD86 via trans-endocytosis (32). Furthermore, CTLA-4 increases the expression of indoleamine 2,3-dioxygenase (IDO) on DC. IDO is an enzyme for tryptophan degradation. Lack of tryptophan results in starvation of other T-cell subsets and therefore, impairs its functionality. For Tregs itself IDO expression is beneficial and leads to expansion of the levels of Tregs (33).

Tregs produce the following cytokines: IL-10, TGF-β and IL-35. These cytokines have an effect on other immune cells mediating immunosuppression. The importance of these cytokines for Treg functionality are shown with in vivo models. Mice lacking TGF-β develop autoimmune disease, which suggest an important role for TGF-β in auto-immunity (34). Mice deficient in IL-10 do not develop autoimmune disease but are more prone to the development of spontaneous colitis (31). An inflammatory environment within the intestine is established prior to the development of colitis in these mice. Lacking IL-10 disturbs the regulated immune response towards gut microbiota. The immune system provokes an increased immune response to the enteric bacteria and commensal bacteria with the proliferation of more Th17/Th1 cells and the production of IL-12 and IL-23 cytokines. The population of CD4+ CD25+ FOXP3+ Treg cells were declined in IL-10 -/- mice. Therefore intestinal homeostasis is disturbed and the animal is sensitive to colitis.



Furthermore, IL-10 -/- mice are hyper responsive to bacterial infections (35). Mice deficient in IL-35 showed decreased capacities of immunosuppression (36).

Besides CTLA-4 and anti-inflammatory cytokines Tregs have many other immunosuppressive mechanisms. Tregs can for example directly kill effector T-cells and other target cells by perforins and granzymes. Treg cells can produce adenosine which is an immunosuppressive molecule. Adenosine is produced by Tregs which express CD39 and CD73. Those molecules facilitate the hydrolysis of ATP/ADP to adenosine. Adenosine mediates immune suppression by the increased production of cAMP. cAMP inhibits the activity of DC and other T cells. Furthermore, Tregs also indirectly affect the balance between T-cell subsets by downregulation of the transcription factors T-bet, IRF-4 and RORyt present on Th1, Th2 and Th17 cells respectively. The diverse mechanisms of immunosuppression act together to obtain immune homeostasis (31).

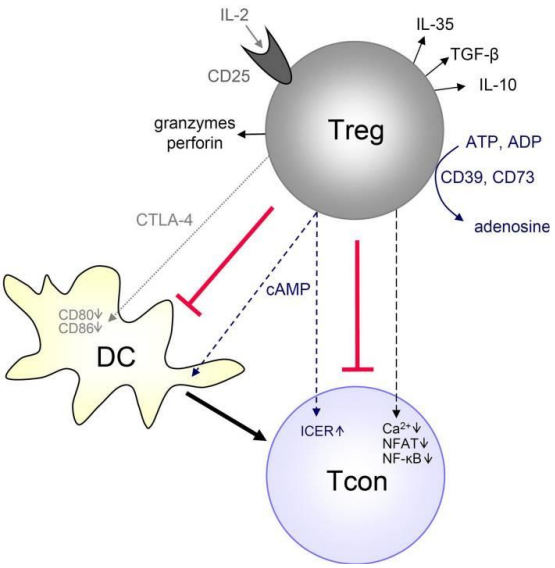


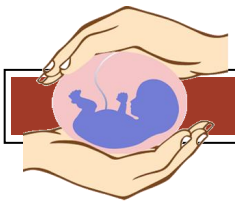
Figure 3: Summary of Treg induced immunosuppression affecting conventional T cells (Tcon). Tregs inhibit Tcon by direct killing via granzymes/perforins, immunosuppression via cytokine production IL-35, TGF-β and IL-10, the production of adenosine and cAMP. Tcons are indirectly inhibited by decreased activity of DC mediated by downregulation of CD80/CD86 via CTLA-4 (31).

4 The role of Tregsin maternal fetal tolerance during normal pregnancy

Pregnancy is a major challenge for the immune system to maintain immune homeostasis in regard to accept the fetus. Nowadays, most researchers do not consider pregnancy as a transient state of immunosuppression alone. They consider pregnancy as a transient immunosuppressive state with fetal-maternal symbiosis (two organisms living together). Therefore, immune adaptations during pregnancy protect the fetus from attack by the maternal immune system together with protecting the fetus from pathogenic infectious disease (37).

4.1 Imbalance of T-cell subsets during pregnancy

During pregnancy there is a shift in the balance between different T-cell subsets.Th17 cells decrease during pregnancy and there is a shift towards more production of Th2 associated cytokines compared to Th1 pro-inflammatory cytokines (38). For a long time it was thought that this Th1/Th2 paradigm was the main reason of maternal fetal tolerance. However, a predominance of Th2 cells is important for a successful pregnancy (39). This is also shown in humans with recurrent abortion, where the Th1/Th2 cytokine ratio was increased (40). In contrast, one study in transgenic mice lacking the production of Th2 cytokines showed that Th2 predominance it is not essential to achieve a successful pregnancy. However, other cytokines were not tested in this study and Th1 cytokines might be downregulated to obtain these results (41).With this in vivo experimental setup pregnancy was still possible. Therefore, the Th1/Th2 paradigm cannot fully explain the phenomenon of tolerance. Suggesting that there must exist another regulatory mechanism of immunosuppression and tolerance induction: Tregs.



Knowledge about the role of Tregs in pregnancy is mostly derived from studies performed in rodents and humans. Tregs can play a local role at the fetal maternal interface within the decidua or act systemically within the periphery.

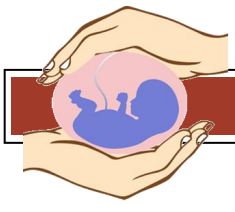
4.2 Peripheral role of Tregs

Expansion of Treg levels during normal pregnancy was first determined in mice. Treg levels were increased already early in pregnant C57BL/6 mice (day 10.5) in different tissues such as spleen, lymph nodes and within the blood compared to non-pregnant mice (42). Tregs during mice pregnancy have a specificity towards Y chromosome-encoded minor Histocompatibility antigens (H-Y). Removal of Tregs in vivo, resulted in an increased mortality of male pups compared to female pups. Indicating, the importance of Treg specificity (43). Analysis of human blood samples during pregnancy also showed increased levels of Tregs (44) (45) (46). Contradictive results are shown by a Treg study in human blood samples which showed no increase of Tregs in the periphery during pregnancy (47). This can be explained by the Treg detection method. Kahn et al. did not found a difference in Treg frequency, but they are the only study using CCR4 as marker during identification (Table 2).

