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Regulatory T Cells and Maternal Immune Tolerance in Pregnancy Associated Breast Cancer - a Murine Model

Dissertation

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Inhaltsverzeichnis

List of Figures

List of Tables

Abbreviations

ADO	adenosine
AMP	adenosine monophosphate
ANOVA	analysis of variance
APC	antigen-presenting cell
ATP	adenosine triphosphate
BD	Becton and Dickinson
CAECAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CCL22	C-C Motif Chemokine Ligand 2
CD	cluster of differentiation
CM	central memory
CTLA-4	cytotoxic T-lymphocyte-associated Protein 4
DNAse	deoxyribonuklease
EAE	experimental autoimmune encephalomyelitis
Eff.	effector
ER	estrogen receptor
EM	effector memory
E2	estradiol
FACS	fluorescence-activated cell scanning
FBS	fetal bovine serum
FoxP3	forkhead-box-protein 3
GATA-3	GATA binding protein 3
gd	gestational day
HBV	hepatitis B virus
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
HGMB1	high-mobility group box 1
Hmox1	heme oxygenase 1
HR	hormone receptor
ICOS	inducible costimulator
IDO	indoleamine 2.3-dioxygenase
IFNγ	interferon γ
i.p.	intra peritoneal
IUGR	intrauterine growth restriction

IVC	individually ventilated cage
Ki-67	Kiel 67. proliferation marker
Lag3	lymphocyte-activation gene 3
LN	lymph node
MHCII	major histocompatibility complex II
mRNA	messenger ribonucleic acid
MUC-1	mucin 1
M1/2	macrophage type 1/2
N	naive
NK cell	natural killer cell
NF-kB	nuclear factor kappa-light-chain-enhancer
NKG2D	natural killer group 2. member D
PABC	pregnancy-associated breast cancer
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PR	progesterone receptor
PRL	prolactin
RBC	red blood cell
RORγT	RAR-related orphan receptor γ
rpm	rounds per minute
SD	standard deviation
SEM	standard error of mean
TAA	tumor associated antigen
TAM	tumor-associated macrophage
Tbet	T-box expressed in T cells
Tcon	conventional T cell
TGFβ	tumor growth factor β
Th cell	T helper cell
Tim3	T-cell immunoglobulin and mucin-domain containing-3
TNM	tumor, node, metastasis
TNFα	tumor necrosis factor α
Treg	regulatory T cell
Tr1	type 1 regulatory cell

1. Objective and Hypothesis

Pregnancy-associated breast cancer (PABC) is an entity with rising incidence as well as high morbidity and mortality. Women affected by PABC show decreased survival with more aggressive and larger tumors, which are more likely to metastasize. The basis for the adverse outcome might lie in pregnancy itself. The implantation and development of a semi allogenic fetus leads to fundamental changes in maternal immunity. In order to prevent fetal rejection, the mother's immune system has to transition to a more tolerant state allowing the fetus to thrive. Regulatory T cells, such as Tregs, T helper cells, and $CD8^+$ regulatory cells, play an important role in this process. By various mechanisms they limit NK cell, effector T cell and macrophage activity. Though beneficial in pregnancy, maternal immune adaption may promote tumor growth and evasion by mitigating key immunological defense mechanisms. Multiple studies already suggested a negative correlation between regulatory T cell prevalence and outcomes in various cancers. Because regulatory T cells take an important role in orchestrating maternal-fetal immune crosstalk as well as tumor immunity, I want to establish the first in-depth phenotyping of this subset in PABC.
In order to achieve this goal, I formulated two main objectives:

I. Establishment of a viable murine breast cancer model during pregnancy focusing on cell line characteristics, tumor morbidity and mortality as well as pregnancy outcome.

II. In-depth immune phenotyping of regulatory T cells to assess their impact on tumor progression within the tolerogenic immune microenvironment during pregnancy.

2. Introduction

2.1. Pregnancy Associated Breast Cancer

Breast cancer is a leading cause for morbidity and mortality worldwide. Over 2 million people were diagnosed in 2020. with 70.000 cases in Germany alone. Besides being the most common malignancy in women, it is also the one accounting for most tumor-related deaths.(Krebshilfe, 2020; WHO, 2021)

With a continuous increase in the incidence of breast cancer in patients under 40 years of age, more women will be affected during a period coinciding with pregnancy. (Guo et al., 2018) Age as a major risk factor for breast cancer also collides with an increasing maternal age. (Martin et al., 2013; Waldenström, 2016; Winters et al., 2017) Already, 1:3000 women are affected by pregnancy associated breast cancer (PABC), which implies diagnosis during pregnancy or up to one year after delivery. (Case, 2016; NCI, 2019)

