Plasticity of $CD4^+$ regulatory T cells and fate of Th17 cells in subsequent pregnancy

Dissertation

zur Erlangung des akademischen Grades eines

Doktors der Medizin (Dr. med.)

an der

Medizinischen Fakultät der Universität Hamburg

vorgelegt von

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aus

Wedel

2025

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Datum der mündlichen Prüfung: 27.03.2026

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1. Introduction

Pregnancy is the life-creating process safeguarding every species from becoming extinct. To be able to undergo this fascinating process of growing another human's life in a woman's body, profound changes need to occur. Visible changes, like expansion of the abdomen and swelling of the breasts for milk production, are accompanied by invisible changes on cellular and molecular levels, namely hormonal changes and adaptations of the immune system. The fetus resembles a semi-allograft, as half of its genetic information is of paternal origin and foreign to the mother. Hence, the maternal immune system reacts to these antigens. However, the fetus is not rejected. An understanding of the immune system herewith is indispensable for feto-maternal research.

1.1 Immune system

In general, a well-balanced immune system is a key factor for overall health. It is essential for defense against pathogens and at the same time for tolerance of the self. Limited productivity of the immune system can lead to diseases such as cancer or infection. In contrast autoimmune diseases or allergic reactions are caused by an overly productive immune system.

The immune system of vertebrates can be divided into an innate and an adaptive arm. The innate immune system, on the one hand, represents the first defense against pathogens. It complies with the rapid need of immune cells but lacks specificity. (Institute for Quality and Efficiency in Health Care, 2006) The main populations are Natural killer cells (NKs), Dendritic Cells (DCs) and macrophages. NKs exhibit direct cytotoxic features inducing apoptosis of targeted cells. Their granules contain perforins and granzymes for defense against tumor and virus-infected cells. (Kim et al., 2000, Hammer et al., 2018) Further, NKs shape the response of the adaptive immune system via cytokine and chemokine secretion. (Anfossi et al., 2006) Multiple receptors regulate activation and inhibition of NKs. The major histocompatibility complex (MHC)I for example is expressed on normal cells. A recognition of the MHCI receptor expressed by NKs leads to an inhibition and a recognition of the self. (Anfossi et al., 2006) DCs are antigen presenting cells (APCs). DCs have the ability of sensing a pathogen and

processing its antigens. An MHC/antigen complex is presented to the T cells of the adaptive immune system, inducing an adequate immune response. (Kotsias et al., 2019) Macrophages are mononuclear phagocytes and exhibit an important role in all stages of immune reactions, from induction to resolution of inflammation. Their various functions in the defense of pathogens, protection of the self, tissue remodeling and reparation is represented by their heterogenicity. (Strizova et al., 2023)

The adaptive arm, on the other hand, which is the focus of the present thesis, has the ability to recognize pathogens via presentation from APCs and to specifically attack them**.** However, the establishment of targeted defense against pathogens, unavoidably consumes additional time, which is partly needed for education of the cells via antigen presentation. Different cell types define the adaptive immune system, namely antibody-producing B cells and T cells. (Institute for Quality and Efficiency in Health Care, 2006) As this thesis focuses on T cells, it is essential to understand their origin and definition. Chronologically, lymphoid precursor cells emerge from the bone marrow and migrate to the thymus, hence naming them T cells. (Institute for Quality and Efficiency in Health Care, 2006) These cells undergo positive and negative selection. (Luckheeram et al., 2012) Cluster of differentiation (CD)4^+ and $CD8^+$ T cells are finally selected*,* MHCII and MHCI specific, respectively. (Murphy and Weaver, 2018) MHC-molecules allow activation by APCs. (Luckheeram et al., 2012) Focusing on $CD4^+$ cells, they can further differentiate into distinct subsets, like T helper (Th) 1 cells, Th2 cells, Th17 cells and the distinct regulatory T cell subsets, like regulatory T cells (Tregs) and T regulatory type 1 cells (Tr1 cells), which have been reviewed. (Luckheeram et al., 2012, Yamane and Paul, 2013) These cells interact with one another via cytokine and receptor signaling. Further, they build balancing pairs, holding up an equilibrium of inflammation and tolerance. On the one hand, there is the Th1/Th2 cell balance. (Thellin et al., 2000) Th1 cells are induced by Interleukin (IL)-12 and Interferon-γ (IFN-γ) and produce IFN-γ, as their main cytokine, which acts pro-inflammatory and suppresses the development of Th2 and Th17 cells. (Luckheeram et al., 2012) Th2 cells produce IL-4 as their main cytokine and are known for their response against extracellular parasites and are causative for allergic inflammation. (Luckheeram et al., 2012) A large quantity of literature is available on these cells and their balance. The Th17/Treg cell balance on the other hand, recently emerged, holding the balance between inflammatory or anti-inflammatory settings. (Nistala et al., 2008, Wang et al., 2024) Over the last

decades, Treg cells gained importance and have become the center of tolerance establishment.

In general, Th17 cells produce IL-17 as their signature cytokine, classifying them as pro-inflammatory. This cytokine can be further divided into diverse subtypes, IL-17A and IL-17F being the most import. (Kolls and Linden, 2004) Th17 cells reside mainly in mucosal sites, maintain mucosal homeostasis, and shield our body against extracellular bacteria and fungi. (Schnell et al., 2023) This protective role is executed by induction of macrophages and epithelial cells besides others, resulting in inflammation of the tissue, as summarized in (Lombardelli et al., 2016). Furthermore, Th17 cells play a role in autoimmune and inflammation related disorders, as reviewed in (Schnell et al., 2023). Additionally, Th17 cells have been mentioned in studies on rejection of transplanted, foreign tissue like corneal or cardiac rejection. (Yuan et al., 2008, Chen et al., 2009) Especially, an imbalance of the pair of Tregs and Th17 cells has been reported, concerning the rejection of transplants and in the incidence of autoimmunity. (Hanidziar and Koulmanda, 2010)

Another subset of interest for this thesis are the Forkhead-Box-ProteinP3^{+} (FoxP3^{+}) Treg cells. A grand amount of research resources has been spent on this cell line and they are the acquainted anti-inflammatory safeguard of the immune system. (Sasaki et al., 2004) They are mostly known for their IL-10 production and the expression of FoxP3. (Hori et al., 2003, O'Garra et al., 2004) They ensure immune homeostasis and prevent our body from overshooting inflammation.

The last subset of interest in this thesis are the Tr1 cells. Tr1 cells are defined as regulatory, IL-10 producing cells, which do not express FoxP3 and are herewith distinguishable from Tregs. (Passerini et al., 2011) Co-expression of CD49b and lymphocyte-activation gene 3 (LAG-3) was shown and specified the lineage profile. Their pivotal role lies in maintaining tolerance, especially in alloimmune response. (Gagliani et al., 2013)

Additionally to the immediate immune response, a memory effect has been proposed for T cells. (Lees and Farber, 2010) This effect describes that cells primarily responding to an antigen evolve into experienced and antigen-specialized cells, building a long-

term immunity instead of dying after defense. (Lees and Farber, 2010) Memory cells present a heterogenic group which is subdivided into central memory (CM) and effector memory cells (EM). EMs exhibit a direct pro-inflammatory effector function and are present in the peripheral tissue. For CMs, secondary exposure to antigen is followed by a differentiation into effector cells. (Sallusto et al., 2004) Immune memory can persist over a long time and was found for effector as well as regulatory cell subsets, bringing up new questions for immune homeostasis. (Ahmed and Gray, 1996, Rosenblum et al., 2011)

1.2 Maternal immune adaptation

Around the 1950s organ rejection was decrypted as immunological incompatibility of donor and recipient. To understand the rapid rejection of transplants from different individuals, tests were performed on skin grafts as reviewed in (Rendell et al., 2020). It was firstly discovered that even a semi-allogenic transplant causes rejection. (Gibson and Medawar, 1943, Rendell et al., 2020) A transplant tolerance model, called the “actively acquired tolerance", was developed. In utero fetal mice were inoculated with cells from an adult animal from another strain, tolerance was established consistently also in adult life. (Billingham et al., 1953) Medawar’s experimental findings were essential for successful transplantation summarized by (Rendell et al., 2020). The question that remained thereafter was the tolerance towards a fetus despite incompatibility of the genetic features towards a mother. How was a tolerance lasting 9-months possible without immunosuppression? (Rendell et al., 2020) Medawar suggested three hypotheses. Firstly, the anatomical and mechanic separation of the fetus from the mother is secured by the placenta. Secondly, the fetus has immature antigens, which stay hidden for maternal immune cells. Thirdly, inertia of the maternal immune system during the process of pregnancy, as reviewed by (Rendell et al., 2020, Wöhrle et al., 2022). Today, all of the proposed hypotheses have been proven incorrect. (Rendell et al., 2020) However new light was shed on pregnancy as an immunological conundrum. The fetus resembles a semi-allograft due to expression of paternal human leucocyte antigen (HLA) by trophoblast cells and is still not rejected. (Papuchova et al., 2019) Maternal APCs process and present the antigens to the mother’s immune cells leading to a recognition without provoking rejection. To prevent a rejection due to an overshooting immune reaction, including an inflammatory

cytokine response, a tolerance establishment and adaptation towards this unique situation of pregnancy is indispensable. (Rendell et al., 2020) A refined crosstalk of trophoblast cells, representing fetal tissue, with the maternal immune cells takes place. Drastic changes in maternal immune cells, their cytokine secretion and further circulating hormones of the mother are observable. (Rendell et al., 2020, Wöhrle et al., 2022) These changes become clinically visible in expectant mothers with previous disease history of chronical autoimmune diseases, which occur in a reduced severity within pregnancy. (Confavreux et al., 1998, Hazes et al., 2011)

In gestation, specific changes lead to a pregnancy-specific composition of innate and adaptive immune cells. After a male's seminal fluid enters the reproductive tract of a woman, immune reactions are activated. (Robertson, 2005) The semen fertilizes the zygote, which then divides multiple times and enters the blastocyst state as summarized in (Wöhrle et al., 2022). In this stadium the implantation takes place. (Hill, 2022) It is partly driven by the trophoblast, which is the outer layer of the blastocyst and can be further divided into villous and extravillous trophoblast (EVT) as reviewed in (Wöhrle et al., 2022). On the one hand, the former does not express MHC class I and II molecules and herewith does not trigger an immune response. EVTs on the other hand, express polymorphic and non-polymorphic HLA molecules (HLA-C, HLA-G and HLA-E), establishing a crosstalk between fetal trophoblast cells and the maternal immune system. (Papuchova et al., 2019, Wöhrle et al., 2022) EVTs get into contact with uterine natural killer (uNK) cells and hence trigger further immune reactions. (Solano, 2019) Mentionable immune cell subsets in the uterine site are uNKs, macrophages and DCs. With 70%, uNKs represent most of the immune cells of an early pregnancy. A steady decrease thereafter marks their importance for implantation. UNKs are less cytotoxic than peripheral NKs and interaction with HLA-C triggers them to produce growth factors and cytokines for remodeling of spiral arteries, as reviewed previously by (Makrigiannakis et al., 2008, Rendell et al., 2020). Similarly, local macrophages take on a tolerogenic phenotype as the M2 phenotype dominates over M1 macrophages. (Solano, 2019) Lastly, DCs are essential for communication between the innate and the adaptive arm. Uterine DCs arrest into an immature and tolerogenic state via IL-10 signaling. (Steinbrink et al., 1997) In contrast to their peripheral counterparts, they reduce antigen presentation and co-stimulatory signals, rather producing anti-inflammatory cytokines. (Blois et al., 2004, Wöhrle et al., 2022)

Further, the adaptive immune system adjusts to pregnancy. As described earlier different T cell balances have been proposed. The Th1/Th2 cell balance shift towards Th2 cells. (Wegmann et al., 1993) The Th17/Treg cell balance also complemented the tolerance establishment of the adaptive immune system in pregnancy, showing increasing Treg numbers as reviewed in (Saito et al., 2010). In the following, the most interesting cell lines for this thesis, are summarized in pregnancy.

There is very little and contradictory data published on the role of Th17 cells in pregnancy. On the one hand, their importance in physiological pregnancy has been shown. Th17 cells have a positive impact on trophoblasts and vice versa. Further, Th17 cells play an important role in regulating trophoblast invasion in late first trimester. (Wu et al., 2014, Wang et al., 2019) The impact of trophoblasts on Th17 cells is an increase in proliferation and apoptosis with a stable differentiation, suggesting a renewal. (Wu et al., 2014) In total, suggesting the creation of a supportive coexistence. On the other hand, they have been measured in increased numbers in pregnancy complications. IL-17 was not only found in cytotrophoblast and syncytiotrophoblast cells in normal human pregnancy but also in miscarriage. (Pongcharoen et al., 2007) Many pathologic conditions, such as preeclampsia and pregnancy loss, have been brought in connection with inflammation and an increase of Th17 cells. (Liu et al., 2017) Until now, the role in abortion is not fully elucidated but it has been suggested that the rather inflammatory Th17 cell subset plays a role in miscarriage and rejection of the fetus. Researchers found increased numbers in spontaneous abortion cases and unexplained recurrent abortion. (Nakashima et al., 2010b, Liu et al., 2011, W. J. Wang et al., 2013) Due to accumulation of IL-17^{+} cells in decidua of spontaneous abortion but not in missed abortion, a role of Th17 cells only in late stage of abortion via neutrophil recruitment and formation of inflammation but not in early stage was suggested. (Nakashima et al., 2010b)

Tregs are indispensable in pregnancy and steadily increase until they peak in the second trimester. (Somerset et al., 2004) They are induced by fetal EVTs, including their IL-10 production. (Svensson-Arvelund et al., 2015) Contact with fetal alloantigen leads to an increase of Tregs, ensuring maternal tolerance towards paternal antigens. (Zhao et al., 2007) Especially in the decidua, Tregs are enriched. (Dimova et al., 2011)

The timepoint of most relevance for Treg accumulation is the transition from an inflammatory setting into a tolerogenic one as reviewed in (Robertson et al., 2018). This is also displayed in their kinetics, as they rise drastically in the first trimester. (Thuere et al., 2007) In pregnancy complication, their role becomes visible once more. In miscarriage, preeclampsia and other complications, low numbers of Treg cells were measured. (Sasaki et al., 2004, Winger and Reed, 2011, Inada et al., 2015, Green et al., 2021)

Recently, the paper 'it is not all about FoxP3' highlighted the importance of other regulatory T cells without FoxP3 expression. These cell lineages also increase in peripheral blood molecular cells (PBMCs) of pregnant women. (Krop et al., 2020) This paper is not about downsizing the importance of $FoxP3^+$ cells, which are undoubtedly indispensable, but rather about raising awareness for the role and importance of other regulatory cell subsets. (Krop et al., 2020) One example, for these cell lines are Tr1 cells**.** It has been postulated, that decidual Tr1 cells express high levels of programmed death protein 1 (PD-1) and granzymes and interact with EVTs. (Salvany-Celades et al., 2019)

Tolerance establishment is not a steady state occurring at a consistent level throughout pregnancy but rather depicts a dynamic process with varying immune profiles. There are different states of pregnancy, which are termed the implantation phase, the placentation phase and the parturition phase. (Green and Arck, 2020) Each phase requires a different immunological setting. In the beginning, inflammation is indispensable for proper establishment and implantation of the oocyte into the uterine wall. Afterwards, a switch into a rather anti-inflammatory immunological setting is highly needed to maintain pregnancy and allow the fetus to grow without disturbances. If this tolerance is not mounted in the placentation phase, pregnancy is in danger of complications or failure. (Green and Arck, 2020) All of these changes underlie certain kinetic schemes, but until today the picture is incomplete. Further research of immunological changes within pregnancy contributes to the comprehension of healthy pregnancy. The act of birth requires a second switch including recruitment of inflammatory immune cells. (Mor et al., 2011) The immunologic mechanisms responsible for these milestones of pregnancy are not fully understood yet. While some timelines of immune cells and their kinetics over the course of pregnancy have been

established, many are still lacking. These adaptations are finely tuned and present a complex network. Hence, one small disturbance can cause immense damage. Therefore, knowledge on all cell subsets involved and their connections to one another are substantial.

1.3 Hormones

The immune system is tightly linked to the hormones circulating through the expectant mother. (Polese et al., 2014) Also the whole adaptation process towards a tolerogenic phenotype is hormonally driven. Hormones such as progesterone and glucocorticoids undergo pronounced changes with advancing pregnancy. (Solano and Arck, 2019) Progesterone is known as the pregnancy hormone, as it has many beneficial effects on pregnancy. It continuously rises throughout pregnancy until levels drop shortly before parturition. (Solano and Arck, 2019) In mice, it was shown that progesterone modulates the immune system towards a Th2 and Treg cell dominated milieu and inhibits Th1 cells. (Miyaura and Iwata, 2002, Mao et al., 2010) In human cord blood results also show a promotion of Treg cells via progesterone, while simultaneously Th17 cells and IL-6 receptors on T cells were suppressed. (J. H. Lee et al., 2011) In total, a favorable, tolerogenic immune milieu is created. (Polese et al., 2014) The effects are not restricted to the adaptive immune system. Also, innate immune cells like macrophages and DCs are set into a tolerogenic state. (Solano and Arck, 2019) Via activation of NK cells, progesterone promotes vascularization of uterine tissue as summarized in (Solano and Arck, 2019). The second hormone of interest is cortisol, which represents the main glucocorticoid (GC). It acts immune-suppressive and anti-inflammatory. (Coussons-Read, 2012) Further, cortisol is notorious as the stress hormone. In pregnancy, glucocorticoids increase 20-fold from middle to late pregnancy, in order to meet energy needs that accrue with advancing pregnancy. (Solano and Arck, 2019) Similarly, the cortisol development shows a two-stage increase. After stagnation around week 26, a second wave of exacerbation is detectable shortly before parturition. The strong immune modulating effects of cortisol are exhibited via the activation of the glucocorticoid receptor on immune cells. (Solano and Arck, 2019) Upregulation tilts the immune system towards a Th2-cell-dominated anti-inflammatory immunity. (Nepomnaschy et al., 2007) A complete differentiation of progesterone and glucocorticoid function is impossible as both share the usage of

glucocorticoid receptors and a similar heritage from pregnenolone as the common progenitor. (Coussons-Read, 2012) Similarities further lie in structural makeup, resembling receptors, nuclear operation and regulation of gene expression, as summarized in (Solano and Arck, 2019). 5-6 % of existent cortisol is not bound to carrier proteins. Only the free hormones have executive functions. The percentage of freely available cortisol increases with pregnancy progression. The same applies for progesterone. Early in pregnancy, progesterone is the predominant hormone, in late pregnancy both hormones are equally present. (Solano and Arck, 2019)

1.4 Maternal immune activation

The course of pregnancy is susceptible to variable factors interfering with the women's and her unborn child's well-being. These factors can either contribute to an improvement of pregnancy or act as a disturbance. Examples for negative influences are medication like acetaminophen, infections, modern lifestyle or stress exposure. (Coste et al., 1991, Lashen et al., 2004, Thiele et al., 2013, Wöhrle et al., 2022)

Stress can be defined as the physical and psychical reaction to a situation that is perceived as non-manageable, endangering a humans' well-being. It is multifaceted and differs from one to another individual, making measurements difficult. (Coussons-Read, 2012, Wöhrle et al., 2022) For differentiation between all the different types and perceptions of stress, the subcategories severity, duration and the different stressors, were established. (Fig. 1) (Wöhrle et al., 2022) Firstly, for severity, it was postulated that minor and major stress ranging from traumatic experiences to daily hassles, has an influence on homeostasis of pregnancy. (Coussons-Read et al., 2007, Coussons-Read, 2012, Coussons-Read, 2013, Wöhrle et al., 2022) Further, it was suggested that sensitivity decreases with pregnancy progression. Equal stress severity is perceived as more stressful in early stages. (Glynn et al., 2004, Coussons-Read et al., 2007, Wöhrle et al., 2022) Secondly, duration of stress distinguishes between acute and chronic stress exposure. Both have been related to spontaneous abortion, a pro-inflammatory cytokine milieu and lack of anti-inflammatory antagonists as listed in (Beijers et al., 2014, Frazier et al., 2018, Wöhrle et al., 2022). Thirdly, the differentiation into stressors presents a very heterogenous group. They can be divided into a psychosocial, an environmental and a cultural type as summarized in (Coussons-

Read, 2012, Beijers et al., 2014, Wöhrle et al., 2022). A range from complete happiness and anticipation to fear of losing the baby leads to onset of pregnancy-specific stress. (Coussons-Read, 2013, Beijers et al., 2014, Wöhrle et al., 2022) Research of the last decades suggests that stress is at least partly causative for many complications. Correlations between negative pregnancy development and recent negative life events were already described. (Neugebauer et al., 1996, Wöhrle et al., 2022) These complications reach from implantation failure, preterm birth, abortion, miscarriage to health problems of the offspring. (O'Hare and Creed, 1995, Qu et al., 2017, Wöhrle et al., 2022) Stress leads to an activation of a reaction of the body, encompassing sympatico activation, endocrinological and immunological changes. (Fig. 2) (Coussons-Read, 2012, Wöhrle et al., 2022) Immune cell changes in stress include a general decrease in leukocytes at the interface. (Antonson et al., 2020). Further, glucocorticoids inhibit proliferation, activation, differentiation and function of most immune cells. Their effect on Tregs for example is either to support their role as suppressive or as helper cells, showing the strong immunomodulatory effect. (Arck, 2001) Today, it is state of the art that $CD4^+$ T-cell subsets undergo proportional and phenotypic changes in stressed women. (Frazier et al., 2018) The physiologically anti-inflammatory setting in pregnancy shifts into an inflammatory one. Innate immune cells start to undergo maturation and leave their anti-inflammatory state. In addition, peripheral cells are recruited and stay inflammatory due to a missing switch into a tolerogenic phenotype. (Kwak-Kim et al., 2014) In contrast to physiological pregnancy, Th2 and Treg cells are smaller in numbers and the inflammatory Th1 and Th17 cells predominate, further leading to a shift in cytokines. (Knackstedt et al., 2005, Kwak-Kim et al., 2014) Nevertheless, an impact of excess GCs was seen in the offspring. (Beijers et al., 2014) Consequences for the offspring are e.g., growth retardation with low birth weight, cardiovascular and maturity problems. (Arck, 2001, Beijers et al., 2014) Today, research makes use of different murine stress models which have gained importance for the exploration of impact of prenatally experienced stress. In this thesis stress application is performed with a sound stress model.