Table 2: Comparison of various studies about Treg frequency during healthy pregnancy

Study	Method of Treg identification	Phase of pregnancy	Outcome Treg frequency
Kahn et al. (47)	CD4+CCR4+CD25^{hi}CD127^{lo} FOXP3+	Third trimester	Similar amount as in non-pregnant controls
Nanan et al. (44)	CD4(+)CD25(high), CD4(+)CD127(low)CD25(+), andCD4(+)Foxp3(+) cells	Third trimester	Increased to non pregnant and PE
Molvarec et al. (48)	CD4+ CD25high FoxP3+	Third trimester	Increased to non pregnant and PE
Mansour et al. (46)	CD4+CD25(bright) FOXP3+	Unknown	Increased to non pregnant and PE

All human studies and most studies in mice do not distinguish natural thymus derived Tregs from induced peripheral Tregs. Although, there is a study available in mice which distinguished the contribution of Tregs with a different origin. They analysed the contribution of thymus derived Tregs by using the Helios marker. Moreover, they transferred thymus derived Tregs from pregnant FOXP3^{GFP} mice to pregnant Raf -/- mice at day 8 of pregnancy. With these experiments they showed that Tregs originating from the thymus increased during day 2 of pregnancy, while they could not trace back the thymus derived GFP positive Tregs at day 10 of pregnancy after adoptive cell transfer. So, thymes originating Tregs are essential for implantation, while later in pregnancy induced peripheral Tregs have more contribution to pregnancy immunology (49). Blood is an easy accessible study material. Therefore, many studies about Tregs are performed by measuring factors/cells in blood. However, it is uncertain if these studies are representative for the local role of Tregs. Tregs from the periphery can migrate to the fetal maternal interface and fulfil a more important local role in the uterus/decidua (50).

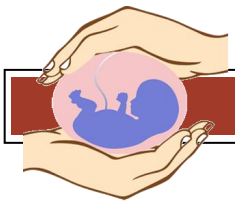


4.3 Local role of Tregs: rodent and human studies

Local levels of Tregs in the decidua are significantly increased during pregnancy in mice and humans compared to a non-pregnant state (42) (51) (52). This increase was measured during the first and second trimester of pregnancy. Ernerudh et al, determined this in first trimester human decidua (53). Most abundant levels of Tregs are found in the decidua parietalis, which lays close to the chorioamniotic membranes of the fetus (54). Moreover, comparing the levels of Tregs localized within the decidua with Tregs in the blood showed that the levels of Tregs in the decidua were much higher in pregnant woman compared to peripheral levels of Tregs (55). Furthermore, Tregs are also present in the uterus before pregnancy occurred studied in mice during the estrous cycle (56). Those tTregs are recruited from the periphery to the uterus during the estrous cycle in mice and reach a maximal level at the moment of ovulation (57). Recruitment of tTregs from the blood is facilitated by local chemokine expression (CCL3,CCL4,CCL22and CX3CL1). tTregs form a tolerogenic environment together with DC and NK cells. At this way the body is prepared for pregnancy, facilitating implantation. CCL4 expression stays high when pregnancy occurs and studies showed that CCL4 induces FOXP3 expression, creating even more Tregs (57).

After implantation foreign paternal antigens are present on fetal cells and presented by DC (58). Naïve CD4+ T-cells recognize those antigens and develop into paternal alloantigen specific pTregs. pTregs are induced rather than activated effector T-cells because the specific microenvironment present during pregnancy. Trophoblasts itself are known to produce TGF-β at constant high levels. TGF-β increases the expression of FOXP3 which directs the naïve CD4+ T-cell towards a Treg phenotype, shown with an in vitro experiment (59). Furthermore, memory Tregs are developed which prepare for a second pregnancy with a fetus expressing similar antigens (60). Evidence for this statement is derived from a study of transgenic mice expressing I-A^b 2W1$S_{55–68}$ antigen. Maternal specific I-A^b 2W1$S_{55–68}$ Tregs developed during primary pregnancy. However, during a secondary pregnancy the I-A^b 2W1$S_{55–68}$ specific memory Tregs were still present and were more rapidly reactivated, a phenomena called priming (61). So, tTregs initiate a favourable microenvironment for pregnancy and are the first regulators of tolerance. Both tTregs and pTregs are required to maintain tolerance and make vascularization possible during gestation. The levels of Tregs reach its max during mid-gestation and decrease before parturition in mice (figure 4) (62).

The importance of Tregs during pregnancy is further strengthened by the performance of Treg depletion/transfer/expansion experiments in mice which all showed pregnancy failure by Treg depletion and pregnancy success by Treg transfer or expansion. An example of one of those experiments showed that a mouse model which is prone to abortion (CBA x DBA/2J model) had decreased levels of Tregs in the decidua. Transferring Tregs from healthy pregnant mice towards pregnant abortion prone mice can rescue rejection of the fetus (63). Another study showed that abortion induced by IL-17 in pregnant CBA/J × BALB/c could also be rescued via adoptive cell transfer of Tregs (64). Suggesting that Tregs are essential for achieving a successful pregnancy. Recent data suggest that transferring Tregs into abortion prone mice results in successful pregnancy by increasing the mast cell population at the fetal-maternal interphase. Uterine mast cells seem to contribute highly to placenta development, angiogenesis and spiral artery remodelling (65).



Other studies examined Tregs in allogenic (genetically distinct) and syngeneic (genetically identical) pregnancies in mice. Aluvihare et al, showed an increase in Tregs in both allogenic and syngeneic pregnancies in all lymph nodes (42). Another study showed similar results. Both mating types resulted in Treg expansion in the lymph nodes of the uterus initiated four days after implantation of the blastocyst. However, syngeneic pregnancies resulted in an increase in Tregs of less extend than allogeneic pregnancies (66). The fact that syngeneic pregnancies still show an increase in Tregs suggests that Tregs also respond independent of paternal alloantigens presented by MHC via self-antigens. This idea was further confirmed by CD25 depletion experiments. Transferring of CD25 depleted cells towards allogeneic pregnant mice resulted in pregnancy failure. Interestingly, syngeneic mice had successful pregnancies without CD25 cell populations (42). The same result was obtained by using an anti-CD25 antibody in allogeneic or syngeneic pregnant mice (62). From these experiments it can be concluded that CD25 cells are responsible for fetal acceptance via immunosuppressive mechanisms including tolerance of self-antigens, fetal-antigens and paternal alloantigens.

