UNIVERSIDADE DE COIMBRA

THE IMPORTANCE, ORIGIN AND TOLERANCE MECHANISMS OF T REGULATORY CELLS DURING PREGNANCY

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IMPORTÂNCIA, ORIGEM E MECANISMOS DE TOLERÂNCIA DAS CÉLULAS T REGULADORAS DURANTE A GRAVIDEZ

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PUBLICATIONS DURING THE DEVELOPMENT OF THIS THESIS

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Woidacki K, Popovic M, Metz M, Schmerse F, Leber A, Linzke N, **Teles A**, Poirier F, Fest S, Jensen F, Rabinovich GA, Maurer M*, Zenclussen1 AC*. Mast cells are essential for uterine tissue remodeling during pregnancy. (Paper in revision)

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Jensen F, Woudwyk M, **Teles A**, Woidacki K, Taran F, Costa S, Malfertheiner SF, Zenclussen A. (2010) Estradiol and Progesterone Regulate the Migration of Mast Cells from the Periphery to the Uterus and Induce their Maturation and Degranulation. PLoS One 5, e14409.

Leber A*, **Teles A***, Zenclussen AC. (2010) Regulatory T cells and their role in pregnancy. Am J Reprod Immunol 63, 445-59 **(shared authorship)**.

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TABLE OF ABBREVIATIONS

ALPS-Autoimmune lymphoproliferative syndrome

APC- Antigen presenting cell

Breg- B regulatory cells

CCR- Chemokine receptor

CTLA-4- Cytotoxic T-Lymphocyte Antigen 4

DC- Dendritic cells

DT- Dyphteria toxin

DTT- Dithiothreitol

E- Embryonic day

E2- Oestrogen

EG- Experimental group

FACS - Flow cytometry

FasL- Fas Ligand

GITR- Glucocorticoid induced tumor necrosis factor receptor family related gene

HBSS- Hank's Balanced Salt Solution

hCG- Human chorionic gonadotropin

HLA- Human Leukocyte Antigen

IDO- Indoleamine 2,3-dioxygenase

IFNg- Interferon gamma

IL- Interleukin

IPEX- Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked

iDCs- immature Dendritic cells

iTreg- induced T regulatory cells

LH- luteinizing hormone

LH/CG- luteinizing hormone/coriogonadotropin

LPS- Lipopolysaccharide

M- Macrophages

M1- Classical macrophage activation path

M2- Alternative macrophage activation path

MCs- Mast cells

MHC- Major histocompatibility complex

MMPs- Metalloproteinases

NK- Natural killer cells

nTreg- natural T regulatory cells

P4- Progesterone

PBS- Phosphate Buffered Saline

PD-1- Programmed cell death 1 pro

PE- Pre-eclampsia

Rag- Recombination activating genes

RPMI- Roswell Park memorial institute medium

RSA- Recurrent spontaneous abortion

SA- Spontaneous abortion

sFlt1- Soluble fms-like tyrosine kinase-1

SLC- secondary lymphoid-tissue chemokine

TCR- T cell receptor

TECK- Thymus-Expressed Chemokine

Teff- T effector cells

TGF-β- Tumor grouth factor beta

Th- T helper cells

TLX- Trophoblast lymphocyte cross-reactive alloantigens

TNF- Tumor necrosis factor

Tr1- T regulatory type 1 cells

Treg- T regulatory cells

uNK- Uterine natural killer cells

ABSTRACT

Pregnancy represents a challenge for the maternal immune system. It has to be alert against pathogens while tolerating paternal alloantigens expressed in fetal structures. T regulatory cells (Treg) are important mediators of tolerance and they have been described as participants in the tolerance mechanisms towards paternal antigens that allow fetal maintenance. However, it is still not clear whether their role is indispensable for the onset, the maintenance of pregnancy or both. Additionally, their origin and mechanisms of action throughout the different phases of pregnancy are issues that still need further clarification.

The main objective of this thesis was to address whether Treg have a decisive role in pregnancy. We first analyzed the population of Treg during the estrus cycle and observed that Treg accumulate in the vaginal fluid and uterus even prior to conception during the receptive phase of the oestrus cycle. Treg depletion after dyphteria toxin (DT) application in a Foxp3DTR model seriously impaired implantation, while their depletion after conception only marginally affected pregnancy by slightly increasing the abortion rate.

These results show that Treg are only essential at the beginning of pregnancy. However, several authors observed an increased in their number thereafter. Thus, we tested the kinetics of Treg during pregnancy and whether Treg at different gestation points are thymic-derived or are converted in the periphery. We reported a general augmentation in the total population of Treg starting before implantation, on day 2 of pregnancy and described that at this time point, in thymus, uterus and uterine draining lymph nodes, Treg exhibit an augmented expression of Helios, a marker that was described to define thymic derived Treg. At later stages, the predominant population is Helios$^-$, suggesting their peripheral conversion. In

fact, we showed that $Foxp3^{+}$ Treg are generated in the periphery in a $Rag1^{-/-}$ model of cell transfer. We confirmed a *de novo* conversion of $CD4^{+}Foxp3^{GFP-}$ into $CD4^{+}Foxp3^{GFP+}$ cells, that was independent of TGF-β and took place at implantation and mid gestation (days 5 and 10 respectively).

To understand the players involved in Treg location during pregnancy we selected several chemokines whose receptors are present at Treg surface and therefore can mediate their migration to the reproductive area. We observed that CCL21 and CCL25 are present in decidua and placenta during pregnancy and are able to attract Treg *in vitro*. We next observed that Treg are almost absent in the uterus of $CCR7^{-/-}$ mice, showing that this receptor determines their homing to the uterus. $CCR9^{-/-}$ mice have normal Treg frequencies but an increased population of T effector cells in the uterus as well as a higher maturation state of dendritic cells from the uterine draining lymph nodes. Similar to what we observed previously by the DT depletion of Treg, the lack of either chemokine receptor critically interfered with implantation. This clearly shows that lack of Treg or an excess of Teff hinders implantation. Our data provides new insights in the generation, origin and function of Treg during pregnancy, essential to understand natural mechanisms of tolerance acquisition.

RESUMO

A gravidez representa um desafio para o sistema imune materno. É necessário que este esteja alerta contra possíveis organismos patogénicos e, ao mesmo tempo, se mantenha tolerante perante os antigénios paternais expressos nas estruturas fetais. As células T reguladoras (Treg) são importantes mediadoras da tolerância e têm sido descritas como participantes nos mecanismos de tolerância para com os antigénios paternais, possibilitando a persistência do feto no útero materno. No entanto, não está ainda esclarecido se o papel destas células é indispensável para o início da gravidez, para a sua manutenção ou ambos. Adicionalmente, a sua origem e os mecanismos de ação das Treg durante as várias fases da gravidez são assuntos que necessitam uma melhor clarificação.

O principal objectivo desta tese foi compreender se as Treg têm um papel decisivo na gravidez. Analisámos inicialmente a população de Treg durante o ciclo do estrus e observámos que estas células se acumulam no fluido vaginal e útero ainda antes da concepção, durante a fase receptiva do ciclo do estrus. A depleção das Treg através da aplicação da toxina da difteria em um modelo Foxp3^{DTR} afetou fortemente a implantação, enquanto que a sua depleção após a concepção apenas afetou marginalmente a gravidez com um aumento mínimo na taxa de aborto.

Estes resultados indicam que as Treg são apenas essenciais no início da gravidez. No entanto, vários autores observaram que o seu número aumenta mais tarde. Sendo assim, testámos a cinética destas células durante a gravidez e se elas são geradas no timo ou convertidas na periferia em diferentes estágios de gestação. Observámos um aumento geral na população total de Treg que começou antes da implantação, no dia 2 de gravidez e descrevemos que, neste período, no timo, útero e nódulos

linfáticos que drenam o útero, as Treg exibiam um aumento de expressão de Helios, um marcador que foi descrito como definindo as Treg com origem no timo. Mais tarde, a população predominante é Helios^{-}, o que sugere a conversão das Treg na periferia. Adicionalmente, através de um modelo de transferência de células em ratinhos Rag1$^{-/-}$, pudemos demonstrar que as Treg são convertidas na periferia. Observámos também que esta conversão *de novo* de células CD4^{+}Foxp3GFP^{-} para CD4^{+}Foxp3GFP^{+} ocorre de um modo independente de TGF-β durante a implantação e a meia gestação.

De forma a entender quais os processos envolvidos na localização das Treg durante a gravidez, selecionámos várias quemoquinas cujos receptores estão presentes na superfície das Treg e que podem assim mediar a sua migração para a área reprodutiva. Observámos que o CCL21 e o CCL25 se expressam na decídua e placenta durante a gravidez e são capazes de atrair as Treg *in vitro*. De seguida, observámos que as Treg estão quase ausentes no útero de ratinhos CCR7$^{-/-}$ demonstrando que este receptor determina a sua localização no útero. Os ratinhos CCR9$^{-/-}$ têm frequências normais de Treg mas exibem um aumento de células T efetoras no útero, assim como apresentam uma maior maturação das células dendríticas dos nódulos linfáticos que drenam o útero. Em semelhança com o que observámos antes, através da depleção das Treg pela aplicação de DT, a ausência de qualquer destes receptores de quemoquina interferiu criticamente com a implantação. Isto demonstra que a falta de Treg ou um excesso de células T efetoras impede a implantação. Os nossos resultados constituem novos dados sobre a origem e função das Treg durante a gravidez, essencial para compreender os mecanismos naturais de aquisição de tolerância.

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INTRODUCTION

1.1- INTRODUCTION

The mystery behind the establishment and evolution of pregnancy has been motivating the curiosity and the imagination of the humanity since always. Nowadays there is still a paradoxical immune question that moves energetic efforts from the scientific community to find an answer:
How can the life of a fetus, an antigenically foreign body which bears antigens from the father, be sustained inside the maternal body for the several months of pregnancy without being rejected by her immune system?

1.1.1- The biology of reproduction:

Many steps are involved in the development of a successful pregnancy, such as proper ovulation and receptiveness of the uterus, fertilization, implantation, placentation, as well as a proper immune response allowing the acceptance of the developing fetus.

1.1.1.1- Ovulation and the cycling uterus:

When females reach sexual maturity, the uterus prepares on cyclic intervals for a possible conception and forthcoming implantation. The state of uterine receptivity results from transformations of the endometrium followed by the oocyte maturation and release from the ovary during the ovulation process (Espey *et al.*, 2004). Each time pregnancy does not occur these transformations are reverted allowing preparation for a future pregnancy. Therefore, this cycle is characterized into pre-receptive, receptive and non-receptive (refractory) phases. Uterine receptivity in mouse and humans is mostly regulated by different levels of the pituitary secreted luteinizing hormone (LH), progesterone (P4) and oestrogen (E2) secreted by the ovary

after stimulation by LH (Fig 1.1) (Wang and Dey, 2006). In humans, ovulation takes place when LH and E2 expression reach their maximum. The reproductive cycle lasts 28 days and is called menstruation. In mice ovulation takes place when LH and E2 expression reach their maximum and P4 levels are augmented (Fig 1.1). Their reproductive cycle lasts 4-5 days and is called oestrus cycle divided in four different phases: diestrus (endometrial desquamation), proestrus (follicular development and proliferation of the endometrium), estrus (ovulation and endometrial receptivity) and metestrus (endometrial differentiation) (Wang and Dey, 2006; Caligioni, 2009).

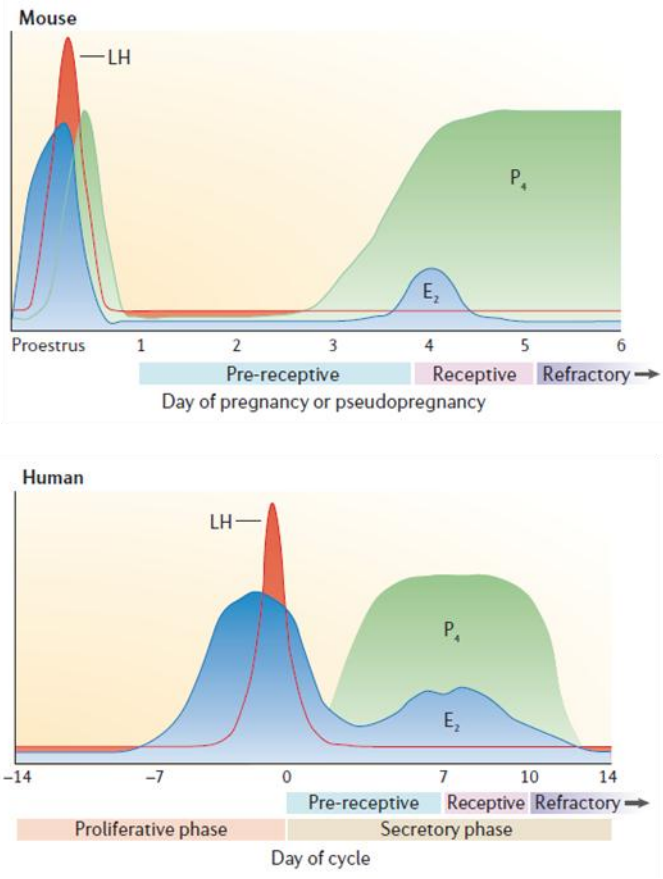

Figure 1.1- Mouse oestrus cycle and human menstruation cycle. The combination of different levels of Luteinizing hormone (LH), Progesterone (P4) and oestrogen (E2) distinguishes the uterine receptive phase preparing the uterus for a possible conception. (Figure from Wang and Dey, 2006)

1.1.1.2- Fertilization and pre-implantation

Mammal fertilization occurs after mating. Male semen, consisting of sperm cells and the seminal fluid which contains a pool of different proteins and bioactive moieties (Cooper 1990; Montagnon *et al.*, 1990) is ejaculated into the vaginal cavity and sperm cells move to the upper vagina (by contraction movements from the myometrium) through the cervix and across the length of the uterus towards the ovum.

When fertilization of the oocyte with the sperm occurs in the oviduct it is followed by the pre-implantation stage which is characterized by continuous mitotic cell division, formation of cell polarity and compaction giving rise to a morula with 8 to 16 cells. The lineage differentiation occurring after the morula stage leads to the edification of the blastocyst that leaves the oviduct entering the uterine lumen. The constitution of the mature blastocyst relies on an outer epithelial trophectoderm (that will develop the future trophoblast cells), the primitive endoderm and the inner cell mass (pluripotent cells that will differentiate into the future embryo). When the endometrium is receptive the blastocyst hatches from the zona pelucida and the implantation can proceed with the trophectoderm contacting the luminal uterine epithelium (Wang and Dey, 2006). A schematic representation of this phase is illustrated in Fig 1.2.

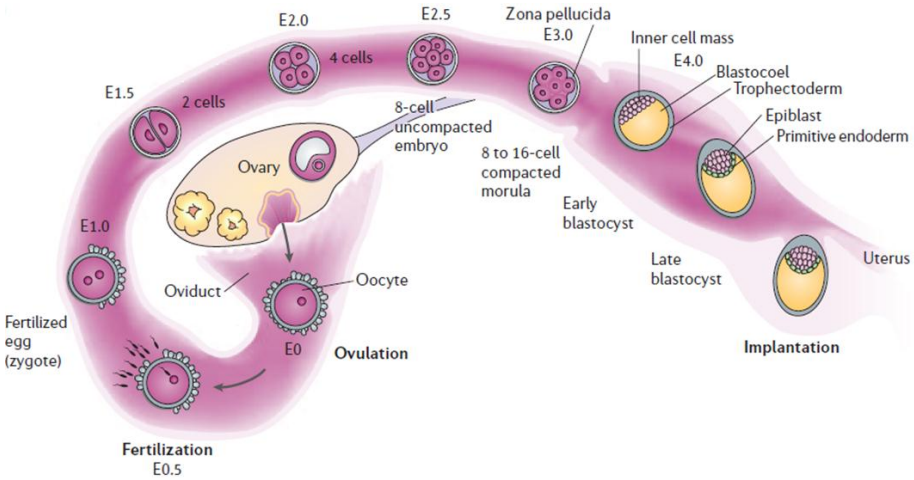

Figure 1.2- Pre-implantation stage in mice. After ovulation and a successful fertilization of the ovum, a series of mitotic divisions occur originating a morula that further develops into a blastocyst. After leaving the oviduct, the blastocyst starts to differentiate and finally hatches to the uterine epithelium starting the process of implantation. E – Embryonic day. (Figure from Wang and Dey, 2006)

1.1.1.3- Implantation

During implantation trophoblast cells anchor into the endometrial stroma and the blastocyst becomes dependent on the maternal environment to further development. The general aspects of the implantation process are similar in humans and rodents (Arvola and Mattsson, 2001) and its success depends on a proper stage of embryo development and the receptivity of the endometrium during the implantation window (Abrahamsohn and Zorn, 1993) which in mice takes place around 4-4.5 days after fertilization (Finn and Martin, 1967; Tabibzadeh and Babaknia, 1995) and its finished at day 5, and in humans it lasts between day 19 and day 24 of a normal 28 day cycle (Bergh and Navot, 1992). The uterine receptivity is the result of an adequate exposure to progesterone and estrogen (Psychoyos, 1976).

Implantation occurs in three main phases: apposition, attachment and invasion.

a) Apposition:

During apposition, the first structural interaction between embryo and mother is established. The accumulation of fluid in the stromal cells conducts to the closure of the uterine lumen increasing the contact between microvilli in the surface of the trophectoderm and uterine epithelial cells. The interdigitation of these cells leads to the formation of smooth mushroom or balloon like projections named pinopodes whose development is dependent on progesterone but is inhibited by estradiol (Martel *et al.*, 1991; Nikas *et al.*, 1995; Singh *et al.*, 1996; Bagot *et al.*, 2001).

b) Attachment:

The communication between the blastocyst and the endometrium becomes stronger and the non-adhesive apical surface of the trophectoderm becomes adhesive and attachment starts controlled by an adhesive-signalling system of several glycoproteins and carbohydrate ligands and receptors expressed by both blastocyst and endometrium. Together, cytokines, integrins, selectins, galectins, proteoglycans, glycosaminoglycans, laminin and collagen help in the transformation of the endometrial tissue which, resembling the transformations of an inflammatory response, experiences an increase in its blood vessels permeability and the recruitment of inflammatory cells producing pro-inflammatory cytokines (Cross *et al.*, 1994; Wang and Dey, 2006).

c) Invasion:

Finally the blastocyst becames very invasive and trophoblast cells invade the surface epithelium by displacement penetration. In this way, surface epithelial cells are displaced from their basal membrane and from each other and they die by autolysis being phagocytized by trophoblast cells. The

trophoblast cells are then exposed to the bare basal membrane and attach to the endometrial epithelium which also starts a decidualization process. During this process the endometrial fibroblastic stromal cells are transformed into the decidua by local modifications of the shape, organization and metabolism and establishing tight junctions with their neighbor cells (Abrahamsohn and Zorn, 1993; Tabibzadeh and Babaknia, 1995).

1.1.1.4- Placentation

Placenta is a vital organ which promotes the interaction of mother and fetus ensuring gas exchanges, hormonal production and secretion, nutrient transport and removal of toxic wastes needed to the fetal survival and growth (reviewed in Pijnenborg *et al.*, 1981; Handwerger *et al*., 2000). Depending on the species, different types of placenta can be formed but early steps in the embryonic development are similar among all vertebrates. In primates and rodents, trophoblasts are invasive cells destroying the maternal vessels to establish themselves. Thus, maternal blood directly contacts the trophoblast cells establishing a hemochorial placenta defined by a single disk-like zone of close contact named discoidal placenta (Benirschke and Kaufmann, 2001).

By mouse embryonic day (E) 3.5 trophoectoderm and the inner cell mass of the blastocyst can already be distinguished and by E4.5, when implantation starts taking place, different trophoblast cell types follow. The mural trophectoderm (trophectoderm layer not in contact with the inner cell mass) differentiates into trophoblast giant cells that stop proliferating while maintaining DNA replication (endoreplication). As a result, these cells become polyploid and exhibit an extremely large cytoplasm secreting a

number of proteins with specialized functions (Zybina and Zybina, 1996; Hu and Cross, 2010).

The proliferation of the polar trophectoderm, overlying the inner cell mass, leads to the differentiation of two diploid cell types, the ectoplacental cone and the extra embryonic ectoderm. The extra embryonic ectoderm further develops to constitute the chorion which is lined up by mesothelial cells. The outer cells of the ectoplacental cone become secondary trophoblast giant cells surrounding the conceptus and the additional cells will form the spongiotrophoblast. The first hematopoietic tissue of the fetus, the visceral yolk sac, emerges from the migration of the extra embryonic towards the inner surface of the visceral endoderm. It originates with blood islands and gives rise to its primitive circulatory system at around E7.5. At E8.5 the chorion contacts with the allantois, a mesoderm-derived finger-like structure from the posterior end of the embryo. This contact induces chorionic folding and labyrinth formation through which fetal blood vessels originating from the allantois grow and give rise to the umbilical cord. As result, a branched structure called labyrinth is created composed of multinucleated syncytiotrophoblast cells (derived from the fusion of trophoblast cells) surrounding the endothelium of the fetal blood vessels and mononuclear trophoblast cells lining maternal blood sinuses. This structure is tightly supported by the compacted spongiotrophoblast layer of non-syncytial cells. The amnion arises from the embryonic ectoderm and mesoderm as a fold which expands forming a thin membrane surrounding the fetus (Benirschke and Kaufmann, 2001). Fig 1.3 shows the early embryonic development of the mouse embryo.

At the end of this differentiation process, three regions can be distinguished in the mature placenta of rodents (Fig. 1.3):

- The labyrinth zone, with both fetal and maternal blood channels participating in gaseous exchange between each other (Brosens *et al*., 1967; Muntener *et al*., 1977).

- Spongiotrophoblast zone, only with maternal blood channels through which maternal blood flows to the fetal side and labyrinth (Muntener *et al*., 1977; Pijnenborg *et al.*, 1981; Cross, 2000)

- The maternal uterine tissue, in close contact with the maternal side of the murine trophoblast giant cell zone (reviewed in Georgiades *et al*., 2002).

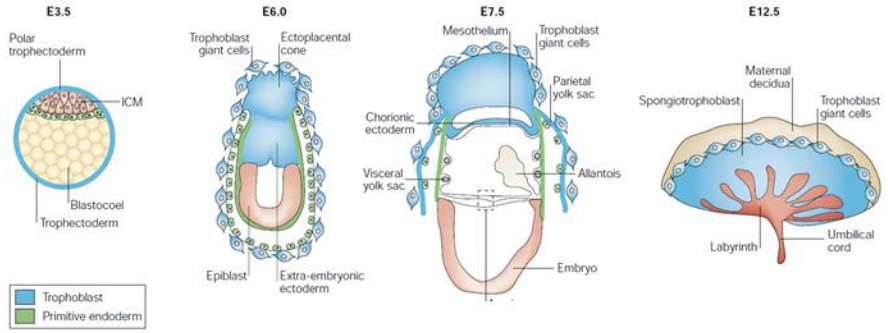

Figure 1.3- Placental development in the mouse. Evolution of different extra-embryonic lineages through different embryonic days (E) giving origin to the final structures of the placenta. (Figure from Rossant and Cross, 2001)

Mouse versus human placentation

The use of human material is limited as an experimental system once it is associated with a series of ethical questions and to difficulties in obtaining samples (reviewed in Georgiades *et al,* 2002). The biologic mechanisms in mice are similar to the ones in humans. Mouse models display short gestation periods, limited space requirements, and are available in a wide range of knockouts being easy to manage. Besides, their genome is also well

characterized (Nagy *et al*., 2003). Mouse models constitute therefore a vital tool in the study of pregnancy. In both species, embryo implantation leads to stromal decidualization-embryo embeded in antimesometrial stroma and placentation is hemochorial (Wang and Dey, 2006). However, mouse and human placenta display a different type of feto-maternal interdigitation and blood flow network. While in humans the placenta is characterized by villi (*villous placenta*) directly surrounded by maternal blood, in mice placentas are formed by interdigitations (*labyrinthine placenta*) and their trophoblast is breached by web-like channels filled with maternal blood and fetal capillaries (Rossant and Cross, 2001). Fig 1.4 shows the comparative anatomy of mouse and human placenta.

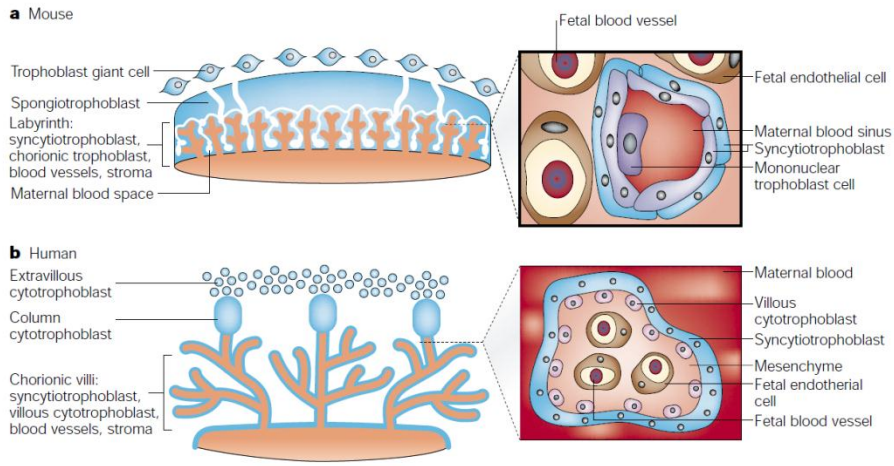

Figure 1.4: Comparative anatomy of the mouse and human placenta (Figure from Rossant and Cross, 2001)**.**

1.1.2 – Infertility and pregnancy complications

Lack of tolerance from the maternal immune system towards the fetus might result in pregnancy complications and events to its termination. Around 70% of all pregnancies fail to go to term. From these, 50-70% are lost during the first month and therefore can be unnoticed (Wilcox *et al.*, 1988). Pathologies

like endometritis, endometriosis, inflammatory pelvic diseases and fallopian tube blockage resulting from inflammatory processes often cause the metamorphosis of the tissues of the reproductive tract and vaginal fluid and are often a cause of infertility in human and animals by a difficult nidation or the encounter of the sperm with the ovum at the moment of conception (Cakmak *et al.*, 2009; Weiss *et al.*, 2009; Gilbert, 2011; Braundmeier *et al.*, 2012; Wiesenfeld *et al.*, 2012).