Fig. 1 Types of stress in pregnancy: Stress in pregnancy was subdivided into different types and perceptions, namely severity, duration and the different stressors. Further this figure displays coping mechanisms. (Wöhrle et al., 2022) Reprinted from Immunology of Recurrent Pregnancy Loss and Implantation Failure, Vol 3, Wöhrle, R., Arck, P. & Thiele, K., Stress-induced immune deviations and reproductive failure, Pages 103-119., Copyright (2022), with permission from Elsevier. DOI: https://doi.org/10.1016/B978-0-323-90805-4.00013-4

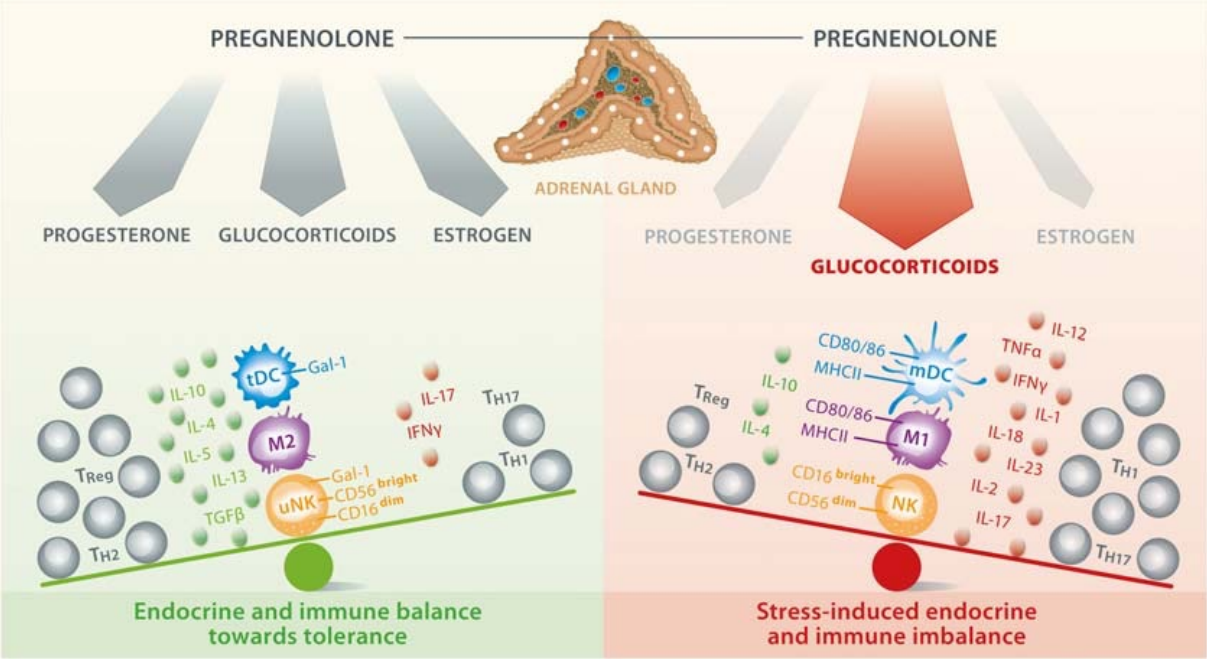

Fig. 2 Hormonal influence on immune cell equilibrium: On the left (green) a balanced hormonal and immunological setup of normal pregnancy is presented, resulting in a tolerance establishment. On the right (red) an immune cell shift due to hormone imbalances in stress is displayed. (Wöhrle et al., 2022) Reprinted from Immunology of Recurrent Pregnancy Loss and Implantation Failure, Vol 3, Wöhrle, R., Arck, P. & Thiele, K., Stress-induced immune deviations and reproductive failure, Pages 103-119., Copyright (2022), with permission from Elsevier. DOI: https://doi.org/10.1016/B978-0-323-90805-4.00013-4

1.5 Mouse models in reproduction

Working with animal models necessarily means following the principles of the 3R Model of replacement, reduction and refinement in animal dependent research. (Arck, 2019) Hence, consideration of advantages and disadvantages of animal research led to the conclusion that the question proposed in this thesis is in high need of the use. There is no replacement yet for this purpose.

Whether the mouse serves as a good model for pregnancy research has already been addressed in the paper “Mouse is the new women?” by David Clark (Clark, 2016). The necessity to use animal models emerges as many experiments and research methods cannot possibly be practiced on humans. For example, examination of uterine cells at different time points of pregnancy requires the sacrifice of the animal to harvest the

organ material. Hence, it is more important to explore the reason for using murine instead of other animal models. Firstly, one advantage is that repetitive testing with different individuals is possible due to inbreeding and inter-individually identical animals. (Clark, 2016) Furthermore, the short gestational time of 18 to 21 days in murine pregnancy is practical and experiments can be performed and replicated within an adequate time frame. (Hill, 2022) A litter size of 6 to 12 pubs, present an attractive number of samples for offspring analysis and experiments. (Hill, 2022) Moreover, immunological techniques are available, and it is scientifically accepted that the human and murine immune system are comparable. (Clark, 2016) Regarding pregnancy, human and mice both share the similarity of a discoidal, hemochorial placenta. (Clark, 2016) The murine placenta resembles the human one more than many other animal species like sheep. (Hemberger et al., 2020) Today, there are many mouse models available for different pregnancy pathologies, e.g. recurrent spontaneous abortion (RSA) (Clark et al., 1980), pregnancy loss (Clark et al., 1993). Mouse models offered priceless insights into human pathologies and the value of mouse data for pregnancy disorders is already confirmed. (Clark, 2014)

For the interpretation of data acquired in a murine pregnancy model, knowledge about murine gestation and the comparison to human pregnancy are indispensable. Fetal development in mice follows a specific timeline, just like in humans. There are milestones that are essential, like implantation or the different steps in embryogenesis of the fetus. These milestones of a physical development occur parallel to immunological adaptations. As described above, after fertilization, the blastocyst is created. (Hill, 2022, Wöhrle et al., 2022) Implantation takes place around gestational day (gd) 4.5 and 5.5. (Clark, 2016) As embryogenesis continues, on gd 6.5, gastrulation occurs. The amnion, chorion and allantois are shaped and the merger of the latter two allows establishment of a placenta. (Hemberger et al., 2020) From gd 10.5 until gd 14.5, the labyrinth becomes increasingly complex, following the heightened need for gas and nutrients of the rapidly growing fetus. Similar to humans, the placenta is crucial in murine pregnancy and secures the survival of embryos in uterine surroundings. (Hemberger et al., 2020) The placenta is an expression of a well proceeding pregnancy and problems in establishment lead to complications. (Wardinger and Ambati, 2022) Gd 15.5, marks the beginning of the third week of gestation, the fetal developmental stage is far progressed as the embryo reaches

Theiler stage 23 of 27 and Carnegie stage 22 of 23. The length of 12-14 mm shows that the last days of gestation register a strong growth reaching 23-27 mm at birth. (Theiler, 1989) Once the fetus has grown and matured enough, the birth process begins. Mice give birth around 19 days after a vaginal plug. (Hill, 2022)

1.6 Immune memory in pregnancy

The importance of an immune adaptation in pregnancy and the memory effect in the situation of re-encounter with a pathogen, derive the question if this effect occurs in secondary pregnancy.

This remembrance of immune changes in general is not a newly described phenomenon as described earlier. $CD8^+$ and $CD4^+$ T cell specification record antigen encounters and adopt a memory phenotype. (Stemberger et al., 2007, Harrington et al., 2008) While after an encounter with the antigen most of the immune cells die, some cells persist and activate a faster response in re-challenge. (Ahmed and Gray, 1996) This effect has been proposed also for pregnancy and was termed the memory effect. (Rowe et al., 2012) In human, $CD4^+$ memory cells persistently effect the immune system after pregnancy, as these cells were measured more than a year postpartum in blood. (Kieffer et al., 2017, Huang et al., 2021) Memory effects have also been described for the uNK cell line of the innate immune system. “Pregnancy trained decidual NK cells” exposed different epigenetic and transcriptomic features in subsequent pregnancies and conducted advantages in placentation. (Gamliel et al., 2018) This long-term effect on immune composition in women, showed differences between normal pregnancy and preeclampsia. Therefore, it was proposed that it represents an evolutionary advantage for future pregnancy. (Huang et al., 2021) It has been clinically observed that there are advantages or disadvantages arising for secondary pregnancy dependent on the outcome of the first one. Recently, a systemic analysis of human clinical data from PubMed, reviewed all observations on birth weight and incidences of gestational complications that accumulated over the last years. (Thiele et al., 2019) On the one hand, pregnancies free from complications have been correlated with a decreased risk for subsequent ones. (Thiele et al., 2019) For example, protection from preeclampsia was seen in multiparous women in case of an undisturbed pregnancy beforehand. This immunological protection was seen in a

partner specific manner. (Trupin et al., 1996) An increase of birth weight was valued as an advantage for the offspring. This is highly relevant and surprising, since the mother's age increases. (Thiele et al., 2019) On the other hand, complications, occurring during the first pregnancy, increase the risk to suffer from complications in subsequent pregnancies. (Thiele et al., 2019) This harmful effect was already described in 1982. (Hathout et al., 1982) Somehow, a woman's body seems to remember changes that have occurred in previous pregnancies and lead to a re-occurrence. (Thiele et al., 2019) For outcome advantages of birth weight increases in secondary pregnancy an immune memory was suggested as one reason. (Thiele et al., 2019) However, the experiments and studies performed in the past years are lacking completion. Especially experimental research is pending and could help to find a connection of the clinical improvement and the molecular mechanisms. In 2012 insights into the differences of the immune system in first compared to second pregnancy was described. This study showed that Tregs increase more rapidly in second pregnancies compared to first ones. (Rowe et al., 2012) Usually, maternal immune cells die after primarily responding to paternal-fetal antigens, however some persistently remain in the body as memory cells. Previously encountered antigens are recognized and a faster response is mounted as reviewed in (Kieffer et al., 2019). Especially in pregnancy an overshooting inflammatory response could be disastrous for maintenance. Th1 and Th17 cells have been described to disturb normal pregnancy and lead to recurrent pregnancy loss. (Raghupathy, 1997, Lombardelli et al., 2016) A memory of these cells could lead to a remembrance of complications and a repetition in subsequent one, as described in human. (Huang et al., 2021) It was suggested that in complications more memory cells of T effector cells result and herewith overshadow a regulatory countermovement. (Thiele et al., 2019) However, also positive effects of this memory effect are observable. Advantages were seen in second pregnancy of women who experienced a previous uncomplicated pregnancy. (Gamliel et al., 2018) Further, rather beneficial features of this "immunological memory of pregnancy (IMOP)" were proposed for late pregnancy and subsequent ones in mice (Rowe et al., 2012) and in human. (Kieffer et al., 2017) A long-time surviving Treg subset was found and proposed to regulate the EM cells in repeated encounter, as reviewed in (Rosenblum et al., 2016). Animal models from the past have shown accumulation of Treg cells and slight persistence of low numbers postpartum. These cells showed a specificity for fetal antigen, a memory phenotype and a positive effect on the resorption

rate. (Rowe et al., 2012) Nevertheless, another possible explanation for the Treg increase can be found in the plasticity from one cell line to another.

1.7 Plasticity of T cells

The phenomenon of plasticity, as the conversion from one to another subset, stirred up the idea of distinct and stable $CD4^+$ subsets. (DuPage and Bluestone, 2016) Firstly described in 1992, reciprocal effects between the two subsets of Th1 and Th2 cells resolved the rule of exclusiveness of these subsets. (Maggi et al., 1992) Later, Th1 cells were even demonstrated to produce IL-4. (Messi et al., 2003) Differences between the subsets were seen as they possess divergent degrees of plastic features. While Th1 and Th2 cells are proposed to be relatively stable, other subsets like Tregs and Th17 cells show a greater susceptibility for transformation. (Zhu and Paul, 2010) Tregs were shown to convert into other subsets like Th1, Th2 and Th17 cells, which has been shown in human and murine data. (Dominguez-Villar et al., 2011, Noval Rivas et al., 2015, Liu et al., 2017) Especially, Th17 cells are notorious to convert into other cell subsets. Their ability to express cytokines typical for other T cell phenotypes, has been shown in mouse experiments of e.g., rheumatoid arthritis, inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE). (Hirota et al., 2011, Hovhannisyan et al., 2011, Komatsu et al., 2014) The change of fate was followed into the direction into subsets such as Th1, Th2, as well as Tr1 and Treg cells. Controlled by cytokines and inflammatory condition among other things. (DuPage and Bluestone, 2016, Liu et al., 2017) A subdivision of Th17 cells into different intermediate subsets like Th17/Th2 and Th17/Th1 cells was postulated as additional cytokine production was measured. The former ones are associated with IL-4 production and the latter with IL-22 and IFN-γ. (Lombardelli et al., 2016) Further, a conversion into regulatory subsets was described to resolve inflammation in the gut in a murine model. (Gagliani et al., 2015) This thesis transmitted the results of Gagliani et al. into a pregnancy model. All experiments in this thesis have been performed using the $Fate^+$ mouse model, which serves for examination of the plasticity of Th17 cells in vivo. It presents a mouse model that exhibits a reporter function and additionally a fate mapping ability. This enables researchers to test for cells that are IL-17 $fate^+$, named ExTh17 cells. Further, in these cells the current expression of IL-10, IL-17A and FoxP3

can be measured. In total, creating a triple reporter mouse for IL-10, IL-17, FoxP3 which further shows a fate mapping ability for IL-17. (Gagliani et al., 2015)

The current knowledge on increased Treg frequencies in secondary pregnancy, raises the question if the Tregs emerge from other cell lines such as Th17 cells via plasticity. And further if this plasticity offers advantages for the outcome of the offspring. This thesis assesses this plasticity in pregnancy, by tracing Th17 cells into the direction of regulatory subsets.

2. Objectives

The aim of this thesis is to attain a more comprehensive understanding of T cell plasticity during gestation. Hereby, Th17 and $CD4^+$ regulatory T cells are examined in the unique immunological setting of first and second pregnancy and exposed to a prenatal stress challenge.

Therefore, we defined the following objectives:

- to identify kinetics of Th17 cells in first and second pregnancies
- to evaluate the fate of Th17 cells transdifferentiating into cells with a tolerogenic phenotype
- to investigate the functional role of Th17 cells and Treg cells in challenged pregnancy

3. Material & Methods

In the following chapter, the material and methods that have been used and performed in the experiments of this thesis, are listed and described in detail. In advance, all experiments have been ethically approved by the German authorities (Behörde für Gesundheit und Verbraucherschutz, Hamburg N20/14).

3.1 Mouse experiment - Experimental setup

3.1.1 Fate^{+} mouse model

All experiments in this thesis have been performed using the Fate^{+} mouse line, kindly provided by Nicola Gagliani. The fate^{+} mouse model serves for examination of plasticity of Th17 cells. The full name of this IL-17A fate reporter mouse line is "IL-17A^{CRE} × Rosa26STOP$^{fl/fl}$YFP(R26^{YFP}) x IL-17A^{Katushka} IL-10^{eGFP} Foxp3^{RFP} triple reporter" mouse model. This fate mapping mouse model enables researchers to track plasticity of Th17 cells in vivo. Fate^{+} mice are triple reporter mice for IL-10, IL-17, FoxP3 and further show a fate mapping ability for IL-17. (Gagliani et al., 2015) For the generation of a mouse model that exhibits a reporter function and additionally a fate mapping ability, two different genetic tools are combined. The reporters, on the one hand, mark cells temporarily, if they express the cytokine or transcription factors at this moment. The fate mapping construct, on the other hand, is induced by the CRE Lox P system. The CRE recombinase gene scissor eliminates the STOP cassette ingrained between two Flox sites. High IL-17 expression of a cell leads to a permanent staining with enhanced yellow fluorescent protein (eYFP). Formerly IL-17 producing cells can be tested for current expression of IL-10, IL-17A and FoxP3. (Gagliani et al., 2015) For the generation of the Fate^{+} mice, firstly, IL-17A^{CRE} mice were generated by inserting a CRE recombinase into the locus of the IL-17A gene. Secondly, they were crossed with Rosa26-eYFP reporter mice, resulting in the generation of the IL-17A fate mouse model. (Hirota et al., 2011) For investigation of regulatory fates of ExTh17 cells, the IL-17A fate mouse was crossed with an IL-17A^{Katushka} IL-10^{eGFP} Foxp3^{RFP} triple reporter mouse creating the Fate^{+} mouse line. (Gagliani et al., 2015) Additionally, control mice of the Black6 lineage served to determine exact gates of the fluorescence marker expression. All mice were kept under 12 h light/dark cycles in the animal facility at the

University Medical Center Hamburg-Eppendorf, Hamburg, Germany (UKE). Further, the animals received food and water ad libitum. The mice were age-matched littermates between 8 and 12 weeks of aged. Female mice were assigned randomly to experimental groups and to Balb/c male mice. Herewith, an allogenic mating model is created, which is needed for an extensive immune reaction. While the majority of mice have been bred and kept in the animal facility of the UKE, C57Bl/6J females were ordered from Charles River (Sulzfeld, Germany). Upon transport to the experimental barrier, female mice were allowed to acclimatize for 2 weeks before mating.

3.1.2 Timed pregnancy

To establish a kinetic scheme of Th17 cells and regulatory T cell subsets, different time points of early and late gestation were investigated. At the age of 6 to 8 weeks females underwent undisturbed pregnancy. After giving birth the offspring were removed to quickly restore to non-pregnant hormone levels. After 14 days at the age of 11 to 12 weeks these females were mated for the second time with the same Balb/c males (bottom timeline). The first pregnancy group joins age-matched at the age of 11-12 weeks. (upper timeline) (Fig. 3) The detection of the first copulation plug in the morning defined gd 0.5 of pregnancy. To confirm pregnancy, weight was measured on gd 8.5 to gd 10.5. Assignment to the groups and males occurred randomly. The number of sacrificed mice in this experiment is a total of 8 and 9 for gd 1.5, 10 and 8 for gd 5.5, 9 and 10 for gd 7.5 and 9 and 11 for gd 15.5, respectively for first and second pregnancy.

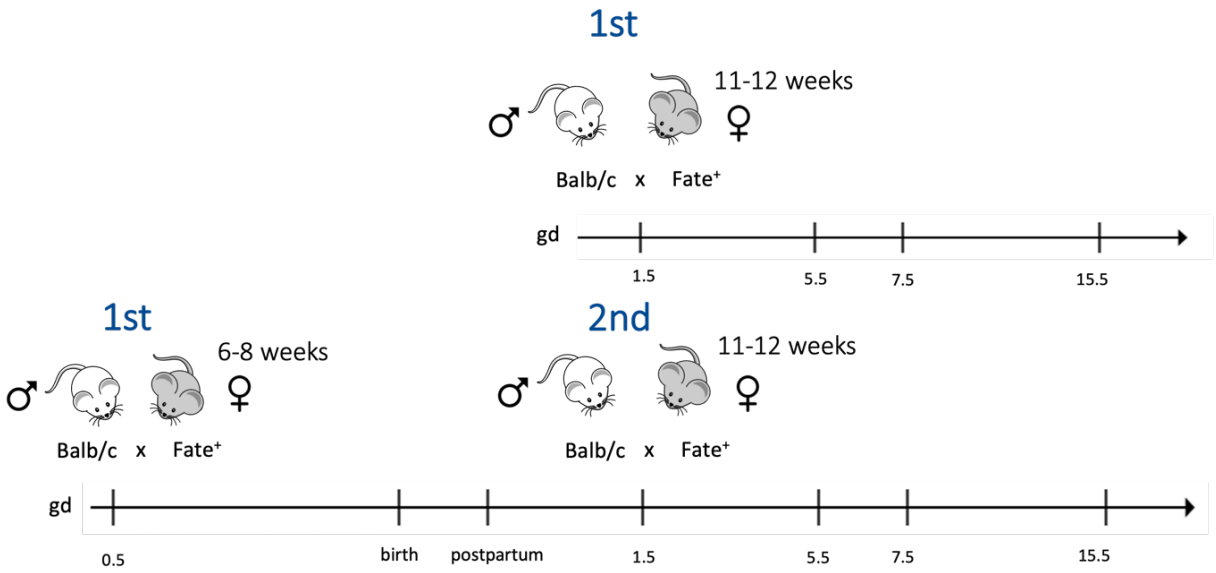

Fig. 3 Experimental setup of the first and second pregnancy mouse model: Allogenic mating of age-matched Fate+ females with Balb/c males was performed once at the maternal age of 11 to 12 weeks (upper timeline) or twice at the maternal age of 6 to 8 weeks in the first and aged matched at 11 to 12 weeks for second pregnancy (lower timeline). Organs were harvested on gestational days 1.5, 5.5, 7.5 and 15.5.

3.1.3 Midgestational stress challenge

The prenatal stress model used in this thesis has been successfully established and utilized by other members in my laboratory. (Solano et al., 2015, Zazara et al., 2018, Wieczorek et al., 2019, Schepanski, 2020) The mice were exposed to sound stress using a rodent repellent device (Wühlmausvertreiber P7901 function vibration, Conrad Electronics SE, Hirschau, Germany) with a frequency of 300 hertz and an interval of 15 seconds. Additionally, an ultrasonic pest repeller (Weitech WK-0220, Wavre, Belgium) was used to emit ultrasound waves. Constant exposure was ensured by placing the devices inside and on top of the cage, respectively. Stress was applied on the days 10.5, 12.5, 14.5 of pregnancy each time for 24 hours. Stressed mice are always compared to a control group, resulting in four different groups in this experiment. Group number 1 and 2 were not stressed and either pregnant for the first or second time. Group number 3 and 4 were both stressed, in first and second pregnancy respectively. (Fig. 4) The number of sacrificed mice in this experiment were a total of 9, 11, 13 and 11, respectively for the above-mentioned groups.

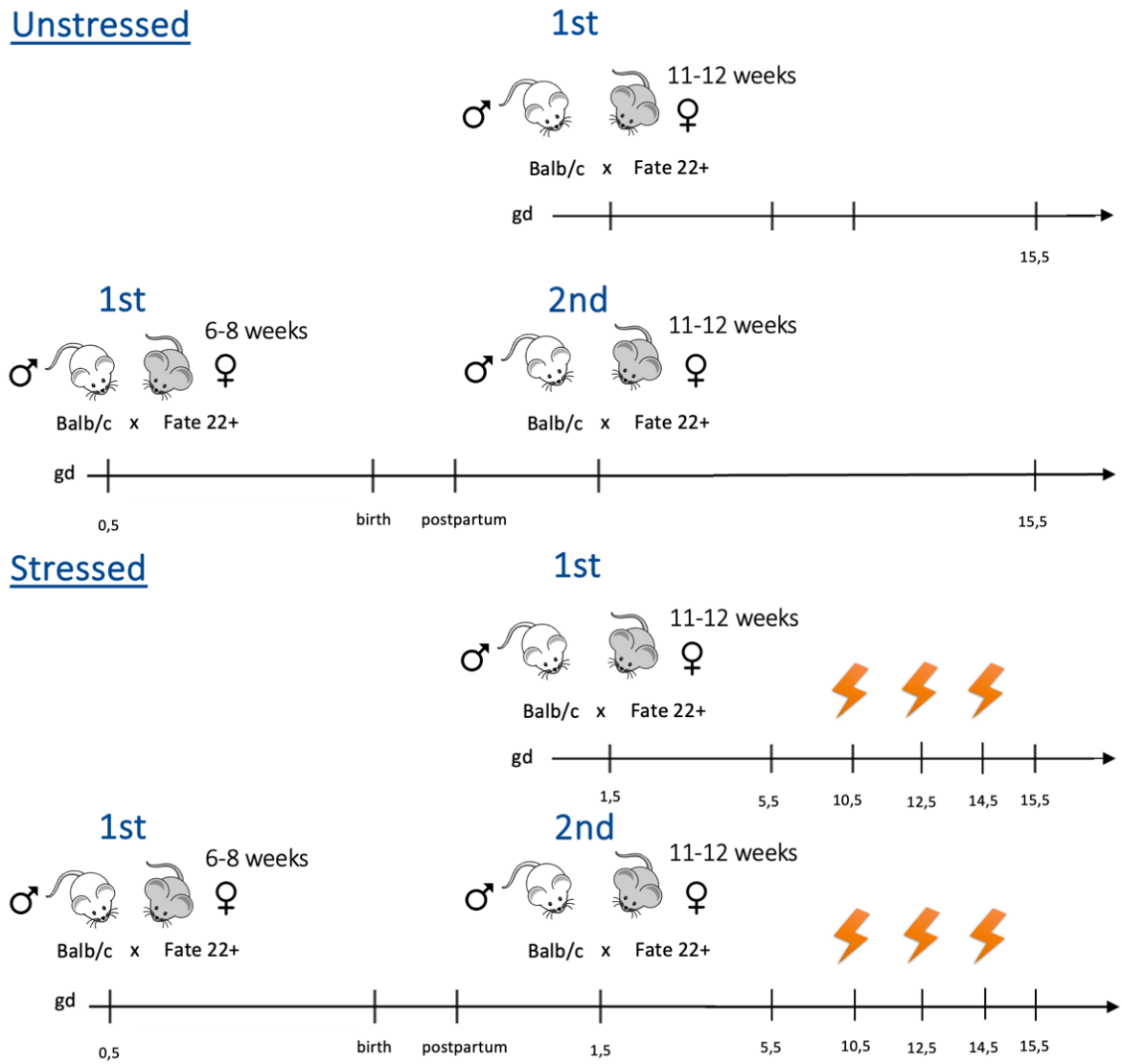

Fig. 4 Experimental setup in the stress mouse model: Age-matched Fate$^+$ females were allogenically mated with Balb/c males once or twice, respectively. Prenatal stress was applicated on gestational days (gd) 10.5, 12.5 and 14.5 in first and second pregnancy. Respective unstressed females served as controls. Organs were harvested on gd 15.5.