Interference with cytokine levels produced by Tregs affect pregnancy success in mice. The most important cytokines during pregnancy are IL-10 and TGF-β which are both known to induce the M2-phenotype of macrophages and contribute to immunosupression. Experiments in the abortion prone mouse model were performed using anti-IL-10 or anti-TGFβ antibodies before mating. This study showed that inhibition of IL-10 before onset of pregnancy increased the rate of pregnancy failure in abortion prone mice. This was not observed by anti-TGF-β treatment (67). Conversely, enhancing Treg development via IL-2 treatment could rescue abortion in the abortion prone mice (66).

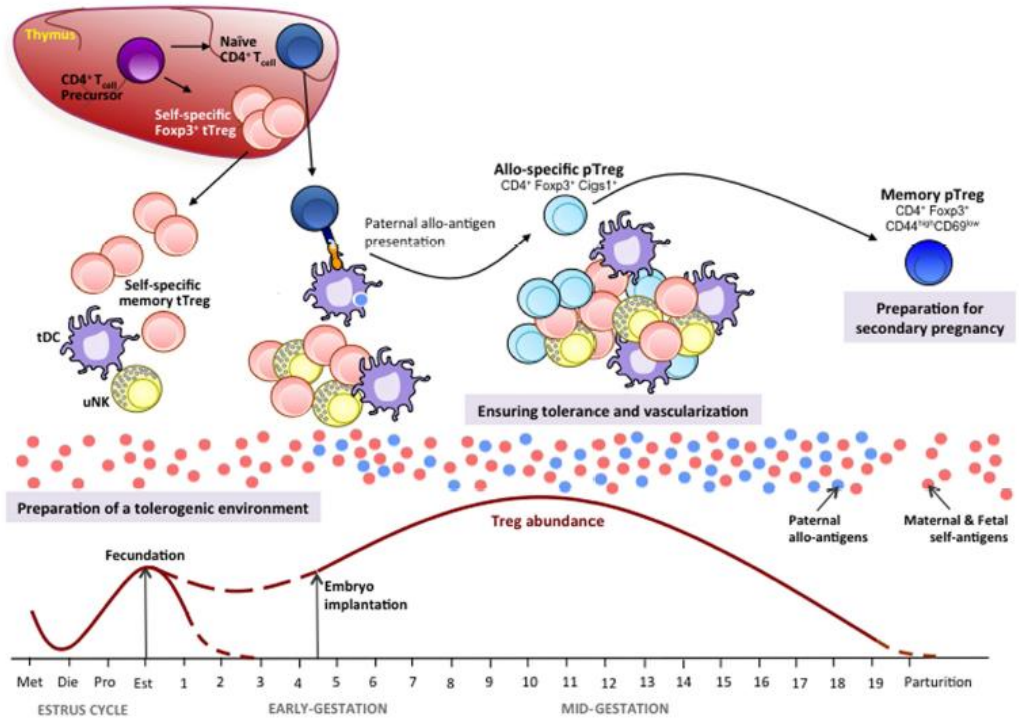
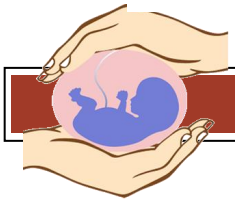


Figure 4: The accumulation of Tregs during pregnancy over time in mice. Even before pregnancy occurs thymus derived Tregs start to expand during the estrous cycle controlled by hormones. tTregs interact with DC and NK cells to make a tolerogenic environment suitable for implantation. Without a further pregnancy, levels of Tregs decrease again. If pregnancy occurs the paternal allo-antigens are presented by DC and recognized by naïve T-cells which results in the generation of allo-specific pTregs. tTregs act together with pTregs to provide tolerance and vascularization. Memory Treg cells develop and can be useful in a secondary pregnancy (62).



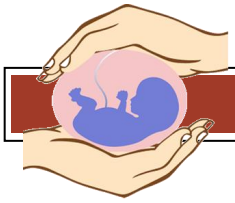
The fact that depletion of IL-10 with antibodies does affect pregnancy, while TGF-β does not is related to the phase of pregnancy investigated during the experiments in abortion prone mice. IL-10 depletion affects pregnancy outcome because this cytokine plays an important role in early pregnancies to promote the proliferation of decidual cells. TGF-β has a more important role during trophoblast invasion and Treg differentiation. Administration of antibodies against IL-10 or TGF-β after mating did not showed any negative effect on mice pregnancy (68). Although, these results are contradictive with the results found in IL-10 -/- or TGF-β -/- models. Anti-IL-10 treatment affects pregnancy while IL-10 -/- does not affect pregnancy. The same is true for TGF-β where TGF-β -/- affects pregnancy, while TGF-β antibody treatment has no adverse effects on pregnancy. Possible explanations for these contradictive findings are as following. Anti-IL-10 treatment may have an effect on abortion prone mice but not on normal wild-type mice as IL-10 knock outs. Anti-TGF-β treatment might have no effect due to inconsistent antibody-antigen binding. Taken together, IL-10 and TGF-β are important factors at early pregnancy, where eliminating these cytokines can have different effect according to the phase of pregnancy, the mouse model used during the experiment and the elimination method used (68).

Recent data suggest a new role for the Notch-1 signalling pathway in maintaining high levels of Tregs during pregnancy. First, in vitro experiments showed that vascular endothelial cells from the maternal part of the decidua were involved in the maintenance of Tregs. Vascular endothelial cells promoted FOXP3 expression and Treg differentiation. They discovered that this process was regulated via Notch signalling. Notch-1 is present on Tregs which interacts with Notch ligands present on vascular endothelial cells. This finding was confirmed by the performance of adoptive cell transfer experiments. The transfer of Notch-1 deficient Tregs to semi-allogenic pregnant mice resulted in abortion. Indicating that Notch-1 is important in Treg expansion (69).