Nearly 60% of reported abortions have been described as having genetic, endocrine, infectious, anatomical, or autoimmune causes (Stray-Pedersen and Stray-Pedersen, 1984; Stephenson *et al.*, 1996).

Pre-eclampsia (PE) is characterized by the onset of hypertension, glomerulonephritis and proteinuria. It affects 5-10% of late pregnancies (reviewed in Robillard, 2002) and is one of the major causes of maternal-fetal morbidity and mortality (Walker, 2000). Possible causes of the development of PE are an insufficient trophoblast invasion or its complete failure in week 16-20 of gestation (Pijnenborrg *et al.,* 1996; Zhou *et al*., 1997) which is thought to be driven by an incomplete tolerance to the fetus as well as a maternal inflammatory response mounted towards the fetal allo-antigens resulting in a generalized endothelial dysfunction (Redman *et al*., 1999).

Spontaneous abortion (SA) affects about 20% of first trimester pregnancies constituting a major clinical problem (Edmonds *et al.*, 1982) with special importance for sub-fertile couples who have already a high failure rate due to infertility.

Recurrent spontaneous abortion (RSA) is diagnosed when women went under three or more spontaneous abortions. It affects 5% of pregnancies under the 20^{th} week of gestation (Roman, 1984) and is suggested to result from immunological disturbances. Primary RSA occurs when all the previous

pregnancies were lost, and secondary RSA when one successful pregnancy preceded several further consecutive abortions (Stirrat, 1990).

Clark *et al.* (1980) introduced a model of murine abortion by observing that in general, CBA/J females mated with DBA/2J males had an abortion rate of about 20-50% while a normal pregnancy resulted when these females were mated with BALB/c males. These different pregnancy outcomes could not result from major histocompatibility factors once both males share the same H-2d major antigens while the female has H-2k (Staats, 1976). This leaded to hypothesize that the most probable explanation for these differences relies in the minor histocompatibility factors of the BALB/c and DBA/2J males (Clark *et al.,* 1980; Chaouat *et al*., 1988).

1.1.3- The immune paradox behind pregnancy

1.1.3.1- The beginning of the reproductive immunology field and the original hypothesis

C. Little was the first, in 1924, to denote the need for the maintenance of maternal tolerance (in Billington, 2003) but it was Peter Medawar, years later, the first to shade this paradoxical question into the light of the scientific concepts of transplantation emerging at that time (Medawar, 1953). Three main hypothesis were first proposed by Medawar as possible explanations for the lack of an immunological reaction against the fetus:

a)- The existence of the placenta that would function as an anatomical barrier physically and totally separating the mother and the fetal conceptus;

b)- The fetus would be antigenically immature, lacking the expression of histocompatibility antigens;

c)- The immune system from the mother would exhibit a passive behavior.

1.1.3.2- Current concepts

Nowadays, as a result of better methodologies available to investigate vital processes, these original hypothesis by Medawar have been unraveled with the progress of our knowledge:

a)- The permeability of the barrier between mother and fetus:

It is true that both mother and fetus differ in the antigen expression, however their circulatory systems are separated from each other (Billington, 2003) and this fact distinguishes the relationship between the maternal immune system and the allogeneic fetus from the common notion of a transplant allograft in which the circulatory system represents a unity. Nonetheless, both humans and rodents have a hemochorial placentation, where the chorion comes in direct contact with maternal blood and thus lack an intact layer of epithelial cells separating mother and the fetus (Wang and Dey, 2006). In this way, trophoblast cells are continuously exposed to potential harmful immune cells. Additionally, it is now well documented the occurrence of microchimerism, a bidirectional trafficking of cells between mother and fetus through the placenta (Fig 1.5) is widely documented. It occurs in both humans and rodents in all stages of pregnancy and can persist many years after delivery (Bianchi *et al.*, 1996; Maloney *et al.*, 1999; Khosrotehrani *et al.*, 2005; Zenclussen *et al.*, 2010) being reported in 61% of adults and 100% of neonates (Dutta *et al.*, 2009). Its prevalence, rarely detected in unfractionated lymphoid tissues, was found in main tissues like

heart, lungs, liver, blood (Dutta *et al.*, 2009) and even in the brain suggesting that fetal cells are also able to cross the blood–brain barrier to enter the maternal brain (Tan *et al.*, 2005). Microchimerism is extended to the immune cells with wide reports of isolated fetal $CD4^+$, $CD11b^+$, and $CD11c^+$ cell subsets in the spleen, lineage-positive cells in the heart (Dutta *et al.*, 2009), T and B lymphocytes, monocytes/macrophages and NK cells in the blood of healthy women (Loubière *et al.*, 2006). Interestingly, a transient population of macrophages from maternal origin was found on the Yolk Sac as early as 7.5 to 8 days of fetal development (Bertrand *et al.*, 2005). Altogether, these findings suggest that there is in fact a permeable barrier between the fetus and the mother resulting ultimately in a cellular cross talk between both individuals.

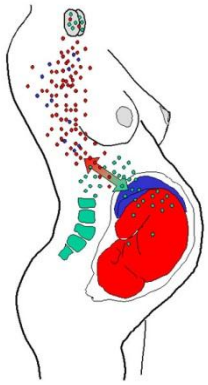

Figure 1.5- Bi-directional microchimerism of maternal, fetal and placental cells. (Figure from Galofré and Davies, 2007).

b)- The uniqueness of the fetal "allograft" histocompatibility antigens

The fetal "allograft" is constituted by the trophoblast cells (the outer layer of the blastocyst that develops into the placenta) and the extra-embryonic membrane. Therefore the events of immune recognition conducting to the tolerance of the fetus gain a special importance at this interface (Billington,

2003). The antigenic nature of the trophoblast exhibits unusual characteristics and it is now accepted that the fetus is not antigenically immature but shows different antigen expression interfering with direct immune responses (reviewed in Szekeres-Bartho, 2002).

Indeed, human trophoblast cells have reduced or absent expression of both classical highly polymorphic MHC class I antigens and MHC class II antigens and express immunomodulatory proteins (Hutter *et al.*, 1996; Petroff *et al.*, 2003). In addition only few human leucocyte antigens (HLA) are expressed at the protein level in the trophoblast cells (Guillaudeux *et al.*, 1995) whereas in the placenta it was possible to find the expression of some non-classical MHC class I antigens, like HLA-E and HLA-G (Kovats *et al.*, 1990; King *et al.*, 2000). Being limited to the gestation period (Carosella *et al.*, 1996), the expression of HLA-G seems to interfere with the maturation of DCs and confer protection against natural killer (NK) cells and cytotoxic CD8T cells, thus providing an early inhibition for both innate and specific immune responses (Rouas-Freiss *et al.*, 1997; Carosella *et al.*, 1999; Kuroki *et al.*, 2007; Gros *et al.*, 2008). Further, it has been demonstrated that in pregnancies with a disparity between maternal and fetal HLA-C there is an augmentation in the population of $CD4^{+}CD25^{bright}$ regulatory T cells suggesting an immunosuppressive role for this HLA (Tilburgs *et al.*, 2009).

Mouse trophoblast lacks expression of nonclassical MHC molecules but expresses MHC class I antigens of both H2-K and H2-D haplotypes (Chatterjee-Hasrouni *et al.*, 1981; Philpott *et al.*, 1988; Madeja *et al.*, 2011). However, the occurrence of an active mechanism of suppression at this local site is suggested from experiments made by inducing the over-expression of H-2 antigens in the murine trophoblast cell populations without resulting in any deleterious effect on pregnancy outcome (Rogers *et al.*, 1998; Shomer *et al.*, 1998).

c)- The active nature of maternal recognition of fetal antigens

The maternal immune system is not ignorant of the fetal antigens as it was long believed. Now it is shown that the immune recognition of fetal antigens is a critical process that determines whether the fetus will be tolerated or rejected by the maternal immune system (Chaouat *et al.*, 1985; Tafuri *et al.*, 1995; Zhou *et al.*, 1998; James *et al.*, 2003; Zenclussen *et al.*, 2010).

Several studies have shown that both maternal T and B cells are aware of and respond to fetal antigens. In a normal pregnancy these cells are in charge of specific tolerance mechanisms (Zenclussen *et al.*, 2007; Moldenhauer *et al.*, 2010; Taglauer *et al.*, 2010).

d)- Other regulators and theories of fetal protection:

- **Induction of apoptosis by Indoleamine 2,3-dioxygenase (IDO) and Programmed Death 1 (PD-1) protein**

Indoleamine 2,3-dioxygenase (IDO) is a tryptophan-catabolizing enzyme expressed by trophoblast cells, macrophages and DCs that has been considered a potential immunosuppressive factor in pregnancy. Munn *et al.* (1998) suggested that through the removal of tryptophan from the placental environment reducing its availability to maternal T cells, IDO could prevent immunological rejection of the fetal allografts. Under certain culture conditions, human macrophages and DCs were able to express IDO inhibiting T cell proliferation by catabolizing tryptophan from the culture medium (Munn *et al.*, 1999; Hwu *et al.*, 2000). IDO was proposed to maintain the opening of blood vessels, act as energy source and confer protection against oxygen radicals in placenta (Bonney and Matzinger, 1998). However, contradictory results were obtained from different models. While mating

combinations between H-2 matched or mismatched IDO deficient mice resulted in a normal pregnancy outcome (Baban *et al*., 2004), there were however no differences in IDO expression levels at the feto-maternal interface when comparing abortion prone and normal pregnant combinations at an early period of pregnancy and only a slight difference was observed at mid-pregnancy (Thuere *et al.,* 2007).

An analogous role is suggested for the protein Programmed Death 1 (PD-1)/B7-H1 axis. PD-1 deficient T cells specific for fetal antigens, accumulate in the uterine draining lymph nodes failing to undergo apoptosis. This suggests a role for PD-1 in maternal-fetal tolerance by inducing apoptosis of paternal antigen-specific T cells during pregnancy and thus controlling their abundance (Taglauer *et al.*, 2009).

- **Fas ligand (FasL)**

The expression of Fas-Ligand was proposed to be involved in the maintenance of the feto-maternal tolerance and its occurrence was described in both embryonic trophoblast and maternal decidua (Bamberger *et al*, 1997; Chatzaki *et al*., 2001). Fas is a membrane protein from the tumor necrosis factor (TNF) and nerve growth factor receptor family expressed in high amounts in activated T and B cells, NK cells, monocytes and macrophages (Nagata, 1994). The interaction of FasL with activated cells bearing Fas induces their apoptosis (Nagata, 1997; Huang *et al.*, 1999). Loss of function mutations in Fas result in an increasing survival of lymphocytes causing autoimmune lymphoproliferative syndrome (ALPS) (Jackson *et al.*, 1999; Magerus-Chatinet *et al.*, 2009). FasL- deficient mice have however normal pregnancies (Hunt *et al*., 1997; Bogovic *et al*., 2005) suggesting a non-redundant role for this molecule in the maintenance of pregnancy.

- **Modification of cellular cytokine mediated response.**

Cytokines are produced by the immune and non-immune cells at the feto-maternal interface and have a role on regulating the mechanisms of pregnancy. It was proposed that a change in the Th1/Th2 balance towards an increasing Th2 response would define fetal acceptance (Lin *et al.,* 1993, Rhagupathy *et al.,* 1997). In consonance, would negatively interfere with pregnancy and lead to spontaneous abortion or preeclampsia (Lin *et al*., 1993; Rhagupathy *et al.*, 2000; Zenclussen *et al.,* 2002, 2003). However, IL-4/IL-10 knockout mice and mice with quadruple IL-4, IL-5, IL-9 and IL-13 gene deletions have normal pregnancy outcomes and IL-12, a Th1 inducer, was found to be substantially expressed in normal pregnancies (Svensson *et al.*, 2001; Fallon *et al*., 2002; Zenclussen *et al.*, 2002). This leads to the conclusion that, while the positive influence of Th2 cytokines and the negative influence of Th1 cytokines in pregnancy are still believed, these cytokines alone do not define the final fate of pregnancy.

-Antibodies

Anti-paternal antibodies can be found in both maternal sera and the feto-maternal interface. They were formerly known as trophoblast lymphocyte cross-reactive alloantigens (TLX) for their cross reactivity with antigens of the trophoblast and it was proposed that such antigens have a central role in maternal allorecognition and blastocyst protection (McIntyre *et al.*, 1983). Studies also suggest that alloantibodies are regular players in a successful pregnancy by means of inhibiting NK mediated cytotoxicity against trophoblast cells (Jalali *et al.*, 1995, 1996). Asymmetric antibodies were distinguished by their molecular asymmetry, due to a high mannose carbohydrate group present in only one of the two Fab regions of the molecule (Margni and Binaghi, 1988), and by being not able to produce

immune reactions to destroy antigens, not forming insoluble complexes with antigen and not fixing complement. They act in a competitive way when mixed with precipitating antibodies of the same specificity (Margni, 1994). They were found in the sera and placenta during pregnancy where they specifically block paternal antigens contributing to the fetal protection (Malan Borel, 1991; Margni and Malan Borel, 1998; Zenclussen *et al.*, 2001).

Together, all the described mechanisms work as a network to mediate the immune tolerance towards the fetus. Compensatory mechanisms may occur in order to protect life by balancing the immunity during pregnancy and ensure the fetal survival while maintaining the functionality of the maternal immune responses towards possible pathogenic factors.

1.1.4- Immune cells at the feto-maternal interface

1.1.4.1- Uterine Natural Killer cells (uNKs)

uNKs constitute a great majority of lymphocytes (65%-70%) in the pregnant uterus in both humans and mice. Their probable origin relies on bone marrow derived leucocytes that migrate to the pregnant uterus. In mice, they proliferate and grow from day 5 to day 7 of pregnancy by an estrogen and progesterone regulated mechanism and acquire cytoplasmic granules while producing IFNγ which induces vascular modifications and contributes to the uNK maturation and senescence. After day 8 of pregnancy apoptotic events lead to a substantial decrease in the uNK population. They have a role on promoting trophoblast migration and invasion and development of spiral arteries and placentation, helping therefore in the establishment of a triumphant pregnancy outcome (Guimond *et al.*, 1998; Ashkar *et al.*, 2000; Greenwood *et al.*, 2000; Ashkar and Croy, 2001). IL-15 knockout mice, which lack uNKs, have however normal litter size despite insufficient spiral arteries

formation (Barber and Pollard, 2003). It was suggested that the pregnancy-specific bias of uNK cells toward HLA-C recognition arises as developing uNK cells interact with uterine stromal cells, which express higher levels of HLA-C during pregnancy (Male *et al.*, 2011).

1.1.4.2- Mast cells (MCs)

Famous for their role in allergic diseases, MCs have been described as important mediators of implantation. Their degranulation results in the release of several factors like histamine, matrix metalloproteases (MMPs), tryptase and vascular endothelial growth factor (VEGF), which are key players in the embryo attachment and uterine invasion and promote angiogenesis during pregnancy. This hypothesis was supported by studies in which the migration of MCs from the periphery to the uterus and their posterior maturation and degranulation was shown to be modulated by estradiol and progesterone (Jensen *et al.*, 2010) This was also observed during the estrus cycle in mice, where the uterine expansion of MCs seems to occur in cycles with a peak during the estrus phase (Woidacki *et al.*, submitted). Additionally, MCs were described as having a role in self-tolerance and tissue protection being intermediaries in T regulatory cell dependent mechanisms of allograph tolerance (Lu *et al.*, 2006). This fact inspired the study of MCs mediated tolerance during pregnancy resulting in the findings that MCs deficient adult mice (C57/BL6J-Kit $^{W\text{-}sh/W\text{-}sh}$ mice) have implantation problems which can be reverted by reconstituting these animals with MCs isolated from the bone marrow of wild type mice (Woidacki *et al.*, submitted).

1.1.4.3- B cells

B cells, classically known as effector cells of the adaptive immune system, act as antigen presenting cells and become plasma cells producing antibodies after antigen presentation. Recently a subpopulation of B cells, the B-1a B cells, together with the auto-antibodies produced by them, was described as having a role on the development of pre-eclampsia (Jensen *et al.*, 2012). In addition, besides their ability to produce antibodies, B cells have the capacity to release cytokines. In this line, an emergent subset of B cells which are able to produce IL-10 were implicated in the maintenance of tolerance and therefore named Breg cells. They are capable of regulating the inflammatory response associated with autoimmune diseases or unresolved infections (reviewed by Mauri and Ehrenstein, 2008) but their role in mediating the feto-maternal tolerance is still unknown.

1.1.4.4- Macrophages (M)

Although some authors claim no differences between the populations of macrophages and dendritic cells (Hume, 2008) the literature is extensive concerning studies of both populations separately. Macrophages are known components of innate and adaptive immunity. They can work as APCs activating T cells and conducting an immune response. The activation of Macrophages is described to occur by two different pathways, the classical (M1) and alternative (M2) resulting in the production of different cytokines and therefore having different outcomes. When interaction with Th1 cytokines or LPS occurs the M1 activation pathway takes place triggering the destruction of microorganisms and tumor cells by means of production of TNFα, IL-12, IL-23 and low amounts of IL-10. Instead, when the interaction occurs with IL-4, IL-10 or IL-13, the M2 activation pathway is settled and high amounts of IL-10 are produced together with low amounts of Th1 cytokines,

helping in the tissue healing and angiogenesis (Stein *et al.*, 1992; Gordon, 2003; Classen *et al.*, 2009).

After natural killer cells, macrophages are the second largest decidual leukocyte population (10-20%) and accumulate close to the implantation site (Tachi and Tachi, 1986; Miller and Hund, 1996). Decidual macrophages were classified as having a M2 phenotype by showing a high production of IL-10 and IDO which confers them an immunosuppressive role. Contrasting with this view, Houser *et al.* (2011) described two unique macrophage populations in the first trimester human decidual tissue which by producing both pro-inflammatory and anti-inflammatory cytokines cannot fit the M1/M2 classification. A population of $CD11b^{+}F4/80^{+}CD11c^{-}$ macrophages was described in the lamina propria expressing several anti-inflammatory molecules, including IL-10 but no or lower amounts of pro-inflammatory cytokines, and found to be able to induce the differentiation of $Foxp3^{+}$ regulatory T cells by a mechanism dependent on IL-10, retinoic acid and TGF-β (Denning *et al.*, 2007). By another hand, an excessive activity of decidual macrophages was connected with preeclampsia (Katabuchi *et al.*, 2003). Further, trophoblast cells are able to recruit and successfully educate monocytes to produce and secrete chemokine profile supporting its growth and survival (Fest *et al.*, 2007) possibly influencing the population of macrophages.

1.1.4.5- Dendritic cells (DCs)

The tolerogenic potential of decidual DCs in pregnancy has been suggested by many but is often contradictory because of their similarities with decidual macrophages. Aditionally, the extreme low number of cells in that tissue makes their study a difficult task. As an example, dendritic cell depletion

results in abnormal placental development supporting the requirement of these cells in the establishment of a successful pregnancy. This is thought to depend on the ability to produce sFlt1 and TGF-β1 which are important regulators of tissue remodeling and angiogenesis. Dcs may also be crucial for decidua formation during implantation in mice (Plaks *et al.*, 2008). Contrasting data from Collins *et al.* (2009) support the theory that after implantation the transformation of the endometrium into decidua works as a physical barrier for the DCs which become entrapped at the feto-maternal interface and cannot contribute as antigen presenting cells (APCs) in the T cell recognition of fetus and placenta. In this way, DCs already present in lymph nodes and spleen would have access to fetal antigens passively shed into the maternal circulation. Additionally, Juretic *et al.* (2004) defend that in human pregnancies, immature dendritic cells (iDCs), together with NK cells, are recruited by the influence of extravillous cytotrophoblast cells at the implantation site. There, if under a dominant Th2 response, these DCs would become tolerogenic, or, in turn, under a strong Th1 response, they would rather become potent APCs (Juretic *et al.*, 2004). However, the fact that Th1 cytokines are of extreme importance during implantation brings this theory a significant revision topic.

1.1.4.6- γδ T cells

γδT cells are a unique population of T cells known to play both an effector and regulatory role in vivo. They are important players of the innate immune system and under episodes of cell infection or stress, they are able to recognize allo and self-antigens and work for the maintenance of tissue homeostasis (Carding and Egan, 2002). Two populations of γδT cells were described in the murine decidua: an early population producing Th1 cytokines, and a later Th2/3 cell subset (Arck *et al*., 1999).These cells have

also been described in the peripheral blood of healthy pregnant women (Szekeres-Bartho *et al.*, 2001). Decidual $\gamma\delta TCR^+$ cells are mostly $CD4^-CD8^-$T cells with cytolytic properties through the expression of perforin, granzyme A and B and FasL. However, the expression of IL-10 and TGF-β also confers them immunomodulatory capacities (Nagaeva *et al.*, 2002; Mincheva-Nilsson, 2003).

1.1.4.7- Regulatory T cells

Regulatory T cells were first described as a population of $CD4^+$ $CD25^+$ T cells actively suppressing self-reactive lymphocytes and participating in the maintenance of immunological self-tolerance (Sakaguchi, 1995). They are now known to have an essential role in preventing autoimmune diseases and inducing tolerance towards allogeneic grafts. Their role in the fetal immune tolerance has been shown by several studies in both mice and humans (Aluvihare *et al.*, 2004; Heikkinen *et al.*, 2004; Saito *et al.*, 2005; Zenclussen *et al.*, 2005). $CD4^+CD25^+$T cells expand in almost all tissues of pregnant mice compared to non-pregnant female mice (Aluvihare *et al.,* 2004; Zenclussen *et al*., 2005). Interestingly, it was observed that this expansion is not continuous throughout pregnancy but it slowly declines from mid-gestation reaching the initial levels, as non-pregnant, after delivery (Zhao *et al.*, 2007). In accordance, a systemic increase of $CD4^+CD25^+$ in the blood of pregnant women was described at the start of pregnancy, peaking in the second trimester and declining to the initial levels once the child was born (Heikkinen *et al.*, 2004; Somerset *et al.*, 2004). Women going under repeated abortions showed a lower frequency of Treg in the blood and these Treg had a lower suppressive capacity compared with normal fertile women (Arruvito et al., 2007).

$CD25^+$ T cells depletion resulted in gestation failure (Aluvihare *et al.*, 2004; Darrasse-Jezè *et al.*, 2006; Shima *et al.*, 2010). After treatment with PC61 anti-CD25 antibody, allogeneic pregnant mice exhibited higher abortion rates followed by an activation of the immune system with expansion of activated $CD8^+$ and $CD4^+$ T cells in the uterine draining lymph nodes. This did not occur in the syngeneic pregnancies (Darrasse-Jezè *et al.*, 2006). Implantation failure was also observed after PC61 induced Treg depletion was performed in the first two days of pregnancy (Zenclussen *et al.*, 2005). Abortion-prone mice which were shown to have fewer $CD4^+CD25^+$ decidual cells, were protected from fetal rejection after the adoptive transfer of Treg from normal pregnant females when the transfer was done in between days 0 and 2 of pregnancy. These Treg were only protective when the donors were pregnant which suggests that Treg are essential in the beginning of pregnancy and they need to be exposed to paternal alloantigens in order to exert a protective effect in vivo (Zenclussen *et al.,* 2005). Accordingly, primary unexplained infertility has been related with a lower expression of FOXP3 mRNA in endometrial tissue of women (Jasper *et al.*, 2006).

The allogeneic dependence of Treg during pregnancy is still a topic under discussion. An initial report from Aluvihare *et al.* (2004) described a systemic expansion in Treg population in both syngeneic and allogeneic pregnancies. However, a more recent report from Zhao *et al.* (2007) shows anti-paternal alloantigen dependent increase in Treg numbers. Another study confirmed an early increase in Treg prior to implantation (Thuere *et al.*, 2007).

Several reports also describe the implications of hormonal fluctuations in the expansion of Treg pool. The induction of Foxp3 was observed *in vitro* in the presence of E2 (Polanczyk *et al.*, 2004; Tai *et al.*, 2008) however there was a poor correlation in vivo when analyzing Treg numbers in lymph nodes of pregnant mice and comparing it with E2 serum or uterine levels (Tai *et al.*,

2008). Additionally, cyclic accumulation of Treg seems to occur during the estrus phase of the oestrus cycle as a result of estrogen induced expression of chemokines which receptors are expressed by Treg (Kallikourdis *et al.*, 2007). A similar process was suggested to occur during the menstrual cycle of women (Arruvito *et al.*, 2007). However, Zhao and colleagues have observed that neither estrogen nor progesterone alone or in combination had an influence on the number of Treg in ovariectomized mice (Zhao *et al.*, 2007). Changes in Treg before implantation could also not be explained thought the effects of hormones alone (Schuurs *et al.*, 1990; Aluvihare *et al.*, 2004).

Human chorionic gonadotropin (hCG) has an important role in the beginning of pregnancy being produced by the blastocyst and by synciotrophoblast cells (Hoshina *et al.*, 1985). The luteinizing hormone/coriogonadotropin (LH/CG) was shown to elicit the migration of Treg *in vitro* and Treg expressed this receptor when exposed to hCG (Schumacher *et al.*, 2008) which suggests that this hormone acts as an attraction factor of Treg to the implantation site during early pregnancy.

1.1.5- Regulatory T cells - Overview

Treg were first described in the 70s as “suppressor T cells” (Gershon *et al.*, 1970). However, their true existence was criticized until 1995 when Sakaguchi and colleagues finally identified them as a specialized T cell subset capable of suppressing self-reactive lymphocytes and participating in the maintenance of immunological self-tolerance (Sakaguchi *et al.*, 1995).