3.1.4 Tissue harvesting

I harvested tissue on gd 1.5, 5.5, 7.5 and 15.5. First, female mice underwent carbon dioxide anesthesia. After 30 to 60 seconds, I collected blood retrobulbarly into an ethylene diamine tetra acetic acid collection tube (EDTA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Afterwards, mice were sacrificed by cervical dislocation,

followed by abdominal and peritoneal opening. Then, I harvested para-aortic lymph nodes, which were kept on ice in sterile Dulbecco's Phosphate Buffered Saline (PBS, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts USA) until further processing. Furthermore, I harvested the uterus excluding the implantations, which were removed before storing the organ in PBS and on ice. (Fig. 5A) On gd 15.5 additionally included the extraction of the implantations to further process the fetuses as well. (Fig. 5B) Firstly, the uterus was taken out of the abdomen in toto and was directly placed in a petri dish with PBS on ice. To extract the fetuses, the amnion case was peeled out of the uterine tissue and subsequently opened. Fetus and placenta were carefully separated and weighted for analyzation of fetal outcome.

3.1.5 Pregnancy outcome

Fetal outcome was measured on gd 15.5 by means of the implantation rate, abortion rate, fetal and placental weight. For the implantation and abortion rate, fetuses and abortions were counted. (Fig. 5A&C) The abortion rate (in %) was calculated by $\frac{\text{number of abortions}}{\text{number of implantations}} \times 100$. Before weighting fetuses and placentas, they were cleaned from uterine tissue and the umbilical cord was discarded.

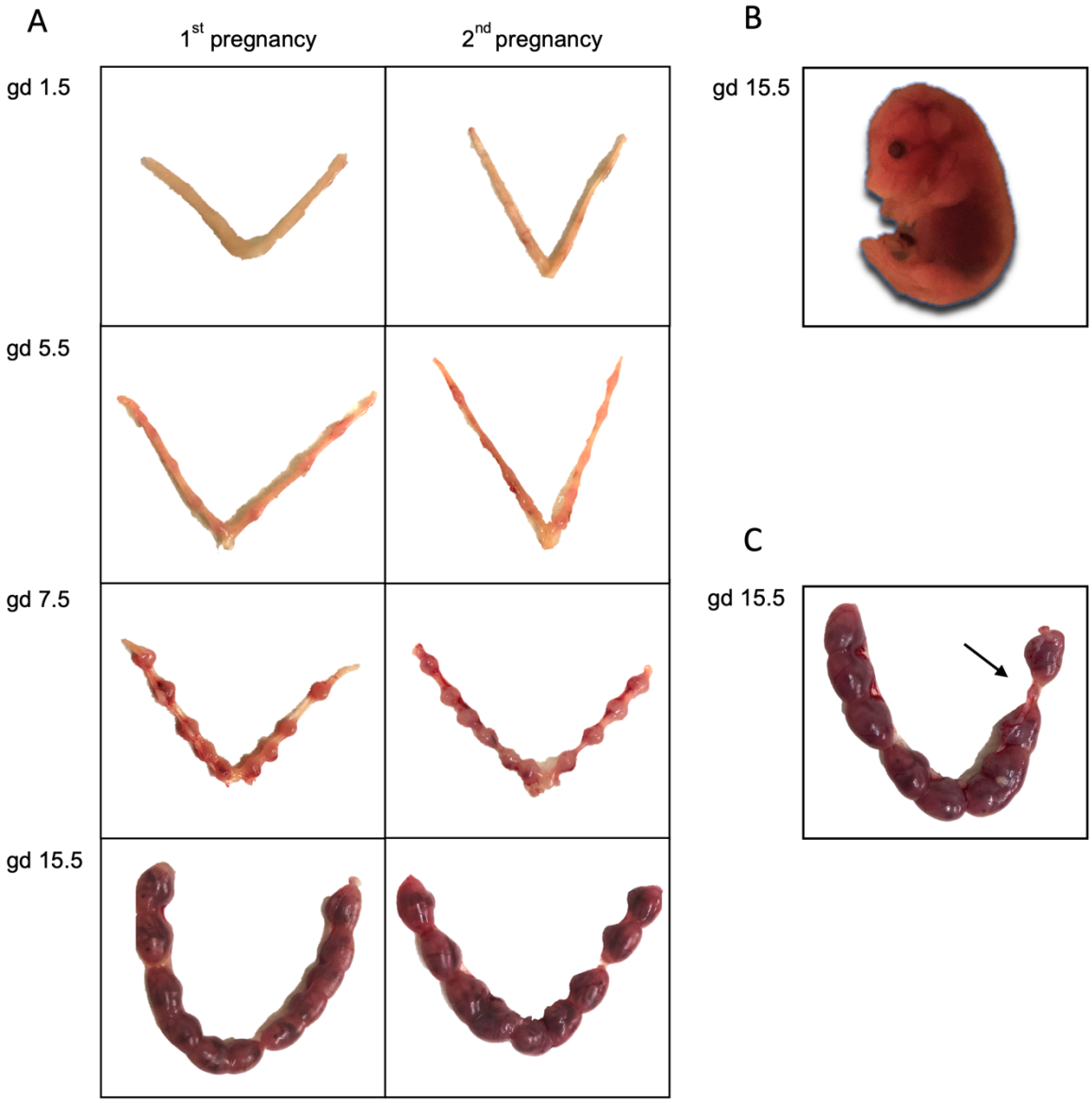

Fig. 5 Harvested uteri and fetus on different gestational days (gd) of first and second pregnancy: The harvest of the uteri was performed on gd 1.5, 5.5, 7.5 and 15.5 of firstly (left) and secondly (right) pregnant mice (A); A fetus is shown on gd 15.5. After an extraction from the uterus and the amnion case a detachment of the placenta was performed (B); The arrow is indicating an abortion of a uterus on gd 15.5 (C)

3.1.6 Tissue processing

For preparation of the tissue for flow cytometry, single cell isolation of the organs was performed. Throughout all procedures storage on ice was ensured. Centrifugation was always carried out at 4°C with 450 g for 8 minutes, unless otherwise specified.

3.1.6.1 Lymph nodes

The lymph nodes (LN) were mashed through a 40 µm nylon cell strainer (Falcon® Corning Inc., Corning, New York, USA) into a petri dish using the plunger of a disposable sterile syringe (Omnifix®-F Luer Solo 0.01-1ml, Braun AG, Melsungen, Germany). After constantly repeated rinsing of the strainer with PBS and a Pasteur pipette (Pastette® 3ml Graduated, Alpha Laboratories Limited, Eastleigh, Hampshire, United Kingdom) the suspension was transferred into a 15 ml falcon tube (CELLSTAR® 15ml/50ml, Greiner Bio-One, Frickenhausen, Germany) Centrifugation was performed as stated above, followed by discarding of the supernatant. In the end only the cell pellet was left, which was resuspended with 1 ml of PBS.

3.1.6.2 Uterus

First, the uterus was transferred into a 1,5ml reaction tube (SafeSeal tube, Sarstedt AG & Co. KG, Nümbrecht, Germany) and then cut into small pieces using small scissors. For further preparation of a single cell suspension, the uterus needs to be digested. Therefore, the shredded uterus was transferred into a 15 ml falcon with an enzymatic digestion solution containing the three enzymes Hyaluronidase (Hyaluronidase from bovine testes, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Collagenase (Collagenase from Clostridium histolyticum, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Bovine Serum Albumin (BSA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), which were solved in Hank's Balanced Salt Solution (HBSS, Gibco, Thermo Fisher Science, Waltham, Massachusetts, USA). Per each 5ml HBSS, I used 1,4 mg Hyaluronidase, 5 mg Collagenase and 5 mg BSA. To activate the process of digestion, the falcon was put into a water bath of 37°C. (Scilogex, Rocky Hills, Connecticut, USA) The bath contained a magnet, while another small magnet was positioned in the tube, to assure a constant mixing of the solution. 20 minutes later, the uterus solution was put on ice, resting for another 2 minutes, allowing the tissue to sink. 3 ml supernatant was then taken onto a cell strainer and rinsed with HBSS into a 50 ml falcon. The 15 ml falcon was refilled with 3 ml HBSS to repeatedly sit in the water bath for 20 minutes. Afterwards, I transferred the whole solution onto the cell strainer and subsequently mashed it with a plunger and washed it with 30 ml HBSS into the 50 ml falcon. Centrifugation was followed by the dismissal of the supernatant and resuspension in 1 ml PBS.

3.1.6.3 Blood

First, 100 µl of the blood samples was transferred into a 1,5 ml reaction tube and directly centrifugated at 13000 rpm for 20 minutes at 4°C. Afterwards the serum was aliquoted into 4 reaction tubes, containing 1.5 µl, 4 µl, 10 µl and the rest and subsequently stored at -20°C for further procession in the future, as described in hormone measurements. In the stress experiment, an additional Fluorescence activated cell sorting (FACS) analysis was performed. The rest of the blood was transferred into a 50 ml Falcon tube. Thereafter, 5ml of red blood cell (RBC) Lysis Buffer (eBioscience™ 1x RBC lysis buffer, Invitrogen by Thermo Fisher Scientific, Life Technologies Corp., Carlsbad, California, USA) were added. With a Pasteur pipette, the blood and the RBC lysis buffer were mixed. After 5 minutes of incubation at room temperature, the lysis was stopped by addition of 30 ml of PBS. A filtration through a cell strainer is performed and followed by centrifugation. Afterwards, the supernatant was discarded, and 1 ml of PBS was resuspended.

3.1.7 Cell counting

Cell counting is required for accurate staining of the cells, in order to ensure an optimal ratio of stain and cells. I counted the living leucocytes with a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) in uterus, LNs and blood using a microscope (Karl Kaps, Asslar/Wetzlar, Germany). I added Trypan Blue staining (0.4 %, Gibco, Thermo Fisher Science, Waltham, Massachusetts, USA) in a 1:10 proportion for the LN and in a 1:50 proportion for the uterus and blood. The counted number allowed conclusion of the total cell number.

3.1.8 Staining

Due to the genetical modifications of the mice used in my experiment, only some antibodies for fluorochrome labelling needed to be added. The integrated markers are IL-17fate, IL-10, FoxP3, IL-17 and IL-22. (Tab. 1) All additional staining has been exclusively performed extracellularly and all organs were treated equally. For staining, I transferred 1 million cells (1.0×10^6 maternal lymph node and uterus cells) of an organ into a FACS tube (Falcon® Corning Inc., Corning, New York, USA). I added 1

ml of PBS for the washing process in the centrifuge. As usual, the supernatant was disposed. Before the actual staining process, a block needed to be added for prevention of unspecific binding. The block consists of rat anti-mouse CD16/CD32 mouse fragment crystallizable block (TrueStain fcX^{TM}, Rat IgG2a, λ, 1:200, BioLegend, San Diego, California, USA) and normal rat serum (1:100, Jackson ImmunoResearch Laboratories West Grove, Pennsylvania, Dianova). Incubation was performed at 4°C in the dark and lasted 15 minutes. Afterwards, the staining with the antibodies was performed without another washing rotation. The antibodies I used are listed in the table 2. Additionally, 0,2 µl dead life stain called eFluor 506 viability dye (D/L, Invitrogen, Thermo Fisher Science, Waltham, Massachusetts, USA) was applied to identify living cells and to exclude dead one via the Fluorochrome AmCyan. After application, the probes again incubated at 4°C in the dark, this time for 30 minutes. Thereafter, a washing rotation with 1 ml of PBS was performed. Centrifugation was followed by resuspension with 200 µl of PBS to prepare the probes for measurement. Furthermore, unstained and fluorescence minus one (FMO) controls were assessed for a negative control and determination of correct gates.

Tab. 1 Reporter and Fluorochromes used for FACS Analyzation: A summary of the Reporter (IL: Interleukin; FoxP3: Forkhead-Box-ProteinP3) and Fluorochromes (FITC: Fluorescein-5-Isothiocyanate; YFP: Yellow fluorescent protein; eFGP: enhanced green fluorescent protein; PE: R-Phycoerythrin; Cy: Cyanine; BV: Brilliant Violet) is listed.

Reporter	Fluorochrome
IL-17fate	FITC/YFP
IL-10	eFGP
FoxP3	PE-Texas Red
IL-17	PE-Cy5
IL-22	BV421(PacBlue)

Tab. 2 Antibodies and Fluorochromes used for FACS Analyzation: A summary of the Antibodies (CD: Cluster of differentiation; gdTCR: gamma delta T cell receptor), Fluorochromes (BUV: Brilliant Ultraviolet; PE: R-Phycoerythrin; Cy: Cyanine; APC: Allophycocyanin), clones, dilutions and sources (Becton Dickinson (BD) Bioscience GmbH (Heidelberg, Germany), BioLegend GmbH (Fell, Germany) and eBioscience Inc. (San Diego, California, USA) is listed.

Antigen	Fluorochrome	Clone	Dilution %	Source
Anti-CD4	BUV395	GK1.5	0,5	BD Bioscience
Anti-CD8	BUV737	53-6.7	0,5	BD Bioscience
Anti-CD3	PE-Cy7	145-2C11	0,5	BioLegend
Anti-gdTCR	APC	eBioGL3	0,5	eBioscience
Anti-CD45	APC-Cy7	30-F11	0,5	BD Bioscience

3.1.9 Flow cytometry

Flow cytometry analysis was performed using the FACSymphony™ A3 (Bioscience, Heidelberg, Germany), which is associated with the DIVA software, both provided by the FACS core unit at the UKE (FACSDiva™ Software version 8.0.1; BD Bioscience, Heidelberg, Germany). The FACSymphony™ A3 has 5 lasers and enables the user to examine up to 30 different parameters. The lasers are set for ultraviolet (355 nm wavelength), violet (405 nm wavelength), blue (488 nm wavelength), yellow-green (561 nm wavelength) and red (637 nm wavelength) light. (BDBiosciences, 2025a) Flow cytometry is a method for cell sorting and analyzation of single cells using fluorescence. Cells are singularly shot with high speed of more than 10.000 cells per second past a laser. The laser reads the staining of the cell. Measurements rely on the emission of optic signals of a cell, as the amount of scattered light correlates with size and complexity of a cell. The measurement allows conclusion about a distinct phenotype of a cell within a heterogenous population. (BDBiosciences, 2025b)

I performed the FACS analysis using FlowJo™ 10.7.1 (Becton Dickinson & Company Tree Star Inc., Ashland, Oregon, USA). On FlowJo™, gates were set according to the staining and additionally, referring to unstained, FMO samples and measurements of Black6 control mice. In the following, the gating strategy for T cell differentiation is

described. First of all, I selected the single cells, excluding double cells. For that purpose, I used the forward scatter, which allows conclusion about the volume of a cell and the side scatter, showing the granularity by radiating a laser in a 90-degree angle. Pursuing the goal of analyzation of different T cell subsets, $CD45^+$ cells were selected. I then continued to investigate the $CD3^+$ cells for further differentiation into $CD4^+$ and $CD8^+$ cells. As the special interest lies in Th17, Treg and Tr1 cells, I focused on the $CD4^+$ Gate, setting a quadruple gate for four different populations. Q1 shows Tr1 cells expressing IL-10 without being $FoxP3^+$. The upper two gates are both positive for FoxP3, on the right with and on the left without current IL-10 expression, can be collectively measured as $FoxP3^+$ Tregs. Q4 in the bottom left is double negative for IL-10 and FoxP3. A deeper look into the $FoxP3^+$ gate reveals my special interest in IL-17 fate expression. In other words, $FoxP3^+$ cells that have been producing IL-17 in the past or ExTh17 cells within $FoxP3^+$ Treg cells. Similarly, Tr1 cells were checked for potential Th17 cell origin. Within the $CD4^+$ cell population, a quadruple gate shows the relationship of current IL-17 production with former production of IL-17. ExTh17 cells are defined as cells, which expressed IL-17 in the past and stopped this production. Further investigations of the fate of these cells concerning their current cytokine production showed IL-10 and FoxP3 expression in these cells. (Fig. 6)

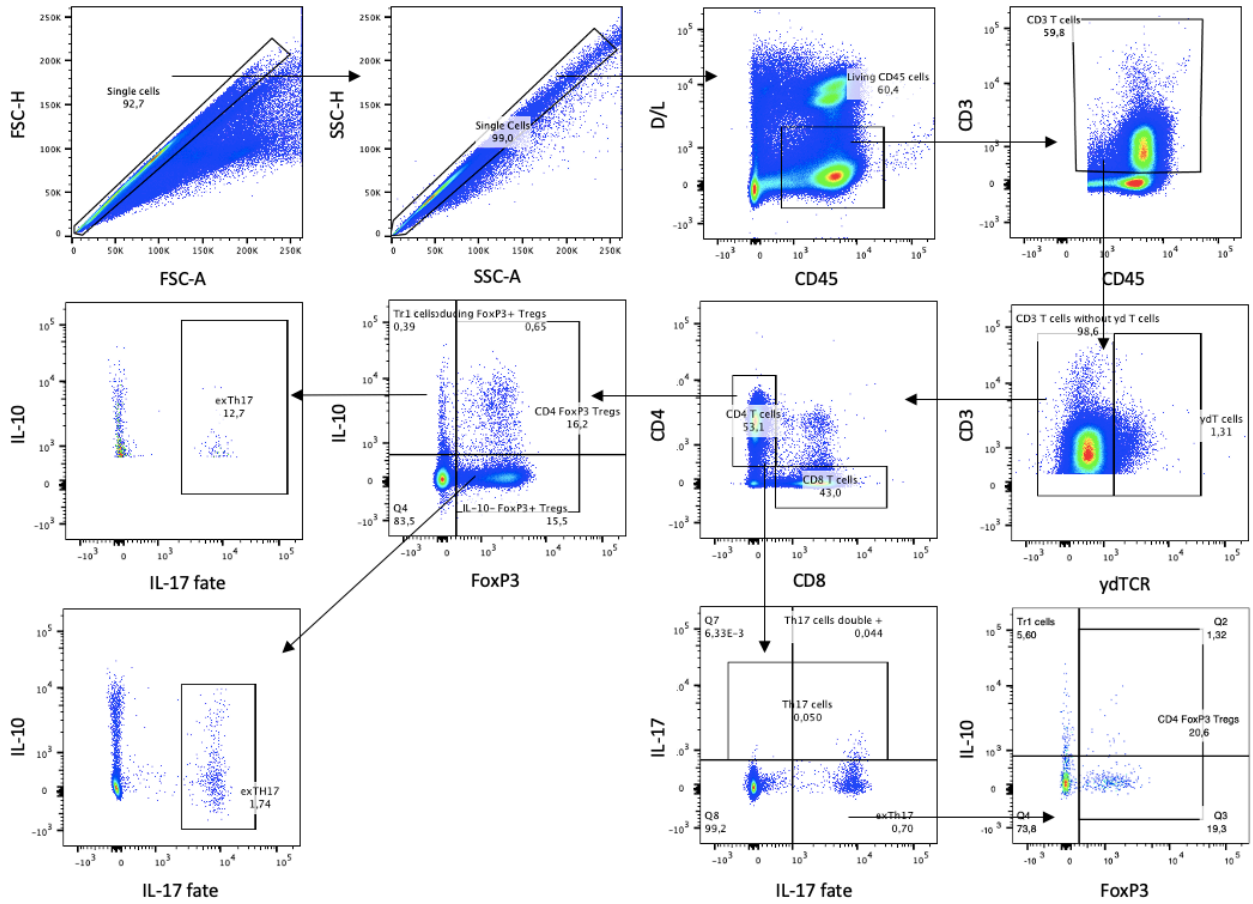

Fig. 6 Gating strategy: After exclusion of doublets and dead cells, living CD45$^+$ cells were gated for CD3$^+$ T cells, and subsequently for CD4$^+$ T cells. CD4$^+$ T cells were then gated for FoxP3 and IL-10 expression. Afterwards, FoxP3$^+$ Treg cells and Tr1 cells were further investigated for ExTh17 cells. CD4$^+$ cells were additionally gated for IL-17 and IL-17 fate expression. ExTh17 cells were gated for FoxP3 and IL-10 expression. The consecutive gating steps are indicated by the arrows in the figure.

3.1.10 Hormone measurement

The plasma samples mentioned in 3.1.4.3 were defrosted for further procession using enzyme-linked immunosorbent assay method (ELISA). The analysis of the pregnancy hormone progesterone was carried out by using a 1:200 dilution of plasma and the ELISA Buffer. Following the manufacturer's instructions, a competitive ELISA (Progesterone ELISA Kit, Cayman Chemical, Ann Arbor, Michigan, USA) was performed on a Nano Quant (Tecan Group AG, Männedorf, Switzerland). After defrosting, the samples for corticosterone measurement were treated with a dissociation reagent. Then, the probes were treated with an assay buffer in a 1:500

dilution. Following the manufacturer's instructions, a corticosterone ELISA (Corticosterone Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, Michigan, USA) was performed. The number of sacrificed mice in the hormone measurement were a total of 6, 6, 8 and 6, respectively for the above-mentioned groups.

3.1.11 Statistical analysis

After FlowJo™ analysis, output data were statistically analyzed in GraphPad Prism Version 9.0 (GraphPad Software, La Jolla, California, USA). First of all, an outlier identification using the ROUT method with a Q of 10%, was performed. This method combines nonlinear regression with an identification of outliers. (Motulsky and Brown, 2006) Afterwards, a normality and lognormality test in form of the Anderson-Darling test, the D'Augustino & Pearson test, the Shapiro-Wilk test and the Kolmogorov-Smirnov test were performed, examining if significance needs to be tested in a parametric or a non-parametric way. Concerning small numbers, the Shapiro-Wilk test is the most accurate one out of all different tests. If the values are not normally distributed, significance is checked with the Mann-Whitney Test for non-parametric groups. Normally distributed, parametric values go into an unpaired t-test, automatically assuming both populations have the same standard deviation. The unpaired t-test, I performed, is two-tailed and works with a confidence level of 95%. The same accounts for the Mann-Whitney test. Statistical significance is herewith achieved if the p-value is <0.05. Significant differences between first and second pregnancies were checked day wise. As the stress experiment compares more than two groups, a one-way-ANOVA replaced the t-test in stress experiments.

4. Results

In the following chapter, the results of this thesis are presented. Parts of the results have already been published in the publication: (Thiele et al., 2025).

4.1 T cell kinetics in physiologic first and second pregnancy

The physiological kinetics of T cells and the role and importance of plasticity in $CD4^+$ T cells are displayed in the context of maternal immune adaptation. The graphs displayed in this thesis rely on data generated using multiple experimental animals. However, for the purpose of demonstration of kinetic curves the individual results were connected.