Studies in human pregnancies showed similar findings as mice studies regarding to Treg levels during the menstrual cycle, pregnancy/abortion. Local levels of Tregs in human pregnancies are determined by researching biopsy material of the uterus/deciduas. They showed an increase in Tregs during pregnancy and accumulation of Tregs during the menstrual cycle. While abortion was associated with decreased amounts of Tregs (52). It was found that Tregs were able to suppress other CD4+ CD25 – T-cells (70). Besides, findings of local levels of Tregs further evidence of the localized action of Tregs at the fetal maternal interface was derived from a study which showed that the levels of Tregs correlated with the amount of HLA-C mismatches. The more mismatches between mother and fetal trophoblast cells, the higher frequency of local Tregs. The Tregs in the deciduas are able to recognize HLA-C. However, they tolerate the presence of fetal HLA-C (54). In mice it was shown that levels of Tregs peak during mid-gestation (71). At the human decidua the levels of Tregs peak during second trimester or third trimester of pregnancy (72). Human studies within the decidua also showed that thymus derived Tregs are particularly important in early pregnancy. Natural tTregs identified with the Helios marker were significantly decreased in decidua of miscarriages, compared to healthy pregnancies. Suggesting tTregs as important regulators of pregnancy maintenance (73).

4.4 Factors influencing Treg levels

The levels of Tregs are regulated by hormones such as estrogen, progesterone and the pregnancy hormone hCG. Estrogen drives FOXP3 expression and expansion of the Treg cell



population (74). Progesterone increases Treg functionality by stimulation of IL-10 expression in Tregs (75). hCG is involved in recruitment of Tregs to the uterus (76). Besides hormone levels, the placenta also secretes important factors including M-CSF, IL-10, TGF-b, and TRAIL which are thought to stimulate the expansion of Tregs towards a tolerogenic environment. This was determined by the use of first trimester human decidual tissue. The placenta secreted factors mentioned before are produced by trophoblasts (77). Furthermore, seminal fluid is mentioned as a regulator of Treg levels. Shima T et al. succeeded in specific isolation of paternal antigen sensitive Tregs based on the recognition of a paternal antigen (MIs Ia) by a specific TCR (Vβ6). They showed an increase in paternal specific Tregs in lymph nodes and uterus of BALB/c x DBA/2 matings. Indicating an important role for paternal specific Tregs in suppression of alloreactive lymphocytes. Immunosuppressive capacity of Tregs was proven in vitro by performing mixed lymphocyte cultures. The role of semen was examined by performing the same mating with infertile DBA/2 males. These matings showed no increase in paternal antigen specific Tregs. Suggesting that normal matings are required for the expansion of these Tregs. Seminal fluid contains TGFβ which probably induces the expansion of Tregs to prepare for pregnancy (58). Another factor present in seminal fluid is CD38 and might also be responsible for Treg expansion since it induces FOXP3 expression (78).

4.5 Critical role of PD1 – PD1L pathway in maternal-fetal tolerance during pregnancy

Recent studies suggest a critical role for PD1 signalling in maternal fetal tolerance. PD1 is a receptor expressed on the different T-cell subsets. PD1 can bind with its ligand PD-L1. Expression of PD1 ligands can be present on a diverse range of cell types including APC and is highly dependent on the microenvironment. Ligand receptor binding results in regulation of the balance between the activation of T-cell subsets and its inflammatory reactions or immune tolerance mechanisms.

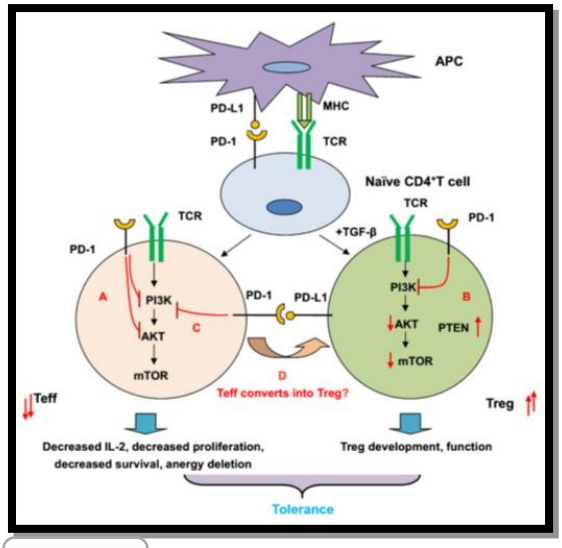


Fig. 5.1

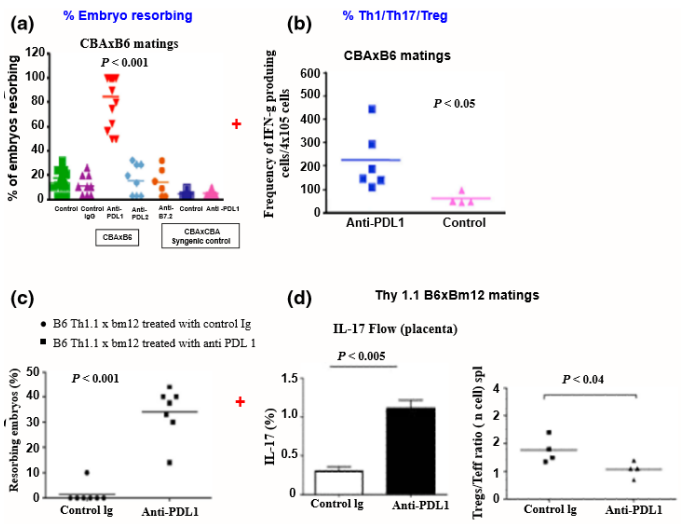
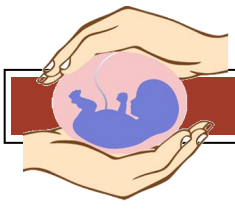


Fig. 5.2

Figure 5: PD-1 receptor ligand interactions are critical for tolerance during pregnancy. Fig. 5.1 shows the mechanism of PD-1 PD-L1 signalling in T-effector cells and Tregs for the induction of tolerance. Fig. 5.2. shows mating experiments in mice. Allogeneic pregnant mice treated with PD-L1 antibodies showed increased pregnancy failure (a/c). Probably caused by increased effector T-cells (Th1/Th17) and decreased amount of Tregs (b,d) (79).