Among a set of several described subsets of Treg, there are three divisions of Treg clearly defined:

1.1.5.1- Type 1 regulatory cells (Tr1)

Tr1 are distinguished by the production of IL-10 and TGF-β (Beissert *et al.*, 2006) together with smaller amounts of IL-15 and IFN-γ (Roncarolo *et al.*, 2001). Their development, like Th1 and Th2 cells, is believed to come from naïve T cells under the stimulation of the local cytokine environment, especially by IL-10 (McGuirk *et al.*, 2002). Most of their immunosuppressive effects are blocked by IL-10 neutralizing antibodies (Roncarolo *et al.*, 2001) suggesting IL-10 as the main mechanism of suppression employed by these cells.

1.1.5.2- T-helper 3 (Th3) cells

Th3 are defined by the production of high levels of TGF-β and low or absent levels of IL-2, IL-4 and IL-10 (Mosman *et al.*, 1996). Their generation is suggested to be stimulated by the cytokine milieu and antigen presentation in the local tissue. Besides characterizing the mechanism of action of these cells, the production of TGF-β seems to promote their development acting like a positive feedback (Daynes *et al.*, 1990; Seder *et al.*, 1998; Weiner, 2001).

1.1.5.3- $CD4^+CD25^+$ T regulatory cells (Treg)

Markers

Treg are distinguished by their characteristic surface markers: the IL-2 receptor CD25 (Sakaguchi *et al.*, 1995), CD127 (human only) (Liu *et al.*, 2006), GITR (McHugh and Shevach, 2002), CTLA-4 (Takahashi *et al.*, 2000) and a unique intracellular marker, X-linked Forkhead box P3 (Foxp3) (Fontenot *et al.*, 2005).

Foxp3 has been validated as the "master switch" for Treg development (Fontenot *et al.*, 2005). Mutations in this gene result in mice with a scurfy phenotype by the development of an autoimmune disease characterized by a hyper-activation of $CD4^+$ Tcells, multi-organ lymphocytic infiltration, enlarged spleen, lymph nodes and liver as well as dermatitis and severe runting (Godfrey *et al.*, 1991; Blair *et al.*, 1994; Kanangat *et al.*, 1996). In humans, FoxP3 mutations have been found in X-linked autoimmune diseases (Bennet *et al.*, 2001).

Origin

$CD4^+CD25^+Foxp3^+$ Treg are now known to be generated in two distinct ways, thymus derived naturally occurring Treg (nTreg) and T reg induced in the periphery (iTreg). While in the thymus Treg are generated from thymocytes expressing TCR with a heightened reactivity for *self*-antigens, in the periphery they are converted from $CD4^+$T cells stimulated with high affinity cognate TCR receptors in the presence of TGF-β and retinoic acid (Chen *et al.*, 2003; Zheng *et al.*, 2004; Kretschmer *et al.*, 2005; Hall *et al.*, 2011). nTreg express glucocorticoid induced TNF receptor family-related gene (GITR) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) on their surface and produce soluble TGF-β and IL-10 (Sakaguchi, 2004). iTreg proliferate after being exposed to antigens in the periphery (von Boehmer, 2003)

Mechanisms of Treg suppression and control

The maturation state of APCs, like DCs, usually through the expression of co-stimulatory molecules (CD80/CD86) and their cytokine profile, is determinant in the outcome of the interactions between DCs and T cells. Conversion of naïve T cells into Treg has been described as resulting from interactions with so called tolerogenic DCs defined as immature or semi-immature DCs by the low expression of IL-12 and TNF-α and variable amounts of MHC class II and

CD80/CD86 (Rutella *et al.*, 2006). Contrasting, the expansion and activation of pre-existing Treg depends on the contact with mature DCs with high expression of MHC class II, CD80/CD86 and IL-12 secretion (Rutella *et al.*, 2006) which seem more active under inflammatory circumstances (Yamazaki *et al.*, 2003).

The diversity of Treg functions is characterized by suppressing proliferation and cytokine production of $CD4^+$ and $CD8^+$ cells (Piccirillo *et al.*, 2001; Mempel *et al.,* 2006) and inhibiting the cytotoxicity of NK cells (Ghiringhelli *et al.*, 2005). They further inhibit APCs such as DCs and macrophages (Cederbom *et al.*, 2000; Mirsra *et al.*, 2004; Taams *et al.*, 2005) and suppress the proliferation of B cells and their immunoglobulin production (Lim *et al.*, 2005).

Treg seem to exert the suppressive actions mostly in a contact dependent manner potentiated by the action of IL-10 and TGF-β and CTLA-4 (Hara *et al.,* 2001; Wahl et al., 2004; Friedline *et al.*, 2009). It was recently shown, however, that Treg have a higher expression of IL-2 receptors that can act as competitors for IL-2 causing its deprivation in adjacent Tcells and thus blocking the IL-2 dependent process of T cell activation and proliferation (de la Rosa *et al.*, 2004; Scheffold *et al.*, 2005).

In addition, IDO expression by tolerogenic DCs is stimulated by receptor-ligand interactions of CTLA-4 expressed on Treg cells and CD80 and CD86 ligands expressed on DCs resulting in decreased activation of T cells (Grohmann *et al.*, 2002). Whether this is relevant *in vivo* it is still a matter of discussion.

Recently, it has been suggested that Treg express IL-9 with important functions in induce suppression by interacting with immunosuppressive MCs (Lu *et al.*, 2006; Eller *et al.*, 2011; Feng *et al.*, 2011; Smith *et al.*, 2011).

1.1.6- Chemokines

The active migration of cells to and within tissues does not occur randomly but results from a coordinated process in which several factors are involved. Among these, chemokines are established key regulators of leucocyte trafficking. They are small secreted proteins grouped into CXC, CC, C and CX3C subfamilies based on the arrangement of the two NH2-terminal cysteine residues. Their effect is mediated by binding to 7-transmembrane-spanning G protein- couple receptors expressed on target cells (Murphy *et al.*, 2000; Zlotnik and Yoshie, 2000).

Treg migration is driven by a dynamic process involving the expression of chemokine ligands within the tissues and their chemokine receptor on the surface of the Treg (Mantovani, 1999; Bono *et al.*, 2007). These factors are critical in the determination of local tolerance mechanisms promoted by Treg in specific tissues. Among the chemokines involved in the process of Treg migration, CCR7 and CCR9 were specially addressed in this thesis.

1.1.6.1- CCR7 and its ligands

Chemokine receptor 7 (CCR7) is expressed on B cells, T cells and DCs. CCR7 and its ligands are involved in the regulation of T cells and antigen presenting cells homing, functional organization of lymphoid organs and thymus compartmentalization once it mediates the migratory events of Tcell development (reviewed in Förster *et al.*, 2008). Thus, it mediates the migration of nTreg to the paracortical areas of lymph nodes under steady state conditions (Ueha *et al.*, 2007)

CCL19 and CCL21 are ligands for CCR7. CCL21 is characterized by a long C-terminal tail containing basic amino acid residues that, by binding to glycosaminoglycans and other molecules seems to be important for efficient presentation of this ligand on the surface of endothelial cells (Gunn *et al.*, 1998; Yoshida *et al.*, 1998). It is also referred as exodus-2 (Hromas *et al.*, 1997) or secondary lymphoid tissue chemokine (SLC) (Nagira *et al.*, 1997) and, since it is unusually constituted by six conserved cysteine residues it has also been specified as 6Ckine (Hedrick and Zlotnik, 1997). In mice, two forms of this chemokine were reported, CCL21-Ser and CCL21-Leu, being encoded by different genes whose products differ by only one aminoacid at position 65 (Vassileva *et al.*, 1999). While CCL21-Leu seems to be expressed in the periphery by initial lymphatic vessels, CCL21-Ser is expressed in lymph nodes including terminal lymphatic vessels of the subcapsular sinus (Chen *et al.*, 2002). CCL19 is also known as EBI-1 ligand chemokine (ELC), macrophage inflammatory protein 3 beta (MIP-3 beta) and exodus-3. It induces the development of dendritic extensions on DCs being therefore implicated on the migratory responses of these cells (Yanagawa and Onoé, 2002).

1.1.6.2- CCR9 and its ligand

CCR9 (Youn *et al.*, 1999) is expressed on most intraepithelial lymphocytes and lamina propria Tcells. It is also expressed by thymic DCs (Vicari *et al.*, 1997) and was recently described in a distinguishable population of immature tolerogenic plasmocytoid DCs (pDC) which were efficient in the induction of Treg (Hadeiba *et al.*, 2008). CCR9 expression was observed on the cell surface of both $\gamma\delta$ and $\alpha\beta TCR^+$ thymocytes playing an important role in T cell development in both T cell lineages (Uehara *et al.*, 2002). In the periphery its expression was found in naive CD8 T cells but not naive CD4 T cells (Wurbel *et al.*, 2006).

CCL25, also known as thymus-expressed chemokine (TECK) (Vicari *et al.*, 1997) is mostly expressed on thymus and has a supporting role on T cell development being involved on the migration, localization and maturation of double positive thymocytes (Youn *et al.*, 1999). Additionally, it participates in the recruitment of lymphocytes, like T cells and IgA-secreting plasma cells to the gut (Zabel *et al.*, 1999; Bowman *et al.*, 2002; Lazarus *et al.*, 2003; Pabst *et al.*, 2004) and is also expressed in the ovaries supporting ovulation (Zhou *et al.*, 2005; Zhou *et al.*, 2009).

1.1.7- Hypothesis:

There are extensive evidences for the expansion of Treg during pregnancy. In addition, wide reports of pregnancy impairment are correlated with diminished Treg populations or an inappropriate Treg function. Together this information suggests a role for Treg in tolerant mechanisms supporting the implantation and/or maintenance of a fetus during pregnancy. However, if Treg have a redundant role in pregnancy establishment and following developmental stages is still poorly understood. Furthermore, the mechanisms behind Treg development and function as well as their origin and kinetics during different stages of pregnancy are questions under debate.

The experiments described in this thesis aim to explore the following hypothesis:

- Treg are essential in pregnancy establishment and/or maintenance.
- The origin of Treg depends on the stage of pregnancy and their expansion is mediated by alloantigens and stimulated by soluble factors that affect pregnancy outcome.

- Treg migrate to the feto-maternal interface under the participation of migratory molecules influencing pregnancy establishment and/or maintenance.

To study the hypothesis above the following aims were addressed:

- To analyze the importance of Treg in the establishment and maintenance of pregnancy as well as their mechanisms of action at the feto-maternal interface.
- To unveil the origin of Treg during different phases of pregnancy as well as the mechanisms behind their generation and action.
- To investigate the factors regulating Treg migration to the feto-maternal interface and their consequent importance in pregnancy establishment and maintenance.

-CHAPTER 2-

MATERIAL AND METHODS

2.1- ANIMALS AND SURGERIES

C57/BL6, CBA/J, DBA2J and BALB/c mice were purchased from Charles River, Germany. Rag1$^{-/-}$ mice were purchased from Jackson laboratory. Foxp3DTR mice (Kim *et al.*, 2007) and Foxp3GFP mice (Fontenot *et al.*, 2005) were kindly provided respectively by Alexander Rudensky (Washinghton, USA) and by Jocelyne Demengeot (Oeiras, Portugal) upon MT agreement. CCR9$^{-/-}$ mice (Wurbel *et al.*, 2001) were kindly provided by Immo Prinz (Hannover, Germany) and CCR7$^{-/-}$ mice were purchased from Jackson Laboratories, USA. These strains were bred and maintained at the Animal Facilities of the Medical Faculty, Otto-von-Guericke University, Magdeburg.

All animals had 6-12 weeks of age at the time of the experiment. They were housed in a barrier facility in a 12:12 hr light-dark cycle and received food and water *ad libitum*. Animal care and experimental procedures were followed upon institutional approval from Berlin (Reg 070/03) and Magdeburg (AZ-42502-2-868) and followed the guidelines of Use of Agricultural Animals in Agricultural Research and Teaching, USA. Exact number of animals used for each experiment is stated in the figure legends.

2.1.1- Matings and determination of day 0 of pregnancy

Animals were mated in cages with 1 male and a maximum of 3 females each. After mating, females were checked daily for vaginal plugs. The day at which the vaginal plug was detected was considered day 0 of pregnancy and the females were separated from the males. The nature of the matings as well as the strained used are stated in the figure legends.

2.1.2- Animal surgeries

Animals were prepared on the indicated gestation day. Blood samples were collected either by heart punction or by retro-orbital puncture after mice were anaesthetized using 250 µl of a solution made of 50 mg/ml ketamin (Pharma Partner, Germany) and 20 mg/ml Rompan (Bayer, Germany) which had been diluted to 1ml using isotone NaCl solution (B/ Braun). Animals were sacrificed by cervical dislocation under anaesthesia.

2.1.3- Monitorization of the oestrus cycle

The vaginal lavage of non-pregnant C57/BL6 female mice was performed with 20 µl of 0.9% sodium chloride every morning in order to identify the phase of their oestrus cycle which was defined by observation of the cellular components of the fresh lavage observed under light microscopy (Axiovert C, Carl Zeiss, Germany; magnification x200). The females were only sacrificed when the stage of the cycle was clear which was further confirmed by light microscopy after additional hematoxylin/eosin staining of the cellular content in the lavage (Axio Observer.A1, Carl Zeiss; magnification x200).

2.1.3.1- Hematoxylin/eosin staining

The slides with the smears from the vaginal lavage were shortly rinsed by dipping in Aqua distillate and covered with a drop of hematoxylin (Fluka Biochemika, Germany) for 1-2 min followed by rinse with warm tap water until blueing under visual check. They were then covered with a drop of eosin (Fluka Biochemika, Germany) for 1-2 min. Further they were dehydrated by dipping in 75% EtOH (Otto Fischer, Germany) (10 dips), 95% EtOH (10 dips), 100% EtOH (10 dips), and 2 times in Xylol (Roth, Germany) 2 minutes each. The slides were mounted with Roti-Histokitt (xylol-soluble

mounting medium; Roth, Germany) and observed under light microscopy (Axio Observer.A1, Carl Zeiss; magnification x200).

2.1.4- Quantification of the number of implantations or resorptions

To clearly quantify the number of implantations on day 5 of pregnancy, mice were anesthetized and injected i.v. with 0,5% Chicago Blue dye (Sigma-Aldrich, Germany) 2 minutes prior sacrificing the animal. For the other pregnancy time points, the females were sacrificed, the uteri removed and the implantation sites together with the resorptions were documented after analysis with the naked eye.

2.1.5- In vivo cell transfer

Cell suspensions of total lymph nodes (LN) from Foxp3GFP mice were enriched for $CD4^+$ T cells by the use of a negative selection kit (Miltenyi Biotec). Treg cells from suspensions of total lymph nodes of congenic C57/BL6 mice were isolated using a $CD4^+CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotec). $CD4^+Foxp3^{GFP}$ T cells or congenic $CD4^+CD25^+$ T cells were further labeled with rat anti-mouse Alexa Fluor 647 conjugated anti-CD4 mAb (Clone RM4-5, BD Pharmingen) and PE conjugated anti-CD25 mAb (Clone PC61, BD Pharmingen) and sorted by flow cytometry on a Diva cell sorter or FACSAria (BD Biosciences). Purified cells were then transferred intravenously into $Rag1^{-/-}$ mice (3×10^5 $CD4^+Foxp3^{GFP-}$ cells and 10^5 $CD4^+CD25^+$ cells). The conversion of $CD4^+Foxp3^{GFP-}$ cells into $CD4^+Foxp3^{GFP+}$ cells was assessed 4–12 weeks later by flow cytometry.

Total thymocytes from Foxp3GFP mice were transferred intravenously into $Rag1^{-/-}$ mice.

2.1.6- Treatments

2.1.6.1- Diphtheria toxin (DT) treatment

Intraperitonal application of 25 μg/kg body weight DT (Sigma-Aldrich, Germany) was made every fourth day after the observation of a plug or preceded by 3 applications before mating. DT treatment resulted in depletion of more than 50% of $Foxp3^{GFP+}$ cells from both uterus and uterine draining lymph nodes in comparison with controls.

2.1.6.2- Vaginal application of TGF-β

As in the report from Clark et al. (2008), 2ng of Human TGF-β1 (MACS, Miltenyi Biotec) in 10 μl of 1% BSA in PBS were applied in the vagina of the animals on the day of mating and every other second day until day 5 of pregnancy.

2.1.6.3- Anti-TGF-β mAb treatment

1 mg of anti TGF-β mAb (clone 1D11 kindly provided by Prof Hideo Yagita, Japan) or Rat IgG (Sigma Aldrich) was injected subcutaneously every 7th day of pregnancy in the reconstituted animals starting on day 0 of pregnancy.

2.1.6.4- IL-2 treatment

Two daily IL-2 injections (IL-2 was cloned by our collaborators in the lab of Enrique Montero, Center of Molecular Immunology, Havana) at different concentrations ($5x10^5$ or $5x10^6$ IU/kg) were administered subcutaneously for five consecutive days starting on day 0 of pregnancy.

2.2- LYMPHOCYTE PREPARATION AND ANALYSIS

2.2.1- Lymphocyte isolation

Tissues were crushed and filtered using a 100 µM cell strainer (BD Biosciences) in a 6 well plate. 5 ml of RPMI medium (Invitrogen, Gibco, Karlsruhe) was added and cells were then transferred into tubes. When the tissues isolated were placenta or blood, up to 50 ml of lysis buffer was added to the tissues and incubated for 10 min at room temperature. The cells were then centrifuged at 1200 rpm for 10 min. The pellet was washed in 20 ml of RPMI at 1200 rpm for 10 min. After discarding the supernatant, the cells were resuspended in 1 ml RPMI (+10% FBS).

2.2.2- Decidual lymphocyte isolation

The isolation of decidual lymphocytes was done as following: decidual tissues were washed with PBS, cut into small pieces and collected in HBSS medium (Invitrogen, Gibco, Karlsruhe) containing 1 mM 1,4-Dithio-DL-threitol (DTT) (Fluka Chemie GmbH, Buchs). After incubation at 37°C for 20 min under mechanical agitation the cells were filtered through a 100 µm net into fresh tubes and centrifuged at 1200 rpm for 10 min at 4°C after which the supernatant was discarded and cells resuspended in 1 ml of RPMI medium (Invitrogen, Gibco, Karlsruhe).

The protocol was repeated twice using HBSS without DTT. At the end, the supernatants were collected and washed with RPMI medium containing 10% FBS and a Ficolite-M solution (Linaris, Bettingen am Main) was added to the tube containing the cells and a gradient separation was performed by centrifugation at 2400 rpm for 20 min without breaks. The middle layer, where lymphocytes were concentrated, was transferred into a new tube and

RPMI + 10% FBS (Cambrex Bio Science Verviers, Verviers, Belgium) was added and centrifuged at 1500 rpm for 10 min at 4°C. The pellet was then resuspended in FACS buffer.

2.2.3- Antibodies (Abs)

The following rat anti-mouse mAbs were used; FITC and Alexa Fluor 647 conjugated anti-CD4 (Clones RM4-4 and RM4-5 respectively; BD Pharmingen), PE conjugated anti-Foxp3 (Clone NRRF-30; eBioscience), APC conjugated anti-Helios (Clone 22F6; BioLegend), PE conjugated anti-I-A/I-E (Clone M5/114.15.2; BD Pharmigen). Hamster anti-mouse APC conjugated CD11c (Clone HL-3; BD Pharmingen) and PE and FITC conjugated anti-CD80 (Clone 16-10A1; BD Pharmigen) mAbs were used. Foxp3 and Helios intracellular staining was performed by using the eBioscience staining set.

2.2.4- Flow cytometry analysis

Expression of each molecule was analysed using FACSCalibur (BD Biosciences) with CellQuest Pro software (BD Biosciences) or Flowjo (Tree Star, Inc.).

2.2.5- Treg MACS isolation

Treg cells were isolated using a $CD4^+CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotec). The $CD4^+$ T cells in MACS Buffer were pre-enriched by indirect magnetic labeling the non-$CD4^+$ T cells with a cocktail of biotin-conjugated antibodies and Anti-biotin microbeads. The cells were labeled in parallel with CD25-PE. Cell suspension was loaded onto a MACS column which was subjected to magnetic field of a MACS separator. The flow output

was enriched in $CD4^+$ T cells while the magnetically labeled unwanted cells were retained in the column. The $CD25^+$ PE labeled cells in the enriched $CD4^+$ T cells were magnetically labeled with anti PE microbeads. Cell suspension was loaded into the column subjected to magnetic field of a MACS separator. The magnetically labeled $CD4^+CD25^+$ were retained in the column while the unlabelled cells flow down. Finally, the column was removed from the magnetic field and labeled cells were eluted as the positively selected cells. The purity was improved by performing another run in a new column and the cells were washed twice and counted.

2.3- CHEMOTAXIS ASSAY

Recombinant murine CCL21 and CCL25 were purchased from R&D Systems (Germany). Total iliac lymph nodes cells were suspended in 100 µl assay medium (RPMI plus 10% FBS) and loaded into 5 mm pore filter transwells inserts (Corning Costar, Cambridge). CCL21 (200 ng/ml) or CCL25 (1000 ng/ml) diluted in assay medium or medium alone in the volume of 600 µl was added to the bottom well, and both migrated cells and cells remaining in the insert were collected after a 120 min incubation at 37°C and stained with rat anti-mouse Alexa Fluor 647 conjugated anti-CD4 (Clone RM4-5, BD Pharmingen). Triplicates of input and migrated $CD4^+Foxp3^{GFP+}$ cells were quantified by flow cytometry and the rate of migration was determined by the analysis of the cell number and frequency in the input and migrated population.

2.4- REAL TIME PCR

2.4.1- RNA extraction and quantification

1 ml of Trizol (Invitrogen, Paisley, UK) was added to a maximum of 100 mg of tissue. The tissues were squeezed using a homogenizator (Ultra Turrax T8) and 200 µl of chloroform (Sigma-Aldrich Chemie GmbH, Steinheim) were added into the tubes. After gently inverting the tube several times by hand to mix it, centrifugation was done for 15 min at 12000 g at 4°C. The upper aqueous phase was collected and transferred into an RNA-se free tube. Cold isopropanol in a ratio 1:1 was added and RNA was allowed to precipitate at -20°C for 10 min. The tubes were centrifuged at 12000 g for 15 min at 4°C and pellets were washed in 500 µl of 80% ethanol. Centrifugation was then done at 7500 g at 4°C for 5 min. Samples were washed twice and the pellets were left to dry for 5 min. RNA was resuspended in a maximum of 30 µl of RNA-se free water and quantification was done spectrophotometrically (absorbance at 260 nm) and diluted to a concentration of 1 µg/µl. The samples were kept at –80°C.

2.4.2- cDNA synthesis

2 µl odT (Amersham Pharmacia, Freiburg) was added to 2 µg of RNA and RNA-se free water was added to a total volume of 18 µl. Samples were incubated at 75°C for 10 min and then left on ice for 2 min before adding a mix of 8 µl of 5x M-MLV RT Buffer, 4 µl RNA-se free water, 4 µl dNTP (2,5 mM) (Amersham Pharmacia, Uppsala, Sweden), 2 µl DNA-se (2u/µl) (Ambion, Huntingdon, UK) and 0.5 µl of RNA-se inhibitor (40u/µl) (Promega, Mannheim). They were then incubated for 30 min at 37°C and 5 min at 75°C. After incubation on ice for 2 min 1 µl Reverse Transcriptase (Promega, Mannheim) and 1 µl RNA-se inhibitor was added to each sample. Following,

samples were incubated at 42°C for 60 min and then 94°C for 5 min and samples were kept at –20°C.

2.4.3- RT-PCR

Amplification was performed by mixing 1 µl DNA (1mg/µl) with 6,25µl SYBR Green Master Mix (Applied Biosystems, Germany), 3 µl primer mix, 0,5 µl of 50nM Fluorescein and 2 µl of RNA-se free water, using a final reaction volume of 13 µl. Initial denaturation was done at 95°C for 5 min followed by a denaturation step of 40 cycles of 45 sec at 95°C. Annealing step followed at 60°C for 60 sec. The reactions were carried out on an iCycler (BioRad, Germany). Each single sample was amplified in duplicates for each primer set. The quantification of cDNA for each sample was normalized to the housekeeper gene β-actin using the $2^{-\Delta Ct}$ methodology to allow the data to be expressed as a fold change relative to the mRNA content of the tissue. All the primers used were previously titrated to set the preferential conditions for the reaction. Forward and reverse primer sequences used are resumed in the Table 2.1.

Table 2.1- PCR Primers used for RT-PCR analysis

Target mRNA	Primer sequence
CCL21	Fw- 5`-CAG GAC TGC TGC CTT AAG TA-3` Rv- 5`-GCA CAT AGC TCA GGC TTA GA-3`
CCL25	Fw- 5`-AAG ACT GCT GCC TGG GTT AC-3` Rv- 5`-TGG CAC TCC TCA CGC TTG TA- 3`
CCL1	Fw- 5`-GTT GCT TCT CAT TTG CGG AG-3` Rv- 5`-GGT GTA GGG CTG GTA GTT TGC-3`
CCL4	Fw- 5` -CTC TCT CTC CTC TTG CTC GT-3` Rv- 5`-CTC-CAA-GTC-ACT-CAT-GTA-3`
CXCL12	Fw- 5`-GAG CCA ACG TCA AGC ATC TG-3` Rv- 5`-CGG GTC AAT GCA CAC TTG TC-3`
CCL20	5`-CCA GGC AGA AGC AAG CAA CT-3` 5`-TCG GCC ATC TGT CTT GTG AA-3`
β-actin	Fw- 5` -GCT TCT TTG CAG CTC CTT GGT T-3` Rv- 5`-GTT GTC GAC GAC CAG CGC-3`

2.5- STATISTICAL ANALYSIS

Groups were compared with GraphPad Prism software. The number of animals/samples used for each experiment is detailed in the figure legends. To determine the normality of the population D'Agostino-Pearson normality test was used. Data representation form and the tests used for group comparison in each experiment are in detail in the figure legends.