$CD3^+$ cells are evaluated within $CD45^+$ cells. In the graph, it is shown that $CD3^+$ cells constitute 50 to 60% of all $CD45^+$ cells in uterus draining lymph nodes. They oscillate around these numbers in first and second pregnancies without significant differences. (Fig. 7A) In the uterus, the frequency of $CD3^+$ cells is significantly increased in second pregnancies compared to first ones in the beginning on gd 1.5 and towards the end of pregnancy on gd 15.5, a range of 5% to 15% is seen. First pregnancies show the lowest level of $CD3^+$ cells on gd 1.5 and continuously rise, peaking at gd 15.5. Second pregnancies display a drop to a level similar to the one of first pregnancies on gd 5.5 and 7.5 and also peak on gd 15.5. (Fig. 7B)

$CD4^+$ cells constitute 20 to 35% of $CD45^+$ cells in the LN. Second pregnancy reaches significantly higher percentages than primigravidae on gd 7.5 and 15.5. In first pregnancies the level stays steadily below 30%. In second pregnancies, the level is lower than the one of first pregnancy on gd 1.5 and rises with advancing pregnancy. The peak is reached on gd 7.5. (Fig. 7C) In the uterus, $CD4^+$ cells make up 2 to 7% of $CD45^+$ cells. A significant increase in second pregnancies compared to first ones is seen on gd 1.5. Throughout the timeline, second pregnancies show higher frequencies than first pregnancies. While first pregnancy shows a constantly low level until gd 7.5, second ones show a decrease from gd 1.5 to gd 5.5. In both groups, the highest percentage is measured on gd 15.5. (Fig. 7D)

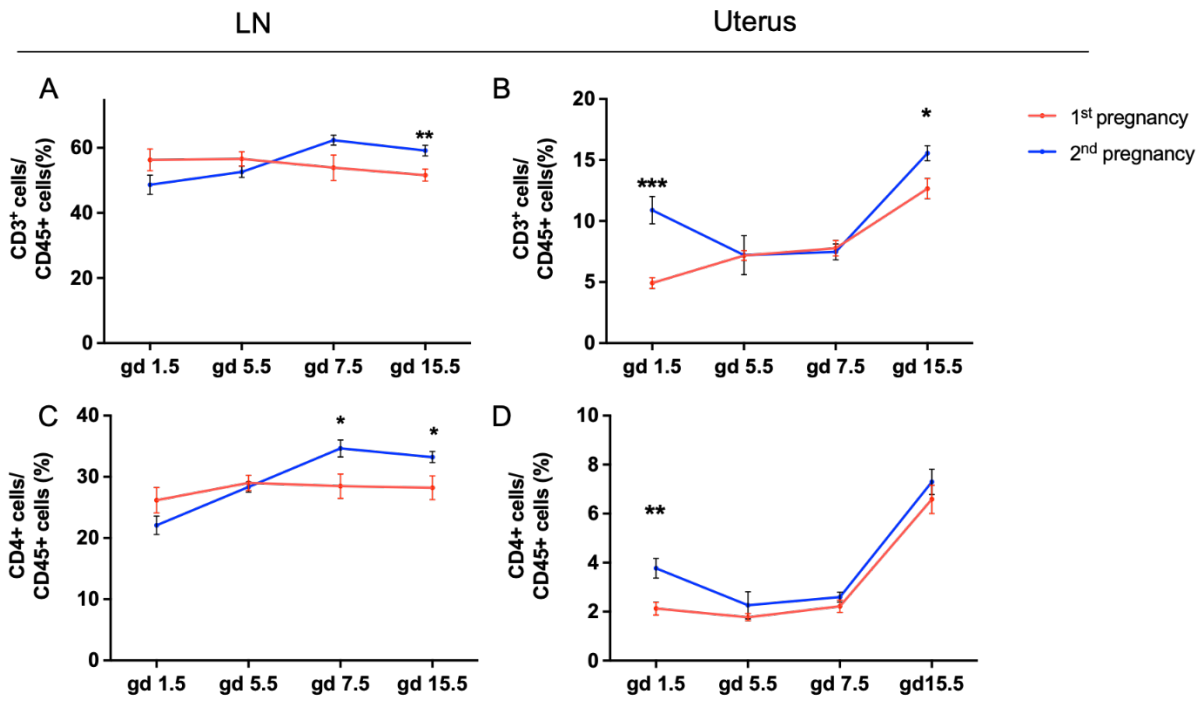

Fig. 7 Increased T cell frequencies in subsequent pregnancies: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and lymph node (LN) and uterus were harvested on gestational day (gd) 1.5, 5.5, 7.5 and 15.5. Flow cytometry analysis of CD3$^+$ cells within CD45$^+$ cells in LN (A) and uterus (B) and CD4$^+$ cells within CD45$^+$ cells in LN (C) and uterus (D) are displayed as a kinetic curve in first and second pregnancies. Data are presented as mean values ±SEM and the statistical significance between first (red) and second pregnancy (blue) using t-test individually per gd (* $p<0.05$, ** $p<0.001$, *** $p<0.001$).

The kinetics of Th17 cells are measured within the CD4$^+$ cells. In the lymph node levels range from 0 to 0.2%. The level of Th17 cells on gd 1.5, 5.5 and 7.5 is significantly higher in second pregnancies compared to first pregnancies. While first pregnancies show a very low signal on gd 1.5, 5.5 and 15.5, increased levels were measured on gd 7.5. Second pregnancies also peak on gd 7.5, dropping to a similar level like firstly pregnant mice on gd 15.5. (Fig 8A)

Locally in the uterus, with a range of 0 to 1.4% of CD4$^+$ cells, Th17 cells show higher percentages than in the lymph node. Second pregnancies show a similar tendency of higher frequencies of Th17 cells compared to the lymph nodes. In primigravidae an increase is detectable on gd 5.5 and the peak on gd 15.5. In second pregnancies, the timeline rises linearly and peaks on gd 15.5. (Fig 8B)

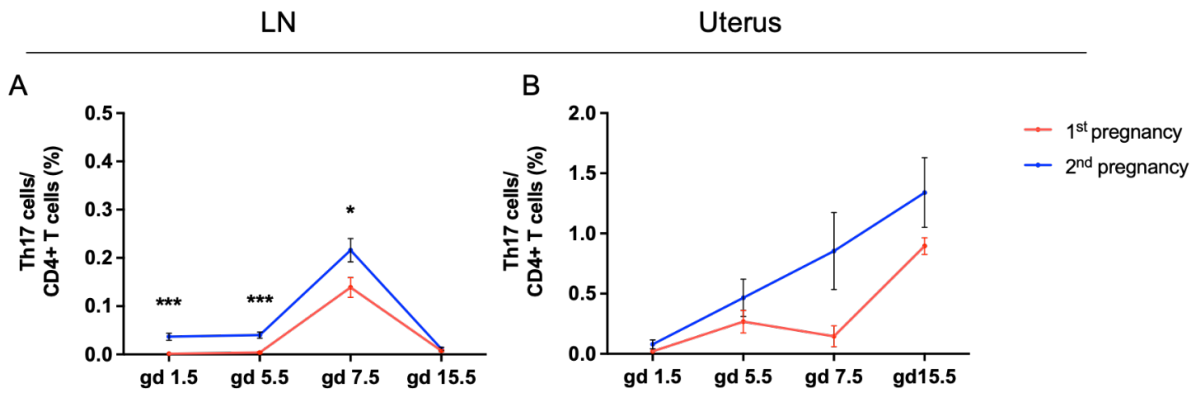

Fig. 8 Increased Th17 cell frequencies in subsequent pregnancies: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and lymph node (LN) and uterus were harvested on gestational day (gd) 1.5, 5.5, 7.5 and 15.5. Flow cytometry analysis of Th17 cells within CD4$^+$ cells in LN (A) and uterus (B) are displayed as a kinetic curve in first and second pregnancies. Data are presented as mean values ±SEM and the statistical significance between first (red) and second pregnancy (blue) using t-test individually per gd (* $p<0.05$, *** $p<0.001$).

The figure shows FoxP3$^+$ Tregs within CD45$^+$ cells. In the LN, Tregs constitute around 3 to 5% of CD45$^+$ cells. A significant increase in Treg numbers in second pregnancy compared to first pregnancies is illustrated on gd 5.5, 7.5 and 15.5. In firstly pregnant mice levels circulate around 3 to 4% throughout pregnancy, in secondly pregnant females similar levels to firstly pregnant mice are observable on gd 1.5, followed by a steady increase with a peak on gd 7.5 and a slight decrease towards gd 15.5. (Fig. 9A) In the uterus, levels range from approximately 0.2 to 1.4%. A significant higher frequency is measured on gd 7.5 in second pregnancy. Gd 15.5 shows a p-value of 0.07. Uterine FoxP3$^+$ cells show similar curves for first and second pregnancy. The curve of second pregnancies is steadily above first-time pregnant mice. From gd 1.5 the levels decrease towards gd 5.5. An increase thereafter peaks on gd 15.5 in both first and second pregnancies. (Fig. 9B)

Further analyzation of Tregs includes the secretion of their signature cytokine IL-10. In the LN, 1.8 to 4.2% of the Tregs secrete IL-10. The highest production occurs on gd 5.5, presenting the only significant difference between first and second pregnancies. On all gds, the curve of secondly pregnant mice is above the first-time pregnant ones. Both first and second pregnancy show similar configuration of the graphs. Evidently,

the lowest production is seen in the beginning on gd 1.5 and towards the end of pregnancy on gd 15.5. (Fig. 9C) In the uterine tissue, IL-10 production ranges from approximately 2 to 18% and no significances were measured. The graph of firstly pregnant mice is slightly above the secondly pregnant ones showing especially high numbers on the peak on gd 7.5 with high scattering rates. The lowest levels are reached on gd 5.5. In second pregnancy, equally gd 5.5 shows the lowest IL-10 secretion, however the peak is reached on gd 15.5. (Fig. 9D)

Investigations of the origin of Tregs is performed using a built-in fate mapping tool. ExTh17 cells, formerly expressing IL-17, are measured within Tregs. In the LN, the frequency of ExTh17 cells ranges from 0.2 to 2% with significantly higher numbers on all gds in second pregnancies. While the frequency of ExTh17 cells in first pregnancy shows a consistently low level, second pregnancy register an increase from gd 1.5 to its peak on gd 5.5. The level steadily decreases towards gd 15.5. (Fig. 9E)

The uterine Tregs show an ExTh17 profile in a range from 2 up to 11%. Second pregnancies constantly show a higher frequency. First pregnancies peak on gd 5.5, second ones on gd 7.5. In both first and second pregnancy, gd 1.5 and gd 15.5 display the lowest numbers of ExTh17 cells. (Fig. 9F)

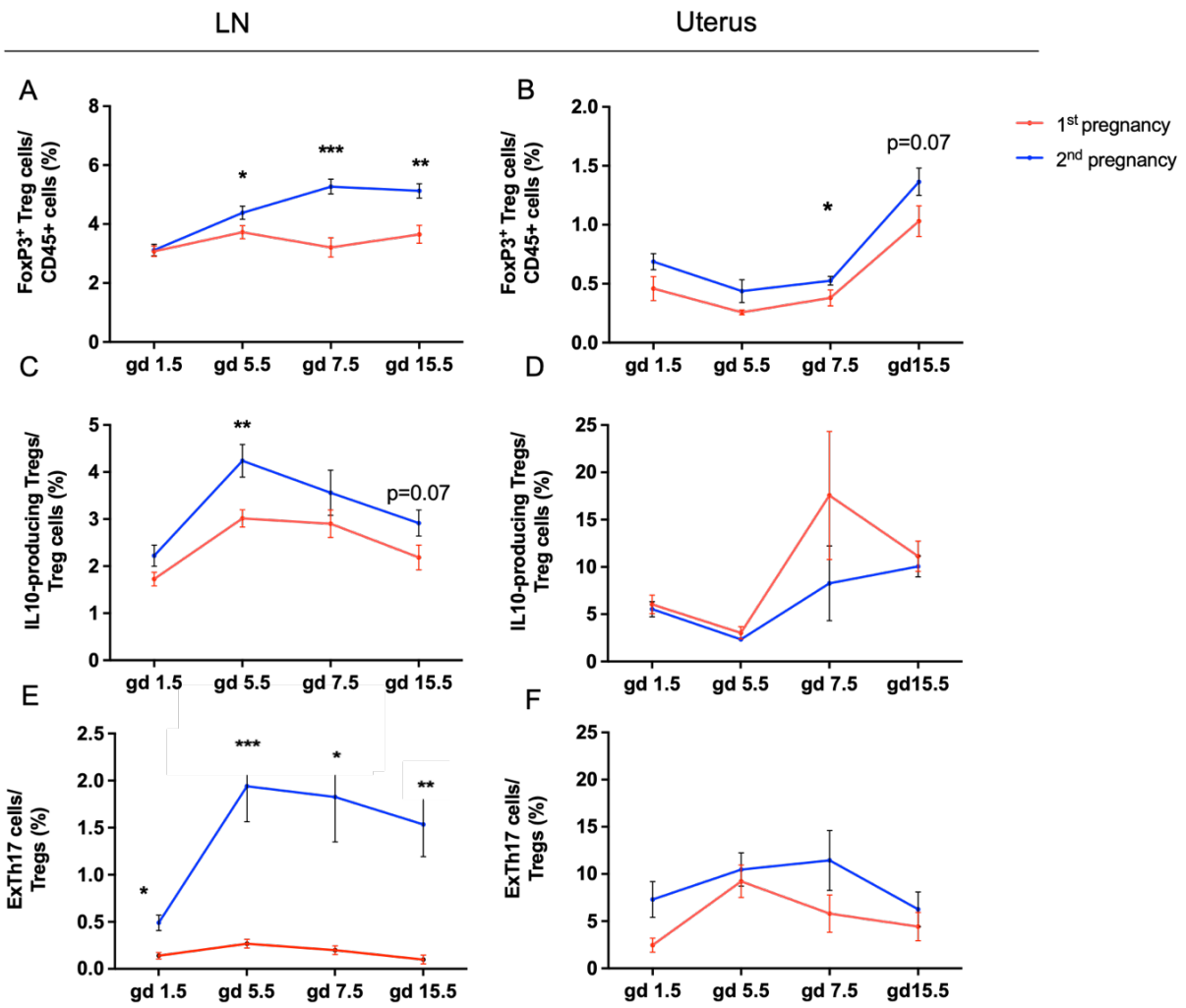

Fig. 9 Increased Treg cell frequencies and expression of an ExTh17-Phenotype of Treg cells in subsequent pregnancies: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and lymph node (LN) and uterus were harvested on gestational day (gd) 1.5, 5.5, 7.5 and 15.5. Flow cytometry analysis of FoxP3$^+$ Treg cells within CD45$^+$ cells in LN (A) and uterus (B) IL-10$^+$ cells within Treg cells in LN (C) and uterus (D), and ExTh17 cells in Treg cells in LN (E) and uterus (F) are displayed as a kinetic curve in first and second pregnancies. Data are presented as mean values ±SEM and the statistical significance between first (red) and second pregnancy (blue) using t-test individually per gd (* $p<0.05$, ** $p<0.001$, *** $p<0.001$).

Tr1 cells display a range of approximately 0.03 to 0.12% of CD45$^+$ cells in the LN. Significant increases of Tr1 cells in subsequent pregnancies compared to first pregnancies are seen on gd 5.5 and gd 7.5. While in first pregnancies a constantly low level of Tr1 cells is measured, second pregnancies show an increase towards gd 5.5 and peak on gd 7.5. The lowest amount of Tr1 cells is measured on gd 1.5 and on gd

15.5 in second pregnancies. (Fig. 10A) In the uterus, a range of 0.1 to 0.6% and no significant differences between first and second pregnancy are observable. The kinetics of a low frequency in the first 3 timepoints (gd 1.5, 5.5 and 7.5) and a rise towards the end of the timeline on gd 15.5 are similar in both, first and second pregnancy. (Fig. 10B)

ExTh17 cells within Tr1 cells in the LN show no difference between first and second pregnancies with a range from approximately 7 to 21%. The kinetic scheme shows a peak on gd 1.5, which is followed by a drop on gd 5.5, reaching the lowest point. A slight increase on gd 7.5 is followed by another drop on gd 15.5. (Fig. 10C) In the uterus, a similar kinetic curve with a range from 1 to 16% is measured. The curve of the secondly pregnant mice is slightly above the one of the firstly pregnant dams without reaching significancy. The highest frequency is measured on gd 1.5 and the lowest on gd 5.5. Some slight increases can be seen on gd 7.5 and towards 15.5. (Fig. 10D)

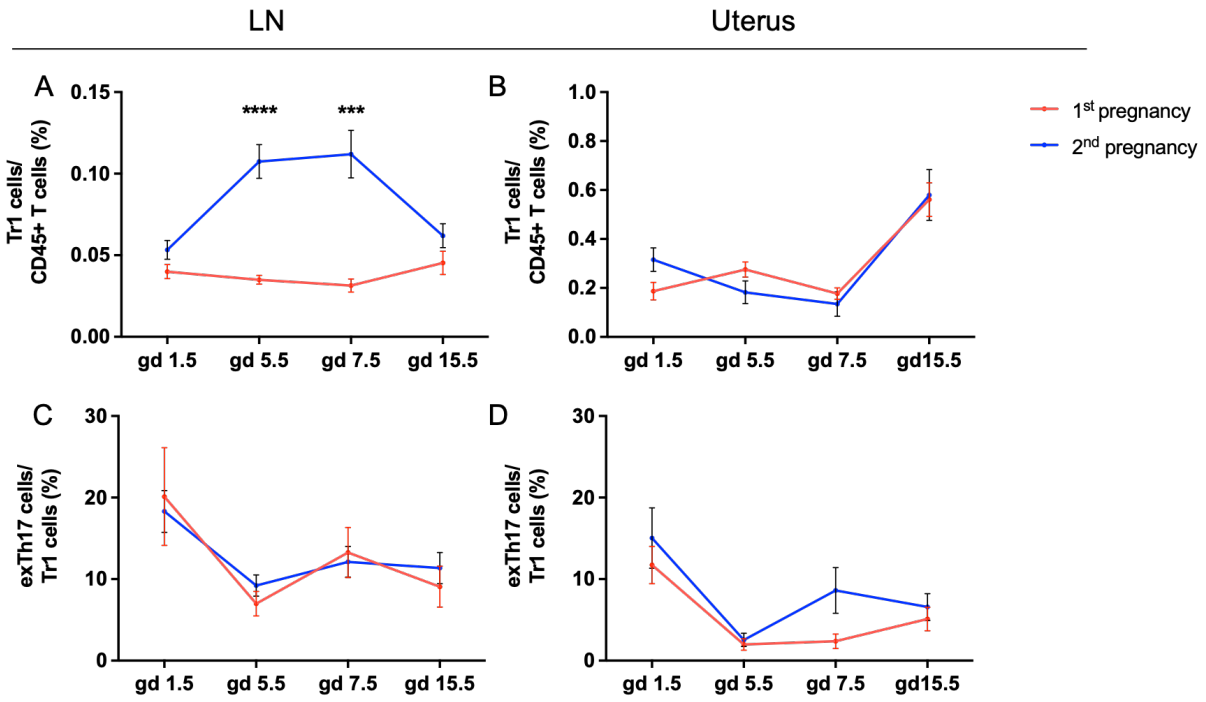

Fig. 10 Increased Tr1 cell frequencies in subsequent pregnancies: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and lymph node (LN) and uterus were harvested on gestational day (gd) 1.5, 5.5, 7.5 and 15.5. Flow cytometry analysis of Tr1 cells within CD45$^+$ cells in LN (A) and uterus (B) and ExTh17 cells in Tr1 cells in LN (C) and uterus (D) are displayed as a kinetic curve in first and second pregnancies.

Data are presented as mean values ±SEM and the statistical significance between first (red) and second pregnancy (blue) using t-test individually per gd (*** $p<0.001$, **** $p<0.0001$).

The subset of ExTh17 cells measures approximately 0.1 to 1.2% of all $CD4^+$ cells in the LNs. Significant differences between first and second pregnancies are observable on all gds. Almost no ExTh17 cells are measurable with consistently low levels in firstly pregnant mice. In secondly pregnant mice, on gd 1.5 the lowest level of the blue kinetic curve is recorded. A rise on gd 5.5 is followed by a drop on gd 7.5. The graph shows its peak on gd 15.5. (Fig. 11A) In the uterine tissue, ExTh17 cells make up 2 to 12% of $CD4^+$ cells. The uterus displays significances between first and second pregnancies on gd 1.5, 7.5 and 15.5. In first pregnancies the lowest point is detectable on gd 1.5. The peak is reached with proceeding pregnancy on gd 5.5. Thereafter, a marginal decrease is measurable, continuously proceeding from gd 5.5 to gd 15.5. In second pregnancies, ExTh17 cell levels steadily rise from gd 1.5 to gd 7.5 and slightly decrease towards gd 15.5. (Fig. 11B)

Within the ExTh17 cell population, FoxP3 expression and the Tr1 population are scanned. Regarding FoxP3 expression in ExTh17 cells in the LN, the graphs of first and second pregnancies cross twice. Percentages of $FoxP3^+$ Treg cells range from approximately 4 to 22%, however on gd 15.5 the difference of first and second pregnancy reaches a p-value of 0.08. First pregnancy shows the highest levels on gd 1.5 and gd 5.5. Thereafter a drop towards the lowest point on gd 7.5 follows. On gd 15.5 a slight increase is detectable. In second pregnancy, gd 1.5 shows insignificantly higher percentages than first pregnancies. The curves firstly cross between gd 1.5 and 5.5, as second pregnancies reach their lowest level. The second crossing of the curves occurs between gd 5.5 and 7.5, as second pregnancy levels stay constant. Towards gd 15.5 the curve reaches a peak. (Fig. 11C) Locally, a range from approximately 5 to 15% is measured and no significances were detected. In first pregnancies, a rise from the lowest percentage on gd 1.5 to the highest one on gd 5.5 is recorded. Towards gd 7.5 the curve drops to slightly higher numbers than measured on gd 1.5, followed by a slight increase towards gd 15.5. In secondly pregnant mice, $FoxP3^+$ Treg cells in ExTh17 cells increase over the course of pregnancy from gd 1.5 to gd 7.5. A slight

decrease is detectable on gd 15.5. The biggest span between first and second pregnancy is traceable on gd 7.5. (Fig. 11D)

The observation of the other regulatory phenotype of Tr1 cells within ExTh17 cells in the LN shows a continuous rise throughout pregnancy in first and second pregnancy with a range of 0.5 to 6%. Second pregnancies show a significantly higher level on gd 5.5. First pregnancy shows lower percentages on gd 1.5, 5.5 and 7.5 and crosses the curve of second pregnancy between gd 7.5 and gd 15.5. (Fig. 11E) In the uterus a range of 0 to 40% is measured. First pregnancies have a significantly higher number of Tr1 cells within ExTh17 cells on gd 5.5. In first pregnancy, lowest frequencies are measured on gd 1.5, thereafter the peak is reached on gd 5.5. A rapid decrease towards gd 7.5 is followed by an extenuated decrease towards gd 15.5. In secondary pregnancy, levels are similar to first pregnancy on gd 1.5 and gd 15.5. In between the peak is reached on gd 5.5 and the lowest point on gd 7.5. (Fig. 11F)

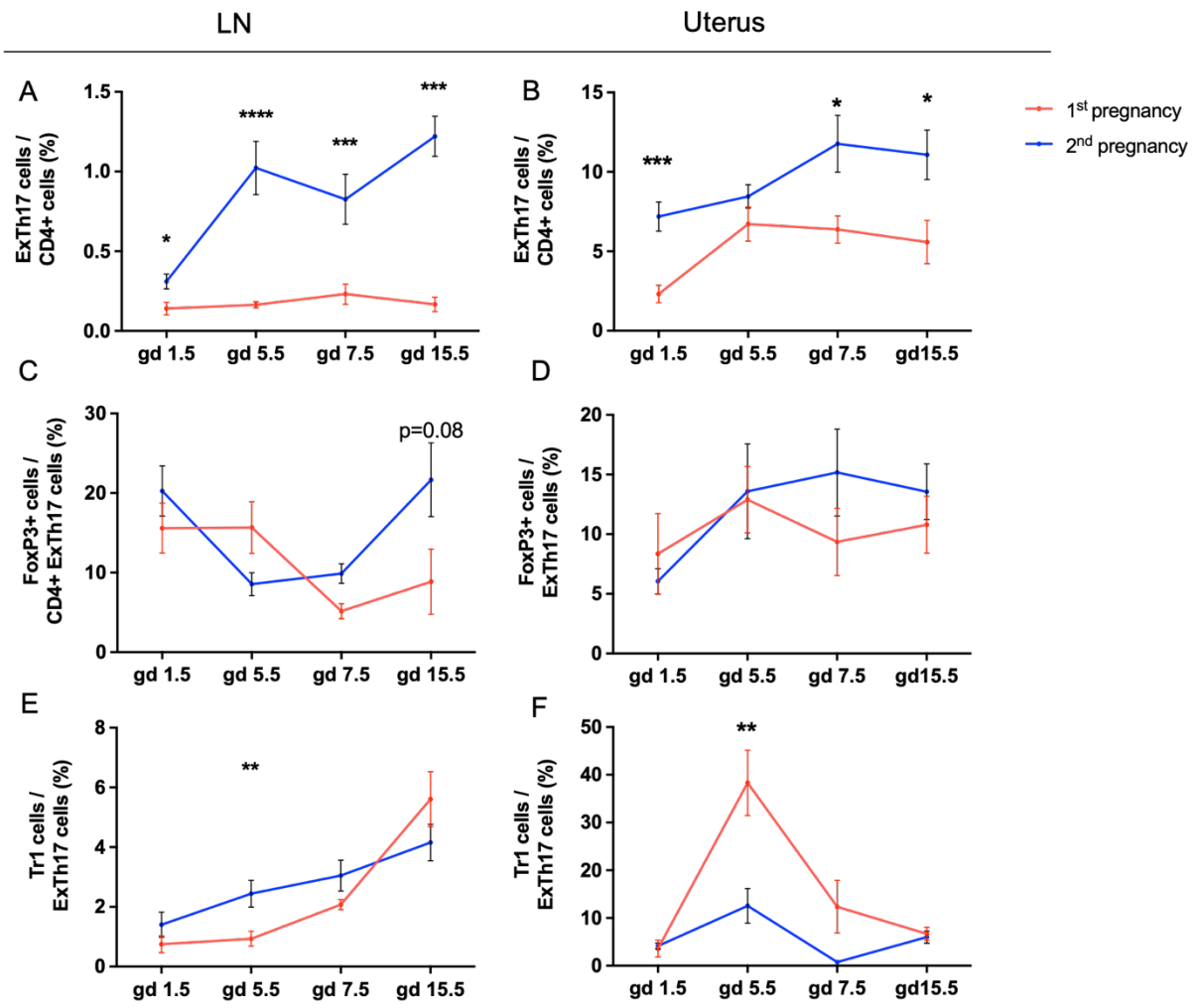

Fig. 11 Increased Ex-Th17 cell frequencies in subsequent pregnancies: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and lymph node (LN) and uterus were harvested on gestational day (gd) 1.5, 5.5, 7.5 and 15.5. Flow cytometry analysis of ExTh17 cells within CD4$^+$ cells in LN (A) and uterus (B) FoxP3 expression within ExTh17 cells in LN (C) and uterus (D) and Tr1 cells within ExTh17 cells in LN (E) and uterus (F) are displayed as a kinetic curve in first and second pregnancies. Data are presented as mean values ±SEM and the statistical significance between first (red) and second pregnancy (blue) using t-test individually per gd (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

4.2 Impact of prenatal stress on first and second pregnancy

The establishment of kinetics of the physiologic course of pregnancy is extended by the examination of the influence of the challenge of stress on pregnancy.