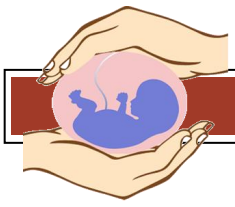
Activation of a naïve T-cell during a normal pro-inflammatory response is followed by an intracellular signalling cascade of the P13K – AKT – mTOR pathway. The interaction of PD1 with PD-L1 ligand induces peripheral tolerance via inhibitory signals towards the P13K-AKT pathway. So PD-L1 is able to directly decrease the activation of naïve T cells. If PD-L1 inhibitory signals are present in combination with TGFβ, the development of Tregs is stimulated. Moreover, Tregs can directly inhibit the functionality of effector T cells such as Th1 and Th17. It is also mentioned in literature that effector T-cells which are inhibited by Tregs can transdifferentiate into Tregs (figure 5.1) (79).

The microenvironment during pregnancy at the fetal maternal interphase probably enhances PD-L1 expression and thereby induces tolerance via Tregs. PD-L1 expression is constitutively present on Tregs and expression is induced on decidual stromal cells and trophoblast cells during pregnancy. In human pregnancies an increase of PD-L1 on decidual stromal cells was already found within the first trimester of pregnancy.

PD-L1 expression on trophoblasts was also present early during pregnancy (month four) in humans. PD-L1 expression was higher in syncytiotrophoblasts compared to cytotrophoblasts. PD-L1 depletion experiments in pregnant mice even gave more evidence for the critical role of the PD1 pathway in pregnancy. Treatment of allogeneic pregnant mice with antibodies directed against PD-L1 showed an increased rate of pregnancy failure (figure 5.2) (80) (81). During antibody treatment these mice had higher levels of IFN-y production associated with higher Th1/Th17 T-cell subsets and a decreased amount of Tregs. Taken together, enhanced PD-L1 expression is critical for a successful pregnancy and maintenance of tolerance, where depletion results in pregnancy failure via an increased inflammatory response mediated by Th1 and Th17 cells.

5 Current knowledge about pre-eclampsia pathology: the role of Tregs

In the previous chapter it is described that the increase in Tregs is required to obtain a successful pregnancy. Pregnancy failure is often associated with a decrease in Tregs. Besides abortion, pregnancy syndromes which occur later during gestation might also be associated with insufficient immunological changes during pregnancy. Where complete failure of immune adaptations in pregnancy will lead to miscarriage, partial failure of these adaptations can result in PE. The pathology of PE is still incompletely understood. The main ideas of PE pathology are that trophoblast invasion occurs insufficiently. Therefore, spiral artery remodelling is incomplete. This results in placenta ischemia. Placenta ischemia is associated with an hypoxia environment with oxidative stress. Moreover the ischemic placenta releases soluble factors also called toxins which can further disturb angiogenesis and inflammation. This is also where the more common name for PE 'toxaemia of pregnancy' comes from. Angiogenesis which is very important for the fetus and placenta is disturbed by an imbalance between pro- and anti-angiogenic factors. Moreover, PE is characterized by chronic inflammation which occurs systemically and within the decidua. Increased inflammation can occur if there is a decrease in regulatory immunity. So, this suggests also an important role for Tregs in PE pathology (82).



5.1 Risk factors for PE associated with Tregs

Some risk factors for PE can be associated with Tregs. One of these risk factors is the fact that PE occurs more frequently in young woman during a primary pregnancy. It is assumed that young woman are less exposed to seminal fluid/sperm compared to older woman before their first pregnancy. This might sometimes also be the case in woman with artificial induced pregnancies or during partner change. Artificial induced pregnancies with in vitro fertilisation (IVF) have a higher success rate when there are more Tregs present in the blood (83). Exposure to seminal fluid is thought to expand Treg cell populations as described earlier in paragraph 4.4. Prolonged exposure to seminal fluid probably reduces the risk for PE, because of the formation of paternal alloantigen specific Tregs (84). Furthermore, some genetic variations related to immunity might be a risk factor for PE. Genetic factors definitely play a role in causing PE since PE is more common if your mother or sister suffered from PE. Deepthi et al. examined the variation between the TGFB1 polymorphism C-509T in a population of pregnant woman undergoing healthy pregnancy compared to PE. Conclusively, a CT genotype can be protective against PE, while a TT genotype increases the risk for PE in this particular study. The TT genotype was associated with TGFb1 overproduction which may contribute to endothelial dysfunction (85).

5.2 Levels of Tregs during PE: studies in humans

Most human studies comparing the amount of Tregs in normal pregnancy with PE in the periphery showed a decreased amount Tregs in PE (86) (87) (88) (89) (90) (91). Decreased Tregs in the blood were also measured in patients with pregnancy induced hypertension (92). However, different studies show different results which is due to the use of different Treg isolation methods and the heterogeneity of PE disease. This will be further explained in chapter 7: discussion. Just as in healthy pregnancies, most studies are performed in blood. A minority of studies also shows a decrease of Tregs within the decidua during PE (93) (94). Decidual Tregs peak approximately at 35 weeks of gestation (figure 6) (94).

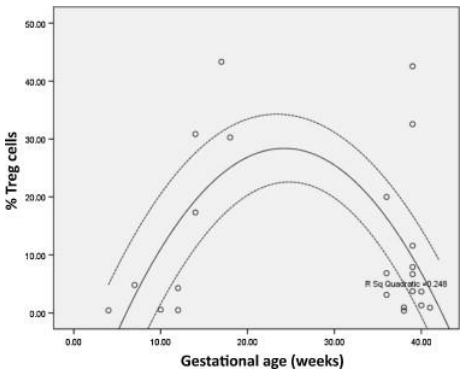


Figure 6: Percentage of decidual Tregs during gestation (94).