-CHAPTER 3-

THE IMPORTANCE OF T REGULATORY CELLS ON PREGNANCY ESTABLISHMENT AND MAINTENANCE

3.1 - INTRODUCTION

It is clear since more than a decade that paternal antigens expressed in fetal structures are not ignored but actively tolerated by the maternal immune system (Tafuri *et al.*, 1995).

Both maternal and fetal regulatory T cells (Treg) have been reported to contribute to the acquisition and maintenance of tolerance during pregnancy by suppressing maternal immune responses against paternal alloantigens in fetal cells (Zenclussen *et al.*, 2005; Mold *et al.*, 2008).

In humans, an increased population of Treg has been associated with a successful pregnancy outcome and augmented abortion rates are coincident with a missing expansion on this special T cell population (Sasaki *et al.*, 2004; Arruvito *et al.*, 2007). However, while it is clear that Treg positively influence the pregnancy outcome, it is still not clear if they have an essential role in the onset and/or maintenance of pregnancy.

Evidences suggest that Treg manifest their function through mechanisms which include the secretion of immunosuppressive soluble factors like IL-9, IL-10 and TGF-β (Kohyama *et al*., 2004; Gangi *et al.*, 2005; Lu *et al.*, 2006; Eller *et al.*, 2011). Some of these were previously shown to influence pregnancy success (Schumacher *et al.*, 2007; Clark *et al.*, 2008), however the mechanisms of action of Treg in pregnancy are still under debate.

The experiments described in this chapter intend to question the dependence of pregnancy on Treg. By using a dyphteria-toxin mediated Treg depletion model the necessity of these cells for either a successful pregnancy establishment or outcome is analyzed. Additionally, the mechanisms of action and regulation of this cell population throughout particular stages of pregnancy are evaluated.

3.2 – KINETICS OF TREG POPULATION DURING THE OESTRUS CYCLE

The implantation of the fetus expressing paternal antigens represents a strong antigenic challenge, which would be expected to result in a fast immunological response. The most expectable way for the immune system to deal with this problem would be to stop such an anti-fetal response as it begins. Therefore it is to hypothesize that mechanisms have evolved that allow the uterus to adapt to a possible implantation event even before fecundation takes place.

The murine oestrus cycle is characterized by four different phases (diestrus, proestrus, estrus and metestrus) preparing cyclically the body for a possible pregnancy which can only take place during the fertile period of the cycle, the estrus phase. This is additionally the only period at which females are sexually receptive to males. As a result of specific hormonal input characterizing each particular phase of the cycle it is to expect that the systemic and uterine populations of Treg are also changing with those hormonal specific changes.

3.2.1- The population of Treg fluctuates during the oestrus cycle peaking at estrus, the receptive phase, in the mucosal tract of the reproductive system

To characterize the behavior of the population of Treg during the estrus cycle the confirmation of different phases of the estrus cycle (diestrus, proestrus, estrus and metestrus) was performed in non-pregnant Foxp3^{GFP} female mice by analyzing a smear of their vaginal lavage under light microscopy (Fig 3.1).

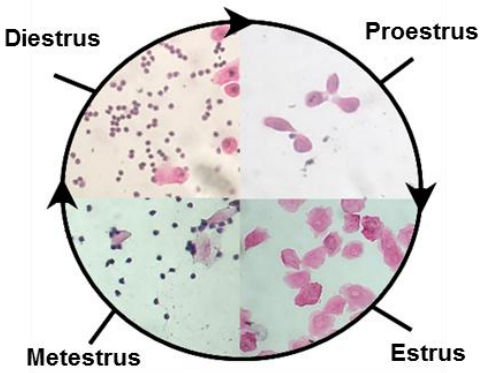

Fig 3.1- Vaginal smears of all four phases of the estrus cycle of non-pregnant females under light microscopy after hematoxylin/eosin staining. Proestrus consists primarily of nucleated epithelial cells. Estrus consists of enucleated cornified cells. Metestrus consists of nearly similar proportion among leukocytes, cornified and nucleated epithelial cells. Diestrus primarily consists of leukocytes. (magnification x200).

The population of $CD4^{+}Foxp3^{GFP+}$ cells was analysed by flow cytometry on the vaginal lavage of $Foxp3^{GFP}$ mice in the different phases of the cycle. An increase in this cell population could be detected at estrus which is the receptive phase of the cycle. Treg frequency decreased again towards metestrus before the restart of a new cycle (Fig 3.2).

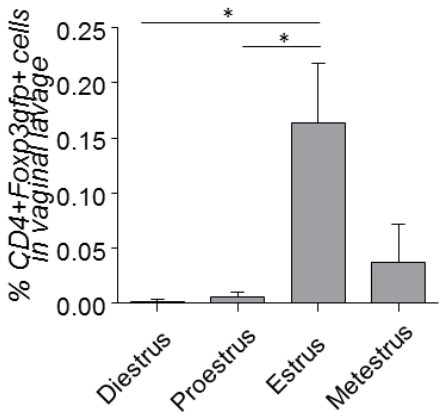

Figure 3.2- Percentage of $CD4^{+}Foxp3^{GFP+}$ cells in the vaginal lavage of female Foxp3GFP mice in different phases of the cycle. (n=5 animals per group) Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05).

The peak of Treg was observed in vaginal lavage but not in other organs as revealed when analyzing the $CD4^+Foxp3^+$ cell populations in thymus and uterine draining lymph nodes of C57/BL6 females in different phases of the cycle (Fig 3.3).

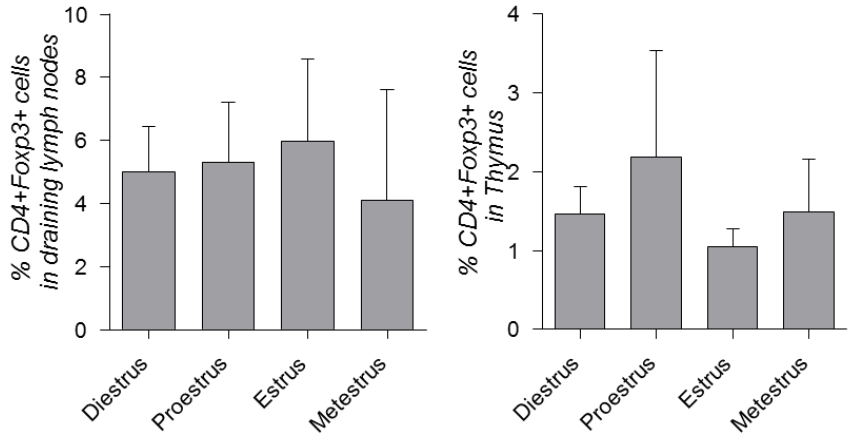

Figure 3.3- ***The population of Treg did not fluctuate in the draining lymph nodes or thymus during the oestrus cycle.*** Percentage of $CD4^+Foxp3^+$ cells of female C57/BL6 mice was analyzed in the draining lymph nodes and thymus during different phases of the estrus cycle (n=4 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test.

Collaborative work by using *in vivo* 2-photon microscopy of the uterus of *Foxp3GFP* animals confirmed a clustering of Treg in specific sites along the uterine tissue during estrus that was not observed during the other phases of the oestrus cycle (data not shown, cooperation with Carlos Tadokoro at IGG, Oeiras, Portugal).

Together, these results suggest that Treg without any antigenic stimulus, accumulate in the reproductive tract of naïve females during the receptive phase of the estrous cycle. This can be interpreted as the preparation from the uterus to allow a fast expansion of antigen-specific Treg if a contact with paternal antigens takes place.

Additionally, it is possible to hypothesize that, through the secretion of anti-inflammatory molecules, Treg present at the moment of conception could also result in the protection of the sperm cells which would then be able to reach the ovum without being attacked by the maternal immune cells present in the reproductive mucosa.

3.2.2- The maturation state of $CD11c^+$ cells fluctuates during the oestrus cycle exhibiting a more tolerogenic state during the receptive phase

DCs maturation state is known to affect the population of Treg (reviewed by Rutella and Lemoli, 2004). Therefore, the co-expression of MHCII and CD80 was analyzed in the gated population of $CD11c^+$ cells in the uterus and draining lymph nodes through the different phases of the cycle in order to address the maturation state of these cells.

We observed that the expression of MHCII and CD80 was lower in proestrus, estrus and metestrus compared to diestrus phase of the cycle. Therefore $CD11c^+$ cells are more tolerogenic in the estrus phase compared with diestrus. (Fig 3.4).

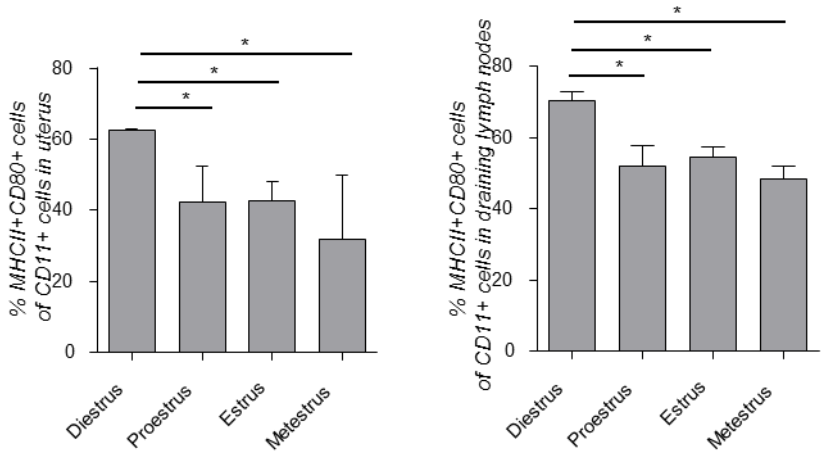

Figure 3.4- ***The population of CD11c$^+$ cells is more tolerogenic during the receptive phase of the oestrus cycle.*** Percentage of MHCII$^+$CD80$^+$ cells gated in the population of CD11C$^+$ cells of female C57/BL6 mice was analyzed in the uterus and draining lymph nodes during different phases of the estrus cycle (n=4 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05).

3.3 – THE IMPORTANCE OF TREG IN PREGNANCY ESTABLISHMENT AND MAINTENANCE

To understand in which regard naturally occurring Treg contribute to pregnancy success and to which extend they are essential for pregnancy establishment and/or maintenance, we employed a well-established model of Treg depletion. Foxp3$^+$ cells can be depleted in Foxp3.DTR mice by application of Dyphteria Toxin (DT) (Kim *et al. 2007.*).

3.3.1- Specific depletion of Treg before fecundation impairs pregnancy establishment

DT was repeatedly applied intra-peritoneally every fourth day to ensure a continuous Treg depletion. We had three different experimental groups (EG) (Fig 3.5). In the control groups, PBS was applied in substitution of DT.

In the first experimental group (EG1), DT application was initiated nine days before the mice were paired (d-9) and continued after plug detection. The number of successful implantations was addressed on day 5 of pregnancy (d5). The importance of the existence of Treg before pregnancy establishment was tested in this group.

In the second experimental group (EG2), DT was first applied immediately after detection of a vaginal plug so that the importance of the Treg population once conception is established could be studied. Here, the number of successful implantations was also recorded at d5.

The third experimental group (EG3) consisted of continuous application of DT from day 0 (vaginal plug detection) until day 14 (d14) of pregnancy. On that day preparation was made and the abortion rate, calculated as the percentage of rejected embryos in the total number of implanted embryos, was registered.

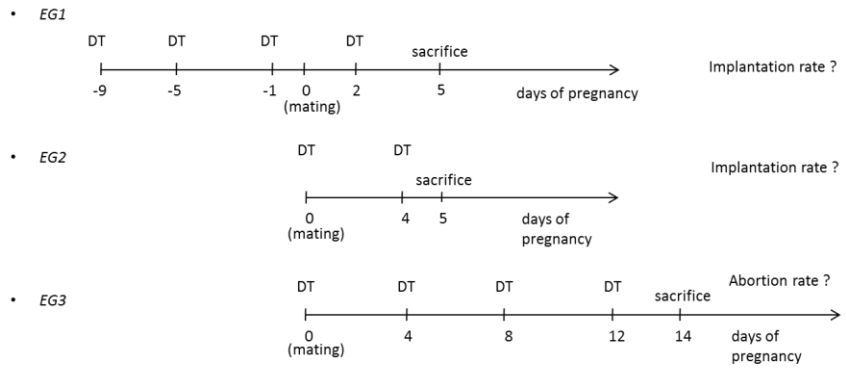

Figure 3.5- Experimental setting of DT mediated Treg depletion in Foxp3DTR mice.
DT was applied intraperitoneally in Foxp3DTR mice at different time points following repeated applications every fourth day in order to ensure a continuous Treg depletion. In the control groups, PBS was applied in substitution of DT. Experimental group (EG). EG1: DT application was initiated nine days before the mice were paired (d -9). The number of successful implantations was addressed on day 5 of pregnancy (d5). EG2: DT application started on the day of mating (d0) and the number of successful implantations was counted on day 5 of pregnancy (d5). EG3: DT application started on the day of mating (d0) and the number of abortions was quantified on day 14 of pregnancy (d14).

Treg depletion before mating (EG1) (DT treatment beginning at d -9) resulted in a lower percentage of females with successful implantations in comparison with the control (Fig 3.6 A).

Treg depletion starting from the day of mating (DT treatment beginning at d0) did not change the success of implantation establishment (EG2). The percentage of females with established implantations after DT treatment was similar to the control group (Fig 3.6 B).

In the experimental group 3, DT application also starting from the day of mating did not result in any deleterious effect on pregnancy outcome. There was a slight increase in the percentage of females with abortions in the treated group (Fig 3.6 C) however it was not significant. Additionally we observed an abortion rate with a mean value of 10% which was not significantly different from the control group (Fig 3.5 D). The uterus, placenta and the embryo were not affected by DT treatment (Fig 3.6 E).

DT treatment resulted in the significant depletion of more than 50% of $Foxp3^{GFP+}$ cells from the uterus and draining lymph nodes in the treated animals compared with the controls in all the experimental groups analyzed.

Together these results suggest that Treg are required for the establishment of pregnancy but not for its maintenance.

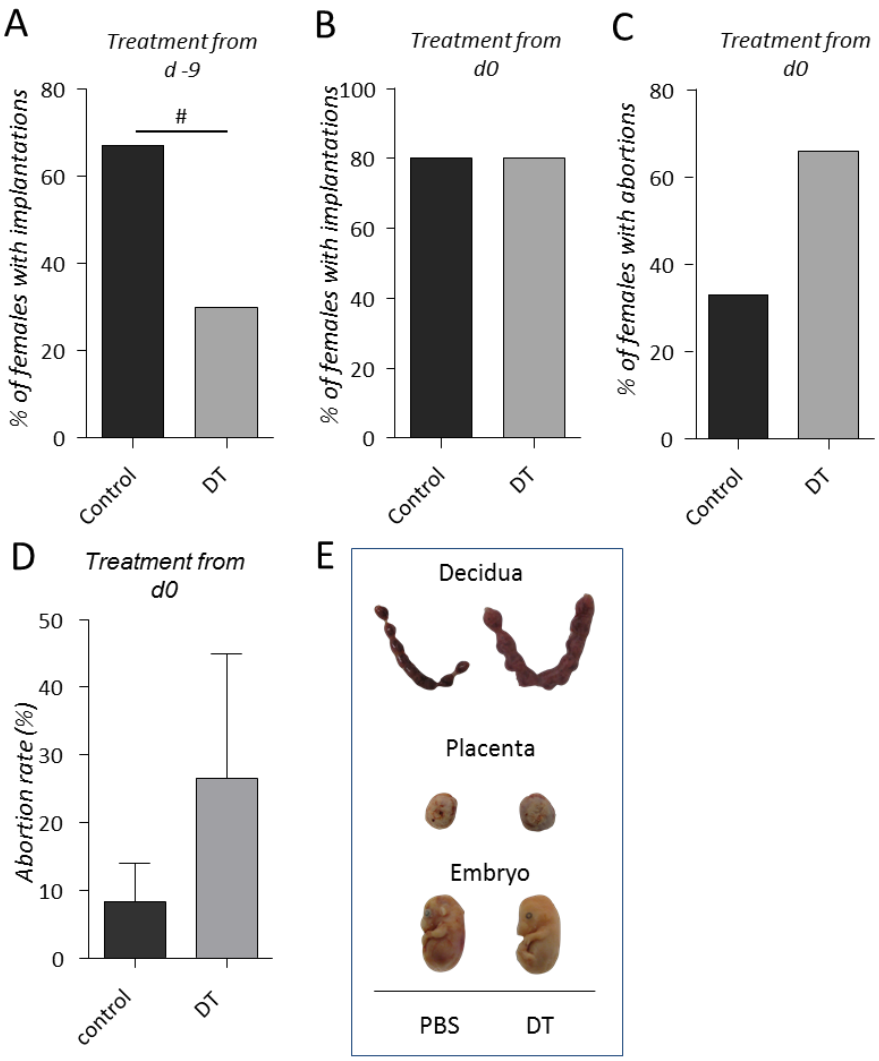

Figure 3.6- Pregnancy establishment was impaired in Foxp3DTR mice by specific depletion of Treg prior to fecundation while Treg depletion at later time points did not affect pregnancy. Foxp3^{+} cells were depleted in *Foxp3DTR* mice by application of dyphteria toxin (DT) every fourth day, starting nine days before mating (d -9) or at the day of mating (d0) with BALB/c males. In control groups, PBS was applied instead of DT. The percentage of females with implantations was detected on day 5 of pregnancy (A and B) and the percentage of females with abortions was observed on day 14 of pregnancy (C). Data are expressed as bars representing the percentage of the total and was analyzed by the Two-sided Fisher's exact test (#: P<0,1). The percentage of abortions was quantified on day 14 of pregnancy (D). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test. Uterus, placenta and embryo were not affected by DT treatment as analyzed on day 14 of pregnancy (E). (n= 5-12 animals per group).

The importance of Treg before conception was confirmed by a collaborative work with the lab of Günter Hämmerling at the German Cancer Research Center in Heidelberg, Germany, with a second model of Treg depletion, the Foxp3LuciDTR mice that express Foxp3 promoter-controlled luciferase, diphtheria toxin receptor, and GFP (Suffner *et al.*, 2010). A significantly decreased implantation success could be noticed in both allogeneic and syngeneic pregnancies. This observation was followed by increased activation of effector T cells and $CD8^+$ T cells in the uterine draining lymph nodes of the Treg depleted animals and an increase in the population of $CD8^+$T cells in the uterine tissue (data not shown) suggesting that the lack of Treg cells induced an inflammatory environment in this action site which is probably exerting a negative influence in the establishment of pregnancy.

3.3.2- Local TGF-β application does not recover implantation loss caused by Treg depletion

Treg requirement for a successful implantation may be due to the secretion of modulatory signals by these cells that would counteract the pro-inflammatory mechanisms characterizing this stage of pregnancy.

Among the mediators secreted by Treg, local TGF-β was chosen as a target of importance because its local application was previously shown to prevent abortions in the CBA/J x DBA/2J combination (Clark *et al.*, 2008).

Therefore, TGF-β was applied intra-vaginally after depletion of Treg so that its effect during implantation could be addressed. Accordingly, Foxp3.DTR mice were depleted of Treg by a continuous DT treatment starting on d -9. On the day of plug detection a vaginal application of TGF-β was performed and followed by two more application every other day. Finally, the number of implantations was counted on d5 (Fig 3.7). As a control group Treg depleted animals were treated with local PBS.

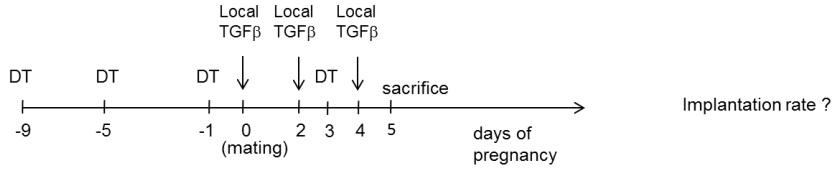

Figure 3.7- Experimental setting of DT mediated Treg depletion in Foxp3DTR mice followed by TGF-β local treatment.
DT was applied intra-peritoneally in Foxp3DTR mice starting from nine days before mice were paired. TGF-β was applied in the vagina on the day of mating and on every other following day. The number of successful implantations was addressed on day 5 of pregnancy (d5). As a control group mice were treated locally with PBS.

The median number of implantations after TGF-β was equal to 0 and was not significantly different from the control group, suggesting that the vaginal application of this molecule could not prevent implantation impairment in Treg-depleted mice.

3.2- DISCUSSION

The experiments described in this chapter show the importance of the preexistence of a Treg population for the onset of pregnancy.

Previous work from Kallikourdis *et al.*, (2007) suggested oscillations in Foxp3 mRNA expression levels in the murine uterus during the oestrus cycle, i.e. they showed a higher amount of transcripts during the estrus phase (fertile period) of the oestrus cycle. This goes in line with the observations done by Arruvito and colleagues (2007), who observed that fertile women experienced an expansion of blood $CD4^+CD25^+FOXP3^+$ in the late follicular phase of the menstrual cycle. This was not observed in patients with a history of recurrent abortions or in postmenopausal women (Arruvito *et al.*, 2007).

Herein, we observed that a similar cyclic expansion of this population is occurring in the vaginal lavage of mice along the oestrus cycle that resulted in a peak during receptivity. This expansion is also coincident with the accumulation of Treg at specific sites along the uterus at the fertile period, just before conception can occur.

Tolerogenic DC subsets are known to promote Treg cell differentiation (reviewed by Steinman *et al.*, 2003). Cyclic alterations in the population of endometrial but not splenic dendritic cells have been described during the oestrus cycle (Zarnani *et al.*, 2007). We found that, in synchrony with the expansion of Treg during estrus phase of the cycle, the population of $CD11c^{+}$ dendritic cells in the reproductive tract and draining lymph nodes showed a more tolerogenic phenotype during the receptive phase of the cycle when comparing with diestrus. It is thus possible that local DCs are participating in the generation and function of Treg in the reproductive tract and mediating the tolerance mechanisms necessary for pregnancy establishment.

Foxp3DTR mice are a well-documented and widely used model of Treg depletion by application of DT toxin (Kim *et al.*, 2007). It could be determined that Treg are in fact essential for pregnancy establishment but not for its maintenance. The depletion of Treg before mating resulted in no pregnancy establishment. Treg depletion at mating did not majorly interfered with pregnancy. It did not interfere with implantation and only induced a marginal augmentation of the abortion rate.

Similar observations were done by our colleagues in both allogeneic and syngeneic pregnancies after depletion in Foxp3.LuciDTR mice (Suffner *et al.*, 2010). This leads to the assumption that these cells may secrete a soluble factor that is necessary for implantation to take place.

Among the soluble factors secreted by Treg, TGF-β was chosen once previous work from Clark *et al.* (2008) suggested that vaginal TGF-β enhances the success of pregnancy in an established model of abortion by promoting a regulatory T-cell response. Additionally, TGF-β is known to be abundant in seminal fluid and this fluid was shown to regulate the accumulation of $Foxp3^+$ regulatory T cells in uterus in the beginning of pregnancy (Guerin *et al.*, 2011).

However, in the present study, the application of vaginal TGF-β just after conception did not result in the rescue of implantations caused by the depletion of Treg. These results are consonant with previous work in which the blockage of TGF-β didn't have a negative effect on pregnancy success in an abortion recovery model promoted by Treg transfer immediately after conception (Schumacher *et al.*, 2007).

Understanding the mechanisms by which Treg exert their influence in the establishment of pregnancy has broad implications in the development of therapeutic strategies for the treatment of infertility and other pregnancy complications.

-CHAPTER 4-

ORIGIN OF T REGULATORY CELLS DURING PREGNANCY

4.1- INTRODUCTION

In the previous chapter evidences of an essential role for Treg in the establishment of pregnancy were shown. Here we aim to study their origin and the mechanisms underlying their generation and effectiveness.

Treg can be either generated in the thymus or in the periphery. Both populations have distinct antigen specificities, T-cell receptor strength and co-stimulatory requirements (Seddon and Mason, 1999; Sakaguchi, 2004; reviewed in Shevach *et al.*, 2006; reviewed in Hall *et al.*, 2011). During pregnancy a hormonal dependent 'involution' of thymus is known to occur in both allogeneic and syngeneic pregnancies (Maroni and de Sousa, 1973; Tibbets *et al.*, 1999; Zoller *et al.*, 2007) but thymus remains active during pregnancy despite suffering several morphological changes (Clarke *et al.*, 1994; Kendal *et al.*, 2000). Zoller and colleagues (2007) showed an enrichment of $CD4^+CD25^+$ Treg in the thymus during late pregnancy suggesting the involvement of thymic generated Treg in this stage of pregnancy (Zoller *et al.*, 2007).

TGF-β is a key factor in the Treg cell commitment. It has been shown to work in the presence of IL-2 in the generation of Treg (reviewed in Horwitz *et al.*, 2003; Zheng *et al.*, 2007) and it has been strongly implicated in the conversion of peripheral $CD4^+CD25^+$ cells into $Foxp3^+$ Treg in both human and mice (Chen *et al.*, 2003; Fu *et al.*, 2004; Zheng *et al.*, 2007). Previous evidences have shown that the intra-vaginal administration of TGF-β resulted in an elevation of a Foxp3 expressing population in the uterine lumen (Clark *et al.*, 2008).

A major role for IL-2 has been proposed in the maintenance of tolerance with different studies revealing a critical role for IL-2 in the generation and maintenance of Treg in both thymus and periphery (Schorle *et al.* 1991,

Kramer *et al*., 1995; reviewed in Nelson, 2004; Setoguchi *et al.*, 2005). While IL-2 by itself is not strictly necessary for natural Treg cell generation (Curotto de Lafaille *et al*., 2004; D´Cruz and Klein, 2005; Fontenot *et al.* 2005), a transient *in vivo* neutralization of circulating IL-2 by an anti–IL-2 monoclonal antibody selectively reduced the number of $CD4^+CD25^+$ Treg but not of $CD4^+CD25^-$ effector cells, eliciting autoimmune diseases in normal naïve mice (Setoguchi *et al.,* 2005).