4.2.1 Hormones

The hormones corticosterone and progesterone are measured in the stress experiments. Progesterone measurements show levels between 100 and 200 ng/ml without reaching any significances. Second pregnancies show a higher level than first ones in control and prenatally stressed dams. Further, an upward trend in stress compared to the control groups is detectable in first and second pregnancy. (Fig. 12A) Corticosterone levels display a mean of about 600 ng/ml in unchallenged pregnancies and around 1000 ng/ml in stressed pregnancies. These changes in corticosterone levels are significant in both, first and second pregnancies. (Fig. 12B)

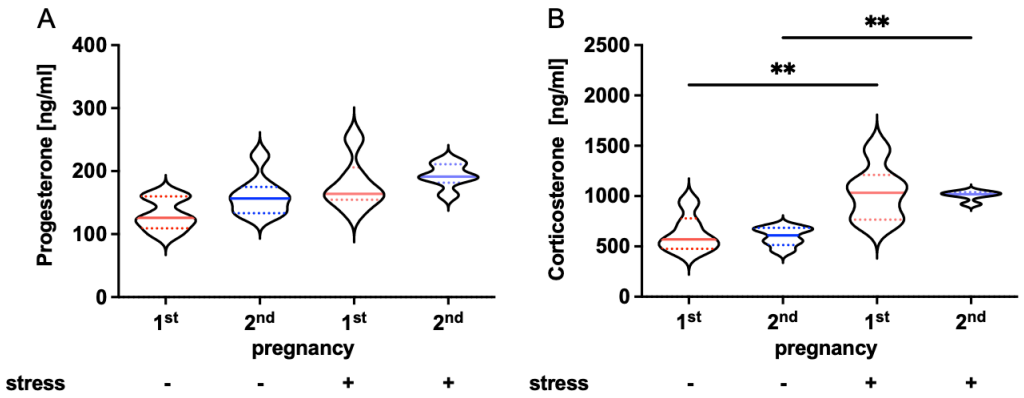

Fig. 12 Increased corticosterone levels after prenatal stress challenge: Fate^{+} mice were mated once or twice to Balb/c, respectively and blood was collected on gestational day (gd) 15.5. Progesterone levels (A) and corticosterone levels (B) in ng/ml in first (red) and second (blue) pregnancies in control (left) and prenatally stressed (right) female mice. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (** $p<0.001$).

4.2.2 Pregnancy Outcome

To explore possible advantages of second or unchallenged pregnancy and to confirm intrauterine growth restriction (IUGR) of the offspring of stressed dams, the fetal outcome is measured. Fetal outcome is assessed by means of implantation, the abortion rate, fetal and placental weight.

The number of implantations amounts to roughly 8 pubs per mother without significant difference between all four groups. The control mice feature slightly higher numbers in firstly pregnant, than in secondly pregnant mice. The stressed groups display a reversed picture. Stressed second-time pregnant mice display the highest implantation rate, while the lowest one is measured in first-time pregnant stressed dams. Hence, the span is increased between the stressed groups. (Fig. 13A)

Next, abortion rates are assessed, showing a range of approximately 0 to 15%. Most abortions manifest in first pregnancies of the control group, significantly more than in normal second pregnancies. In contrast to the unchallenged counterparts, stressed first-time pregnant mice show lower abortion rates, while a slight increase is seen in the offspring of second-time pregnant, stressed dams. All in all, the very low abortion rate of secondly pregnant mice is prominent. (Fig. 13B)

Fetal weight shows a range from 380 to 420 mg. Significances are detectable between secondly pregnant control animals and secondly pregnant stressed mice and between the stressed groups. In total, the highest fetal weight was measured in prenatally stressed second pregnancy. Comparison of the parities shows a trend towards an increased weight of pubs from secondly pregnant mice in the control groups. The span is extended in the stressed groups. Prenatally stressed first pregnancies show a decreased weight compared to the control group, while stressed second pregnancies register a significant increase compared to the controls. (Fig. 13C)

Placental weight ranges from 105 to 115 mg. In the control groups, a higher weight was observed in second pregnancies. This tendency is attenuated between prenatally stressed first and second pregnancy. Both stressed groups indicate a lower weight in stress. Further, the span between first and second pregnancy is reduced. (Fig. 13D)

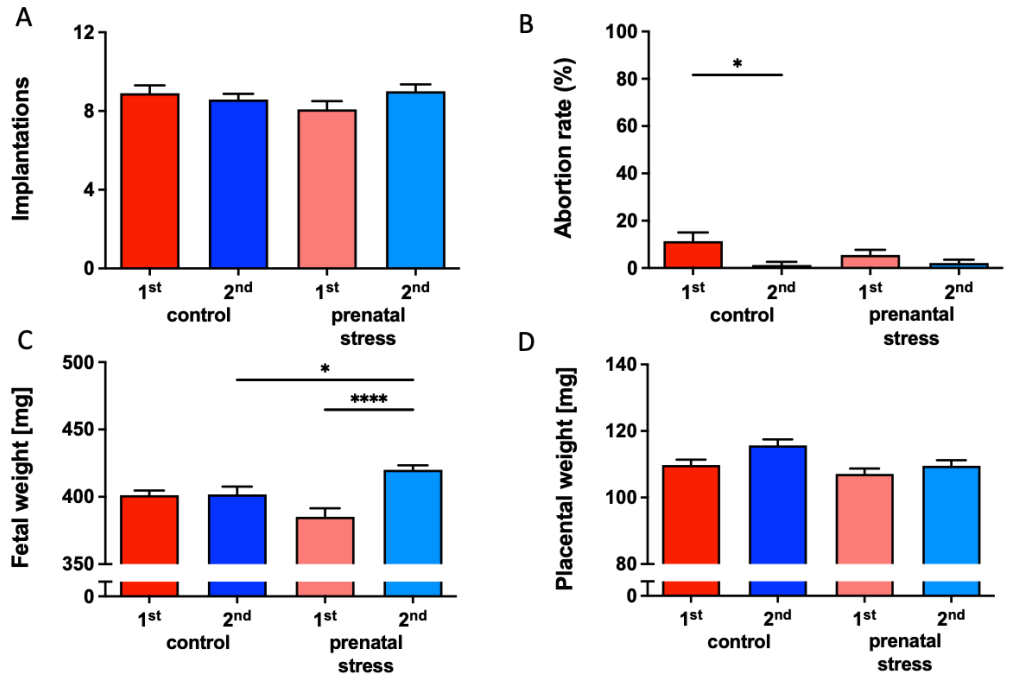

Fig. 13 Fetal weight is differentially affected by prenatal stress challenge in first and second pregnancy: Fate^{+} mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Uterine tissues including fetal implantations were harvested on gd 15.5. Fetal outcome is measured in implantation (A) and abortion rate (B), fetal (C) and placental weight (D). Each graph shows data for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (* $p<0.05$, **** $p<0.0001$).

In the following results of investigations of the functional role of Th17 cells and regulatory T cells are displayed for challenged pregnancy. The graphs are designed in a violin plot to show the distribution more accurately. The controls are displayed on the left side of each figure for first and second pregnancy. On the right side, the stressed groups are presented.

The first figure shows a contemplation of CD3^{+} cells within CD45^{+} cells. In the LN, levels range from 50 to 60%. Significant differences are detectable between first and second pregnancy in the control groups. CD3^{+} cells are higher in second pregnancy in comparison to first ones in both stressed and control groups. In prenatally stressed

pregnancy, the numbers are generally elevated compared to unchallenged equivalents. While unchallenged first pregnancies show the lowest percentage, stressed second pregnancies show the highest one. (Fig. 14A) In the uterus, a range of approximately 11 to 20% is measurable. A trend of a rise in $CD3^+$ cells in second pregnancy compared to first ones becomes apparent. Further, a tendency of elevated numbers in stress is recognizable. (Fig. 14B)

The population of $CD4^+$ cells are displayed within the parent population of $CD45^+$ cells. In the LN, the levels range from 25 to 35% and significant differences are measured between first and second control animals. In the stressed counterparts, a similar increase in stressed pregnancy is detectable without exceeding significant levels. Comparing unchallenged first with prenatally stressed first pregnancies, the trend points towards higher percentages in stress. For second pregnancies, this trend is not noticeable, rather the tendency of a slight decrease. (Fig. 14C) In uterine tissue, levels stay constant in all groups. (Fig. 14D)

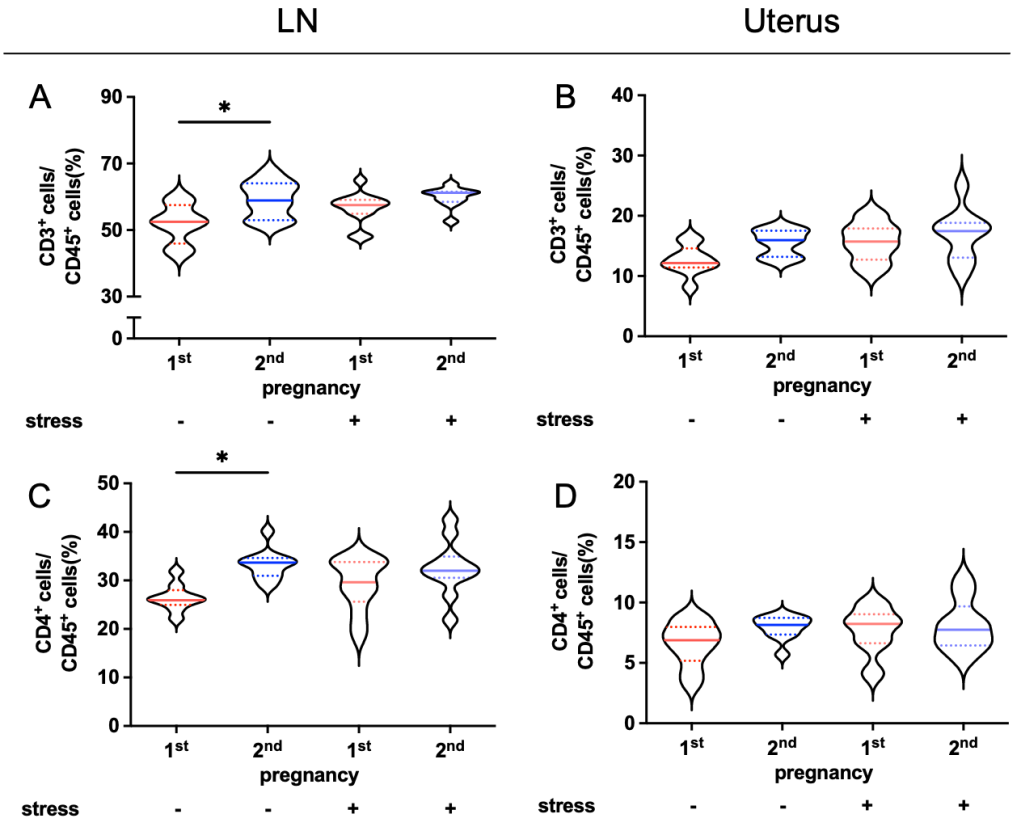

Fig. 14 T cell frequencies in prenatal stress challenge: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Lymph node (LN) and uterus were harvested on gd 15.5. Flow

cytometry analysis of CD3$^+$ cells within CD45$^+$ cells in LN (A) and uterus (B) CD4$^+$ cells within CD45$^+$ cells in LN (C) and uterus (D) are displayed for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (* p<0.05).

Next, Th17 cells within CD4$^+$ cells are displayed. In the LN, a range from approximately 0 to 0.01% is measured and no significant differences are seen between all groups. No changes are exhibited neither between first and second pregnancy nor in stress. (Fig. 15A) A similar picture of stable percentages of Th17 cells within the CD4$^+$ population is recognizable in the uterus. The percentages are measured around 1% independent of the circumstance of first or second and stressed or unstressed pregnancy. (Fig. 15B)

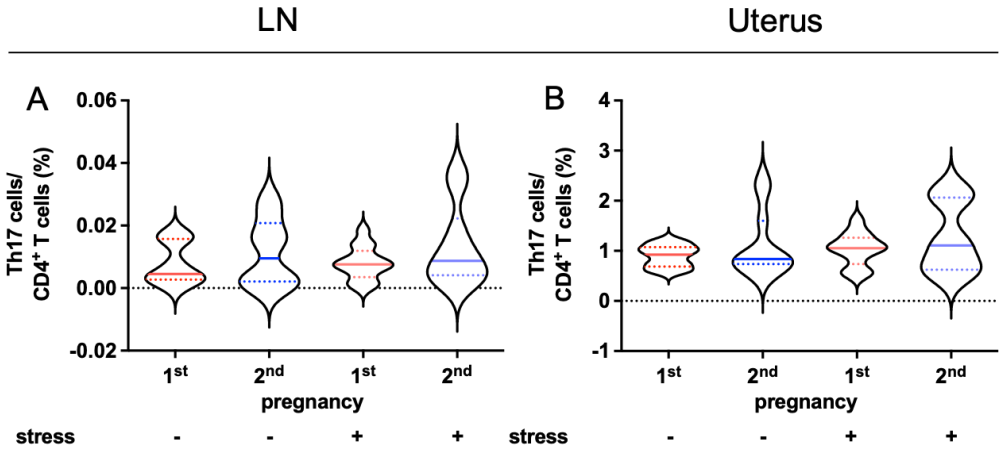

Fig. 15 Stable Th17 cell frequencies in prenatal stress challenge: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Lymph node (LN) and uterus were harvested on gd 15.5. Flow cytometry analysis of Th17 cells within CD4$^+$ cells in LN (A) and uterus (B) are displayed for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (* p<0.05, ** p<0.001, *** p<0.001).

The first regulatory phenotype of interest is the FoxP3$^+$ Treg population, which is analyzed within CD45$^+$ cells. For the FoxP3$^+$ cells in the LN a range of approximately

4 to 5.5% is displayed. Significant changes are seen between first and second pregnancy with and without stress challenge. No significant changes are demonstrated between control and stressed groups. (Fig. 16A)

The population of FoxP3$^+$ Treg cells in the uterus shows a range from 1 to 1.5% without significant differences. However, a tendency similar to the changes in the LN is seen, as an increase in second pregnancy compared to the first one is displayed. Again, changes concerning the stress provocation are not detectable. (Fig. 16B)

The anti-inflammatory capacity of FoxP3$^+$ Treg cells is monitored via the IL-10 cytokine expression. In the LN, levels of 2.5 to 3.5% and no significances between all populations are measurable. Further, firstly pregnant, unchallenged dams display lower frequencies. (Fig. 16C) In the uterus, levels shortly below 2% are measured. Equal frequencies within the control group and the stressed group are displayed. A slight decrease of the IL-10 expression in stressed compared to control populations is detectable. (Fig. 16D)

Percentages of ExTh17 cells traced within the FoxP3$^+$ Tregs show levels of approximately 0 to 1.5%. Visibly, a change occurs in secondly pregnant mice. While almost no ExTh17 cells are measured in firstly pregnant mice, significant increases are seen in second pregnancies, regardless of the stress challenge. A tendency of slightly higher increases in stressed mice compared to unchallenged secondly pregnant mice is observable. (Fig. 16E) Locally, in the uterus, frequencies show a range of 4 to 5%. While secondly pregnant mice in the control group display higher levels than firstly pregnant dams, the stressed dams do not show this tendency. Differences between firstly pregnant controls and firstly pregnant stressed mice are measured. In secondary pregnancy, this tendency is not observable. (Fig. 16F)

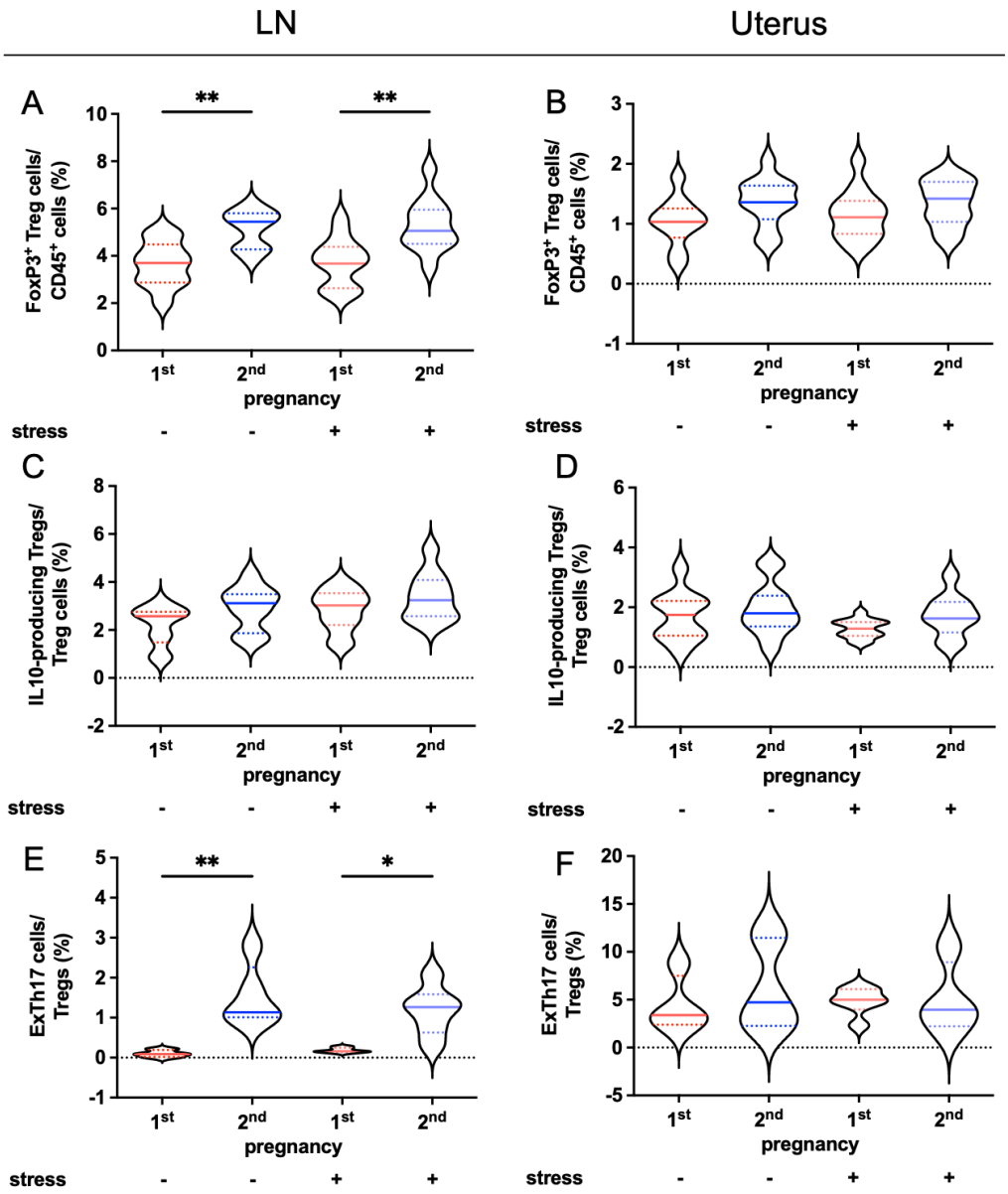

Fig. 16 Increased Treg cell frequencies and ExTh17 cell phenotype in subsequent pregnancies in prenatal stress challenge: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Lymph node (LN) and uterus were harvested on gd 15.5. Flow cytometry analysis of FoxP3$^+$ Treg cells within CD45$^+$ cells in LN (A) and uterus (B) IL-10$^+$ cells within FoxP3$^+$ Treg cells in LN (C) and uterus (D), and ExTh17 cells in FoxP3$^+$ Treg cells in LN and uterus (F) are displayed for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (* $p<0.05$, ** $p<0.001$).

The second regulatory subset, the Tr1 cell phenotype, is evaluated within the $CD45^+$ population. In the LN, frequencies between 0.04 and 0.06% are measured. Significant differences are measurable between first and second prenatally stressed pregnancies. This tendency is also detectable in the unchallenged correlate, however without exceeding significance. In unchallenged and stressed first pregnancies, Tr1 cells show lower levels than second pregnancies. The stressed group shows a trend of exhibiting higher numbers than unchallenged equivalents. (Fig. 17A) For the uterus, a range of 0.3 to 1% and no significant differences are measurable. Here, the first three groups of first and second pregnancy in the control groups and stressed first pregnancy share similar percentages. Only the group of stressed secondly pregnant mice shows a tendency of higher numbers. (Fig. 17B)

The ExTh17 cells within the Tr1 subset show a similar trend in the LN like the Tr1 cells. Levels of 5 to 25% are measured. In second pregnancies, a higher percentage of Tr1 cells formerly expressed IL-17 than in primigravidae, uninfluenced by prenatal stress. This trend is significant for the stressed groups and insignificant for the control group. Further, a trend of higher frequencies of ExTh17 cells in stressed second pregnancies compared to the unchallenged correlate is observable. Primigravidae show similar levels, unchanged by stress exposure. (Fig. 17C) The uterus shows a range from 2 to 20% without significant changes over all populations. However, the tendency of higher percentages in the second pregnancy groups again signs off, especially in the prenatally stressed groups. (Fig. 17D)

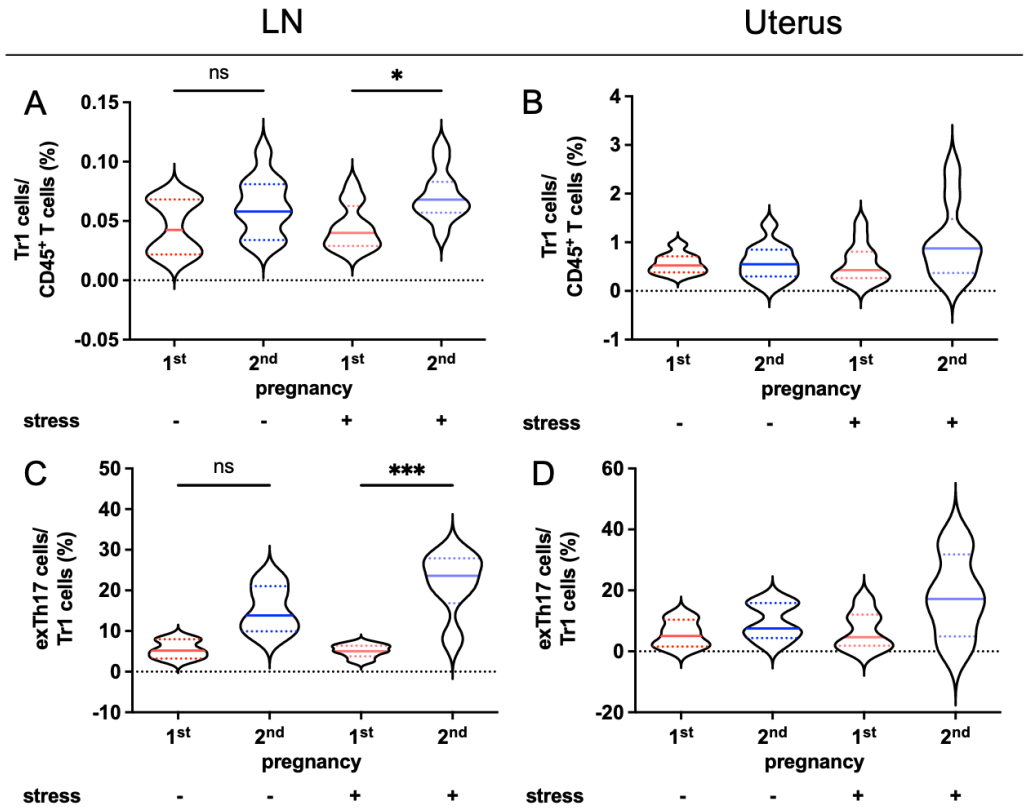

Fig. 17 Increased Tr1 cell frequencies in subsequent pregnancies in prenatal stress challenge: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Lymph node (LN) and uterus were harvested on gd 15.5. Flow cytometry analysis of Tr1 cells within CD45$^+$ cells in LN (A) and uterus (B) ExTh17 cells within Tr1 cells in LN (C) and uterus (D) and Tr1 cells within ExTh17 cells in LN (E) and uterus (F) are displayed for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (* $p<0.05$, *** $p<0.001$).