5.3 Tregs and the RUPP rat model of PE

Studies about PE are also performed in animal models. Many models of single gene knock outs exist for PE. However, the most extensively used model for PE is a reduced uterine perfusion pressure (RUPP) rat model. RUPP can be induced in rats by placing clips around the abdominal aorta during pregnancy (figure 7). This surgery will cause a reduction in uterine blood flow with 40%. At a similar way RUPP can also be induced in other animals than rats such as dogs, primates and rabbits. However, pregnancies in rats and primates are thought to be most representative for human pregnancies. Therefore, the rat model is most widely used. The RUPP rat model of PE covers many characteristics of the disease pathology of PE in humans. These rats have hypertension, proteinuria, impaired renal function and intra uterine growth restriction of the rat pups. Moreover, RUPP rats have just as humans with PE

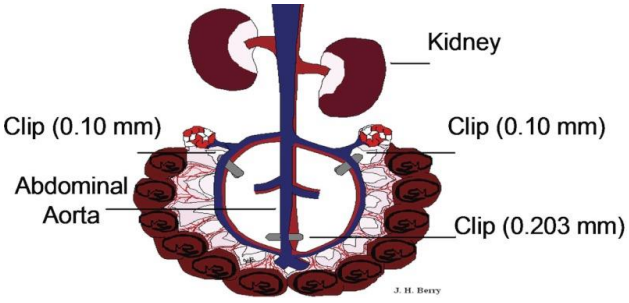


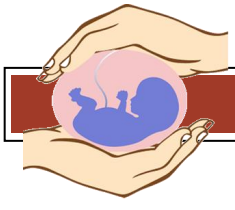
Figure 7: The induction of RUPP in rats via clipping of the abdominal aorta during pregnancy (95).

increased levels of oxidative stress, endothelin-1, placental hypoxia-inducible factor-1α (HIF-1α), angiotensin 1 receptor (AT1) auto antibodies and increased inflammatory cells and cytokines (TNF-α, IL-6, CD4+ T cells).A total increase in CD4+ T-cell subsets is mainly caused by an increase in Th17 cells. Treg cells are decreased in the RUPP model of PE. Moreover, decreased levels of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) are present, while soluble fms-like tyrosine kinase-1 (sFIt-1) levels are increased which indicates the disturbed angiogenesis and placentation in PE. So, RUPP rats highly mimic PE at multiple ways what makes it a more interesting model to study PE compared to other animal models of PE (95). The RUPP model and its use for the investigation of new treatments for PE will be discussed in chapter 6.

5.4 The role of Treg deficiency in PE pathology

Treg deficiency in PE can contribute to pathology at multiple ways. Firstly, Tregs act together with NK-cells in the uterus and EVT cells to regulate the balance between EVT invasion and EVT apoptosis. During PE an increased amount of EVT apoptosis is observed. So, lacking Tregs can contribute to insufficient trophoblast invasion associated with incomplete remodelling of spiral arteries (72). Incomplete spiral artery remodelling results in reduced blood flow to the placenta. Placenta's of woman with PE are often smaller leading to reduced growth of the fetus. Secondly, a lack of Tregs can result in failure of maternal-fetal tolerance. Breakage of maternal fetal-tolerance is due to the expansion of alloreactive effector T-cells (Th1 and Th17). Thirdly, an imbalance between pro-inflammatory (TNF-a, IL-6, and IL-17) and anti-inflammatory cytokines (IL-10, IL-4) is reported for PE which can be caused by Tregs. Inflammation contributes to endothelial dysfunction in PE which is associated with the sudden rise in blood pressure as the main symptom of PE.

The findings of changed levels of T cell subsets during PE pathology suggest dysfunction of the PD1-PD-L1 pathway during PE (see paragraph 4.5). However, changes in PD1 levels or its ligands were not observed within the peripheral blood samples of patients with PE compared to healthy pregnancies in the third trimester (48). This finding could mean that PE pathology is unrelated to PD1 signalling. However, this is unlikely because PD1 signalling is highly implicated in the regulation of Tcell subsets including the levels of Tregs. Therefore, further studies about the topic of PD1 signalling should be performed in different settings (periphery/decidua/stage of pregnancy) to be sure of the role of PD1 signalling in PE pathology.



6 Future prospects: the use of Tregs for therapy

6.1 Pre-eclampsia

Recent studies examined the expansion of Tregs for therapy of PE in the RUPP rat model. During the clipping procedure an intraperitoneal pump was implanted for the delivery of IL-10 to the RUPP rat model. At this way the rats were treated with 2.5 ng/kg/d IL-10. IL-10 is an anti-inflammatory cytokine which might be able to restore the immunological imbalance during PE pathology. IL-10 is known to downregulate inflammatory cytokines and transcription factors. Besides inhibiting the proliferation of inflammatory cells IL-10 is also reported as activator of FOXP3 expression which facilitates Treg expansion. Therefore, IL-10 was used to induce expansion of Tregs in the RUPP model. The results of this study are shown in figure 7. IL-10 was indeed able to significantly expand the population of Tregs in the RUPP model of PE (figure 8 A).

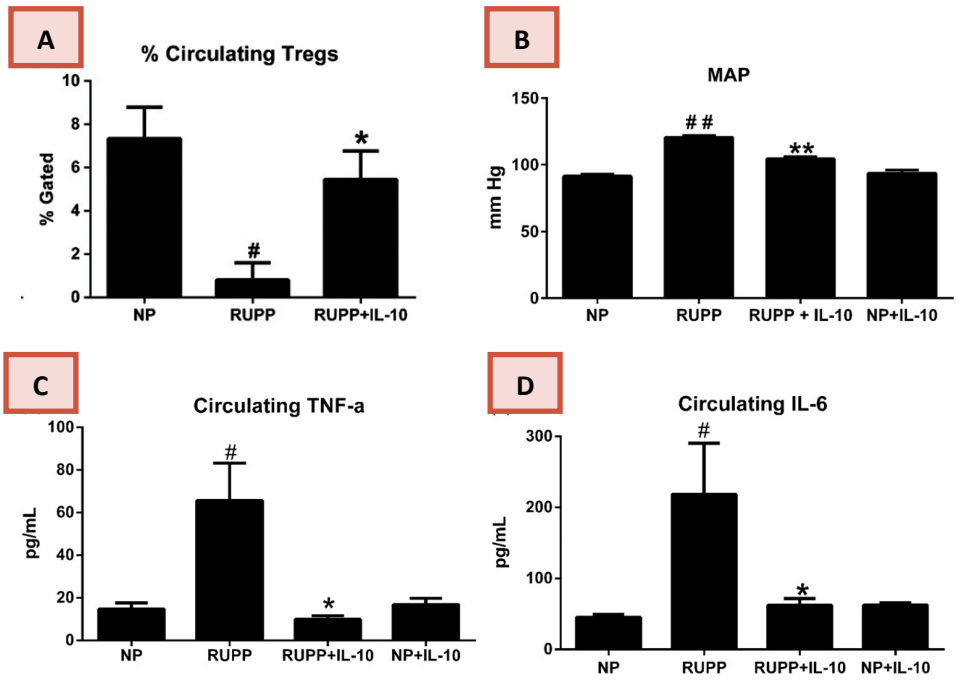
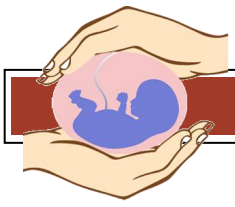