In addition to the rising incidence, outcomes are worse in pregnant women experiencing breast cancer compared to their non-pregnant counterparts. It was shown that overall as well disease-free survival, defined as time until relapse or death, were reduced with hazard ratios of 1.44 and 1.6. respectively. (Azim et al., 2012) Consistently with decreased survival, Johansson et al. reported larger tumors and a higher number of regional lymph node metastases in pregnant women. Distant metastases were also found to be more common in PABC, though nonsignificant with very few reported cases. (Johansson et al., 2018) In an institution-based cohort PABC tumors also showed higher pathological stages and invasiveness grade. (Gooch et al., 2020)

In regard to hormonal changes during pregnancy, particular emphasis should be paid on tumor subtypes. Based on the St. Gallen classification, breast tumors are classified according to their status of hormone receptor (HR) sensitivity as well as overexpression for the growth factor HER2 and the proliferation maker Ki-67. While Luminal A and B tumors are positive for the estrogen receptor (ER) and may be positive for the progesterone receptor (PR), luminal B also shows a high expression for Ki-67. The HER2 over expression subtype is negative for hormone receptors and triple negative tumors do not show overexpression of HER2 or hormone receptor sensitivity. (Goldhirsch et al., 2013) Therefore, only luminal

type breast cancers can be influenced by pregnancy-related hormone surges in estrogen and progesterone.

Especially for progestin, a synthetic analogue of progesterone, a negative effect on breast cancer is well established. Menopausal supplementation of progestin, to alleviate symptoms of hormonal depletion, is related to an increased risk for breast cancer. (Chlebowski et al., 2020) Recently, a case-cohort study with a twelve year follow-up reported that women with elevated levels of circulating progesterone were also found to be at increased risk for breast cancer with a hazard ration of 1.16. (Trabert et al., 2020) While estrogen supplementation does not seem to have the same effect as progesterone on breast cancer risks, the reduction of it is strongly related to improved outcomes in luminal type cancers. (Chlebowski et al., 2020) The modulation of estrogen is a key mechanism in the treatment of HR positive breast cancers. Blockage of estrogen production is either achieved with aromatase inhibitors or by the use of selective estrogen receptor inhibitors e.g. Tamoxifen. (McAndrew & Finn, 2020)

Nevertheless, hormonal changes during pregnancy come short of explaining decreased survival in PABC patients when considering tumor subtypes in this specific population. In a Swedish cohort, ER positive tumors were significantly less common during pregnancy and up to 6 months postpartum. The same was observed for the PR with an association remaining up to 5 years after delivery. Additionally, the number of triple negative tumors increased from 23% in the nulliparous group to 31% in pregnant and 36% in 0-6 months postpartal women. (Johansson et al., 2018) Similar findings were reported in smaller cohorts. (Gooch et al., 2020; B. Y. Han et al., 2020)

2.2. Maternal Immunity

In order to allow fetal implantation as well as growth, a well-orchestrated balance between inflammation and immune-tolerance is required. For this purpose, effector cells, such as $CD8^+$ cells or macrophages, are under the influence of regulatory cells, e.g. $FoxP3^+$ Tregs or T helper cells, capable of modulating effector cell activation and activity. Beyond immune-regulation, various of these cells also possess an effector phenotype, e.g. TH1 cells.

2.2.1. How FoxP3$^+$ Tregs Modulate Maternal Immunity

Beginning with conception, the maternal immune system has to undergo significant changes in order to allow the semi allogenic fetus to thrive and prevent rejection. As shown in a murine, model these immune modulations are even preceded by an increase of regulatory T cells (Tregs) in the uterus during the estrous cycle in anticipation of implantation.(Kallikourdis & Betz, 2007). Tregs, which are identified by their cell surface expression of CD4 and CD25 along with the co-expression of their master regulator Forkhead-Box-Protein P3 (FoxP3) mostly exert tolerogenic immunosuppressive functions. The majority of Tregs matures in the thymus after different selection processes and travels to secondary and non-lymphoid tissues. (Richards et al., 2015; Teles et al., 2013) A smaller portion matures in the periphery by stimulation of naïve CD4$^+$ cells via TGFβ and interleukin-2 (IL2). (Zheng et al., 2004)