ExTh17 cells are also analyzed within the CD4$^+$ population. In the LN, frequencies of approximately 0.1 to 1.2% are measured. Significantly higher numbers are detectable in second pregnancies compared to primigravidae in unchallenged and stressed condition. Very low numbers of ExTh17 cells are measured in both groups of firstly pregnant mice. Differences between control and stressed groups are not detectable. (Fig. 18A) Similar tendencies acquire for the uterus. Here, a range of approximately 6 to 11% is detected. Second pregnancies show higher percentages, however no

significance is reached locally. Further, no differences are observable between prenatally stressed and unchallenged conditions. (Fig. 18B)

ExTh17 cells are further analyzed for FoxP3 expression. In the LN, levels of 10 to 20% are measured and differences are insignificant. The lowest level is detected in firstly pregnant control mice, while the highest value is seen in secondly pregnant control dams. The prenatally stressed groups show similar frequencies. (Fig. 18C) Similarly, the uterus shows no significances and ranges from approximately 4 to 12%. The lowest FoxP3 expression is measurable in first pregnancy of the control group and the highest expression in second pregnancy of the control group, as well as in firstly pregnant stressed dams. (Fig. 18D)

Tr1 cells within the ExTh17 subset in the LN range from approximately 2 to 8% and do not reach any significances. A trend of higher Tr1 cells within the ExTh17 cells can be seen in first compared to second pregnancies in both, unchallenged and prenatally stressed mice. A comparison of the control with the stressed group shows a tendency of higher Tr1 cell frequency in stressed second pregnancy than in the unchallenged counterpart. (Fig. 18E) In the uterine tissue, the data shows no significant changes either. A range of approximately 5 to 9% is apparent. First-time pregnant mice show a tendency for an increase towards second pregnancy in the control group, while a decreased Tr1 cell percentage within ExTh17 cells is seen in primigravidae in stress. Tr1 cells stay at a constant level in stressed dams independent of the stress condition. (Fig. 18F)

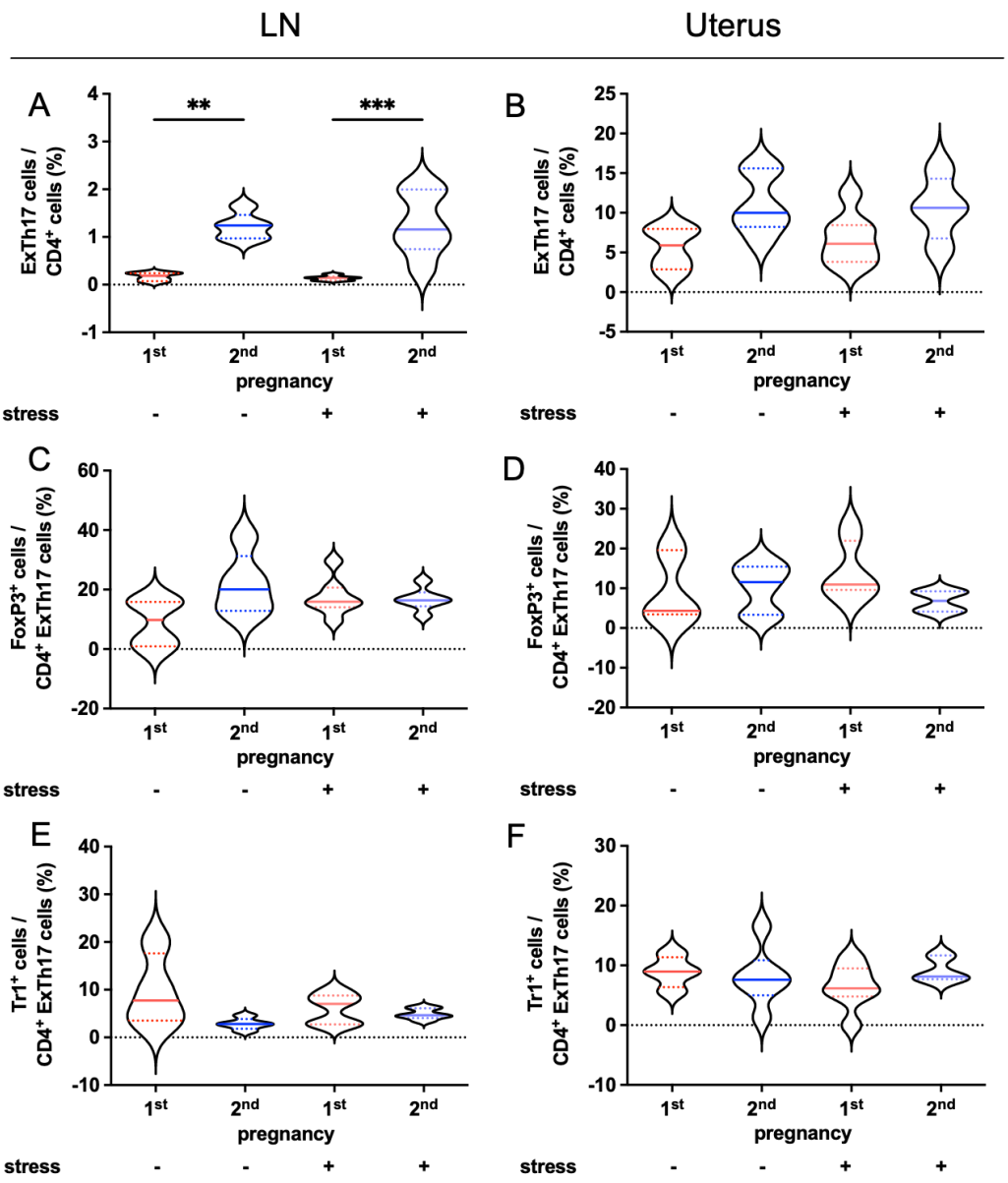

Fig. 18 Increased ExTh17 cell frequencies in subsequent pregnancies in prenatal stress challenge: Fate$^+$ mice were mated once or twice to Balb/c, respectively, and stress was applicated on gestational day (gd) 10.5, 12.5 and 14.5. Lymph node (LN) and uterus were harvested on gd 15.5. Flow cytometry analysis of ExTh17 cells within CD4$^+$ cells in LN (A) and uterus (B) FoxP3 expression within ExTh17 cells in LN (C) and uterus (D) and Tr1 cells within ExTh17 cells in LN (E) and uterus (F) are displayed for control (left) and prenatally stressed (right) female mice and a comparison between first (red) and second (blue) pregnancy. Data are presented as mean values ±SEM and the statistical significance between groups was assessed using One-way-ANOVA (** $p<0.001$, *** $p<0.001$).

In the stress experiments, additionally to the uterine and the LN tissue, PBMCs are analyzed with flow cytometry. The graphs for this data are not displayed, as no significant differences are found between all groups. (Tab. 4.2.1)

Tab. 3 T cell frequencies assessed in PBMCs on gestational day 15.5 in first and second pregnancy in control and prenatal stress challenged dams

Population	1st	2nd	1st + stress	2nd + stress
$CD3^+$ in $CD45^+$	36,68 ± 2,014	35,39 ±1,889	37,24 ± 2,169	35,02 ± 1,027
$CD4^+$ in $CD45^+$	19,18 ± 1,385	18,99 ± 1,344	18,96 ± 1,043	19,26 ± 0,7926
$CD4^+$in $CD3^+$	52,07 ± 1,620	53,38 ± 1,317	51,02 ± 1,031	55,12 ± 2,300
$FoxP3^+$Tregs in $CD45^+$	1,578 ± 0,1555	1,379 ± 0,1107	1,287 ± 0,1004	1,272 ± 0,1174
$FoxP3^+$Tregs in $CD4^+$	8,143 ± 0,2610	7,310 ± 0,4720	6,853 ± 0,6214	6,568 ± 0,4396
ExTh17 in $FoxP3^+$ Tregs	0,4740 ± 0,07859	0,7138 ± 0,08838	0,7767 ± 0,07274	0,6600 ± 0,09320
$IL\text{-}10^+$ $FoxP3^+$ Tregs in $CD4^+$	0,1880 ± 0,005831	0,1525 ± 0,01346	0,1350 ± 0,02141	0,1633 ± 0,01498
$IL\text{-}10^+$ $FoxP3^+$ Tregs in $FoxP3^+$ Tregs	2,177 ± 0,1738	2,144 ± 0,2115	1,932 ± 0,1567	2,507 ± 0,2956
ExTh17 in $CD4^+$	0,2767 ± 0,02525	0,3350 ± 0,05418	0,2883 ± 0,02960	0,2167 ± 0,01667
$FoxP3^+$ Tregs in ExTh17	6,117 ± 0,5822	9,063 ± 1,289	9,103 ± 1,265	8,276 ± 1,008
Tr1 in ExTh17	1,548 ± 0,5897	1,974 ± 0,6037	2,183 ± 0,4788	2,172 ± 0,4304
Tr1 in $CD45^+$	0,08167 ± 0,01046	0,07500 ± 0,01069	0,07500 ± 0,01648	0,1117 ± 0,02880
Tr1 in $CD4^+$	0,4267 ± 0,04310	0,3925 ± 0,04204	0,4017 ± 0,09181	0,4380 ± 0,06931
ExTh17 in Tr1	4,300 ± 0,5628	3,790 ± 0,5510	3,140 ± 0,7347	2,847 ± 0,5387

5. Discussion

The aim of this thesis is the comparison of immunological adaptation in first and second pregnancies, focusing on Th17 cells and regulatory T cell subsets. A specific interest lies in the plasticity of Th17 cells and the consequences for reproductive success. Further, the effect of stress is evaluated. The first key finding is that Th17 cells exist in low frequencies in pregnancy. Additionally, formerly IL-17 producing ExTh17 cells are exclusively present in secondary pregnancy. These cells partly transform into Treg and Tr1 cells. The plasticity into other subsets remains to be studied. Lastly, in secondary pregnancy the fetal outcome was not impaired as observed in first pregnancy with prenatal sound stress. Could plasticity be a simultaneous mechanism contributing to advantages of second pregnancy besides the memory effect?

Measurements of the Th17 cell population enabled an establishment of a kinetic scheme over the timeline of pregnancy. A special interest lies in the inflammatory timepoints, as Th17 cells are regarded as inflammatory cells. Especially on gd 7.5, an increase is observed in the LN. This increase in Th17 cells might be explainable as an over-hanging effect of the implantation, occurring around gd 5.5. This thought is supported by the theory of positive effects of Th17 cells on neovascularization and interactions with EVTs. (Nakashima et al., 2010b, Wu et al., 2014) Minuscular numbers of Th17 cells are measured in the LN in late pregnancy (gd 15.5), suggesting that a recruitment of Th17 cells to the LN is more relevant in earlier pregnancy. Herewith, comparing the inflammatory timepoints, an increase of Th17 cells in the LN can be seen at implantation, but is not measurable close to labor. This physiological decrease towards the end of pregnancy might suggest less importance and availability of Th17 cells in the LN and further a recruitment to local sites. However, labor takes place on gd 18.5 or 19.5, the measurement on gd 15.5 might be too early to already detect inflammatory signals of the inflammatory phase. Absence of Th17 cells might be a representation of the tolerogenic setting. All in all, these findings underline the importance of a kinetic timeline for interpretation of pathologic results, while an increase of Th17 cells on gd 15.5 might induce complications, Th17 cells are physiologically present in early pregnancy LNs. It would be interesting to look at additional gds before and after gd 15.5, to see at which timepoint the frequency of Th17 cells decreases and if further increases occur after this gd. Remaining questions are

reasons for a decrease towards the end of pregnancy and if this decrease in the LN stays steady until labor or if the Th17 cell population increases in the last days of gestation.

In the uterus, the signal of IL-17-staining shows low levels in early pregnancy. A small increase is measured on gd 5.5, which is followed by a drop on gd 7.5. These measurements support existing data reporting very low numbers of Th17 cells in the decidua of first trimester. (Mjosberg et al., 2010) The peak is reached on gd 15.5 locally. The data suggests a recruitment of Th17 cells to the uterus, hinting towards the establishment of inflammation towards parturition. Another mouse experiment from 2003 also showed IL-17 expression at the feto-maternal interface on multiple days. The measured levels between days 6.5 to 10.5 of gestation were low, but detectable in glands and basal proliferative stroma. By gd 12.5, no signal of anti-IL-17 staining was left in the decidua. (Ostojic et al., 2003) However, the presented study does not look further than gd 12.5 and herewith the inflammatory phase of birth is not displayed. Additionally, the mouse model used in this thesis has genetical mechanisms to mark IL-17, eradicating problems with staining.

Despite its pro-inflammatory activity, positive reports for the role of Th17 cells in pregnancy are available. IL-17 still has pleiotropic effects like neovascularization or pro-angiogenic capacities. Via IL-17, Th17 cells were attributed to inhibit apoptosis and promote proliferation, growth and invasion of trophoblasts in the first trimester and contribute to augmentation of EVT cells. (Nakashima et al., 2010b, Wu et al., 2014) Similarly, these neovascularization features can be seen in tumorous masses. (Asadzadeh et al., 2017) These reports also support the presence of Th17 cells in the implantation phase in this thesis. Furthermore, it was propounded that decidual Th17 cells have no pathogenic role in pregnancy. Human decidual Th17 cells produced more IL-4, IL-17A, IL-17F and IL-22, than the ones found in PBMCs. A benefit for pregnancy maintenance and for implantation was suggested, proposing a switch into the Th17/Th2 kind. (Lombardelli et al., 2016) Other human studies found higher numbers of IL-17 in healthy pregnancy PBMCs than in spontaneous abortion cases. (Kaminski et al., 2018) These studies agree with the results presented in this thesis, as Th17 cells are measurable in the LN and the uterus. Further, it can be proposed that Th17 cells are physiologically present and important for pregnancy. More data on Th17 cell levels

in healthy human pregnancy can be found within studies researching pathologies, as a measurement of regular pregnancy is displayed in the control group. For example, in a single-time observation of unexplained RSA, Th17 cells were present in the control group. (Liu et al., 2011) One limitation presented by comparison of human and murine data is the inclusion of pregnant women regardless of the number of previous pregnancies. Furthermore, uterine tissue of healthy women is difficult to obtain. Measurements in the decidua are possible but studies on material from fetal demise are rare. Nevertheless, important information can be learned and a careful comparison to animal models can be made. A kinetic study on Th17 cells in PBMCs of healthy pregnant and non-pregnant women showed a significant increase in first trimester and a decrease back to frequencies similar to non-pregnant women in third trimester. (Wu et al., 2014) Therefore, in human peripherally low frequencies were measured in advanced pregnancy, concordant with the data of this thesis on gd 15.5 in the LN. However, other studies showed that in normal pregnant women peripheral lymphocytes show no significant change in IL-17 production in the first, second and third trimester, also when compared to the proportions in healthy non-pregnant women. (Nakashima et al., 2010a) A possible explanation for these differences is the tissue of investigation. Results from PBMCs might differ from measurements of the decidua. Various studies showed that Th17 cells represented a higher proportion of $CD4^+$ cells in the decidua than in PBMCs. (Nakashima et al., 2010a, Wu et al., 2014) Higher proportions of Th17 cells in the decidua than in PBMCs might sound disadvantageous, however, considering the unsterile milieu, immune defense stays inevitable. (Saito et al., 2010) My data contributes to these observations and theories, as locally higher percentages of Th17 cells were measured.

Nevertheless, pregnancy research has focused on the pathologic role of Th17 cells, as multiple studies measured increased Th17 cell proportions in peripheral blood in women suffering from Recurrent pregnancy loss (RPL) and unexplained RSA. (Wang et al., 2010, Liu et al., 2011) Th17 cells might have a positive and a negative role in pregnancy, especially overshooting numbers can be harmful. Furthermore, the progress of pregnancy and the time point of immune cell measurements might be important for the interpretation. Another study found that inevitable abortion cases showed an IL-17 accumulation in decidual tissues in the late, but not in an early stage of pregnancy. (Nakashima et al., 2010b) In conclusion, Th17 cells are part of

physiological pregnancy and cause of various complications. The physiologic availability of Th17 cells in pregnancy is displayed in this thesis and an assumption of a way less pathogenic role of Th17 cells, than the one prevailing, could be assumed. This cell lineage is measurable in murine pregnancy in LN and uterus. Certainly, the description of pregnancy related problems caused by Th17 cells cannot be denied. Therefore, the balance of Th17 and Treg cells is of highest actuality and a disequilibrium might cause problems.

The balance of Tregs and Th17 cells joined the Th1/Th2 balance and contributes to the equilibrium of pregnancy. Therefore, results of this regulatory subset in pregnancy are additionally highlighted. The link of Th17 cells and Tregs was shown in a study on unexplained RSA. Besides an increase of IL-17 and Th17 in peripheral blood and decidua, lower numbers of Treg cells were seen. (Liu et al., 2011) It has been postulated, that an imbalance between the ratio of Th17 cells and Treg cells leads to RPL. On the one hand, the ratio between IL-17$^+$ T cells and FoxP3$^+$ Treg cells and the level of IL-17$^+$ T cells was increased in this pathology. On the other hand, FoxP3$^+$ Treg cells showed a decreased level. (S. K. Lee et al., 2011) These findings support the theory of a disruption of the Th17/Treg balance, which was also described for other inflammatory contexts. (Nistala et al., 2008)

Physiologically, Tregs' kinetics show a steady increase in the LN until gd 7.5 and only slightly decrease on gd 15.5. The increase towards gd 7.5 in the LN goes hand in hand with previous studies. In human PBMCs, a steady increase was shown, which results in a peak in second trimester. (Somerset et al., 2004) EVT induction and reaction to fetal alloantigen can be related to this early increase. (Zhao et al., 2007, Svensson-Arvelund et al., 2015) It has been postulated that Tregs are most important at the switch from an inflammatory to a tolerogenic setting, showing the biggest increase in first trimester in human and mice, reviewed in (Robertson et al., 2018) Other studies on mice also suggest importance of CD25$^+$ Treg cells in early pregnancy, while no effect of their depletion of CD25$^+$ Tregs was seen in late pregnancy. (Shima et al., 2010) Earlier murine kinetic studies regarding Treg cells have shown that Tregs expand in pregnancy, especially when compared to abortion prone counterparts. This augmentation was already seen before implantation. (Thuere et al., 2007) Tregs are already present in the uterus on gd 1.5, showing higher levels before implantation than

after. It has been postulated that this increase can be seen shortly after the semen enters. (Thuere et al., 2007) Leading to the conclusion that seminal fluid triggers the expansion of Tregs showing an early tolerance establishment. (Robertson et al., 2009) Next to seminal fluid, alloantigen led to expansion of Tregs in early murine pregnancy. (Zhao et al., 2007) These findings support the hypothesis of a Th17/Treg balance, as higher numbers of Th17 cells are measured in the LN of early murine pregnancy. Both cell lineages rise simultaneously, and a balance is established.

Further, Tregs are measurable in high frequencies in both tissues on gd 15.5, suggesting an importance for late pregnancy. Analogically to the data of this thesis, murine kinetics of Tregs, in uterus draining LN showed a continuous increase on a timeline from gd 5.5 to 16.5, only showing a small decrease on gd 16.5. (Chen et al., 2013) In contrast, other murine experiments showed that after the expansion of Tregs in early pregnancy, a decrease was measured from mid gestation onward, which resulted in non-pregnant level at term. (Zhao et al., 2007) Human blood investigations also showed highest Treg frequencies in the first trimester, numbers decreased in third trimester, but stayed measurable. (Heikkinen et al., 2004, Seol et al., 2008) At term these human studies showed high Treg proportions locally, as 14% of the decidual $CD4^+$ cells were Tregs (Heikkinen et al., 2004) Published murine data also highlighted the importance of Tregs for late gestation. The presence of Tregs on gd 14.5 was shown, while in abortion models these cells were diminished. A Treg transfer from a normally pregnant mouse was able to prevent abortion. (Zenclussen et al., 2005) As the data of local sites in this thesis shows high frequencies in both, Tregs and Th17 cells, in late gestation, overshooting Th17 cells might be responsible for the abortions occurring in the above-mentioned model. The combination of low Treg frequencies and normal or high Th17 cell numbers might lead to a disruption of balance. Again, the importance of the balance of Treg and Th17 cells could explain physiological equilibrium. Another possible explanation for the variation in Treg measurements in late gestation, might be the act of labor, as non-labor women at term have higher Treg numbers. These changes at the onset of labor were speculated to occur via hormonal impact. (Schober et al., 2012) Furthermore, the comparison of human and murine studies could be causative for different results. The time range of trimesters is wide and sometimes the numbers of cases are small, especially in human studies.

Another key finding of this thesis is the depiction of differences between first and second pregnancies. These differences are detectable on various gds and propose that changes occurring during first pregnancy have lasting effects for subsequent ones. The immune system seems to remember the influence of previous pregnancies and adapts quicker in subsequent ones. The remembrance of immune changes and adoption a memory phenotype has been described earlier. (Stemberger et al., 2007, Harrington et al., 2008) This effect has been proposed also for pregnancy and was termed the memory effect. (Rowe et al., 2012) In further immunological studies, additionally an increase of effector $CD4^+$ cells in pregnancy was described. These cells remained increased more than a year after parturition. Hereby, short term effects, but also persistency was demonstrated. (Kieffer et al., 2017) The memory effect has been suggested to be responsible for lower complication rates and increased birth weight in second pregnancies, despite an advanced age of the mother. (Kieffer et al., 2017, Thiele et al., 2019, Thiele et al., 2025) The control groups of the stress experiment of this thesis display a normal pregnancy outcome in first and second pregnancy. While the birth weight in second pregnancy was insignificantly elevated compared to first pregnancy, the abortion rate showed a significantly lower frequency in second pregnancy, supporting the effect of an improved birth outcome in subsequent pregnancies.

Immune cell differences are observable for Th17 cells in second pregnancy in both organs. Significant differences in the LN can be seen on gd 1.5, 5.5 and 7.5. In the uterus, the signal of IL-17-staining displays slightly elevated numbers in second pregnancy. Also, for this subset of $CD4^+$ effector cells the memory effect has been described in other contexts. Studies on human blood have found Th17 cells acting as memory cells in candida infection and identified the C-C chemokine receptor type (CCR) 6 and CCR4 as true memory markers on Th17 cells. Further, it was proposed that they retain the cytokine production. (Acosta-Rodriguez et al., 2007) Additionally, a local accumulation was described, as higher proportions of $CD4^+$ and $CD8^+$ memory cells were measured in the decidua than in blood of healthy pregnancy. (Powell et al., 2017) A difficulty of analyzation of available literature on previous pregnancy is, that most of the human data does not consider a women's parity, as for example in this study nulliparous women are compared to pregnant women. (Somerset et al., 2004)

One might suspect that an immune reaction that is faster and stronger, as described for memory cells, might be harmful in subsequent pregnancy. This question has been approached for other cell lines of the adaptive immune system, for example the $CD8^+$ cells. Murine studies on their persistence after parturition and the immune memory in subsequent pregnancy have shown that a memory pool is created after primary pregnancy. However, in this mouse model the effect and mechanisms of the response of $CD8^+$ T cells in subsequent pregnancy were shown to be blunted. In secondary pregnancy, a failure of re-expansion and an upregulation of PD-1 and LAG-3 was recorded, which create a hyporesponsive “exhaustion prone” $CD8^+$ cell pool. (Kinder et al., 2020) Another murine study on $CD8^+$ memory cells showed that re-encounter with antigens with skin transplants were rejected quicker after first pregnancy, while second pregnancies were lacking this effect. Tolerance was induced by dysfunction and failing at exhibiting effector functions. (Barton et al., 2017) If these inactivation processes also apply for $CD4^+$ cells, remains to be explored. Further the possibility of a pregnancy supportive role of Th17 cells, which has been elucidated earlier, might represent advantages of higher frequencies in early subsequent pregnancy.