Figure 8: The effect of IL-10 infusion on RUPP rats of PE. IL-10 treatment induces the expansion of Tregs (A) and decreases the mean arterial pressure (MAP) (B). IL-10 decreases the formation of the pro-inflammatory cytokines TNF-α and IL-6 (C,D) (96).

Moreover the blood pressure was decreased due to IL-10 treatment and almost restored back to normal (Figure 8 B). IL-10 treatment also significantly decreased the formation of pro-inflammatory cytokines TNF-α and IL-6 (Figure 8 C,D). Furthermore, AT1 auto antibody production and oxidative stress levels were decreased upon IL-10 treatment of the RUPP model (96). So this study suggest that indirect expansion of Tregs via IL-10 is a potential new treatment against PE to restore immune homeostasis. Another study in the RUPP model directly increased the amount of Tregs by transferring Tregs from non-pregnant to pregnant RUPP rats (97). Direct expansion of Tregs also showed a decrease in blood pressure in the RUPP model, indicating Tregs as a good target for future Treg based immunotherapy of PE in humans.



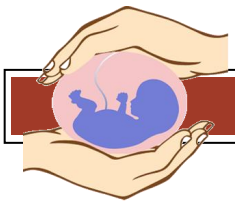
6.2 Using the knowledge of pregnancy immunology against transplant rejection

As Treg based immunotherapy in humans does not yet exist for pregnancy complications including miscarriage, PE and infertility, Treg based immunotherapy is highly under investigation in clinical trials in the field of transplantation research. A fetus is known as natures allograft and tolerated via the immune adaptations during pregnancy including an important role for Tregs. Since it is known that Tregs are immunosuppressive cells and regulators of tolerance the idea was present to use Tregs for therapy against organ transplant rejection and graft versus host disease (GvHD) (98).

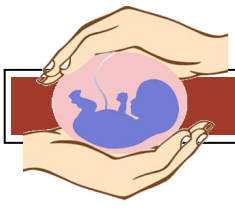
Treg based immunotherapy starts with the isolation and sometimes the in vitro expansion of Tregs. During isolation and expansion it is important that Tregs keep their functionality. Good manufacturing practice (GMP) protocols are available for Treg isolation, expansion and for the production of antigen specific Tregs. However, one big issue in this research is that Treg cells are highly heterogeneous and it is not known which specific subtype of Tregs is the best to use for Treg based immunotherapy and from which source the cells should be isolated. Different subtypes of Tregs are for example FOXP3 negative Tregs, FOXP3+ Tregs, T regulatory type 1 (Tr1) cells and tissue resident Tregs. Different subtypes of Tregs probably have different mechanisms of immunosuppression/tolerance (98).

The first clinical trial studies in humans with Tregs were performed to prevent GvHD in patients which received an allogeneic haematological stem cell transplantation (HSCT) because of a haematological cancer. Six studies are performed to examine Treg based immunotherapy for GvHD (99) (100) (101) (102) (103) (104). A great advantage for future research is that all those studies showed that it was safe to transplant Tregs in human patients. However, the groups of patients tested in these early clinical trials were quite small. Three of those studies showed a positive effect and were able to diminish or even prevent the occurrence of GvHD due to Treg infusion. Besides GvHD, more recent studies focus on the role of Tregs in solid organ transplantation. A study about kidney transplantation suggest that transplant rejection occurs more frequently in kidney transplant recipients with low FOXP3+ Treg cell populations compared with patients with higher Treg cell populations (105). Therefore, monitoring the peripheral levels of Tregs after transplantation might be a tool for the future to predict transplant rejection. Although, more research is required.

At the moment, clinical trials are also ongoing to investigate Treg based immunotherapy for solid organ transplantation of the liver or kidney. The different studies use different methods including: polyclonal expanded Tregs, ex vivo expanded autologous polyclonal Tregs or donor-specific expanded Tregs. Treg infusion was combined with or without the administration of the immunosuppressive drug rapamycin to the patients. Rapamycin is known to stimulate the in vitro expansion of Tregs while it inhibits the differentiation and proliferation towards other T-cell subsets. The results of these clinical studies are not known yet. Therefore, many questions remain about Treg based immunotherapy. What is the best subtype, source of Tregs and how long do the cells survive in the host, what is the exact mechanism of action and is it possible to create a local increase in Tregs similar as tissue resident Tregs. Tregs are a very heterogeneous cell population which can probably also transdifferentiate back to other T-cell subtypes under inflammatory conditions. Until now this is the main concern about Treg based therapy.Although, future Treg based immunotherapy is promising and probably a good treatment against



transplant rejection and this will hopefully reduce the necessity of immunosuppressive drugs which are normally used after transplantation (98).



7 Discussion/conclusion

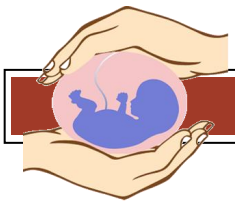
This review has given an overview about the literature describing the role of Tregs in healthy pregnancy and PE. A majority of articles describes the essential role of the increased population of Tregs during normal pregnancy and the decreased population of Tregs during PE. Therefore, it can be concluded that Tregs are essential cells for achieving a successful pregnancy. Although, studies about Tregs in pregnancy and PE do not always give similar results regarding to Treg percentages and immunosuppressive activity. Rahimzadeh et al, found with the aid of writing a systematic review (2015) that the inconsistency among study results is due to the use of different markers for Treg detection. They compared different studies of Tregs in PE and concluded that all studies using CD4+ CD25+ FOXP3+ or CD4+ FOXP3+ markers gave similar results. These studies all found a decrease in peripheral Tregs during PE (106) (table 3).