Studies in mice show that CD11c$^+$ antigen presenting cells (APCs) recognize parental antigens in the seminal fluid and migrate to the uterus draining lymph nodes. Subsequently, male alloantigen specific Tregs expand in response to low major histocompabiltiy complex II (MHCII) expression on these APCs. (Raker et al., 2015; Robertson et al., 2009; Shima et al., 2015; Shima et al., 2020) While the function of Tregs seems to be less pivotal in late pregnancy, they are essential for implantation and maintenance in the early phase of pregnancy. (Shima et al., 2010) Allogeneic pregnant mice which were depleted of Tregs by treatment with a monoclonal CD25 antibody present gestation failure and increased resorption rates mid-pregnancy. Interestingly, Aluvihare et al. additionally demonstrated an increase in alloantigen independent Tregs as a consequence of pregnancy itself. (Aluvihare et al., 2004)

Data from studies in humans also showed the increase of peripheral highly potent effector Tregs (CD4$^+$CD45RA$^-$FoxP3$^+$) which were non-paternal antigen-specific of fetal alloantigens. (Tsuda et al., 2019)

2.2.2. Novel Players in Pregnancy: Type 1 Regulatory Cells

A distinct and highly potent population of CD4$^+$ regulatory cells are type 1 regulatory (Tr1) cells. They are FoxP3$^-$, but co-express Lag3 and CD49b in mice as well as humans. (Gagliani et al., 2013) Similarly to Tregs, they produce high amounts of IL-10 and TGFβ. Recently, they have first been described in human

decidua, where they demonstrated to cause effector T cell suppression. (Salvany-Celades et al., 2019) Additionally, they are capable of hydrolization of ATP to immunosuppressant adenosine via their ectonucleotidases CD39 and CD73 causing metabolic disruptions in effector cells. (Schuler et al., 2014)

2.2.3. Classic T Helper Cell Dichotomy in Pregnancy

Another key player in gestational immune tolerance are T helper cells. In this study I focused on the well-established subtypes Th1 Th2 and Th17.

Changes in Th1/Th2 dichotomy commence during peri-implantation period and shift the immune environment within the fetal-maternal interface toward Th2-associated cytokine predominance. Circulating syncytiotrophoblast-derived microparticles stimulate a mild systemic inflammatory reaction with production of pro-inflammatory cytokines, such as IL-12 and TNF-α, but low levels of IL-18. Decreased IL-18 limits production of IFN-γ, mainly produced by Th1 cells. (Wang et al., 2020) Other cytokines produced by Th1 cells include TNF-α and IL-2. Predominance of cell-mediated Th1 immunity demonstrated fetal rejection/resorption in murine models as well as obstetric complications in humans. (Kwak-Kim et al., 2003; Lin et al., 2009; Renaud et al., 2011) Conversely, Th2 mediated humoral immunity and production of anti-inflammatory cytokines such as IL-4. IL-5. IL-10 and IL-13 demonstrated to be essential for successful pregnancies, summarized in figure 1. This paradigm is supported by observations of ameliorating Th1 and Th17 -type autoimmune disorders but worsening of Th2-type autoimmune disorders during pregnancy. (Abu-Raya et al., 2020)

Th2 predominant immunity seems to persist up to 4 weeks postpartum with a consequent reestablishment of a Th1 predominant state. (Sykes et al., 2014)

Th17 cells increase within the decidua as part of the defense mechanism against extracellular microbes, but demonstrated reverse pregnancy outcomes in excessive levels. (W. J. Wang et al., 2010) However, in absence of pathologies levels of Th17 cells in the periphery did not differ between pregnant and non-pregnant women.(Nakashima et al., 2010)

2.2.4. Immune Tolerance beyond CD4: CD8+ Regulatory Cells

A novel player in pregnancy immune tolerance are CD8+ regulatory cells. They have already demonstrated to possess a potent immunoregulatory phenotype in various models such as skin graft rejection or Graves' disease (Liu et al., 2019; Saitoh et al., 2007) In murine models, $CD8^+$ $CD122^+$ regulatory cells demonstrated to act through IL-10 production and thereby decreasing proliferation of $CD8^+$ $CD122^-$ cells and IFN-γ production.(Endharti et al., 2005) However, there is minimal evidence of their role in pregnancy. In a murine model investigating intrauterine growth restriction, fetal weight as well as placental vascularization improved significantly upon $CD8^+$ $CD122^+$ cell transfer. (Solano et al., 2015) These findings indicate an important role of these regulatory cells in pregnancy.