There are contradictory data regarding the participation of antigen-dependent and independent mechanisms for Treg expansion during pregnancy (Darrasse-Jèze *et al.*, 2006; Tilburgs *et al.*, 2009; Khan and Baltimore, 2010). While previous studies support a hormonal-driven influence on Treg expansion (Aluvihare *et al.* 2004; Polanczyk *et al.*, 2004; Tai *et al.*, 2008), others have given further evidence that this expansion is rather caused by alloantigen recognition than by hormonal impact (Zhao *et al.*, 2007; Tilburgs *et al.*, 2009).

The experiments described in this chapter aim to describe the distribution of the population of Treg during different phases of pregnancy and research distinct possibilities for their origin. Further, the mechanisms behind their origin and action in different pregnancy stages are addressed.

4.2 – KINETICS OF TREG DURING PREGNANCY

Following the confirmation that Treg are essential for pregnancy establishment but not for its maintenance and taking into account the fact that there are different stages during pregnancy with different tolerance requirements, the question of whether there are different Treg subpopulations at different pregnancy periods influencing the pregnancy outcome was addressed.

The population of $CD4^{+}Foxp3^{+}$ Treg was analyzed in the uterus, uterine draining lymph nodes, thymus, blood and mesenteric lymph nodes of C57/BL6 mated BALB/c females on days 0, 2, 5, 8, 10 and 12 (d0, d2, d5, d8, d10 and d12) of pregnancy.

The results obtained showed a similar oscillatory pattern in the Treg populating the pregnant uterus, the uterine draining lymph nodes, peripheral blood and the mesenteric lymph nodes, augmenting as early as d2, decreasing towards d8 and showing a new increase on d10 (Fig. 4.1A).

In thymus, Treg showed a significant augmentation on day 12 of pregnancy but no major oscillations before that time point (Fig 4.1A). The weight of thymus slightly and not significantly increased until d8 showing an abrupt and significant decrease from day 10 of pregnancy (Fig 4.2B).

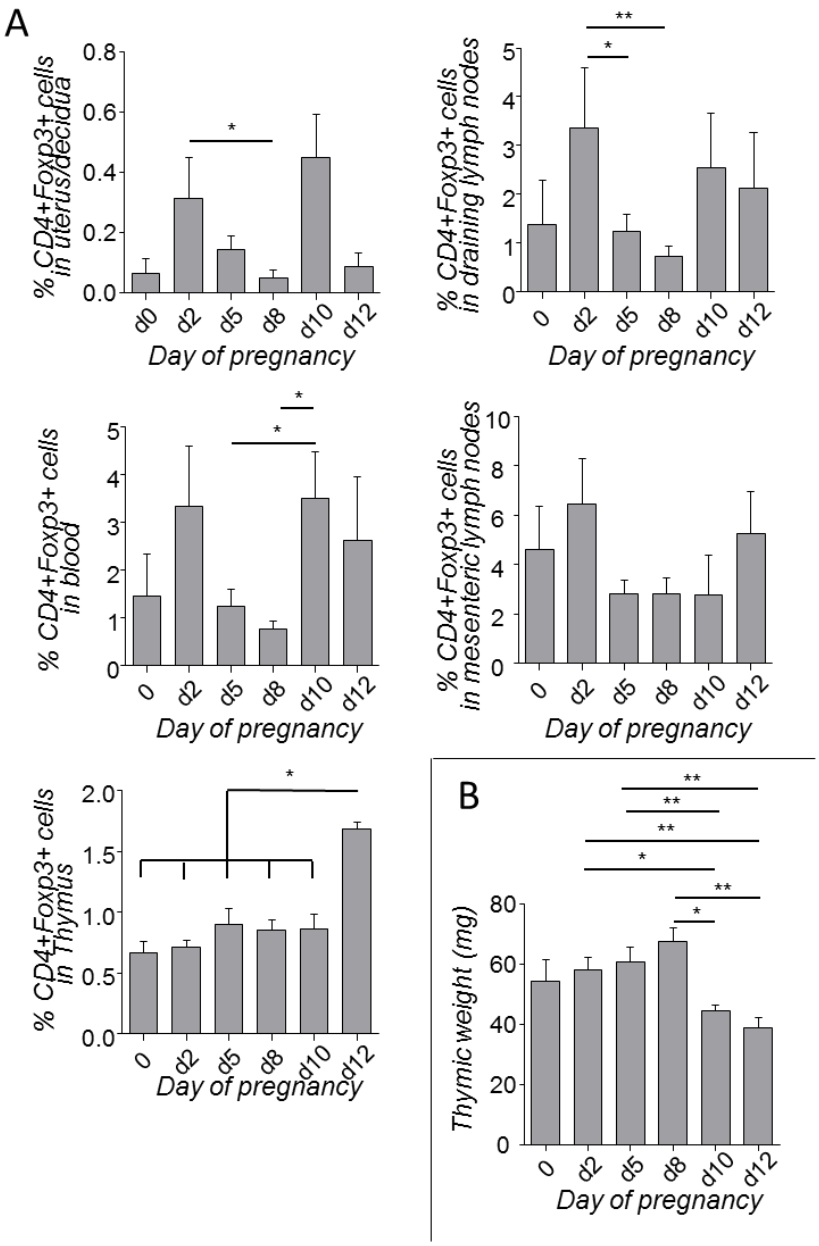

Figure 4.1- **Treg kinetics shows oscillatory changes during pregnancy.** BALB/c females were mated with C57/BL6 males and the population of $CD4^{+}Foxp3^{+}$ Treg was analyzed in the uterus/decidua, uterine draining lymph nodes, thymus, blood and mesenteric lymph nodes on days 0, 2, 5, 8, 10 and 12 of pregnancy (d0, d2, d5, d8, d10 and d12) (A). Thymic weight (mg) was measured at the same time points of pregnancy (B). (n=5 animals per group). Data are expressed as means with SEM (*:P ≤0.05; **P≤0.01).

4.3– ORIGIN AND MECHANISMS OF GENERATION OF TREG DURING PREGNANCY

4.3.1- Helios$^+$ Treg distribution suggests an expansion of thymic derived Treg in the beginning of pregnancy and the occurrence of *de novo* conversion of peripheral Treg after implantation

Helios, a member of the Ikaros transcriptor factor family, was recently described as a marker for thymic derived Treg *in vivo* (Thornton *et al*, 2010). Having the kinetics of Treg during pregnancy in mind we analyzed Helios expression in the CD4$^+$Foxp3$^+$ population to understand the origin of Treg during the different stages of pregnancy.

The expression of Helios was analyzed in CD4$^+$Foxp3$^+$ Treg from thymus, uterine draining lymph nodes and uterus of BALB/c mated C57/BL6 females. Non pregnant (NP) as well as d2, d5 and d10 pregnant females were included in this study.

The population of Helios$^+$ Treg augmented in all analyzed organs on d2 after conception (Fig 4.2 A), even before implantation suggesting that the pre-implantatory population of Treg is mainly of thymic origin.

At d5, just after implantation takes place, there was a significant decrease in the frequency of Helios$^+$ Treg in the draining lymph nodes accompanied by a concomitant increase in the population of peripheral de novo generated Helios$^-$ Treg (Fig 4.2 B). This suggests that Treg at this stage of pregnancy are predominantly converted in the periphery.

In the uterus and thymus, the population of thymic derived Helios$^+$ Treg decreased from d2 onwards reaching the initial values, as in the NP stage, on

d10. Contrasting, there was a new augmentation on the population of Helios^{+} Treg of thymic origin in the uterine draining lymph nodes (Fig. 4.2 A)

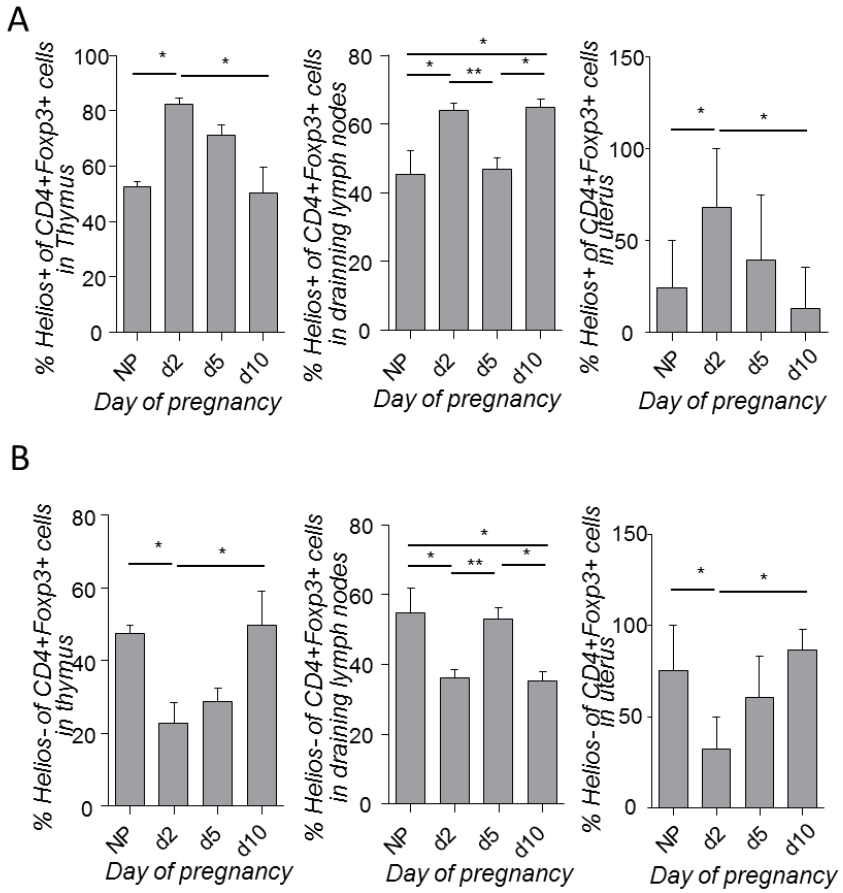

Figure 4.2- **The expression of Helios^{+} in Treg changes during pregnancy.** The percentage of Helios^{+} (A) and Helios^{-} (B) of CD4^{+}Foxp3^{+} cells was analyzed in the thymus, uterine draining lymph nodes and uterus of non-pregnant (NP) C57/BL6 females and BALB/c mated C57/BL6 females on days 2, 5 and 10 of pregnancy (d2, d5, d10) (n=4-5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05; **P≤0.01).

4.3.2- Migration of thymic Treg to the periphery is similar in both non pregnant and day 10 pregnant mice

The data previously described suggests that at d10 Treg from the lymphoid organs draining the uterus are mainly from thymic origin. To additionally explore this hypothesis, thymocytes from d8 pregnant Foxp3GFP mice were intravenously transferred into d8 pregnant Rag1$^{-/-}$ animals. The location of migrating Foxp3^{GFP+} cells was traced on thymus, uterine draining lymph nodes and uterus two days later, on d10. As a control group, cells from non-pregnant Foxp3GFP mice were equally transferred into non pregnant Rag1$^{-/-}$ animals. Thymic Treg were able to migrate to all analyzed organs in both non pregnant and d10 pregnant mice. No differences were found in the frequency of migrated cells between the two groups of animals to the specified tissues (Fig. 4.3).

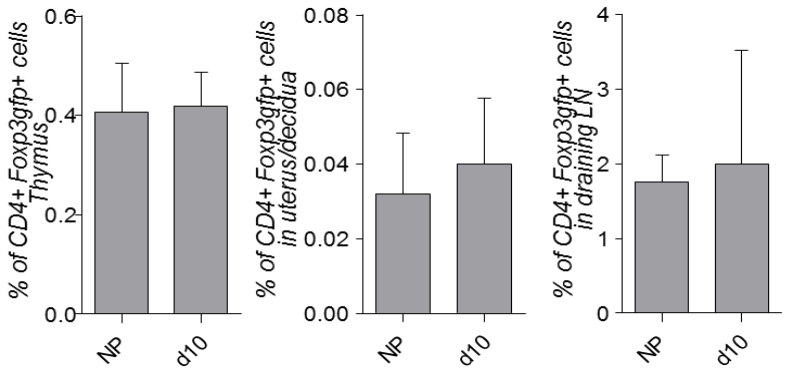

Figure 4.3- **After reconstitution of Rag1$^{-/-}$ mice with thymocytes from Foxp3GFP mice Treg cells presented the same migratory phenotype in both non pregnant and mice on day 10 of pregnancy.** Thymocytes isolated from 8 days pregnant *Foxp3GFP* mice were transferred into Rag1$^{-/-}$ animals at the same day of pregnancy and their localization was traced on day 10 (d10) of pregnancy. As control, thymocytes isolated from non-pregnant *Foxp3GFP* mice were transferred into non pregnant (NP) Rag1$^{-/-}$ animals and traced two days after transference. CD4^{+}*Foxp3*$^{GFP+}$ cells were analyzed in thymus, uterine draining lymph nodes and uterus/decidua (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test

4.3.3- At mid and late pregnancy Treg convert from Foxp3$^-$ into Foxp3$^+$ cells in the periphery in a TGF-β independent way

To investigate whether peripheral conversion of naive T cells into Treg takes place, we made use of Rag1$^{-/-}$ mice which are deficient in the recombination-activating genes, Rag1 (Mombaerts *et al.*, 1992) required for initiating the process of V(D)J recombination during lymphocyte maturation and as a result these mice lack mature T and B lymphocytes. Therefore, we employed this lymphopenic mouse model to visualize the conversion of Foxp3$^-$ cells into Foxp3$^+$ cells by expression of GFP (Sun *et al.*, 2007). Shortly, Rag1$^{-/-}$ female mice were transferred with CD4$^+$Foxp3^{GFP-} cells from Foxp3GFP mice and CD4$^+$CD25$^+$ cells from normal C57/BL6 WT mice were added to avoid autoimmunity. If Foxp3$^-$ cells convert into Foxp3$^+$ cells this change can be easily demonstrated by flow cytometry. After allowing reconstitution for a period of 4 weeks, the animals were paired with BALB/c males and CD4$^+$Foxp3^{GFP+} cells were traced in several organs on pregnant females on both d5 and d10 (Fig 4.4). Non-pregnant reconstituted animals were used as a control.

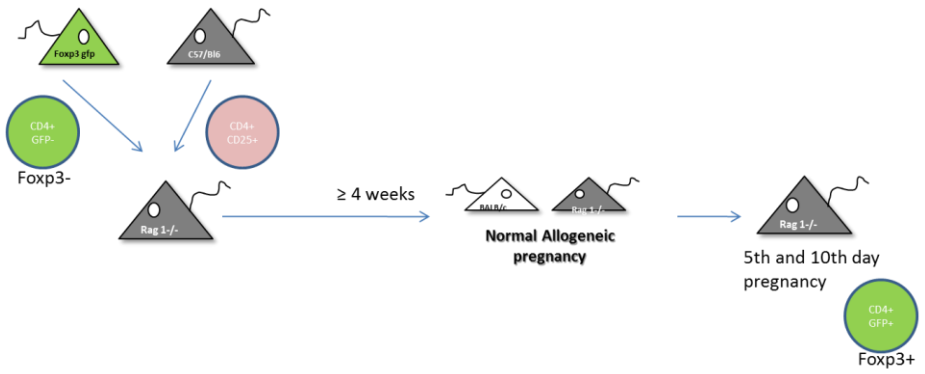

Figure 4.4- Reconstitution of Rag1$^{-/-}$ mice with CD4^{+}Foxp3^{GFP-} cells from Foxp3^{GFP} mice and CD4^{+}CD25^{+} cells from wild type mice. Rag1$^{-/-}$ female mice were transferred with CD4^{+}Foxp3^{GFP-} cells from Foxp3GFP mice and CD4^{+}CD25^{+} cells from normal C57/BL6 WT mice were added to avoid autoimmunity. After allowing reconstitution for a period of 4 weeks, the animals were finally paired with BALB/c males and CD4^{+}Foxp3^{GFP+} cells were traced in several organs on both d5 and d10 pregnant females. Non pregnant reconstituted animals were used as a control.

Compared to the non-pregnant control group, an increased conversion of Foxp3^{-} into Foxp3^{+} cells could be observed in the uterine draining lymph nodes of the reconstituted Rag1$^{-/-}$ females on both d5 and d10 (Fig 4.5 A and B). This augmentation in the pregnancy-mediated conversion could also be detected in a significantly manner in the mesenteric lymph nodes and in the blood on d5 but not in other lymph nodes nor in the spleen (Fig 4.5 B).

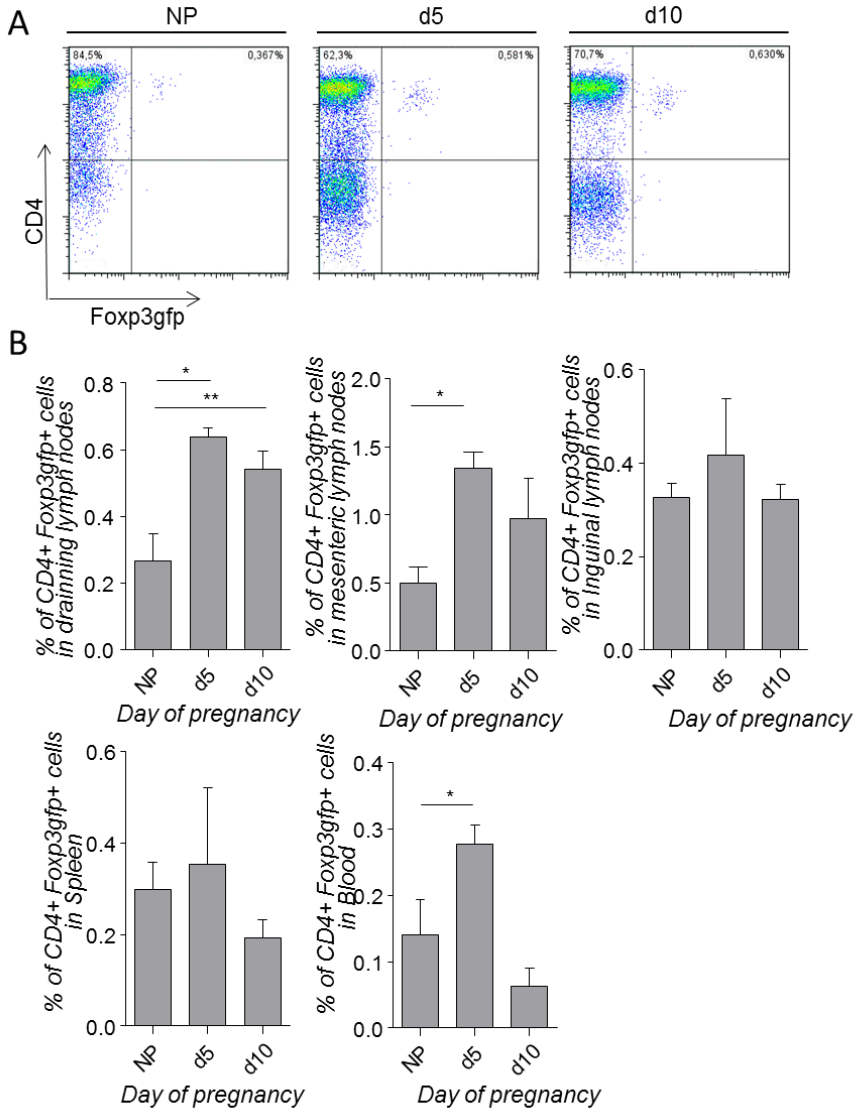

Figure 4.5- *de novo* conversion of Treg occurs in peripheral organs on day 5 and 10 of pregnancy. Rag1$^{-/-}$ mice were transferred with CD4^{+}Foxp3^{-} cells from Foxp3GFP mice together with CD4^{+}CD25^{+} cells from WT (C57/BL6). Conversion of CD4^{+}Foxp3^{-} into CD4^{-}Foxp3^{+} cells was traced by *de novo* expression of CD4^{+}Foxp3^{GFP} Treg on the reconstituted mice on days 5 (d5) and 10 (d10) of pregnancy and non-pregnant (NP) mice in the uterine draining lymph nodes (A and B), mesenteric lymph nodes, inguinal lymph nodes, spleen and blood (B) (n=4-5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P <0.05).

TGF-β is known as an important player in the mechanism of Treg conversion (Chen *et al.*, 2003; Horwitz *et al.*, 2003; Fantini *et al.*, 2004). Therefore, in order to analyze the functional needs of this cytokine in the Treg conversion observed in our model, a neutralizing antibody against TGF-β was injected subcutaneously in pregnant reconstituted $Rag1^{-/-}$ on d0 and d7. Animals were sacrificed on d12 so that the influence of TGF- β in their pregnancy outcome could also be correctly addressed by analyzing both, the abortion rate and implantation number. The control animals were injected with IgG.

The results show that Treg conversion in the uterine draining lymph nodes was not influenced by the blockage of TGF-β (Fig. 4.6 A) predicting that it occurs independently of this molecule. Additionally, neither the abortion rate (Fig 4.6 B) nor the implantation number (4.6 C) was affected by the anti TGF-β treatment providing further evidences that it has no influence on the pregnancy outcome.

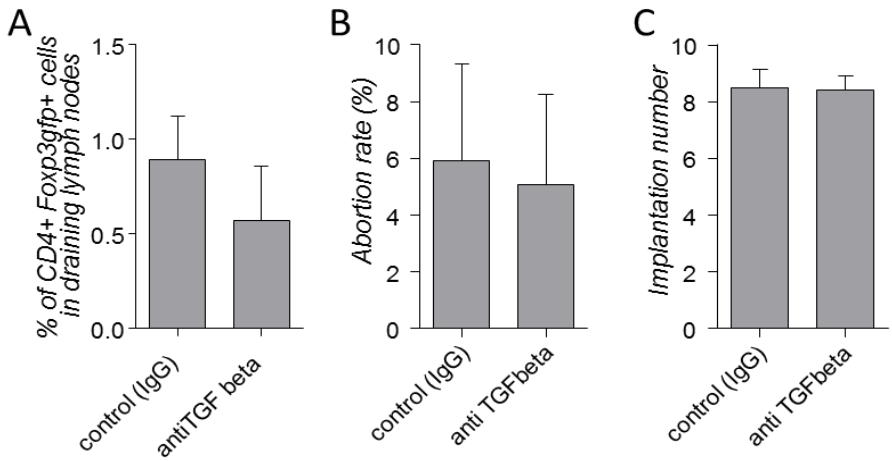

Figure 4.6- **Anti-TGF-β did not show any effect on the de novo conversion of Treg in the draining lymph nodes during pregnancy.** A neutralizing antibody against TGF-β was injected subcutaneously in pregnant reconstituted $Rag1^{-/-}$ on day 0 and day 7 of pregnancy. As a control group animals were injected with IgG. The percentage of $CD4^{+}Foxp3^{GFP+}$ cells was analyzed on the uterine draining lymph nodes on day 12 of pregnancy (d12). (A) Additionally, abortion ratio (B) and Implantation numbers (C) were observed on the same animals. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test.

4.3.4- IL-2 treatment can induce maternal tolerance towards the fetus

IL-2 is strongly implied in both thymic generation and peripheral maintenance of Treg being an essential factor for their homeostatic recovery (Almeida *et al.*, 2002; Liston *et al.*, 2007; Suffner *et al.*, 2010). In the current study, it was proposed to investigate whether IL-2 would avoid abortion in a well-known abortion-prone mouse model consisting of DBA/2J-mated CBA/J females because of its known effect in Treg. Pregnant females received two daily IL-2 injections at different concentrations ($5x10^5$ or $5x10^6$ IU/kg) which were administered subcutaneously for five consecutive days starting on day 0. As a control group, mice were injected with PBS. The population of $CD25^{+}$ cells and the abortion rates were analyzed on d14

The results obtained demonstrated that IL-2 treatment was effective in decreasing the abortion rates in the abortion-prone animals (Fig 4.7 A). Together, an increase in the population of $CD25^+$ cells in both thymus and uterine draining lymph nodes could be observed (Fig 4.7B) giving further evidences that the population of $CD25^+$ Treg expanded by the application of IL-2 in a dose dependent matter influencing the pregnancy outcome.

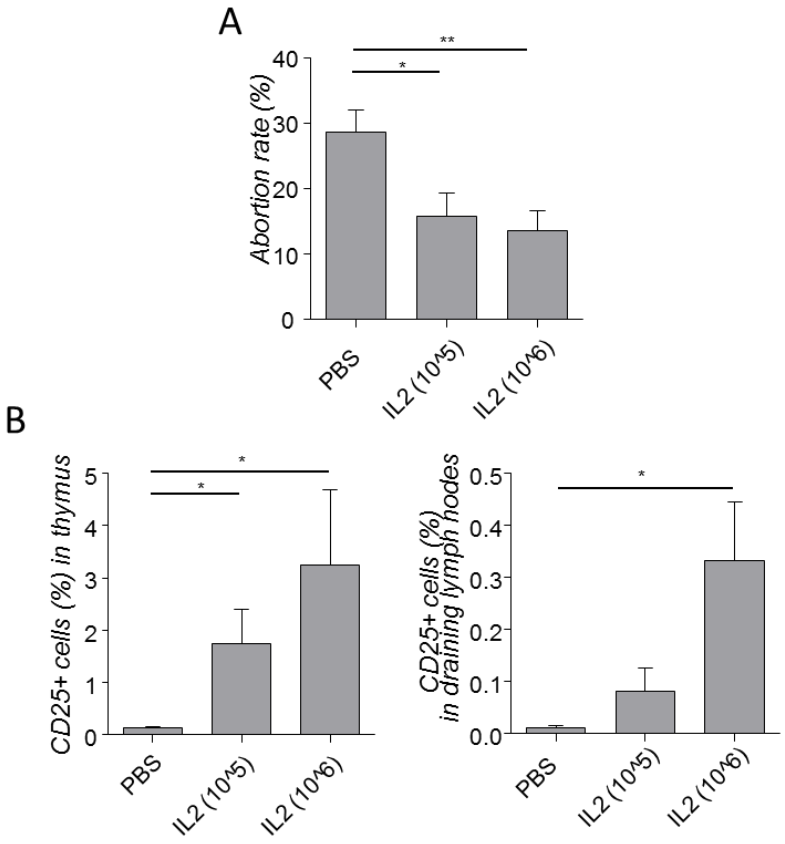

Figure 4.7- IL-2 treatment induces maternal tolerance reducing the abortion rates in abortion-prone mice. DBA/2J mated CBA/J females were treated subcutaneously twice a day with different dosis of IL-2 every day for 5 consecutive days starting from the day of mating. Control animals received PBS. Abortion rate (A) and the percentage of $CD25^+$ cells in the thymus and uterine draining lymph nodes (B) were measured on d14. (n= 5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P <0.05; **:P<0,01).