Table 3: Comparisons of studies about Treg frequencies (106)

Sources	Markers	Changes in frequency of *Tregs* *(PE, NP or non-P were compared)*[†]	References
Umbilical cord blood	CD4+CD25+	PE > NP	[92]
	CD4+FoxP3+	NS	
	CD4+CD25highFoxP3+	NS	
	CD4+CD25highCTLA4+	NS	
	CD4+CD25highGITR+	NS	
	CD4+CD25highCTLA4+GITR+	NS	
Decidua	FoxP3+	PE < NP	[15]
	FoxP3+CD4+	PE < NP	
	CD4+FoxP3+	NS	[80]
	[‡]CD4+Helios-FoxP3+	PE < NP	
Peripheral blood	CD4+FoxP3+	PE <NP	[80,84,89,93]
		PE < non-P	
	CD4+FoxP3+	PE <NP	[91]
		NP> non-P	
	CD4+Helios-FoxP3	PE < NP	[80]
		NP > non-P	
	CD4+Helios+FoxP3+	NS	
	CD4+CD25+	NS	[28,29,91–93]
	CD4+CD25+	PE >NP	[81]
	CD4+CD25high	PE < NP	[15,28,89,90]
	CD4+CD25high	NS	[81]
	CD4+CD25high	[§]NS(PE/non-P)	[89]
	CD4+CD25high	PE < non-P	[15]
	CD4+CD25high	PE < non-P	[81]
	CD4+CD25low	NS	[28]
	CD4+CD25+FoxP3+	PE < NP	[83,85–87,93]
	ratio of Th17/CD4+CD25+FoxP3	PE >NP	[82,85]
	CD4+CD25+FoxP3high	PE < NP	[87]
	CD4+CD25+FoxP3low	PE >NP	
	CD4+CD25highFoxP3+	PE < NP	[15,83,91]

This also states the importance of using the Treg specific intracellular marker FOXP3 for the detection of Tregs. Using FOXP3 as a marker has in a technical point of view some disadvantages. FOXP3 is an intracellular marker and can only be used for identification of Tregs via intracellular FOXP3 stainings. This procedure requires cellular fixation/permeabilization which results in dead cells which are not suitable to use for other molecular techniques for example RNA isolation.Despite its technical challenges FOXP3 is the only known specific marker for Tregs identified. Therefore, studies which did not used FOXP3 cannot be compared with the more recent studies (107).

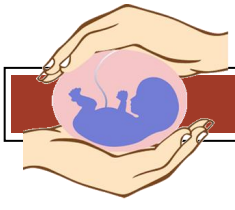
Moreover, studies using additional markers such as CD127 and CTLA-4 or even more markers are more likely looking at subpopulations of Tregs and not to the total pool of Tregs. Since, recent findings indicated the high amount of heterogeneity among Tregs cells. Many subpopulations of Tregs exist and Tregs are highly plastic. Many studies do not consider the heterogeneity of Tregs and only look at total Treg pools. The more recent studies are more focussed on Treg subpopulations, because it is most likely that different subpopulations have different mechanisms of regulating immune homeostasis which are not known yet. It is also still not possible to make a distinction between human natural and peripheral Tregs based on



marker expression. It would be helpful to develop a way to distinguish these cells in humans in the future to obtain more knowledge about the contribution of these distinct cells during normal and complicated pregnancies in humans (108).

Despite the marker problem in Treg research, many things are clear about the role of Tregs in pregnancy. Tregs play a local and peripheral role. Tregs play a major role in acceptance of a semi-allogenic fetus by inducing maternal fetal tolerance towards self-antigens, paternal alloantigens and fetal antigens. Tregs create a local tolerant environment, via different mechanisms. Tregs inhibit the proliferation of other T-cell subsets and secrete anti-inflammatory cytokines. Many independent studies in mice and humans state the importance of Tregs for a healthy pregnancy. In PE Tregs contribute to disease pathology via failure of maternal fetal tolerance, failure of spiral artery remodelling and the induction of chronic inflammation what causes endothelial dysfunction. Therefore, a logical thought is to treat pregnancy complications such as PE by expanding the population of Tregs via Treg based immunotherapy. In a rat model of PE expansion of Tregs was already able to diminish disease severity and lower the blood pressure (96). Results are promising but, more research is required to apply Treg based immunotherapy in humans.

The knowledge about Tregs derived from pregnancy studies can also be used for another application: transplant rejection. Clinical trials of Treg based immunotherapy are already completed for hematopoietic stem cell transplantation which indicates safety of Treg therapy. Furthermore, clinical trials for Tregs as therapy against solid organ transplant rejection are running at the moment. Taken together, it can be concluded that Tregs are crucial for pregnancy success and they are a great promising tool for future therapy against pregnancy complications as PE and also against transplant rejection.



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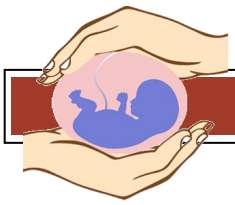

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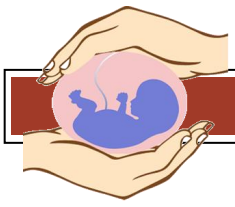

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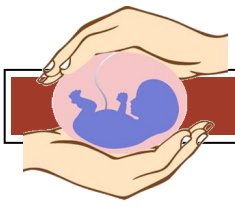

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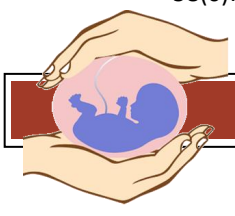

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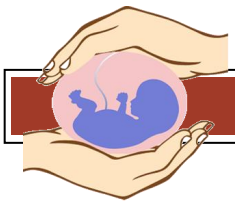

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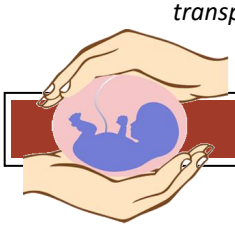

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