Also, for the innate immune system “trained immunity” as a model similar to plasticity has been proposed for uNK cells of subsequent pregnancy. (Gamliel et al., 2018) Secondly pregnant women displayed a different type of NK cells than primigravidae, again leading to the postulation that remembrance of pregnancy makes subsequent ones more robust. It was proposed to reside in the decidua as an epigenomic memory. (Goldman-Wohl et al., 2019) Epigenetics describe changes in the genome as for example DNA methylation and can be induced by environmental stimuli like stress or nutrition as summarized by (Ragini et al., 2023). Via epigenetic changes, trained immunity might present an advantage for subsequent pregnancy due to improvement of the placentation process, as suggested in (Gamliel et al., 2018). Therefore, an investigation of epigenetic changes in subsequent pregnancy could be of interest in the future. Also, the increase of Tregs or the plasticity of Th17 cells in secondary pregnancy might be linked to adaptations of the genome in first pregnancy. Supporting this hypothesis, it has been suggested that due to the methylation status of T and NK cells in pregnancy, long-term modifications can be induced and were proposed to be an evolutionary advantage of subsequent pregnancy. (Huang et al., 2021) However, epigenetic memory could also present a disadvantage in cases of pregnancy

complications, as modifications differed in preeclampsia and normal pregnancy. (Huang et al., 2021) Also the risk for pregnancy complications has been postulated to be transmitted cross-generationally and has been linked to the predisposition to disease for the offspring as summarized by (Katzmarski et al., 2021, Lodge-Tulloch et al., 2022).

An accumulation of effector memory cells in blood and decidua after pregnancy might not be disastrous for subsequent pregnancy if a regulatory memory hampered the impact of effector memory. Proposing the same mechanism of an immune balance accounts for both the untrained immune system and for memory cells in pregnancy. This balance might be equally important for a tolerance establishment in subsequent pregnancy. A murine model for secondary pregnancy found specific Tregs that accumulate in pregnancy, remain postpartum and rapidly expand in secondary pregnancy, herewith building a protective suppressive memory repertoire. Accelerated kinetics of Tregs were measured in subsequent pregnancy. (Rowe et al., 2012) This paper proposed that the generated Treg cells are fetal-specific, representing experienced Treg cells. (Rowe et al., 2012) The results for the first regulatory subset of Tregs in this thesis confirm these measurements, as significant increases of Tregs are detectable in second pregnancy on gd 5.5, gd 7.5 and gd 15.5 in the LN and on gd 7.5 in the uterus. These measurements could further validate earlier findings of increases in second pregnancy from other members of my lab. Similarly, especially in the LN, significant increases in second pregnancies were found on various gds. (Thiele et al., 2025) This suppressive T cell memory effect was firstly presented in a murine model. Re-exposure with antigen led to an enhanced response and a faster activation. Due to its specificity, a clonal selection concept was postulated. (Loblay et al., 1978) Another murine model, evaluated the effect of infection on first and second pregnancy. It was shown that a previous uncomplicated pregnancy protects from infection-triggered abortion. Further Treg depletion led to less fetal wastage in subsequent pregnancy than in first ones. (Gregory et al., 2021) It was further shown, that Tregs form a memory pool after acute viral infection. These cells can expand rapidly in re-encounter and further show a large amount of IL-10 secretion and suppressive capacities. (Sanchez et al., 2012) Also in this thesis, the IL-10 expression of Tregs was increased in the LN of secondary pregnancies, reaching significance on gd 5.5. It can be proposed that a higher production is mounted to oppose inflammation caused by

implantation. This opposition could be more efficient in secondly pregnant mice, facilitating the introduction of the growth and tolerance phase of pregnancy. The importance of this cytokine for second pregnancy should be evaluated in future studies. (Thiele et al., 2025)

Antigen-specific expansion of Tregs represents these regulator memory cells, which similarly to the effector memory cells, escape apoptosis and persist after resolution of the immunological event. However, the identification of memory Tregs (mTregs) remains a challenge, as the commonly used definition of memory cells presents some differences of the regulatory counterpart. (Rosenblum et al., 2016, Thiele et al., 2025) Firstly, the memory concept of prior activation, persistence and enhanced activity in re-exposure is not completely fulfilled by mTregs, as a steady persistence has not been demonstrated yet. Secondly, effector memory cells are defined by phenotypic markers like CD44, which Tregs commonly express also in non-memory state. (Rosenblum et al., 2016) This issue was addressed recently and potential markers for the detection of mTregs were identified. Bioinformatic analysis of Tregs from parous mice were distinguished from naïve Tregs. Especially programmed death-ligand 1 (PD-L1), C-X-C chemokine receptor 4 (CXCR-4), CD20, CD80 and CD81 are potential candidates. (Thiele et al., 2025) Further studies are needed to test these markers as a mTreg detection method.

In this thesis, an enhancement of the Tr1 cell population was further demonstrated in secondary pregnancy, showing significantly higher frequencies on gd 5.5 and gd 7.5. A memory effect for this T cell subset has been described before. (Bollyky et al., 2011) Also, the establishment of a Tr1 cell memory pool from human naive precursor cells was demonstrated in cell culture. (Charles et al., 2019) These memory cells could be responsible for the increase in second pregnancies. However, other studies propounded that Tr1 cells do not exhibit a memory function, as in re-challenge of allergic airway inflammation, re-establishment of tolerance failed. (Yadava et al., 2019) In general, only a small amount of data is available for the regulatory memory, creating a research task for the future. Another possibility for increased numbers of regulatory cells, apart from a memory establishment, is the conversion from the effector memory cells. This thesis proposes a second mechanism acting simultaneously to the memory effect and contributing to the outcome advantages of secondary pregnancy as

suggested in (Thiele et al., 2025). This phenomenon is described in the following parts of this thesis and has been named plasticity.

Plasticity is a recently arising immunological concept and has been shown in different contexts like the gut (Hovhannisyan et al., 2011, Gagliani et al., 2015, Harbour et al., 2015) and in autoimmune diseases like arthritis. (Korn et al., 2009, Komatsu et al., 2014) Plasticity was also made visible in vivo by generating a fate mapping mouse model. (Lee et al., 2009, Hirota et al., 2011) which has also been used in this thesis. In the experiments in an EAE mouse model, Th17 cells showed the ability to express cytokines typical for other T cell phenotypes, dependent on the inflammatory state, its pathogenesis and the feature of chronicity. (Hirota et al., 2011) Different research fields can be used as a comparison for pregnancy. Especially cancer research is commonly used, as the tumor also creates an environment which is rich in vascularization and tolerates the proliferating foreign tissue, as summarized by (Hur et al., 2023) An ambiguous role of neovascularization as a pro-tumorous and the inflammation as an anti-tumorous process were described. (Asadzadeh et al., 2017) Similar to pregnancy, the role of Th17 cells in cancer has not been elucidated to full extend and accumulation can mean good and bad prognosis. Plasticity was seen in tumorous processes as well, showing a conversion of Th17 cells towards Tregs and Th1 cells in human cancer cells. It was postulated that Th17 cells exhibit a dual function of effector and regulatory cell. (Ye et al., 2011) This proposition is especially interesting for the data of this thesis, as Th17 cells were traced in the direction of regulatory subsets.

Plasticity has many facets and directions and has been described for multiple cell subsets, e.g., Th1, Th2, Th17, Treg and Tr1 cells. Transdifferentiation from one subset into another and vice versa, was linked to instability of expressed genes as reviewed in (Liu et al., 2017). For example, Tregs show the ability to convert into Th1 and Th17 cells. (Liu et al., 2017) The other way around, Th17 cells change their fate into subsets such as Th1, Th2, as well as Tr1 and Treg cells. All controlled by hormones, cytokines and inflammatory condition. (Liu et al., 2017) The milieu defines the direction of plasticity, making it interesting for pregnancy as changes are frequently present. Especially Th17 cells were found to exhibit plastic features towards other cell subsets. It was shown that the fate of ExTh17 cells is determined by the inflammatory setting and condition, deciding the direction of plasticity. A chronic inflammatory milieu for

example led to a conversion into IFN-γ producing cells, while in acute settings IL-17A production was shut down, demonstrating plasticity of Th17 cells towards Th1 cells. (Hirota et al., 2011) This plasticity towards Th1 cells was further shown in a murine model. The resulting cells were described as IL-17/IFN-γ double-producing cells. (Lee et al., 2009) In self-limiting inflammation, IL-10 production of ExTh17 cells is increased, showing a Tr1 phenotype due to reprogramming of the transcriptional profile. (Gagliani et al., 2015) In earlier studies, it has already been shown that Th17 cells exhibit plastic features in pregnancy. In human decidua, differentiation into Th17/Th2 cells has been suggested as one mechanism for conversion into harmless Th17 cells. Th17 cells that do not exhibit detrimental but rather beneficial effects on pregnancy. (Lombardelli et al., 2016) Arising questions for feto-maternal immunology concern the presence and the extend of plasticity in pregnancy and the importance for maintenance of pregnancy. Further fate exploration of Th17 cells into cells with a tolerogenic phenotype and a possible guidance of pregnancy-specific changes by plasticity are of interest. In this thesis, the presence of plasticity of Th17 cells is shown in pregnancy. Th17 cells are examined for the conversion into regulatory cell subsets. Low levels of plasticity of Th17 cells towards Treg cells are detectable in the LN throughout the timeline of murine pregnancy. In the uterus, a higher frequency of Th17 cell origin was found in Tregs, hinting towards a higher plastic feature of Th17 cells locally. A fast conversion of Th17 cells into their regulatory counterpart might enable a quick resolution of an inflammatory into a tolerogenic milieu. On gd 5.5 and 7.5 5 to 11% of the local Tregs formerly produced IL-17. These high percentages might partly represent the pregnancy specific Treg increase. Herewith plasticity might be a mechanism which supports pregnancy establishment and enables the required quick change of milieu.

Commonalities between Th17 and Treg cells are the above-mentioned functional antagonism, as the Th17/Treg balance and their similar inheritance pathway from the same progenitor of naïve $CD4^+$ cells. Further, both are induced to differentiate by transforming growth factor beta (TGF-β). TGF-β is an inductor of FoxP3 expression and herewith essential for Treg differentiation. Also, Th17 polarization is mediated via TGF-β signaling, merely Th17 cells additionally need IL-6. (Bettelli et al., 2006) This close relationship and balancing system of these two cell lines is pronounced if a conversion in one and another is possible, resulting in more flexibility and adjustment options. This knowledge highlights the importance of the Th17/Treg balance and offers

potential for research and possible treatment opportunities. Reports of an anti-inflammatory action of plasticity are available. For example, plasticity of Th17 cells was shown to ensure homeostasis in the bowel by producing IL-10 in a murine model. (Xu et al., 2020) Other studies stated that Th17 cells express an IL-10 receptor and herewith are controlled by Treg and Tr1 cells. Experiments of IL-10 blockage led to a Th17 increase and treatment with IL-10 to a decrease of Th17 cells. (Huber et al., 2011). The interplay of Th17 cells and Tregs seems to include a conversion into one and another, supporting the hypothesis that plasticity could be a possible mechanism impacting pregnancy. Especially in secondary pregnancy significant increases were seen concerning the ExTh17 frequency within Treg cells. The plasticity might offer advantages of the quick resolution of inflammation and the faster recruitment of Treg cells. The mechanism of plasticity could be responsible for advantages in secondary pregnancy and might be involved in the improvement of the fetal outcome. (Thiele et al., 2025)

The susceptibility of Th17 cells towards plasticity could have different explanations. One proposition is based on the instability of its transcription factor. While Th1 and Th2 cells are completely regulated by T-box expressed in T cells (T-Bet) and GATA3, making them a master transcription factor, which is essential and sufficient, RAR-related orphan receptor (ROR)γ is not sufficient for complete Th17 differentiation. (Ciofani et al., 2012) Multiple regulators like Signal transducer and activator of transcription 3 (STAT3) for Th17 transcription were identified, describing a connected and complicated network without one master regulator. (Ciofani et al., 2012) Further, the expression of RORγ has no stabilization via positive feedback loops and additionally susceptibility to environmental influences enables plasticity. (Ciofani et al., 2012) However, Th17 cells are not a randomly converting cell subset. Fos like 2 (Fosl2), which is known as a determinant of cellular plasticity, was found as one regulator, stabilizing Th17 cells from overshooting plasticity. (Ciofani et al., 2012) Unpublished data is supposed to show that even after sufficient priming of Th17 cells, adoption of another fate was easily induced by cytokine exposure, while maintenance of the IL-17 production was seen. (Zhu and Paul, 2010) Th17 cells are proposed to be plastic at every stage of their differentiation. An explanation for the pronounced plastic susceptibility of Th17 cells compared to Th1 and Th2 cells, might be a missing terminal differentiation. (Zhu and Paul, 2010)

However, Th17 cells are not the only subset exhibiting plastic features, as they were also described for Tregs from human in cell culture. They can similarly stop their expression of FoxP3 and convert into other cell lines. An IL-17 production and a loss of their suppressive function was further described. (Beriou et al., 2009) Ex vivo experiments on human cells, showed that mTregs secreted IL-17 and further expressed RORγt. (Ayyoub et al., 2009) This direction could be researched in pregnancy in future studies. The hypothesis of a closer relationship than the Th17/Treg balance can be proposed. Th17 cells and Tregs might be able to convert into their counterpart. An open pathway into both directions could be an important mechanism regulating inflammation and tolerance. In this thesis the origin of Tregs was traced and the plasticity of Th17 cells was examined. Significant differences in ExTh17 cells within the Treg cell population between first and second pregnancy are measured. Small frequencies of Tregs emerge from Th17 cells in the LN of first pregnancy throughout the whole timeline. The rise of Treg cells in second pregnancies can be explained by plasticity of Th17 cells towards this regulatory phenotype as the rise of 2% within the Treg population in secondary pregnancy on gd 7.5 could explain the ExTh17 percentage within second pregnancies. The hypothesis that plasticity might contribute to the advantages of second pregnancies over first pregnancies can be made. The plasticity might display an opportunity for fast conversion from one cell line into the other. This mechanism would be helpful for resolution of displacements and excessive inflammation. It was shown in murine models that Treg cells can be converted into Th17 cells. (Xu et al., 2007) This finding was followed by research on human Tregs' ability to differentiate into Th17 cells, including the expression of RORγ and CCR6. (Koenen et al., 2008) Hence, a plasticity from and towards Treg cells has been described before. Another explanation for the existence of the ExTh17 Tregs is the presence of double positive cell subsets. The main transcription factor of Th17 cells was found in Treg cells, resulting in FoxP3$^+$ RORγt$^+$ T cells. These cells resembled Tregs and Th17 cells in transcriptome and epigenetics and were found in mouse models of colitis. FoxP3$^+$ RORγt$^+$ T cells were potent Treg effector cells with anti-inflammatory function. (Yang et al., 2016) Other studies also found Tregs that co-express RORyt under IL-6 exposure, creating double positive BiTregs as an independent cell lineage, which acts as an effector. IL-6 was herewith found as a pleiotropic cytokine, regulating the Th17/Treg balance by favoring Th17 cells over Tregs. (Hagenstein et al., 2019) A bifunctional nature was described to this distinct

subset, which exhibits anti- and pro-inflammatory activities. (Kluger et al., 2016) In future studies both transcriptional factors, as well as IL-6 should be included in the staining to further analyze the subset measured in this thesis and to understand if these cells are a distinct subset or if they truly convert into Treg cells. Furthermore, additional markers should be assessed. For example, CD39 and CD73 were identified as surface markers on $CD4^+$ and Treg cells, correlating negatively with Th17 frequencies in a study on allergic asthma. (L. L. Wang et al., 2013) In continuation, bioinformatic analysis by colleagues from my laboratory led to the proposition of a participation of CD73 in the transdifferentiation of Th17 cells towards Tregs. (Thiele et al., 2025) Also PD-1/PD-1L was suggested to be involved in conversion of effector T cells into regulatory subsets, as shown for Th1 cells and suggested as a possible mechanism in pregnancy. (Amarnath et al., 2011, Thiele et al., 2025) Future studies should include these markers, to examine plasticity more specifically.

Concerning the Tr1 subset, similarly ExTh17 cells are measured throughout pregnancy, in both LN and the uterine tissue high frequencies are detectable, especially in very early pregnancy on gd 1.5. Approximately 20% of Tr1 cells in the LN on gd 1.5 formerly produced IL-17. However, interestingly, the plasticity differences between first and second pregnancy in $FoxP3^+$ Treg cells are not measurable in Tr1 cells. ExTh17 phenotype expression within Tr1 cells is similar in first and second pregnancies. The increase of Tr1 cells in the LN in second pregnancy probably has a different origin than the plastic transformation. These findings lead to the conclusion of an importance of Th17 plasticity for the subset of Tr1 cells, which is shown in first pregnancies and validated to the same extent in second ones. Herewith, it does not lead to the improvement of subsequent pregnancies but could be important for pregnancy and the anti-inflammatory recruitment in general. Other fate-mapping studies of the intestine already provided evidence that in inflammatory settings Th17 cells may lose IL-17A expression and transdifferentiate into Tr1-like cells, producing IL-10. (Gagliani et al., 2015) These converting cells were equipped with signaling for TGF-β and the aryl hydrocarbon receptor (AhR). (Gagliani et al., 2015) Hence, the pathogenic setting is opposed with IL-10 production and conversion into Tr1 cells, resulting in resolution of inflammation. These studies are supported by the findings of this thesis. Another study found out that ligand-activated transcription factor AhR regulates Th17 cell differentiation besides regulatory T cells in mice. (Quintana et

al., 2008) The data of this thesis shows that plasticity from Th17 cells to Tr1 cells is measurable in pregnancy and occurs in first and second pregnancy to a similar extent. In future studies the transcription factor AhR could be of interest to explore a possible link between these subsets in the presented model and context of pregnancy.

Also, ExTh17 cells within $CD4^+$ T cells show differences between first and second pregnancies. While only small frequencies are measured in the LN over the course of pregnancy of first-time pregnant mice, significant increases are recorded in second pregnancy on all gds. Further, in the uterine tissue, gd 1.5, 7.5 and 15.5 display a significant increase in second pregnancy. The assumption of higher conversion rates and plasticity in both tissues in subsequent pregnancies could be causative for improved pregnancy outcome in subsequent pregnancy. However, the results for ExTh17 cells reveals, that the regulatory subsets are not the only cells Th17 cells convert into. The regulatory subsets display less than 30% in the LN. A possible pathway for the remaining 70% of ExTh17 cells would be the cease of production of IL-17 without the adoption of another phenotype. The limitation of IL-17 might still indicate a benefit for pregnancy. Cells that did convert into regulatory phenotypes represent an advantage for the second pregnancies' outcome and cells that stopped IL-17 production might support this effect. However, in another possibility ExTh17 cells adopt other phenotypes, which are not evaluated in this setting. Regulatory subsets might only make up a fraction of the plasticity of Th17 cells. Hence, the question arises which cells ExTh17 cells convert into. Other possible plasticity routes of Th17 cells are Th1 and Th2 cells. Other conversion options could be beneficial or destructive for the pregnancy outcome.

A subdivision of the Th17 cell subset dependent on additional cytokine production and marker expression, resulted in the establishment of intermediate subsets like Th17/Th2 and Th17/Th1 cells in investigations of human decidual cells. (Lombardelli et al., 2016) These findings further showed that Th17 cells exhibited a beneficial role in pregnancy, as the Th17/Th2 phenotype even executed a crucial role in implantation. (Lombardelli et al., 2016) This information leads to the proposition that the ExTh17 cells that are not assigned to a subset in this thesis might be of Th17/Th2 phenotype and might contribute to an advantage for the pregnancy outcome in secondary pregnancy. However, a switch towards the Th17/Th1 subset might represent a pregnancy

unfavorable option, as excessive inflammation might be delirious for the outcome. A classification of Th17 cells into inflammatory pathogenic and anti-inflammatory non-pathogenic ones has been suggested before for mice and humans. (Lee et al., 2012, Hu et al., 2017) Prospectively, this classification of Th17 subsets might enable an interpretation of their pathogenicity in pregnancy. To address the identification of the remaining cells Th17 cells converted into, different approaches on multiple levels of cell differentiation could be used. Additionally, to the cytokine exposure and transcription factor measured in this thesis, more cytokines like IFN-γ and TGF-β could be assessed. Further, the transcription factors of Th17, Th1 and Th2 cells could be of interest. Further steps on molecular level could present a solution, for example the chromatin landscape or epigenetics.

In conclusion, differences on cellular and molecular level between pathogenic and non-pathogenic Th17 cells could be responsible for a switch from protection to rejection of pregnancy. Hence, the identification of plasticity towards Th1 and Th2 cells in addition to the regulatory subsets, is of high interest to understand the influence of Th17 cells and plasticity on pregnancy. Knowledge on these plastic adaptations could possibly enable a therapeutic intervention for pregnancy complications in case of a pathogenic phenotype of Th17 cells. Plasticity might represent fast conversion and improvement of pregnancy adaptation, especially in subsequent pregnancy. These advantages might result from resolution of excessive inflammation and a quicker tolerance establishment. The pathogenic and non-pathogenic phenotypes should therefore also be assessed in secondary pregnancy, as plasticity seems to be more pronounced. All in all, Th17 cells and their role in pregnancy remain cryptic. Their plasticity towards anti-inflammatory subsets offers a quick adaptation and shows that a clear characterization into anti- or pro-inflammatory is impossible.

Lastly, the effect of stress exposure on pregnancy including its outcome and its immunological deviations was examined. In this thesis, fetal weight, placental weight, abortion rate and implantation rate are evaluated on gd 15.5 in the stress experiment. First, assessment of fetal weight shows significant increases in stressed second pregnancies compared to stressed first ones. For the control groups a small increase in second pregnancy is also displayed. A significant increase of fetal weight in secondary pregnancy was also shown in different human studies as summarized in

(Thiele et al., 2019). Moreover, the span between first and second pregnancy widens in stress, which might highlight the effect of resistance towards stress in second pregnancy. The impact of stress on first pregnancy further imposes with a decrease of the birth weight and result in an IUGR. This birth weight decrease in stressed mice has been shown before. (Solano et al., 2015, Wieczorek et al., 2019) This impact represents the susceptibility to stress which only occurs in first-time pregnant dams. Prenatal stress perception encourages complications like the result of low-birth-weight infants in humas exposed to a hurricane during pregnancy. (Xiong et al., 2008) Results of this thesis show the ineffectiveness of stress on subsequent pregnancies, which even show a significant increase in birth weight in stress, despite decreasing weight in first pregnancies. These findings are supported by another study performed in my laboratory. Birth weight reduction occurred in pubs from stressed animals on gd 15.5. Second pregnancy was guarded from this effect. (Thiele et al., 2025) In total, a destructive effect of stress on the outcome of pregnancy was confirmed in this thesis. An IUGR only occurred in first pregnancy. Further, a protection from these destructive effects of stress in secondary pregnancy is proposed. These results raise the question of why a second pregnancy protects against an IUGR in stress. Resulting in the hypothesis, that an unencumbered first pregnancy establishes an advantage and protection against stress in a subsequent pregnancy. Possible explanations for these advantages are e.g., improvement of placentation, implantation, hormonal and immunological adjustments remembered from or learned in first pregnancy. Immunologically reasons such as Treg increases and plasticity might also acquire for stress prevention besides an improved outcome in regular pregnancy.