4.4- ANTIGEN SPECIFICY OF TREG DURING PREGNANCY

4.4.1- Paternal alloantigens influence the expansion of Treg after implantation

Past reports are divergent while relating to the participation of antigen-dependent and independent mechanisms for Treg expansion during pregnancy (Darrasse-Jèze *et al.*, 2006; Tilburgs *et al.*, 2009; Khan and Baltimore, 2010). To clarify the extension of paternal antigen influence in the population of Treg during pregnancy in this study, the expansion of Treg on both allogeneic and syngeneic pregnancies was analyzed on d5 (just after implantation takes place) and d12 (when placentation is completed). Either Foxp3GFP or C57/BL6 female mice were used and mated with C57/BL6 males or BALB/c males for the establishment of syngeneic or allogeneic pregnancies, respectively.

At day 5 and 12 the frequency of $CD4^{+}Foxp3^{+}$ cells in allogeneic pregnancies was found to be higher than the syngeneic ones (Fig 4.8 A and B). Interestingly, this was true for the mesenteric lymph nodes on day 5 (Fig 4.8 A) and additionally for the thymus and blood on day 10 of pregnancy (Fig 4.8 B) but surprisingly, no significant differences could be found in the uterine draining lymph nodes, despite of an existing trend on day 5 of pregnancy (Fig 4.8 A) suggesting that the presence of paternal antigens after implantation has a time-dependent influence in the organs of the pregnant female.

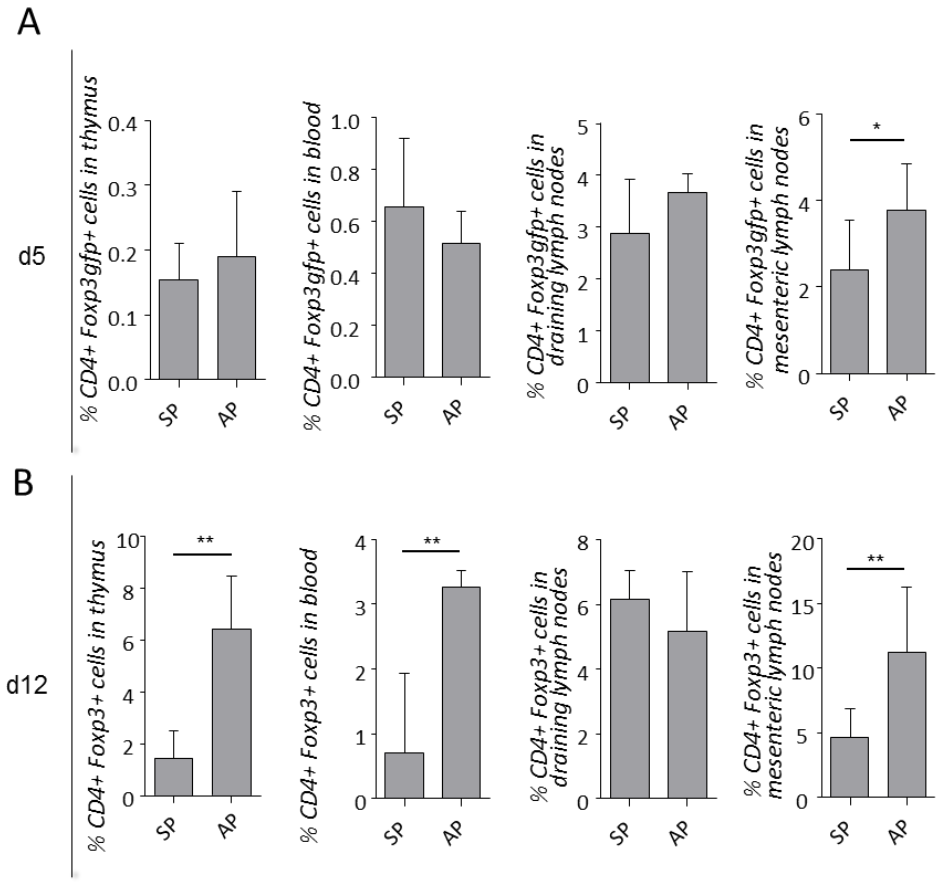

Figure 4.8- The population of Treg during pregnancy shows differences in the expansion in both allogeneic and syngeneic pregnancies. Foxp3GFP mice were paired with both C57/BL6 or BALB/c males and the population of $CD4^{+}Foxp3^{GFP+}$ cells was analyzed in thymus, blood, uterine draining lymph nodes and mesenteric lymph nodes on day 5 of pregnancy (d5). The same procedure was done with C57/BL6 females and the population of $CD4^{+}Foxp3^{+}$ cells was analyzed on day 12 of pregnancy (d12). (n=4-5 animals per group). Data are expressed as medians with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05; **P≤0.01).

4.5- DISCUSSION

The kinetics of Treg during pregnancy and the mechanisms of generation and conversion of this cell population were here analyzed in different pregnancy time points.

Besides Treg lack a pivotal role once pregnancy is established, their kinetics provides a clear evidence of their dynamic presence at later stages. In the last years it has become clear that in addition to naturally occurring Treg the phenomenon of peripheral conversion of Treg from Teff cells is well documented in several models (Chen *et al.*, 2003; Kretschmer *et al.*, 2005; Zheng *et al.*, 2007).

The exposure of female reproductive tract to semen is known to elicit immune responses in human and mice (Pandya *et al.*, 1985; Johansson *et al.*, 2004; Sharkey *et al.*, 2007). The augmentation in the population of Treg here observed at the beginning of pregnancy (d2) supports the hypothesis of us and others that semen induces immune tolerance from the moment of mating (Thuere *et al.*, 2007; Robertson *et al.*, 2009; Zenclussen *et al.*, 2010).

In contrast, a general decrease in the total population of Treg that was noticed from d5 can be explained by adaptation changes occurring during implantation, which is established just before this time point, and which lead to the development of angiogenesis, which is *per se* similar to an inflammatory process (Sherer and Abulafia, 2001; Fiedler *et al.*, 2006).

At d8 the total population of Treg is at lowest. This time point is indicative of when the labyrinth starts forming and angiogenesis is the key event herein. It is also the starting point when the circulatory system of the fetus is formed and both blood from fetus and mother make exchanges at the spiral arteries (Brosens *et al*., 1967; Muntener *et al*., 1977; reviewed in Moffett and Loke, 2006).

A new augment in Treg frequency could be observed from d10 in all the tissues analyzed except the thymus. The thymic distribution of Treg only depicted a significant augmentation on day 12 of pregnancy. This can be in part explained by the well-known process of thymic involution that occurs during pregnancy in a hormonal dependent way (Tibbets *et al.*, 1999) and that starts around day 10 of pregnancy resulting in changes in the distribution of the thymocytes (Clarke *et al.*, 1994; Kendal *et al.*, 2000). Our results are also in concordance with observations from a recent study describing an enrichment of $CD4^+CD25^+$ Treg in the thymus during late pregnancy (Zoller *et al.*, 2007).

The ability to discriminate between thymic origin and peripheral *de novo* converted of the different Treg populations co-existing in the system has long been a very difficult issue by the lack of a proper marker of differentiation. However, in 2010 Thornton and colleagues proposed Helios as a marker for thymic-derived naturally occurring Treg (Thornton *et al.*, 2010).

Thymic-derived $Helios^+$ Treg happened to be the predominant population in thymus, uterus and respective draining lymph nodes during early pregnancy. This points to a mainly thymic derivation of Treg in the system during the period previous to implantation. In turn, it emphasizes the theory of thymic Treg having an important participation in pregnancy establishment. Several studies confirmed an increase of Treg already on day 2 of pregnancy in blood, lymph nodes, and thymus (Aluvihare *et al.*, 2004; Zenclussen *et al.*, 2005; Thuere *et al.*, 2007) and particularly, the affluence of $Foxp3^+$ Treg in the uterus and draining lymph nodes during the peri implantation phase has been documented in mice (Guerin *et al.*, 2011). Hence, an increased CD25 expression in $CD4^+$ cells was described at day 2.5 after conception in several lymphoid tissues (Aluvihare *et al.*, 2004). The seminal fluid but not sperm

itself was described as influencing the expansion of Treg cells in the uterine draining lymph nodes at an early time point (Robertson *et al.*, 2009; Guerin *et al.*, 2011). This also goes in accordance with previous findings in which Treg derived from spleen and thymic compartment of normal pregnant mice could avoid abortion in animals with an abortion susceptibility caused by mating pair differences in the minor histocompatibility complex (abortion prone model of pregnancy (Clark *et al.*, 1980; Chaouat *et al.*, 1988) only when transferred from day 0 to day 2 and not later time points of pregnancy suggesting an important role for thymic derived Treg at the beginning of pregnancy (Zenclussen *et al.*, 2005, 2006; Schumacher *et al.*, 2007; Wafula *et al.*, 2009)

At day 5 of pregnancy, just after implantation takes place, the population of thymus derived Treg declines. This phenomenon was followed by the augmentation of Helios^{-} Treg population, which are most probably *de novo* converted peripheral Treg. By using a Rag1$^{-/-}$ lymphopenic mouse model of transfer, the conversion of Treg in the periphery at this time point could be confirmed. Treg conversion was significantly higher in the uterine draining lymph nodes, blood and mesenteric lymph nodes of d5 pregnant animals compared with non-pregnant animals.

Helios^{+} Treg show a second peak of increase at d10 in the uterine lymph nodes. Additionally, pregnancy-induced peripheral conversion of Treg in the Rag1$^{-/-}$ pregnant animals at this time point took place in the uterine draining lymph nodes but not in the other organs suggesting that both thymic derived and peripheral converted Treg populate these local lymphoid organs on this time point.

The population of Helios^{+} Treg in the thymus at this late pregnancy stage shows a decrease to values similar to the ones obtained in the non-pregnant

animals. This gives strength to the hypothesis that Treg are derived from the thymus at the start of pregnancy (d2) and migrate to the periphery during the following days, at least before d10. By analyzing the migration of $Foxp3^{GFP+}$ on $Rag1^{-/-}$ transferred with total thymocytes from Foxp3GFP mice on d10 it was not possible to observe an increased migratory rate in the pregnant mice compared with non-pregnant controls. This suggests that at this time point Treg at the periphery might have both thymic and peripheral origin.

A population of $Helios^{-}$ Treg, hence *de novo* converted, could also be observed in the thymic tissue during the different time points analyzed. The existence of a similar population was also previously described by Thornton *et al.* (2010). These authors further stated that they could not observe this population in the thymus from young mice (3–7 d old) which had exclusively $Foxp3^{+}Helios^{+}$ cells, therefore suggesting that these $Helios^{-}Foxp3^{+}$ are cells that were induced in the periphery and entered the thymus by recirculation (Thornton *et al.*, 2010). Other publications recently presented evidences for a population of $Helios^{+}$ cells generated in the periphery in both *in vivo* and *in vitro* (Gottschalk *et al.*, 2012) questioning the specificity of this marker for thymic derived Treg. A very recent work from Samstein *et al.*, (2012) shows that Treg that accumulate in the decidua require CNS1, essential for the conversion of peripheral Treg, for their development, giving a strong support to the theory of a pregnancy induced peripheral de novo conversion here presented.

Paternally-derived antigens present in fetal structures can be processed by maternal APCs and prone a specific Treg population which would allow the maternal tolerance in the presence of allo-antigens (Zenclussen *et al.*, 2010). However, there are still no clear conclusions regarding the participation of antigen-dependent and independent mechanisms for Treg expansion during

pregnancy. We have shown a higher Treg augmentation in allogeneic pregnancies compared with syngeneic ones. This could be observed immediately after implantation (d5) and in mid-pregnancy (d12) giving further evidence that the total expansion of Treg was stimulated by male alloantigens. Interestingly these observations were extended to tissues like the mesenteric lymph nodes which are not at the feto-maternal interface. A further explanation for this interesting observation might rely on the microchimerism phenomenon which is known to occur during pregnancy. Supporting this hypothesis it was already published that paternal-derived antigens can be detected at early pregnancy stages in several lymphoid organs and tissues like heart, lungs, liver and blood of pregnant females (Khoshroterani *et al.*, 2005; Dutta *et al.*, 2009; Zenclussen *et al.*, 2010). Our results are in concordance with a report from Zhao *et al.* who observed that a Treg expansion was augmented in the presence of fetal alloantigens (Zhao *et al.*, 2007). Contrasting, an older report suggested the alloantigen independence of $CD4^+CD25^+$ Treg expansion during pregnancy (Alluvihare *et al.*, 2004).

The strong hormonal changes that occur during pregnancy were further suggested as affecting Treg expansion (Aluvihare *et al.*, 2004). This hypothesis was also supported after the observation that physiological doses of estrogen mediate Treg expansion as well as the conversion of naïve T cells into Treg (Tai *et al.*, 2008). Contradictory data came from reports that neither estrogen nor progesterone alone or in combination had an influence on the number of Treg in ovariectomized mice (Zhao *et al.*, 2007). Additionally we have previously observed that the number of Treg increases in lymph nodes of females mated with intact or vasectomized males but could not be detected when pregnancy was mechanically induced as in the case of pseudo-pregnancy (Thuere *et al.*, 2007).

During the elaboration of this thesis we tried to analyze the peripheral conversion of Treg in syngeneic pregnancy in the $Rag1^{-/-}$ cell transfer model in the same way we did with allogeneic pregnancies. However this could not be achieved due to problems related with the maintenance of the $Rag1^{-/-}$ mice while the experiments were running. It would be of interest to study if the spatial patterns of thymic generation and peripheral conversion of Treg are an allogeneic dependent event in order to clarify the events behind the origin of Treg during pregnancy.

Unlike in other models (Sun *et al.*, 2007) the blockage of TGF-β did not exert any effect on the *de novo* conversion of Treg on the uterine draining lymph nodes during pregnancy suggesting that it occurs independently of this molecule. It has been described that mice which have $CD4^+$ cells that do not respond to TGF-β by expressing a dominant negative TGF-β receptor (dnTGFβr) still have similar levels of peripheral Treg compared with the wild type (Fahlen *et al.*, 2005) which may indicate a non-redundant role of TGF-β in the process of *de novo* generation of Treg.

A major role for IL-2 has been proposed in the maintenance of tolerance (Nelson, 2004). It appears to play a significant role in both thymic development and peripheral maintenance of Tregs (Schorle *et al.*, 1991; Setoguchi *et al.*, 2005). The importance of this cytokine was further potentiated by experiments showing that the number of peripheral Treg depend on the number of IL-2 producing cells. The data described in the present chapter showed that abortion prone mice treated with proven dosis of IL-2 could prevent abortive resorptions. This was followed by a dosis dependent increase in the population of $CD25^+$ cells in both uterine draining lymph nodes and thymus supporting the hypothesis that IL-2 prevented abortion through stimulating the expansion of Treg. A more reliable analysis

conclusion about an increase in the population of Treg would be done by analyzing the population of $CD4^{+}Foxp3^{+}$ cells, however at the time we realized this experience we didn't have access to the Foxp3 staining. Supporting this suggestion it has been recently demonstrated that after the use of an anti-CD25 antibody in pregnant mice, the population of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg was depleted. Pregnancy was impaired when the treatment was applied at the peri-implantation period (at day 2.5 of pregnancy), having no effect once implantation occurred (Shima *et al.*, 2010) which goes in line also with the results obtained in the chapter 3 of this dissertation.

-CHAPTER 5-

INFLUENCE OF MIGRATORY MOLECULES DURING PREGNANCY

5.1- INTRODUCTION

In the previous chapters the importance and origin of Treg during the different stages of pregnancy was discussed. It was shown that a pre-existing population of Treg at the beginning of pregnancy is essential for pregnancy onset but not maintenance while both thymic derived and *de novo* converted Treg bloom locally with the progress of pregnancy.
Tregs can act in both paracrine and cell-contact dependent ways, their cellular localization is then essential for their functional ability of suppressing specific immune responses (Siegmund *et al.*, 2005; Zhang *et al.*, 2009). This brings up the question on how are Treg recruited to work at the tissues were they are needed upon the different immunological requirements characterizing different stages of pregnancy.

Chemokines are known coordinators of leukocyte migration through the complex network system involving the lymphoid organs and peripheral tissues (Viola *et al.*, 2008). Interactions between the chemokine receptors expressed on the leukocyte surface and the ligands expressed on the target tissues constitute the basic concept of leukocyte migration to their site of action. Chemokine receptors respond classically to more than one ligand (Mantovani, 1999). This enables the coordination of an unrestrained system in which a leukocyte with a specific chemokine receptor can have a variable response towards the set formed by the several ligands existing in a specific tissue. Thus, the migratory outcome resultant from the different chemokine ligand/receptor established interactions is a complex issue to predict (Mantovani, 1999; Viola *et al.*, 2008).

Originally associated with immune responses of T effector cells during the process of Inflammation and viral clearance (Baggiolini, 1998) chemokines

have been additionally associated with the process of suppression by mediating the local recruitment of Treg cells to their site of action (Navarrete, 2009). Treg express several chemokine receptors whose ligands are expressed at the fetal–maternal interface (Pollard *et al.*, 1999; Robertson *et al.*, 1998; Wood *et al.*, 1999; Kallikourdis *et al.*, 2007; Guerin *et al.*, 2011).

Chemokine ligands such as CCL22, CX3CL1, CCL3, CCL4, and CCL5 were shown to be expressed in mouse uterus in a fluctuation pattern during the estrus cycle. This pattern was suggested to be synchronized with oscillations in Foxp3 mRNA expression levels occurring in the same tissue which might indicate a possible correlation of these chemokine ligands with an increasing population of Treg in the cycling uterus (Kallikourdis *et al.*, 2007).

Nevertheless, from these ligands only CCL4 showed high expression in the d10.5 pregnant uterus. Chemokine receptor CCR5, which is a receptor for the CCL4 ligand, is reportedly expressed by Treg in the gravid uterus facilitating the selection and retention of Treg expressing CCR5 in the implantation site (Kallikourdis *et al.*, 2007). This marker was claimed to be associated with a highly suppressive phenotype and may be a marker for those cells that have been activated by paternal alloantigen (Kallikourdis *et al.*, 2007).

The chemokine ligand CCL19 (MIP3beta) whose receptor CCR7 is also found on Treg, was also identified on glandular and luminal uterine epithelial cells and its maximum expression was found after the exposure to seminal fluid and sperm. A role for this chemokine on the recruitment of Treg into the implantation site was suggested, indicating the accumulation of Treg in the uterus before the implantation takes place (Guerin *et al.*, 2011).

Together, it seems that the expression of chemokines is tightly related with the mechanisms occurring at a given point in the local tissue being slightly different on the non-pregnant and pregnant uterus.

In this chapter it is discussed whether chemokines are involved in the recruitment of Treg into the feto-maternal interface at different stages during pregnancy. The temporal expression of different chemokine ligands at the feto-maternal tissues in different pregnancy stages is addressed as well as the migratory potential they exhibit towards their specific receptors expressed on Treg. Additionally, the *in vivo* effect of chemokine deletion is studied in genetic deficient animals to address the question whether this alone is sufficient to interfere with pregnancy onset or maintenance.

5.2- CHEMOKINE EXPRESSION AT THE FETO MATERNAL INTERFACE

To address the participation of different chemokine ligands in the migration of Treg into the feto-maternal interface we started by analyzing the mRNA levels of chemokines whose receptors are expressed in the surface of Treg. The levels of CCL21 (SLC or 6Ckine), CCL25 (TECK), CCL1 (TCA-3), CCL4 (MIP1β), CXCL12 (SDF1) and CCL20 (MIP3α) mRNA were then assessed by qRT-PCR in both sampled decidual and placental tissues from C57/BL6 mated BALB/c females on days 0, 2, 5, 8, 10 and 12 of pregnancy (Fig 5.1).

5.2.1- CCL21 and CCL25 mRNA expression increases at the feto-maternal interface as pregnancy advances.

Analysis of mRNAs expression of chosen chemokines in placenta and decidua confirmed their presence at the feto-maternal interface. Kinetic analysis at different pregnancy days revealed that CCL21 expression augmented in the decidua from day 8 of pregnancy onwards and was maintained high until day 12 and CCL25 expression increased in placenta on day 12 of pregnancy (Fig 5.1 A-B).

Increased mRNA levels in the placenta were also noticed for CCL1 and CCL4 at day 10 of pregnancy and for CXCL12 at day 12 of pregnancy (Fig 5.1 C-E). The expression of CCL20 showed no variation in the referred tissues during any of the analyzed time points (Fig 5.1 F).

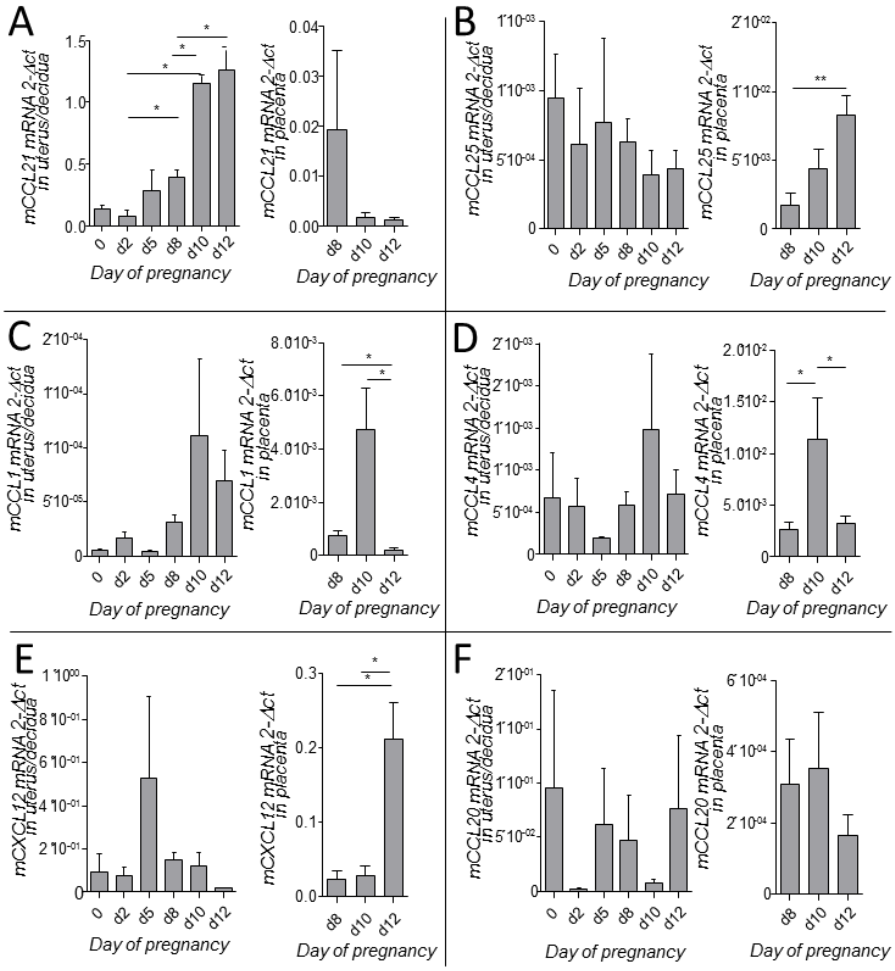

Figure 5.1- mRNA expression of chemokine ligands CCL21 and CCL25 increase at the feto maternal interface during pregnancy. BALB/c females were mated with C57/BL6 males and the expression of CCL21 (A), CCL25 (B), CCL1 (C), CCL4 (D), CXCL12 (E) and CCL20 (F) mRNAs in the tissues was analyzed on days 0, 2, 5, 8, 10 and 12 of pregnancy (d0, d2, d5, d8, d10 and d12) in the uterus/decidua and on days 8, 10 and 12 of pregnancy (d8, d10 and d12) in placenta. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05; **P≤0.01).

5.3- CCL21 AND CCL25 CHEMOKINE LIGANDS CAN ATTRACT TREG FROM THE FETO-MATERNAL INTERFACE

5.3.1- Treg from the uterus and draining lymph nodes express CCR7 and CCR9 chemokine receptors for CCL21 and CCL25, respectively.

To establish a potential correlation between the CCL21 and CCL25 ligands and the localization of Treg at the feto-maternal tissues, the expression of their specific chemokine receptors, CCR7 and CCR9, was analyzed on the population of $CD4^{+}Foxp3^{GFP+}$ cells from the uterus and uterine draining lymph nodes of either non pregnant (NP) or BALB/c mated pregnant Foxp3GFP females on day 12 of pregnancy (d12). The results indicate that both receptors are expressed in Treg of uterus (A) and the uterine draining lymph nodes (B) of both non-pregnant (NP) and pregnant animals (d12) (Fig 5.2).

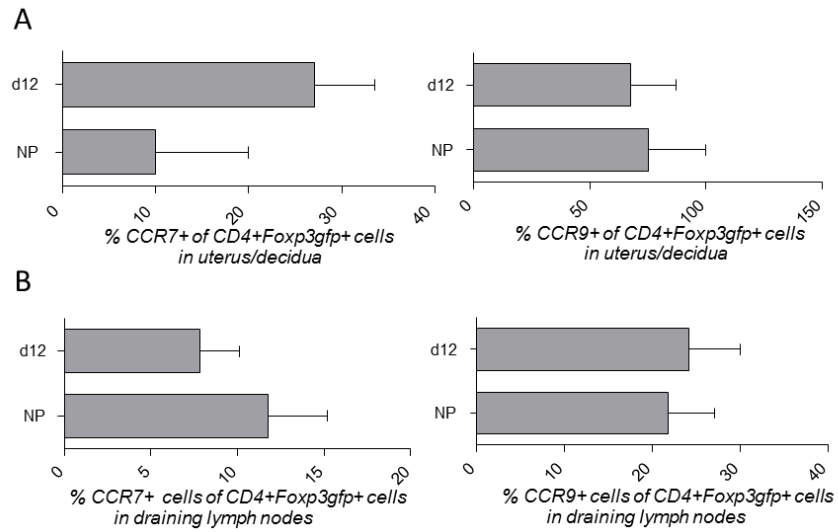

Figure 5.2- Treg at the feto-maternal interface express the chemokine receptors CCR7 and CCR9 regardless of the pregnancy state. The expression of CCL21 and CCL25 specific chemokine receptors, respectively CCR7 and CCR9, was analyzed in the gated population of $CD4^{+}Foxp3^{GFP+}$ cells from the total lymphocytes from uterus (A) and uterine draining lymph nodes (B) of either non-pregnant (NP) or BALB/c mated pregnant Foxp3GFP females on day 12 of pregnancy (d12). (n= 4 animals per group) Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test.

5.3.2- Treg from the uterine draining lymph nodes of both non pregnant and pregnant mice migrate *in vitro* towards CCL21 and CCL25.

Knowing that Treg express the receptors for CCL21 and CCL25 we next examined whether Treg with the potential to migrate to the feto-maternal interface were able to migrate in response to these chemokine ligands.

To answer this question, and assuming that Treg might migrate from the lymphoid organs draining the uterus to this site of action, chemotactic assays were performed with isolated cells of the uterine draining lymph nodes from Foxp3GFP non-pregnant or d12 pregnant females (mated with BALB/c males). We tested whether isolated Treg were able to migrate upon CCL21 or CCL25 murine recombinant chemokine placed at the bottom of transwell migration chambers (Fig 5.3).

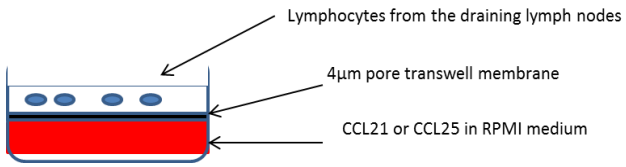

Figure 5.3- Experimental setting of the assay to analyze the migration of Treg towards the chemokine ligands. Using total isolated cells from the uterine draining lymph nodes of $Foxp3^{GFP}$ non-pregnant mice or mice at day 12 of pregnancy, the migration of Treg towards both recombinant CCL21 and CCL25 was analyzed after 120 minutes of incubation by determining the relative number of $CD4^{+}Foxp3^{GFP+}$ cells present in the lower part of a two-chamber transwell system, referred to the total number of $CD4^{+}Foxp3^{GFP+}$ cells in both upper and lower parts of the chambers. Spontaneous migration was subtracted.

The results showed that Treg from the uterine draining lymph nodes from both, pregnant and NP mice migrated towards the chemokine gradient of CCL21 and CCL25. This migration was slighter higher, but not significantly, for Treg from the pregnant animals in comparison with the NP ones (Fig 5.4).

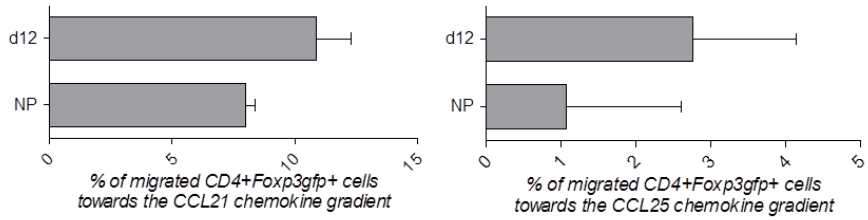

Figure 5.4- Treg from the uterine lymph nodes of both NP and pregnant animals migrate towards the ligands CCL21 and CCL25. The percentage of $CD4^{+}Foxp3^{GFP+}$ cells migrating towards the CCL21 or CCL25 gradient was analyzed by the use of migration assays with cell from the uterine draining lymph nodes of Foxp3GFP non-pregnant mice (NP) or mice on day 12 of pregnancy (d12). Data are expressed as means with SEM of three experiments (n=3 animals per group) done in triplicates each and was analyzed by using the Mann-Whitney-U test.

5.4- *IN VIVO* IMPLICANCE OF CCR7 AND CCR9 CHEMOKINE RECEPTORS EXPRESSION FOR PREGNANCY ESTABLISHMENT AND MAINTENANCE

Having shown that CCL21 and CCL25 are expressed at the feto-maternal interface and can attract Treg from the uterine draining lymph nodes *in vitro* and that uterine and draining lymph nodes Treg express CCR7 and CCR9 we next addressed the influence that this interaction would exert on pregnancy establishment and maintenance. For this we analyzed reproductive features in knockout mice for the CCR7 and CCR9 receptors (Förster *et al.*, 1999; Wurbel *et al*., 2001).

5.4.1- CCR7

5.4.1.1- CCR7 deletion derives in the absence of Treg in the pre-conception uterus.

In the non-pregnant wild type animals the population of Treg expresses CCR7 and migrates towars the CCL21 ligand. Here, the populations of $CD4^{+}Foxp3^{+}$ Treg and $CD4^{+}Foxp3^{-}$ Teff in the uterus of non-pregnant $CCR7^{-/-}$ mice were analyzed in order to address the effects of the absence of this receptor on

these T cell populations. Non pregnant C57/BL6 mice were used as control group.

The results showed that lack of CCR7 derived in the absence of $CD4^+Foxp3^+$ Treg (Fig 5.5A). The population of $CD4^+Foxp3^-$ Teff cells in uterine tissue of the $CCR7^{-/-}$ mice did not significantly change in comparison to wild type (Fig 5.5B).

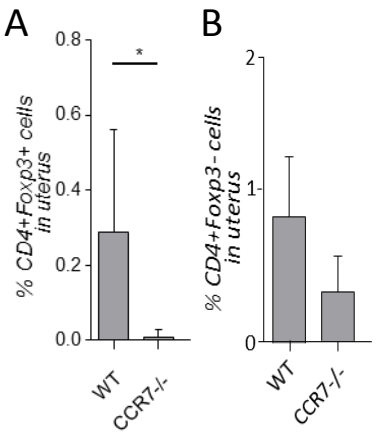

Figure 5.5- Lack of CCR7 leads to the absence of Treg without affecting significantly Teff in the pre-conception uterus. The percentage of $CD4^+Foxp3^+$ cells (A) and $CD4^+Foxp3^-$ cells (B) was analyzed in the uterus of C57/BL6 (WT) and $CCR7^{-/-}$ females (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P <0.05).

5.4.1.2- CCR7 absence compromises pregnancy establishment

To analyse the effects of CCR7 absence in the establishment of pregnancy CCR7 knockout females were paired with both syngeneic (C57/BL6) and allogeneic (BALB/c) males. The number of plug detections, after mating, that resulted in a real pregnancy establishment twelve days later, was quantified as a marker of a successful conception/implantation. Mated C57/BL6 female animals were used as control.

The results demonstrated that the number of plug detections that did not result in pregnancy was higher in pregnancies from the knockout mice than wild type ones. This consequently revealed a lower percentage of CCR7$^{-/-}$ females with implantations on these animals that was significant in allogeneic pregnancies of CCR7$^{-/-}$mice when compared with the wild type counterparts (Fig 5.6).

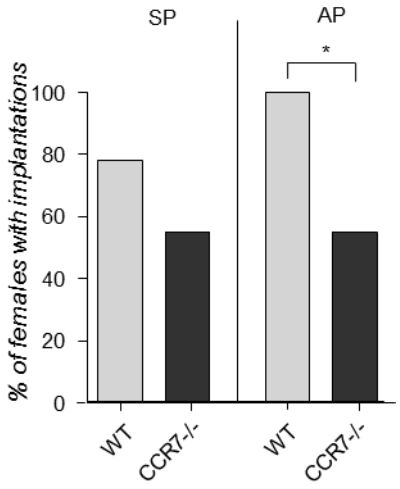

Table 5.6- Implantation is impaired in CCR7$^{-/-}$ mice. The percentage of females with implantations was measured by the presence of fetal implants 12 days after the observation of a plug in CCR7$^{-/-}$ mated syngeneically (BALB/c males) or allogeneically (C57/BL6 males). Controls consisted of C57/BL6 females mated with the same mating pairs (n=5 animals per group). Data are expressed as bars representing the percentage of the total and was analyzed by the Two-sided Fisher's exact test (*: P<0,05)

5.4.1.3- CCR7 absence do not significantly alter the uterine Treg population in mid pregnancy

In wild type animals, Treg from the pregnant uterus also express the CCR7 chemokine receptors and migrate towards CCL21 ligand. To address the importance of this chemokine receptor in the homing or proliferation of Treg at the maternal-fetal interface during the pregnancy state, the population of

$CD4^+Foxp3^+$ Treg was analyzed on the pregnant knockout mice in both allogeneic and syngeneic established pregnancies and compared with the wild type counterparts. The population of Treg from the knockout pregnant mice is lower in comparison with the wild type corresponding groups however this different is not significant (Fig 5.7).

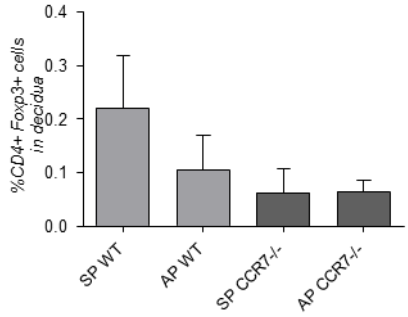

Figure 5.7- The absence of CCR7 do not significantly influence the uterine population of Treg on day 12 of pregnancy. CCR7-/- and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. The percentage of $CD4^+Foxp3^+$ cells was analyzed in the decidua on day 12 of pregnancy. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05).

5.4.1.4- CCR7 deletion is related with changes in the abundance of $CD49b^+$ cells in antigenic dependent way at the feto-maternal interface during pregnancy.

Given the increase expression of CCL21 mRNA observed on day 10 and 12 of pregnancy in either decidua or placenta it was to expect that these chemokines are exerting a local effect at the feto maternal interface on this time point. Therefore, and since CCR7 is also expressed in the surface of other immune cells, the effect of these chemokines on the maturation state of $CD11c^+$ cells and on the population of $CD49b^+$ cells (Fig 5.8 B) was analyzed at the feto maternal interface of the pregnant knockout mice in both allogeneic and syngeneic pregnancies and compared to the ones obtained in the pregnant wild type mice.

No significant differences were observed in the maturation of $CD11c^+$ cells. Additionally, the population of decidual and placental $CD49b^+$ cells in the wild type syngeneic pregnancies was significantly diminished this was not be observed in the $CCR7^{-/-}$ animals.

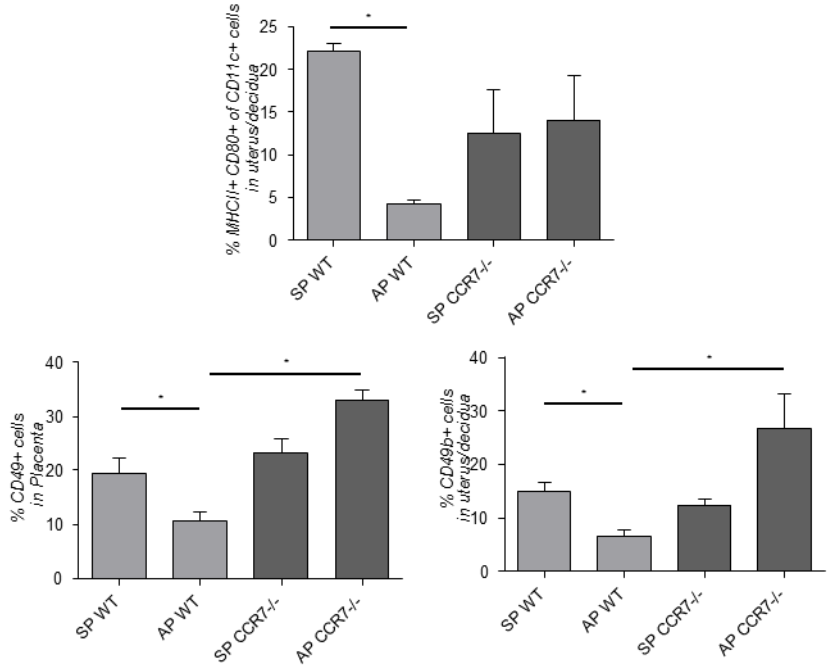

Figure 5.8- The absence of CCR7 chemokine receptor influences the maturation of dendritic cells and cause changes in the abundance of NK cells in an antigenic dependent way at the feto maternal interface on day 12 of pregnancy. $CCR7^{-/-}$ and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. The percentage of $MHCII^+CD80^+$ of $CD11c^+$ cells was analyzed in the decidua (A) and the percentage of $CD49b^+$ cells was analyzed in both decidua and placenta (B) on day 12 of pregnancy. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05; **P≤0.01).

These data further indicated the occurrence of antigenic dependent changes in different immune cell populations during pregnancy that seem to be dependent on CCR7.

5.4.1.5- CCR7 absence does not significantly interfere with the outcome of pregnancy once it is established

It could be observed that, besides the decreased implantation success, in some animals however pregnancy could be established. Thus, it was possible to analyze the importance of CCR7 in the pregnancy outcome of those pregnant animals. With that purpose, abortion ratio and implantations number were analysed on day 12 of pregnancy of both allogeneic and syngeneic CCR7$^{-/-}$ pregnant mice. Pregnant C57/BL6 animals were used as control.

We observed that either the abortion rate or implantation numbers of the knockout animals were similar to the control mice in both allogeneic and syngeneic pregnancies. This predicts that the lack of CCR7 do not affect pregnancy outcome once it is established (Fig 5.9).

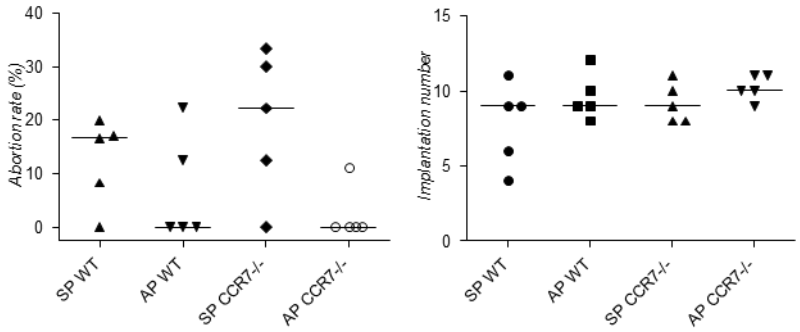

Figure 5.9- Once pregnancy is successfully established, CCR7$^{-/-}$ animals have normal abortion ratios and implantation number in both allogeneic and syngeneic pregnancies. CCR7$^{-/-}$ and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. Abortion rate and the implantation number were observed on day 12 of pregnancy. (n=5 animals per group). Data are expressed as medians and were analyzed by using the Mann-Whitney-U test.

5.4.2- CCR9

5.4.2.1- CCR9 deletion leads to an augmentation of Teff in the pre-conception uterus.

As it was observed, Treg from the non-pregnant wild type animals expressed CCR9 and were responsive to CCL25. To study the effects of the absence of CCR9 on the uterine T cells we analyzed the populations of $CD4^{+}Foxp3^{+}$ Treg and $CD4^{+}Foxp3^{-}$ Teff from the uterus of non-pregnant $CCR9^{-/-}$ mice. Non pregnant C57/BL6 mice were used as control group.

The absence of CCR9 resulted in an increment on the population of Teff. No significant changes could be observed without in the population of Treg in the uterus of the knockout animals (Fig 5.10 A and B).

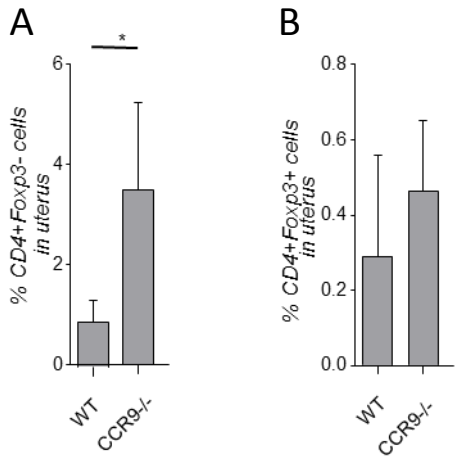

Figure 5.10- Lack of CCR9 leads to an augmentation of Teff without affecting Treg in the pre-conception uterus. The percentage of $CD4^{+}Foxp3^{+}$ cells (A) and $CD4^{+}Foxp3^{-}$ cells (B) was analyzed in the uterus of C57/BL6 (WT) and $CCR9^{-/-}$ females (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P <0.05).

5.4.2.2- Absence of CCR9 resulted in an incremented maturation of dendritic cells from the uterine draining lymph nodes.

CCR9 expression was shown to define a population of highly tolerogenic plasmocytoid dendritic cells that are potent inducers of regulatory T cell function (Hadeiba *et al.*, 2008). In addition, it was also described that $CCR9^{low}$ DCs are more mature that $CCR9^{high}$ and act as more potent stimulators of T cells (Drakes *et al.*, 2009).

Since the population of Teff cells was found to be higher in the uterus of non-pregnant $CCR9^{-/-}$ mice and this can be caused by an higher maturation state of DCs (thus, less tolerogenic), we analyzed the expression of $MHCII^{+}CD80^{+}$ on the gated population of $CD11c^{+}$ cells from uterine draining lymph nodes to addressed their maturation state. As expected, a higher percentage of $MHCII^{+}CD80^{+}$ cells of the $CD11c^{+}$ cells was observed in the analyzed uterine lymphoid organs of $CCR9^{-/-}$ mice comparing with the wild type control (Fig 5.11).

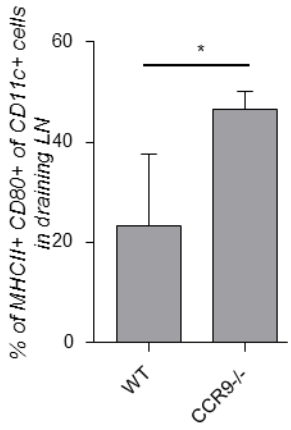

Figure 5.11- $CD11c^{+}$ population in the uterine draining lymph nodes of $CCR9^{-/-}$ mice exhibit a higher maturation phenotype. The percentage of $MHCII^{+}CD80^{+}$ of $CD11c^{+}$ cells was analyzed in both non pregnant $CCR9^{-/-}$ and C57/BL6 (WT) females (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P <0.05).

5.4.2.3- CCR9 absence negatively influences pregnancy establishment

CCR9 knockout females were paired with both syngeneic (C57/BL6) and allogeneic (BALB/c) males. The number of plug detections, after mating, that was confirmed as a real pregnancy after 12 days, was quantified as a marker of a successful conception/implantation. Mated C57/BL6 female animals were used as control.

We observed a lower percentage of pregnant females after plug detection on the CCR9$^{-/-}$ animals when compared with the wild type. This difference was significant in the allogeneic pregnancies (Figure 5.12).

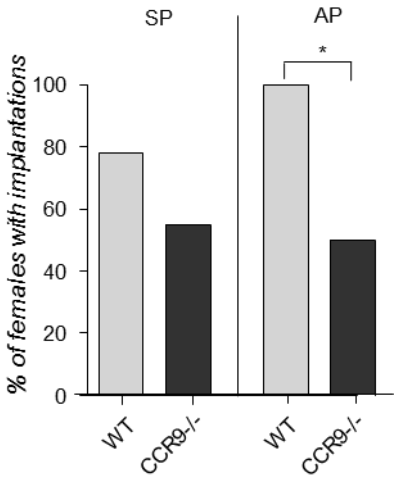

Figure 5.12- Implantation is impaired in CCR9$^{-/-}$ mice. The percentage of females with implantations was measured by the presence of fetal implants 12 days after the observation of a plug in CCR9$^{-/-}$ mated syngeneically (BALB/c males) or allogeneically (C57/BL6 males). Controls consisted of C57/BL6 females mated with the same mating pairs. (n=5 animals per group). Data are expressed as bars representing the percentage of the total and was analyzed by the Two-sided Fisher's exact test (*: P<0,05)

5.4.2.4- CCR9-/- mice show a normal uterine Treg distribuition in mid pregnancy.

Having observed that in wild type animals, Treg from the pregnant uterus express the CCR9 chemokine receptors and migrate towards the CCL25 ligand we analyzed the importance of this chemokine receptor in the homing or proliferation of Treg at the feto-maternal interface during the pregnancy state. The population of $CD4^{+}Foxp3^{+}$ cells was analyzed on allogeneic and syngeneic established pregnancies of $CCR9^{-/-}$ mice and compared with the wild type counterparts. However, there were no detectable differences in the populations of Treg from any of the knockout pregnant mice group in comparison with the corresponding wild type groups (Fig 5.13).

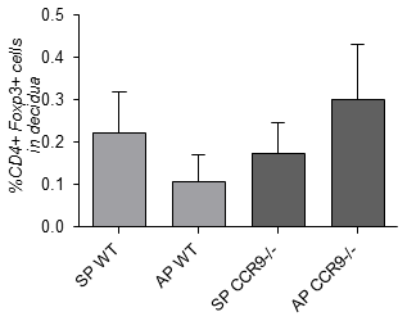

Figure 5.13- The absence of CCR9 chemokine receptor do not significantly influence the population of Treg on day 12 of pregnancy. $CCR9^{-/-}$ and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. The percentage of $CD4^{+}Foxp3^{+}$ cells was analyzed in the decidua on day 12 of pregnancy. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05).

5.4.2.5- CCR9 absence influences the the maturation of dendritic cells and cause changes in the abundance of CD49b+ cells in an antigenic dependent way at the feto maternal interface during pregnancy.

The higher expression of CCL25 mRNA at the placenta on day 12 of pregnancy led us to the hypothesis that this chemokine might exert a local effect in other immune cells. Considering this assumption, the maturation state of $CD11c^+$ cells the population of $CD49b^+$ cells were analyzed at the feto maternal interface of the pregnant knockout mice in both allogeneic and syngeneic pregnancies and compared to the ones obtained in the pregnant wild type mice.

Comparing with the results obtained in the wild type correspondent pregnancies, is highlighted an increased maturation of $CD11c^+$ cells at the decidua in the allogeneic pregnancies of $CCR9^{-/-}$ mice that contrast with the lower maturation these cells exhibit in the syngeneic pregnancies. Unexpectedly, the population of $CD11c^+$ cells from the $CCR9^{-/-}$ syngeneic pregnant mice is less mature than the wild type controls (Fig 5.14 A). Additionally, contrasting with the wild type, in the absence of CCR9 there are no differences in the population of $CD49b^+$ cells in between allogeneic and syngeneic pregnant animals.

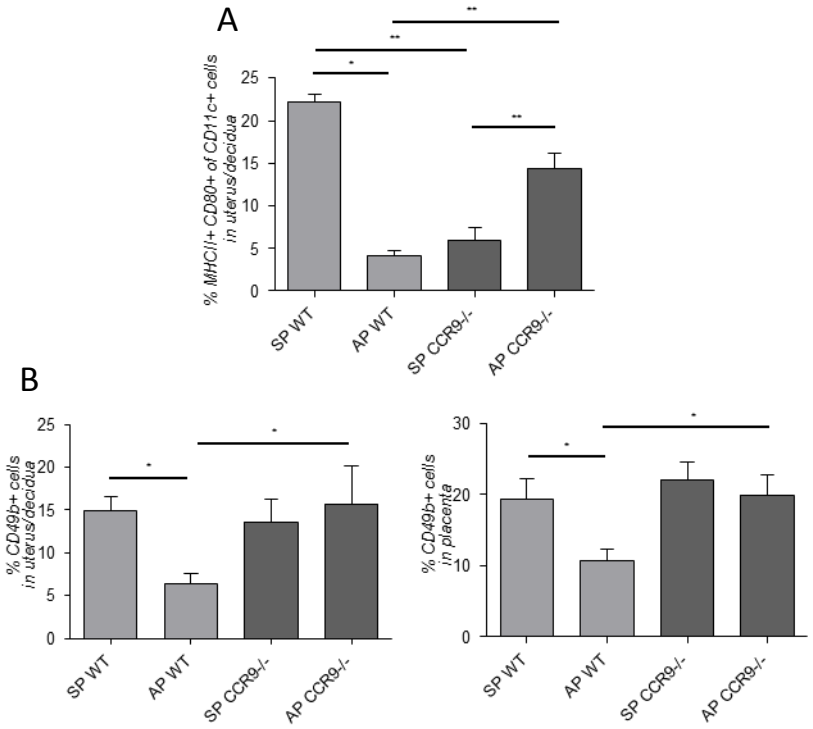

Figure 5.14- The absence of CCR9 chemokine receptor influences the maturation of dendritic cells and causes changes in the abundance of NK cells in an antigenic dependent way at the feto-maternal interface on day 12 of pregnancy. $CCR9^{-/-}$ and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. The percentage of $MHCII^{+}CD80^{+}$ of $CD11c^{+}$ cells was analyzed in the decidua (A) and the percentage of $CD49^{+}$ cells was analyzed in both decidua and placenta (B) on day 12 of pregnancy. (n=5 animals per group). Data are expressed as means with SEM and were analyzed by using the Mann-Whitney-U test (*:P ≤0.05; **P≤0.01).

These data further indicated the occurrence of antigenic dependent changes in different immune cell populations during pregnancy that seem to be dependent on CCR9.

5.4.2.6- Absence of CCR9 does not influence pregnancy outcome if implantation took place.