The importance of a sufficient supply of the fetus with nutrients, growth factors, hormones, oxygen besides others is secured by a functional placenta. A sufficient blood flow can only be secured in a fully developed placenta. To monitor this development, placental weight was measured and compared. The placental weight shows a tendency of an increase in second pregnancy independent from the stress challenge. Moreover, a trend of lower placental weight is pictured in stress. These trends underline the hypothesis of an improved fetal and placental development in second pregnancy. An increased supply allows quicker growth, suggesting an advantage of second pregnancy. Further, the effect of stress might cause the tendency of lower placental weight in stress. It was postulated that growth hormone secretion is

hampered and the placental perfusion is restricted in stress, leading to maturation and malnutrition issues and consequently to low birth weight. (Knackstedt et al., 2005) Herewith, besides birth weight decreases, an alteration of placental evolvement under the influence of stress is apparent. In future studies, a structural analysis of placental tissue might be of use for further differentiation of the influence of stress.

It was shown that fetal abortion and implantation rates were similar in stress. (Zazara et al., 2018) The two parameters of abortion and implantation rates are also assessed in this thesis and present similar results. Reasons for the stability of these two parameters in stress could be the timepoint of stress application. As implantation occurs approximately on gd 5.5 the stress applied from gd 10.5 to gd 14.5 might not be interfering with these parameters. The same model could be repeated with stress application occurring earlier in pregnancy to test if the influence on abortion and implantation rate leads to a different outcome like failure or reduction.

In summary, fetal birth and placental weight display a resistance of second pregnancies against external stressors. Stress application affects firstly pregnant mice significantly greater magnitude regarding the outcome. These results corroborate statements of fewer complications occurring in second pregnancies. Additionally, it was summarized that complications in first pregnancy, result in higher risk for repetition of these complications in second pregnancy. (Thiele et al., 2019) This statement could be researched in future experiments by testing second pregnancies that experienced stress exposure in previous gestation. Furthermore, the effect on the offspring would be an interesting supplementation to the here presented data. An irregular formation of the immune system has been described in offspring of stressed mothers. (Pincus-Knackstedt et al., 2006)

One possible reason for the absence of an IUGR in second-time pregnant mice is an improvement in hormonal adjustments. This hypothesis was addressed in measurements of corticosterone and progesterone levels in maternal serum. Hormone levels of corticosterone in maternal serum show an elevation in stressed animals. It is significant in first as well as in second pregnancies, meaning that the stress application was successful. An increase of cortisol in stress is a well-known and broadly researched. Measurements of daily cortisol in the urine of rural Mayan women for

example showed cortisol increases in stress. (Nepomnaschy et al., 2004) Normal levels of GCs in pregnant mice are dependent on the gestational advance, as levels continuously rise with an intensified increase in late gestation. (Wieczorek et al., 2019) For gd 15.5, around 500 to 600 ng/ml were measured in control animals, similarly to the data presented in this thesis. (Wieczorek et al., 2019) In addition to their increase, GCs' importance for organogenesis illustrates the physiological need of GCs in pregnancy. However, excessive levels were associated with significant changes in menstrual hormone profile. (Nepomnaschy et al., 2004) Further, disruptions in hormonal levels were reported to disturb implantation. (Baird et al., 1999) Excessive levels are even more perilous for the offspring. GCs for example induce immune diseases later in life by disturbing the programming of the fetal hypothalamic pituitary adrenal axis. (Solano et al., 2016) Therefore, a protection of the fetus from excessive GCs is of high importance. A mechanism to shield the fetus is the glucocorticoid barrier in the placenta, the 11β-hydroxysteroid dehydrogenase type 2 (11ßHSD2). This barrier is exceeded if too high levels of GCs circulate in the mother, resulting in GCs entering the fetal blood system. (Solano et al., 2016) The measurements in this thesis show similarly elevated levels in stressed first and secondary pregnancy, however the fetal effect of an IUGR is decreased in secondary pregnancy. One reason for this uninfluenced outcome in second pregnancy might be lower concentrations of GCs reaching the fetus. This effect could be attributed to higher concentrations of 11ßHSD2, leading to less GCs crossing the placenta towards the fetus. Herewith proposing a protective effect of 11ßHSD2, shielding second pregnancy from an IUGR. Further studies could include measurements of 11ßHSD2 and fetal glucocorticoid concentrations.

Negative effects of cortisol in pregnancy have been described to a huge extend. Already in the 90s, studies described the result of spontaneous abortion. (Neugebauer et al., 1996) Especially during early pregnancy and placentation phase, an increase of maternal cortisol levels showed a correlation with the risk of miscarriage. (Nepomnaschy et al., 2006) Opposing these studies, the trend of slightly elevated levels of corticosterone in second pregnancies compared to first ones is measured in this thesis. A less pathogenic role could prevail if the increase does not overflow a critical level. Measurements were made on gd 15.5, presenting a timepoint close to term where GCs physiologically increase. (Fowden et al., 1998) Further, this increase

is crucial for development processes and administration of GCs presents a treatment for pre-term birth, as it supports the lung maturation of the fetus. (Papageorgiou et al., 1979, Jobe et al., 1993) Small increases might have a supportive effect on maternal adaptations to pregnancy. Herewith, leading to the proposition that slightly higher numbers of GCs in second pregnancy might even present an advantage, if they do not exceed a horrendous amount.

Besides changes in GC levels, also lower progesterone levels have been described in stress. A hormonal imbalance is induced, which leads to a shift from progesterone towards GCs. (Arck, 2001) This imbalance could interfere with maturation of the fetus and maintenance of pregnancy, as progesterone is indispensable. (Csapo and Pulkkinen, 1978, Couzinet et al., 1986) Via inhibitory feedback loops, increasing cortisol levels might result in a progesterone decrease in mice. (Nepomnaschy et al., 2007) Additionally, a pregnenolone steal has been suggested for stress. It describes a lack of pregnenolone as a common progenitor in the synthesis of the steroid pathway of cortisol and progesterone. (Solano and Arck, 2019) As both hormones access the same precursor hormone, excessive production of cortisol might lead to a reduction of the availability for progesterone, resulting in a displacement of these two hormones. (Solano and Arck, 2019) An imbalance of GCs and progesterone might cause severe disruption of pregnancy. The fetus is undernourished, as vascularization is restricted, explaining the onset of an IUGR. (Solano and Arck, 2019) However, in the presented experiments no change was detectable within the GC measurements. Possibly, already physiologically elevated GC level in secondary pregnancy result in a decreased fold-change of GC due to prenatal stress, which might explain the absence of IUGR normally observed in first pregnancy. Furthermore, the balance in second pregnancy in stress might be shifted towards progesterone, still representing a beneficial outcome. All in all, the hormone measurements in this thesis did not supply conclusive results for a hormonal impact on an IUGR in first-time pregnant stressed dams, absent in second pregnancy. A possible explanation might be a high distribution of values. Similar progesterone results have been described before. (Szenci et al., 2011) Here, fluctuation and individual differences in progesterone levels were accused of masking progesterone increases. (Szenci et al., 2011) In the future, experiments could be repeated, to verify results. Furthermore, another explanation for the absence

of an IUGR in second pregnancy in stress might be an improved immunological adaptation.

Prenatal stress challenge is notorious for disturbance of pregnancy maintenance and further for impairment of fetal development by causing endocrinological and immunological changes. (Solano and Arck, 2019) For a long time, stress research in pregnancy lacked methods for stress replication in experimental setups. In the last decades, a variety of basic science mouse models and human study designs were established, and biomarkers were identified. In this thesis, these models now help with the examination of the topic of the immune disruption, caused by prenatally experienced stress. The impact of stress on the immune system is displayed by Th17 and regulatory T cell changes. Furthermore, the plasticity of these cell subsets towards one another in stress challenged pregnancies is examined. An increase of Th17 cells in pregnancy complications has been elucidated in full extend throughout this thesis. It remains unclear if stress also influences this population. The increase of this cell subset in multiple complications might anticipate an increase in stress, too. However, in the LN no increase of the Th17 population is seen in stress. Even though Th17 cells were accused of inducing different gestational problems, these results suggest that Th17 cells are not causative for stress induced problems at this timepoint of pregnancy. It would be interesting to look at the transcriptional profile and cytokine expression, exploring if the phenotype of Th17 cells changes. A differentiation into pathogenic and non-pathogenic Th17 cells could be of interest in future studies. Stress could possibly lead to a displacement of an anti-inflammatory subtype with an inflammatory one. Hence, stress would not affect the number of Th17 cells but may promote a shift towards a pathogenic phenotype. Another explanation might be no participation of Th17 cells within the processes occurring in stress. Furthermore, the gd 15.5 might be too late in gestation to see the influence of stress within the immune cells. A switch towards the needed parturition triggering inflammation occurs physiologically.

Another possible explanation for an absence of an IUGR in second pregnancy, could be a Treg increase. The results presented in this thesis show that the increase in Treg cells described for second pregnancies, were not altered by stress exposure, and stayed significant in the LN. These results confirmed observations from another mouse model. (Thiele et al., 2025) The hypothesis that an immunologic benefit of subsequent

pregnancies stays stable in stress can be proposed. Additionally, stress did not interfere with the plasticity of Th17 cells into Treg cells in second pregnancy. This effect remained as pronounced in the stressed second pregnancy group. In the future, further investigations on different timepoints of pregnancy should be performed. It is unclear, if the steadiness of the immune cell subsets of Th17 cells and Tregs relies on the advance of pregnancy.

Tr1 cells display significantly higher frequencies in the LN in secondly pregnant stressed dams, than in firstly pregnant stressed mothers. In the control groups, significance was not reached between second-time pregnant mice compared to first-time pregnant mice. This significant change could be interpreted as a more pronounced susceptibility of firstly pregnant mice towards stress, resulting in an extended span between first and second pregnancy in stressed than in unchallenged mothers. A possible explanation is the advantage of a memory of immune cells in second pregnancy. This experiment suggests advantages of physiologic second pregnancy also apply for challenged pregnancy. Furthermore, this increase might be caused by the effect of plasticity of Th17 cells towards Tr1 cells. ExTh17 within the Tr1 cells are significantly higher in the LN in the secondly pregnant stressed group. In stress, an adaptation might be needed more heavily to oppose the inflammatory setting, created by the stress reaction.

Locally in the uterus, trends of an increase of Tregs and Tr1 cells including their former-IL-17 expression in second pregnancy are stable in stress. Less concise results in this organ compared to the LN could be caused by differences in the procession. Firstly, the procession of the tissue is more complex, as the uterus needs to undergo enzymatic digestion. Further, cell numbers are lower after erasing non lymphatic cells through cleaning processes. The analyzation of tissue resident cells and an eradication of circulating cells could be achieved in future experiments. Tissue residence can be marked with in vivo immune cell labelling. (Zazara et al., 2022) The usage of an intravenous staining could distinguish between immune cells that are present in vasculature and those residing outside intact endothelium. This staining was for example tested on the parenchyma of the lung. A protocol, where the Anti-CD45.2 monoclonal antibody is applicated intravenously 2-3 minutes before sacrifice of the mouse should be followed. (Anderson et al., 2012, Anderson et al., 2014)

In the stress experiment, next to LN and uterus, PBMCs are examined. Concerning e.g., Th17 cells, Treg cells, Tr1 cells no changes are measured between all groups. Also, in other studies no change of the Th17 cell population in the PBMCs was measurable. (Nakashima et al., 2010a) A pronounced pregnancy adjustment might occur in regional LNs and uterus. However, the majority of human data presented in this thesis analyzes immune cells in the blood as it is ethically more difficult to obtain other tissues from healthy, pregnant women. These issues do not acquire for mouse experiments, emphasizing the necessity of murine models. However, humans and mice still differ. Obviously, all collected data from mouse experiments need to be further validated in human clinical trials and studies.

All in all, the measurements of hormones, Tregs or plasticity of Th17 cells obtained in the stress experiment do not explain the absence of an IUGR in second-time pregnant stressed dams sufficiently. One consideration could be that fetal weight is further dependent on cardiac, metabolic, and hemodynamic changes, as for example the cardiac output increases in second pregnancy as the heart is trained from the first pregnancy. (Campbell and MacGillivray, 1972, Clapp and Capeless, 1997) Therefore, the immunological changes in second pregnancy do not solely constitute to the improvement. However, besides the performed experiments, complementary research should include a more detailed look into the functionality and epigenetic testing of T cells. Additionally to the frequency measurements obtained in this thesis, their function could explain advantages detected in stressed second pregnancy. The function of Tregs, could be assessed via cytokine expression. Next to IL-10, the secretion of TGF-β, and interactions via cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and PD-L1 could be helpful to characterize functional changes, as summarized in (Thiele et al., 2025). Furthermore, future studies could assess data on Th1, Th2 cells and gamma delta T cells. Especially, gamma delta T cells could represent an interesting subset to investigate, as their role in pregnancy was proposed to be beneficial. (Yang et al., 2022) Another aspect to be considered is the timespan of stress application. Several days of stress might be too long to have a distinguishable impact on T cell frequencies between all groups. In future studies an examination could be performed after one day of stress application. Further, woman, who experienced complications in first pregnancy, had an elevated risk to repeatedly suffer from complications in subsequent

pregnancy. (Thiele et al., 2019) It would be interesting to also evaluate a reaction in mothers that are exposed to stress in first pregnancy and thereafter undergo second pregnancy in a similar murine model in future studies.

In summary, this thesis confirms elevated $CD4^+$ Treg cell frequencies in subsequent pregnancy in an alternative mouse model. Further, pro-inflammatory Th17 cells were measured at low frequencies in first and second pregnancy. However, exclusively in second pregnancy increased frequencies of formerly IL-17-producing ExTh17 cells were detected and showed the ability to transdifferentiate into $CD4^+$ Treg and Tr1 cells, herewith exhibiting a regulatory phenotype. Hence, in addition to the mechanism of an adaptive immune memory, plasticity of T cells might contribute to an improved fetal outcome in second pregnancy. Further, a prenatal stress challenge did not affect fetal outcome of second pregnancy, as an IUGR, normally observed in first pregnancies affected by prenatal stress challenge, was absent. Future studies should assess whether memory acquisition and plasticity lead to an epigenetic change in subsequent pregnancies. Additionally, the extend of plasticity should be evaluated towards other T cell subsets to identify a potential network of transdifferentiation, including the expression of transcription factors and secretion of cytokines. Moreover, additional gestational days and a focus on uterine tissue-resident immune cells could amend the presented data herein. Overall, the data presented in this thesis expand the state of knowledge on feto-maternal immunology and contribute to the long-term goal of determining immunological targets and developing therapy methods for pregnancy complications.

6. Abstract

As the fetus exhibits paternal genetic features immunologically foreign to the mother, a successful pregnancy requires precise adaptations of the maternal immune system. An improved maternal immune adaptation was observed in second pregnancy, along with an improved fetal outcome, if the first pregnancy went medically uneventful. These beneficial effects in second pregnancy could be attributed to an immune memory established in first pregnancy, as increased frequencies of $CD4^+$ regulatory T (Treg) cells were detected in second pregnancy. However, T cells are also characterized by their ability to display plastic features and to transdifferentiate into other phenotypes. The impact of T cell plasticity on pregnancy and its contribution to an improved pregnancy outcome remains to be identified, especially in respect to prenatal challenges such as maternal stress perception.

Using a fate mapping mouse model, $CD4^+$ Treg cell, T helper (Th)17 cell, ExTh17 cell and regulatory Type 1 T (Tr1) cell frequencies in uterine tissue, lymph nodes and peripheral blood were analyzed by flow cytometry on different gestational days during first and secondary pregnancy to assess their plasticity during gestation. Additionally, first and second pregnancies were exposed to a prenatal sound stress model to evaluate pregnancy outcome.

Th17 cells were detectable in low frequencies in first and second pregnancies. Exclusively in second pregnancy, ExTh17 cells could be detected and were observed to now display a regulatory phenotype ($CD4^+$ Treg and Tr1). Vice versa, increased $CD4^+$ Treg cell frequencies were confirmed in second pregnancy and approx. 2% of these cells expressed an ExTh17 cell phenotype. Interestingly, increased Tr1 cell frequencies observed in subsequent pregnancies, could not be attributed to T cell plasticity. Lastly, second pregnancy showed an improved pregnancy outcome which was not affected by intrauterine growth restriction (IUGR) in response to prenatal stress normally observed in first pregnancies.

In summary, this thesis suggests that T cell plasticity might contribute to a beneficial maternal immune adaptation and an improved pregnancy outcome in subsequent pregnancies. Future studies on subsequent pregnancies should further specify plasticity of Th17 cells, aim at epigenetic changes and identify reasons for a protection from an IUGR in stress.

Zusammenfassung (Deutsch)

Da der Fetus väterliche genetische Merkmale aufweist, die der Mutter immunologisch fremd sind, sind für eine erfolgreiche Schwangerschaft präzise Immunanpassungen unerlässlich. Bei Zweitgravidität wurde eine Verbesserung der Immunanpassung und des fetalen Ergebnisses beobachtet, sofern die erste Schwangerschaft medizinisch unauffällig verlief. Diese positiven Effekte könnten auf ein in der ersten Gravidität etabliertes Immungedächtnis zurückzuführen sein, da in der zweiten Schwangerschaft erhöhte $CD4^+$ regulatorische $FoxP3^+$ T (Treg)-Zellfrequenzen nachgewiesen wurden. Zusätzlich wird T-Zellen die Fähigkeit in andere Phänotypen zu transdifferenzieren zugeschrieben. Die Auswirkung von T-Zell-Plastizität auf Schwangerschaft und der Beitrag zu einem verbesserten Ergebnis muss noch ermittelt werden, insbesondere bezüglich pränataler Herausforderungen, wie maternaler Stresswahrnehmung.

Anhand eines Fate-Maus-Modelles wurden $CD4^+$ Treg-Zell-, T Helfer 17 (Th17) Zell-, ExTh17 Zell- und regulatorische Typ 1 (Tr1) Zellfrequenzen in Uterus, Lymphknoten und Blut an verschiedenen Gestationstagen bei Erst- und Zweitgebärenden mittels Durchflusszytometrie ausgewertet, um die Plastizität in der Schwangerschaft bewerten zu können. Zusätzlich wurde die Schwangerschaft Stress ausgesetzt und nach Erfolg des fetalen Ergebnisses ausgewertet.

Th17-Zellen sind in niedriger Frequenz in Erst- und Zweitschwangerschaften messbar. Ausschließlich in Zweitschwangerschaften wurden ExTh17-Zellen nachgewiesen und gezeigt, dass diese einen regulatorischen Phänotypus annehmen ($CD4^+$ Treg und Tr1). Umgekehrt, wurden erhöhte $CD4^+$ Treg-Zellfrequenzen in zweiten Schwangerschaften bestätigt und ca. 2% dieser Zellen wiesen einen ExTh17-Zell-Phänotypen auf. Interessanterweise konnten erhöhte Tr1-Zellfrequenzen in Zweitschwangerschaften nicht auf die T-Zell-Plastizität zurückgeführt werden. Zuletzt zeigten zweite Schwangerschaften ein verbessertes fetales Ergebnis, welches nicht von intrauteriner Wachstumsretardierung (IUWR) als Reaktion auf pränatalen Stress betroffen war, wie es bei Erstgebärenden normalerweise beobachtet wird.

Alles in allem, suggeriert diese Arbeit, dass T-Zell-Plastizität zu einer Verbesserung der maternalen Immunanpassung und des fetalen Erfolges in nachfolgenden Schwangerschaften beitragen könnte. Zukünftige Studien zu Zweitgravidität sollten die Plastizität von Th17-Zellen weiter spezifizieren, auf epigenetische Veränderungen abzielen und die Gründe für einen Schutz vor einer IUWR bei Stress ermitteln.

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8. List of abbreviations

NK	Natural killer cells
DC	Dendritic cells
MHC	Major histocompatibility complex
APC	Antigen presenting cell
CD…	Cluster of differentiation …
Th	T helper
Treg	Regulatory T
Tr1	T regulatory type 1
IL-…	Interleukin …
IFN-γ	Interferon-gamma
FoxP3	Forkhead-Box-ProteinP3
LAG-3	Lymphocyte-activation gene 3
CM	Central memory
EM	Effector memory
HLA	Human leucocyte antigen
EVT	Extravillous trophoblast
uNK	Uterine natural killer
PBMC	Peripheral blood molecular cell
PD-1	Programmed death protein 1
GC	Glucocorticoid
RSA	Recurrent spontaneous abortion
gd	Gestational day
IMOP	Immunological memory of pregnancy
EAE	Experimental autoimmune encephalomyelitis
eYFP	Enhanced yellow fluorescent protein
UKE	University Medical Center Hamburg-Eppendorf
EDTA	Ethylene diamine tetra acetic acid
PBS	Dulbecco's Phosphate Buffered Saline
LN	Lymph node
BSA	Bovine Serum Albumin
HBSS	Hank's Balanced Salt Solution
FACS	Fluorescence activated cell sorting

RBC	Red blood cell
D/L	Dead life stain
FMO	Fluorescence minus one
FITC	Fluorescein-5-Isothiocyanate
YFP	Yellow fluorescent protein
eFGP	Enhanced green fluorescent protein
PE	R-Phycoerythrin
Cy	Cyanine
BV	Brilliant Violet
gdTCR	gamma delta T cell receptor
BUV	Brilliant Ultraviolet
APC	Allophycocyanin
BD	Becton Dickinson
ELISA	Enzyme-linked immunosorbent assay
IUGR	Intrauterine growth restriction
RPL	Recurrent pregnancy loss
CCR6	C-C chemokine receptor type 6
mTregs	Memory Tregs
PD-L1	Programmed death-ligand 1
CXCR	C-X-C chemokine receptor
TGF-β	Transforming growth factor beta
T-Bet	T-box expressed in T cells
ROR	RAR-related orphan receptor
STAT3	Signal transducer and activator of transcription 3
Fosl2	Fos like 2
AhR	Aryl hydrocarbon receptor
11ßHSD2	11β-hydroxysteroid dehydrogenase type 2
CTLA4	Cytotoxic T-lymphocyte-associated protein 4

9. List of figures

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11. Pre-publications

Wöhrle, R, Arck, P & Thiele, K 2022, Stress-induced immune deviations and reproductive failure. in J Kwak-Kim (Hrsg.), Immunology of Recurrent Pregnancy Loss and Implantation Failure. 1 Aufl., Bd. 3, Elsevier, London, S. 103-119. https://doi.org/10.1016/B978-0-323-90805-4.00013-4

Thiele, K, Urbschat, C, Riquelme, JIA, Ahrendt, LS, Wöhrle, R, Schepanski, S, Eckert, JJ, Becht, E, Qi, M, Alawi, M, Becker, M, Gagliani, N, Mittrücker, H-W, Diemert, A & Arck, PC 2025, 'Pregnancy-acquired memory CD4+ regulatory T cells improve pregnancy outcome in mice', NAT COMMUN, Jg. 16, Nr. 1, S. 6522. https://doi.org/10.1038/s41467-025-61572-w

12. Erklärung des Eigenanteils

Im Folgenden erkläre ich meinen Eigenanteil an dieser Dissertation.

Ich habe meine Dissertationsarbeit in dem Zentrum für Geburtshilfe, Kinder- und Jugendmedizin/ Klinik und Poliklinik für Geburtshilfe und Pränatalmedizin unter der Betreuung von Prof. Dr. Petra Arck und Dr. Kristin Thiele durchgeführt.

Die Konzeption habe ich mit Unterstützung von Prof. Dr. Petra Arck und Dr. Kristin Thiele erarbeitet.

Sämtliche Versuche und die statistische Auswertung sind nach Einarbeitung durch Dr. Kristin Thiele von mir durchgeführt worden.

Das Manuskript dieser Dissertation habe ich selbstständig verfasst.

Teile der Arbeit wurden in der Publikation „Pregnancy-acquired memory $CD4^+$ regulatory T cells improve pregnancy outcome in mice" (Thiele et al., 2025) veröffentlicht.

13. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Das gilt insbesondere auch für alle Informationen aus Internetquellen.
Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum Unterschrift

14. Acknowledgement

I would like to acknowledge the people who supported me during the time of my dissertation.

First, I would like to express my gratitude for my doctoral supervisor Prof. Dr. Petra Arck. I thank her for the support, knowledge and feedback, as well as the opportunity to participate in the Graduiertenkolleg “Hamburg School of Immunity and Infection”.

Moreover, I would like to acknowledge my supervisor, Dr. Kristin Thiele, who provided support and guidance throughout my thesis. I am grateful for her advice, time and encouragement, her scientific expertise and feedback.

Furthermore, I want to thank the whole department of feto-maternal medicine at the UKE for creating a nice working environment and for the support in technical questions.

At last, I would like to thank my family and friends who supported me emotionally and encouraged me throughout all steps.