Besides the low rate of implantation in CCR9$^{-/-}$ mice, in some animals pregnancy was established. We therefore analyzed the importance of CCR9 in the pregnancy outcome of those pregnant animals. For that, abortion ratio and implantations number were analysed on day 12 of pregnancy of both allogeneic and syngeneic CCR9$^{-/-}$ pregnant mice. Pregnant C57/BL6 animals were used as control.

We could observe that both abortion rate and implantation numbers of the syngeneic or allogeneic pregnant knockout animals was similar to the wild type mice independently of the alloantigen (Fig 5.15).

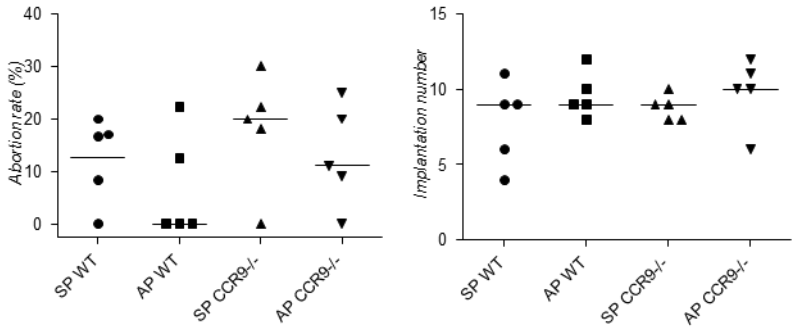

Figure 5.15- Once pregnancy is established, CCR9$^{-/-}$ animals have normal abortion ratios and implantation number in both allogeneic and syngeneic pregnancies. CCR9$^{-/-}$ and C57/BL6 (WT) females were paired with either C57/BL6 (syngeneic pregnancy = SP) or BALB/c (allogeneic pregnancy = AP) males. Abortion rate and the implantation number were observed on day 12 of pregnancy. (n=5 animals per group). Data are expressed as medians and were analyzed by using the Mann-Whitney-U test.

5.5- DISCUSSION

Migration of Treg is known to take place through the action of chemoatractants. Hormones like the human chorionic gonadotropin were shown to attract Treg into the feto-maternal interface during early human pregnancy (Schumacher *et al.*, 2009). Aditionally, chemokines are widely known participants in the process of Treg migration (Bono *et al*., 2007). The aim of this chapter was to evaluate the contribution of chemokines in the recruitment of Treg to the feto-maternal interface during the progress of pregnancy. By using qRT-PCR the relative mRNA amount of different chemokines whose receptors are known to be expressed in the surface of Treg was quantified at several pregnancy time points (d0, d2, d5, d8, d10, d12) in the decidua and placenta.

Treg act in a local way, therefore the coordination of their migratory events to the action site is an essential process. During pregnancy different gestational steps occur characterized by specific temporal processes taking place with the requirement of distinct environments. We therefore analyzed different periods of pregnancy taking into account the developmental processes occurring at these time points: conception (day 0), pre-implantation (day 2), implantation (day 5), labyrinth formation and vascularization (day 8), placental development (day 10), complete formation of the chorioallantoic placenta (day 12).

Several chemokine ligands were selected for analysis by having the common characteristic that their receptors were previously described to be involved on the Treg localization to specific tissues. These included CCL21, TCA-3 (CCL1 homologous in mice), CCL4, CCL25, CXCL12 and CCL20 which correspondent receptors are, respectively CCR7, CCR8, CCR9, CXCR4 and CCR6 (Iellem *et al*., 2001; Zou *et al.*, 2004; Schneider *et al.*, 2007; Yamazaki *et*

al., 2008; Wermers *et al.*, 2011). They were all expressed at mRNA levels in decidua and/or placenta.

Of the analyzed ligands, CCL21 and CCL25 were selected by having a significant mRNA expression increase in either decidua or placenta at one or more of the analyzed time points. Accordingly, mRNA expression of CCL21 had a 16 fold increase in decidua 10 days after conception and this increase was maintained until day 12 of pregnancy. CCL25 showed a 5 fold increase in placenta on day 12 of pregnancy. These increases correlate with the general increase on the population of Treg in the different tissues and specifically on decidua and draining lymph nodes, of the pregnant females on day 10 of pregnancy.

Hypothesizing that Treg migrate to the lymphoid organs draining the feto maternal interface and from there to the local tissues we further analyzed the migratory potential of the Treg from the iliac lymph nodes towards the ligands CCL21 and CCL25 found to be expressed on the decidua and placenta. This analysis was performed with both cells from NP and d12 pregnant animals. The results revealed that although the expression of CCR7 and CCR9, the respective chemokine receptors for CCL21 and CCL25 ligands, on the surface of Treg did not change with pregnancy progression, the percentage of Treg migrating towards the ligands only slightly increased, but not significantly, in the Iliac lymph node cell population from the animals on pregnant compared to NP ones. This goes in accordance with the results obtained in chapter 4. Treg from NP animals equally express both CCR7 and CCR9 receptors and migrate *in vitro* towards the ligands thus, even the expression of CCL21 and CCL25 increases at the feto-maternal interface with the progression of pregnancy it is possible that these ligands are participating in the establishment of pregnancy at the start, by influencing the population

of Treg that was shown to be essential for this process at the start of pregnancy.

Chemokine receptors, especially CCR7 and CCR9, expressed in Treg (Schneider *et al.*, 2007; Wermers *et al.*, 2011), were described as major players in the acquisition of tolerance mechanisms (Worbs and Förster, 2007; Hadeiba *et al.*, 2008; Drakes *et al.*, 2009). To address the participation of these receptors on regulation and maintenance of a successful pregnancy, $CCR7^{-/-}$ and $CCR9^{-/-}$ animals were employed.

CCR7 is required for the homing of T cells and DCs to the lymphoid organs and epithelial tissues under both inflammatory and steady state environmental conditions (Förster et al., 1999; Ohl et al. 2004). In the uterus of $CCR7^{-/-}$ mice the Treg population was drastically reduced further suggesting a role for this receptor in the homing of Treg to the uterine epithelial tissue even before conception takes place. In fact, we observed that $CCR7^{-/-}$ animals had a significantly lower implantation rate when compared to wild type controls. Interestingly, Guerin et al. (2011) already proposed a role for this chemokine in the recruitment of Treg into the peri-implantation site by reporting the presence of another CCR7 ligand, CCL19, in glandular and luminal uterine epithelial cells, and showing its maximum expression after being exposed to seminal fluid and sperm (Guerin *et al.*, 2011).

Lack of CCR9 in the NP animals resulted in a significant increase in the uterine population of CD4 effector cells. A higher maturation state of DCs in the lymphoid organs draining the uterus was observed in these animals as well. As previously described in other mucosal environments (Hadeiba *et al.*, 2008; Drakes *et al.*, 2009), CCR9 can act as a regulator of inflammatory states (Wermers *et al.*, 2011). Consequently, it is not surprisingly that animals

lacking CCR9 also showed a poor implantation success which can be attributed to the excessive inflammation occurring in its absence.

Thus, Treg absence or increased presence of effector T cells in uterus might be interfering with ovum fecundation. The impairment of Treg function and an excess of inflammatory cells and molecules is often the cause of infertility in the impaired endometrial function observed under inflammatory states like endometritis (Czernobilsky, 1978; Gilbert, 2011). Therefore it is possible that a similar environment is occurring in the $CCR7^{-/-}$ and $CCR9^{-/-}$ animals which might explain their reduced implantation rates.

Despite impaired implantation, $CCR7^{-/-}$ and $CCR9^{-/-}$ mice presented normal pregnancy with low abortion rates, suggesting that once the implantation is successfully established, the lack of these chemokine receptors does not affect pregnancy outcome, resulting in normal syngeneic and allogeneic pregnancies.

However, the higher CCL21 and CCL25 mRNA expression observed at the feto-maternal interface at later time points of pregnancy suggest a role for these chemokines in these tissues after implantation is settled. While the decidual population of Treg did not show any differences in the knockout animals in comparison with the wild type ones in either syngeneic or allogeneic pregnancies, the population of both and decidual and placental $CD49b^{+}$ cells or the maturation state of the population of decidual $CD11c^{+}$ cells showed to be regulated by the CCR7 and CCR9 chemokine receptors in an antigenic dependent way. This may interfere with the fine regulation of other processes which escape the scope of this thesis.

The results presented in this chapter allow the assumption that although pregnancy is possible and apparently normal in the absence of CCR9 or CCR7, it establishment is severly impaired in the absence of these chemokines and $CCR9^{-/-}$ and $CCR7^{-/-}$ mice show consequently a decreased fertility. Through interaction with their chemokine ligands, CCR7 and CCR9 participate in the homing or proliferation of Treg and Teff cells in the uterus possibly affecting the regulatory mechanisms in this tissue before implantation. The mechanisms for recruitment of Treg cells to this action site are likely different after embryo implantation takes place once no differences could be observed in the Treg population caused by the lack of either CCR7 or CCR9 later on pregnancy. Other immune cell populations seem however to be affected in an antigenic dependent way by these chemokine receptors in the course of pregnancy.

-CHAPTER 6-

FINAL REMARKS AND FUTURE PERSPECTIVES

6.1- FINAL REMARKS AND FUTURE PERSPECTIVES

With the evolution of placental pregnancy a dramatic challenge is presented to the maternal immune system by the continuous exposure to the paternal antigens expressed by the semi-allogeneic fetus.

Treg are known suppressors of a wide variety of auto-immune responses (Sakaguchi, 2004), they are shown to mediate natural tolerance to transplants (Kingsley et al., 2002) and seem to play an important role on the maternal immune tolerance towards fetal alloantigens (Aluvihare *et al.*, 2004; Rowe *et al.*, 2011). The lineage-specific forkhead transcription factor Foxp3 is now accepted as the master-regulator of Treg development and function (Hori *et al.*, 2003; Khattri *et al.*, 2003; Fontenot *et al.*, 2005). A link between the appearance of $Foxp3^+$ expression and an invasive placentation has been recently suggested giving strength to the hypothesis of an essential role for Treg in a successful pregnancy (Andersen *et al.*, 2012; Samstein *et al.*, 2012). In fact, there are evidences suggesting a requirement for Treg prior to implantation (Zenclussen *et al.*, 2005). While only their beneficial effect in pregnancy has been shown (Zenclussen *et al.*, 2005; Schumacher *et al.*, 2007; Shima *et al.*, 2010; Samstein *et al.*, 2012) no scientific evidences confirmed their indispensability for pregnancy establishment.

We confirmed the existence of Treg in uterus and vaginal fluid of non-pregnant females and documented the cyclic expansion of this population in the vaginal fluid of mice during the oestrus cycle that resulted in a peak during the receptive period (estrus phase). Treg clustering in uterus during this phase was further confirmed in the uterine tissue by our collaborative work with the laboratory of Carlos Tadokoro, IGC, Oeiras. By using 2-photon microscopy, Treg were shown to gather at specific sites along the uterus just before conception. These data are consistent with a recently published work

(Guerin *et al.*, 2011) where, by IHC, it was also observed a distribution of individual or clustered Treg throughout the endometrial tissue with fewer cells in the myometrial tissue of mice during the estrus phase. Also supporting our observations, other studies have reported a higher amount of Foxp3 mRNA transcripts during the estrus phase of the oestrus cycle in mice (Kallikourdis *et al.*, 2007) and an expansion of $CD4^+CD25^+FOXP3^+$ in blood of fertile women occurring during the late follicular phase of the menstrual cycle (Arruvito *et al.*, 2007). However, this study is the first to prove the oscilations of Treg at the celular level rather than the transcripts.

Our results are, in addition, the first to describe that Treg accumulation during the receptive phase of the estrus cycle is not only occurring in the uterus but they also appear in vaginal lumen. Treg in the vaginal tract may therefore constitute the primary "tolerance line" at the moment of conception, helping in the survival of sperm cells armed with male antigens, which could further move straight through the cervix and across the uterus to meet the oocyte in the *ampulla* of the fallopian tube.

While the cyclic augmentations of Treg were informative of their importance for pregnancy establishment, the results obtained after the depletion of Treg in the Foxp3DTR mice demonstrated that Treg must be present at the moment of conception for implantation to occur but are dispensable for pregnancy maintenance. These findings were further confirmed in a second model of Treg depletion, the Foxp3.LuciDTR mice (Suffner *et al.*, 2010) by collaborative work with the lab of Günter Hämmerling, DKFZ, Heidelberg. A severely impaired implantation was also observed in these mice when $Foxp3^+$ cells were depleted from the system before mating the animals. This was observed in both allogeneic and syngeneic pregnancy combinations, which leads us to the assumption that the absence of Treg would affect an antigenic-independent process at this particular time point. This may occur

because the absence of Treg fosters a massive inflammation that hinders implantation.

Results from our cooperation with the laboratory in Heidelberg have further shown that the decreased fertility success after Treg depletion was characterized by activation of Teff cells in the uterine draining lymph nodes and an accumulation of $CD8^+$cells in the uterus. This suggests that the depletion of Treg provokes an inflammatory response on the uterine region. This might have a negative influence in the physiological conditions necessary for the sperm to move towards the ovum.

Implantation is also a known inflammatory process and therefore it is tempting to hypothesize that Treg are required to counteract this process during this stage. Consequently, the absence of Treg would change the immune balance and result in an exacerbate inflammation with a negative effect on pregnancy.

The present study was the first to demonstrate that pre-existence of a population of Treg before the conception is essential for a proper pregnancy establishment. This population seems therefore to control the activation of Teff cells and the accumulation of $CD8^+$ cells in the uterus which might have a deleterious effect on the fertilization or implantation process. Further experiments to address the levels of inflammatory cytokines after Treg depletion as well as the histological changes in the uterus should provide a better clarification of this process.

It was reported that vaginal delivery of TGF-β at the time of pairing increased Treg numbers and reduced abortion in CBA/J x DBA/2J mouse model (Clark *et al*., 2008). TGF-β intravaginal administration did not improve implantation success after Treg depletion in our model. Thus, Treg depletion seems to

affect the local tissues previous to conception, therefore it might be worth to study the outcome of TGF-β application before mating the animals. In addition, the action of other Treg-associated soluble factors such as IL-10 or IL-9 should be characterized as they may be also implied in the process of fertilization and/or implantation.

Treatment with an anti-CD25 monoclonal antibody (Shima *et al.*, 2010) also resulted in implantation failure only if the treatment was applied at day 2.5 of pregnancy. These authors further observed an increased resorption rate after anti-CD25 treatment which was significant in the allogeneic pregnancies but not in the syngeneic ones even when the treatment was applied during the peri-implantation and post-implantation period. Although the model used was different, our results contrast with the reported outcome since we observed that depletion of Foxp3+ Treg after the onset of pregnancy did not provoke any effect on the implantation rates or resulted in significant changes of the abortion rate. Our work is nevertheless consistent with another study (Samstein *et al.*, 2012) published very recently, in which $Foxp3^{+}$ Treg deletion once allogeneic pregnancy is established caused the rejection of around 10% of the fetuses. However, contrasting with the view of these authors, we consider that this low percentage of rejection is not representing a significant effect considering that 90% of fetal survival and 10% of abortion is the usual abortion rate in allogeneic combination (Thuere *et al.*, 2007).

We analyzed the kinetics of Treg in a normal allogeneic pregnancy and investigated their origin during different stages of pregnancy by quantifying the percentage of $Helios^{+}$ cell, of thymic origin (Thornton *et al.*, 2010), within Treg or by directly tracing peripheral conversion or thymic derived migration in a lymphopenic mouse model transferred with either peripheral $CD4^{+}Foxp3^{GFP-}$ cells or thymocytes from Foxp3GFP mice. Additionally, the

mechanisms behind their origin and action were addressed by analyzing the influence of IL-2 on pregnancy outcome in an abortion prone mouse model and the effects of male allo-antigens in the proliferation of Treg.

The kinetics of Treg documented that total Treg augment at the beginning of pregnancy on day 2 after mating which was suggested to be elicited by the seminal fluid (Thuere *et al.*, 2007; Robertson *et al.*, 2009; Zenclussen *et al.*, 2010). At day 5 of pregnancy there was a general decrease in this population which might be motivated by the inflammatory process of implantation (Sherer and Abulafia, 2001; Fiedler *et al.*, 2007) established just before this time point. At day 8, the population of Treg showed the lowest percentage of the analyzed time points probably because this stage is characterized by the formation of the invasive labyrinth (Brosens *et al*., 1967, Muntener *et al*., 1977). A new expansion of total Treg was further observed from day 10 of pregnancy.

Helios^{+} Treg, proposed to represent thymic derived Treg, were the predominant population at early pregnancy. This suggests a thymic derivation of Treg at the uterine region before implantation takes place. At day 5 of pregnancy, just after the establishment of implantation, this population of thymic derived Treg diminishes and is followed by the augmentation of Helios^{-}, thus peripheral converted Treg. To address whether in fact a conversion took place, we next analyzed whether Foxp3^{-} cells convert into Foxp3^{+} because of pregnancy. Some authors question the validity of Helios as marker for thymic Treg indicating that their expression in peripheral induced Treg might be reflecting the context of stimulation during Foxp3 induction (Gottschalk *et al.*, 2012). However, Thornton *et al.* (2010) observed that young mice (3–7 d old) had exclusively Foxp3^{+}Helios^{+} and they could not detect a population of Helios^{+} cells in the thymus from these animals, suggesting that Helios^{-}Foxp3^{+} cells observed in thymus of adult mice

are cells that were induced in the periphery and entered the thymus through recirculation (Thornton et al., 2010). Supporting the existence of peripheral conversion of Treg during pregnancy we confirmed, by the *de novo* expression of GFP in Teff cells transferred in a $Rag1^{-/-}$ lymphopenic mouse model, an augmented conversion of Treg at day 5 of pregnancy in the uterine lymph nodes, blood and mesenteric lymph nodes. This could also be observed later, on day 10 of pregnancy. The percentage of migrated $Foxp3^{GFP+}$ cells from the thymocytes transferred to $Rag1^{-/-}$ lymphopenic mice is similar to the one observed in non-pregnant mice. This suggests that Treg proliferate in the thymus at the beginning of pregnancy and migrate to the periphery on the following days. The peripheral conversion of Treg also augments after implantation takes place and is maintained until later in pregnancy. In line with our results, a very recently published report (Samstein *et al.*, 2012) elegantly showed that peripheral converted Treg and not thymic derived Treg are responsible for the avoidance of fetal resorption, an event taking place at mid pregnancy, after implantation. The importance of peripheral converted Treg or thymic derived Treg for implantation was however not addressed in the referred study.

We observed an increased systemic expansion of Treg in allogeneically pregnant females just after implantation (d5) and mid pregnancy (d12) stages suggesting that Treg expansion on these pregnancy time points is alloantigen-dependent. Our results contrast with an initial report suggesting the alloantigen independence of Treg expansion during pregnancy (Alluvihare *et al.*, 2004) but they are in concordance with a more recent report showing that Treg expansion was augmented in the presence of fetal alloantigens (Zhao *et al.*, 2007). Besides, a solely hormonal effect on Treg expansion as originally suggested (Alluvihare *et al.*, 2004) is discarded by the work of Zhao *et al.* (2007).

It is of further interest to study if the same spatial pattern of thymic generation and peripheral conversion of Treg is an allogeneic dependent event. Both, the analysis of Helios^{+} Treg and the *de novo* conversion and migration of CD4^{+}Foxp3^{GFP+} cells transferred to Rag1$^{-/-}$ could be addressed in order to approach this issue.

Unlike in other models (Sun *et al.*, 2007) the blockage of TGF-β did not exert any effect on the de novo conversion of Treg on the uterine draining lymph nodes during pregnancy suggesting that it occurs independently of this molecule. The improved pregnancy outcome in abortion prone mice after treatment with IL-2 and the subsequent observed increase in the population of CD25^{+} cells suggest a role for this cytokine in the expansion of Treg during pregnancy. IL-2 is important in both thymic development and peripheral maintenance of Treg (Schorle *et al.*, 1991; Setoguchi *et al.*, 2005). As CD25 is an activation marker for all CD4^{+} cells, there is a limitation of using this maker in the identification of Treg once a CD25^{+} population can be composed of both activated T cells (Caruso *et al.*, 1997) and Treg (Sakaguchi *et al.*, 1995). The influence in the population of CD4^{+}Foxp3^{+} cells should be therefore addressed in a future study in order to make clear interpretations of our results. However, a recent study has shown that following treatment with an anti-CD25 monoclonal antibody the depletion of CD4^{+}CD25^{+}Foxp3^{+} could be observed and this resulted in the impairment of pregnancy (Shima *et al.*, 2010). These results are in concordance with our results and suggest that CD25, the IL-2 receptor, has an essential role in the maintenance of pregnancy through the action of Treg.

We next aimed to elucidate the mechanisms of recruitment of Treg to the reproductive tract during pregnancy. Hormones, like HCG, were proposed to influence the migration of human Treg (Schumacher *et al.*, 2008) but we here

concentrated mainly on chemokines and their receptors. The levels of mRNAs of several chemokines whose receptors are known to be expressed in Treg were analyzed in both uterus/decidua and placenta of allogeneic pregnant mice at different stages of pregnancy. The mRNA expression of CCL21 and CCL25 showed to be augmented in the decidua and in the placenta during pregnancy. Their chemokine receptors CCR7 and CCR9 were further expressed in the population of Treg from the lymph nodes draining the uterus from either non-pregnant or pregnant animals. Their *in vitro* migration towards the recombinant ligands however occurred in a similar extension in both non pregnant and pregnant mice.

Since CCR7 and CCR9 were previously described as playing a major role in tolerance mechanisms and were in particular related with Treg influence (Worbs and Förster, 2007; Hadeiba *et al.*, 2008; Drakes *et al.*, 2009) we found of interest to address their participation in the maintenance of a successful pregnancy by making use of $CCR7^{-/-}$ and $CCR9^{-/-}$ mice.

CCR7 deficiency derived in the absence of Treg but not Teff in uterine tissue of non-pregnant mice as compared to wild type animals. Thus, this chemokine receptor might be involved in the homing or proliferation of Treg to the uterus. The absence of CCR7 resulted, similar to what we observed after depletion of Treg by DT treatment, in implantation failure. In consonance, it has been reported that CCR7 has an important function in mediating Treg tolerance mechanisms in a transplantation model (Jin *et al.*, 2009) and Treg from CCR7 mice are less effective than their wild-type counterparts in preventing the development of inflammatory bowel disease (Schneider *et al.*, 2007). Others, have additionally described that the absence of CCR7 results in a defective trafficking of Treg to the exocrine organs impairing protection from autoimmunity into mice with autoimmune exocrinopathy resembling Sjögren's syndrome (Ishimaru *et al.*, 2010).

The lack of CCR9 resulted in significantly augmented frequencies of uterine $CD4^{+}Foxp3^{-}$ Teff cells, whilst comparable Treg frequencies could be observed in relation to wild type mice. Further, the DCs from the uterine draining lymph nodes of the $CCR9^{-/-}$ mice showed a higher maturation state. This goes in hand with the proposed role of CCR9 in the maintenance of a tolerogenic population of DCs previously observed in other mucosal environments (Hadeiba *et al.*, 2008; Drakes *et al.*, 2009). $CCR9^{-/-}$ mice also showed impaired implantation. In accordance with this observation, CCR9 was reported to have a regulatory role in other models like in the prevention of chronic ileitis in mice (Wermers *et al.*, 2011).

Thus, resembling what we observed after Treg depletion, either the absence of Treg or more effector T cells in the reproductive tract resulted in a reduced implantation success.

Despite the implantation impairment observed in both $CCR7^{-/-}$ and $CCR9^{-/-}$ mice, the few animals in which implantation was successful had apparently normal pregnancies both in syngeneic and allogeneic matings. Normal frequencies of decidual Treg in mid pregnancy where observed. However, the lack of these chemokine receptors resulted in alloantigen dependent variations in the distribution of $CD49b^{+}$ cells and the maturation state of $CD11c^{+}$ cells at the feto-maternal interface suggesting that CCR7 and CCR9 might also be influencing other immune cells during the pregnancy state.

It is possible that Treg migration, accumulation and function are not exclusively regulated by one single chemokine ligand or receptor. To draw deeper conclusions in the following of this study, we are now establishing a colony of double CCR7/CCR9 knockout animals to address the influence of

both receptors together on the population of Treg and on pregnancy establishment and maintenance.

In a very recent publication Nancy *et al.* (2012) showed strong evidences of a chemokine gene silencing in the decidual stromal cells of pregnant vs. non-pregnant animals due to a potential epigenetic modification of the decidual tissue that would be responsible for limited Teff trafficking. The authors proposed this as a major mechanism of fetal survival. Nonetheless, we were able to find effector cells in the uterus of pregnant animals if CCR9 is deleted as well as in the uterus of Treg-depleted animals. In addition, the referred work focus on the post-implantation period, which doesn't preclude the importance of Treg at earlier time points to the establishment of pregnancy as our results here suggest.

The results presented in this dissertation contribute to the understanding of the Treg mediated mechanisms during gestation. We also had a specific focus on the influence that particular chemokines connected with Treg migration or expansion had on pregnancy establishment and maintenance.

In particular, we found that:

- Treg oscilates during the estrus cycle, accumulating in the uterus and in the vaginal fluid during receptivity;
- Treg depletion before conception impairs fecundation or implantation because of increased inflammation;
- Treg depletion once pregnancy is established only marginally affected the abortion rates;
- Conversion of Foxp3+ cells occurs in the periphery after implantation.

- CCR7 dictates the homing or proliferation of Treg in the uterus and its absence derives in impaired implantation;
- CCR9 deficiency results in accumulation of Teff cells in uterus and more mature DCs in the draining lymph nodes which results in impaired implantation;

By adding novel elements to the complex network of information about the immune tolerance mechanisms supporting pregnancy, the data here published represent an advance in the knowledge of the immunology of reproduction. Treg are involved in an array of pregnancy complications and thus constitute an excellent therapeutic target to avoid them. Understanding the complex mechanisms influencing Treg numbers, function and migration can contribute to the future development of new protocols for improving human fertility.

-CHAPTER 7-

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7.1- REFERENCES

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