2.2.5. Non-Regulatory Lymphocytes and Pregnancy

In order to gain a broad insight into tumor immunology during pregnancy, I deemed it necessary to examine lymphocyte activity beyond regulatory cells. A well-established method to distinguish functionally distinct subsets of T cells is by heir expression of the surface markers CD44 and CD62L. First described for $CD8^+$ cells this nomenclature is now applied on $CD4^+$ cells as well. Based on the mentioned markers, T cells can be identified as naïve ($CD44^-$, $CD62L^+$), central memory ($CD44^+$ $CD62L^+$), effector ($CD44^-$, $CD62L^-$) or effector memory ($CD44^+$, $CD62L^-$) subsets. (Mousset et al., 2019; Sbierski-Kind et al., 2018) Both effector memory (T_{EM}) and central memory(T_{CM}) $CD4^+$ and $CD8^+$ cells, which are derived of their respected naïve precursors (T_N), show high anti-tumor activity, although differing in their site of residence. While T_{EM} are localized in the periphery, T_{CM} reside in secondary lymphoid organs where they await reactivation by antigen exposure. (Gray et al., 2018; J. Han et al., 2020) Recently, $CD44^-$ $CD62L^-$ T_{EFF} CD8+ cells have also been identified as being particularly effective in restoring anti-tumor immunity.(Nakajima et al., 2021) Besides their role in malignancies these subsets have also demonstrated influences on pregnancy. The function of $CD4^+$ memory cells is not yet fully understood and strongly depends on whether a previous pregnancy took place, since these cells are mainly induced by paternal-fetal antigens. However, a tight regulation of memory $CD4^+$ cells seems necessary, since both higher and lower

than normal proportions have been associated with preeclampsia and recurrent pregnancy loss. (Kieffer et al., 2019; Nguyen et al., 2017; Ramhorst et al., 2003) Memory $CD8^+$ cells have been found in higher (T_{EM}) and lower(T_{CM}) proportions in the decidua compared to peripheral blood. Their Influence in pregnancy however, has not been fully elucidated. (Feyaerts et al., 2017)
Due to a high degree of overlap between the phenotypes of the classification based on CD44 and CD62L between $CD4^+$/$CD8^+$ cells and Tregs, this scheme has recently also been used for regulatory cells. However, there are some distinct variations. In contrary to the anti-cancer role in the previously mentioned cell types, $CD44^+$ Tregs represent a potent immunosuppressive phenotype capable of creating a tolerogenic environment in cancers. (Bollyky et al., 2009; Darrasse-Jèze et al., 2009) CD62L, on the other hand, can be applied in the same way as in $CD4^+$/$CD8^+$ cells in order to distinguish naïve and central memory ($CD62L^+$) from effector and effector memory ($CD62L^-$) phenotypes. (Ambada et al., 2017)

2.3. Pregnancy Hormones and Immunity

In humans, Treg levels seem to be highly correlated with estrogen levels. Unlike postmenopausal and recurrent spontaneous abortion affected women, healthy premenopausal women showed an increase in Tregs during their menstrual cycle which correlated with estrogen levels (Arruvito et al., 2007) A model for inflammatory bowel disease in mice suggested estrogen action through ERβ. After administration of an ERβ agonist, Treg levels increased and inflammation improved.(Guo et al., 2019) Unlike for estrogen, the influence of progesterone on Tregs remains uncertain. *In vitro* experiments demonstrating progesterone effects via the glucocorticoid receptor demonstrated increased cell death for conventional $CD4^+$ cells but not for Tregs. (Hierweger et al., 2019) Consistently, treatment with progesterone in humans aiming to decrease preterm labor, led to increased levels of peripheral Tregs in pregnant women.(Areia et al., 2016)
For Tr1 cells, there are currently no studies available on their interaction with progesterone and only one indicating a positive correlation with estradiol (E2), a major estrogen in nonpregnant women based on IL-10 gene expression. (Tai et al., 2008)

Influences of pregnancy hormones on various T helper cells have been well established. A murine multiple sclerosis model (EAE) demonstrated inhibitory action of E2 through the ERα receptor on Th1 and Th17 cells. (Lélu et al., 2011) Additionally for RORγt, the master transcription factor for Th17 cells, a direct negative effect of ERα on the promoter region was demonstrated.(Chen et al., 2015) On the other hand, *in vitro* experiments showed increased GATA-3 mRNA levels, the master transcription factor for Th2 cells, upon addition of E2.(Lambert et al., 2005) In humans, measured cytokine levels in postmenopausal women who have undergone estrogen replacement therapy suggested a decreased Th1/Th2 balance, resembling a similar trend in pregnancy.(Xia et al., 2009) Similar observations were made for dydrogesterone, a pharmaceutical progesterone. *In vitro* it showed to decrease Th1 and increase Th2 cytokine levels. After addition of progesterone-receptor antagonist mifepristone the effect was reversed, suggesting direct action through the PR. (Raghupathy et al., 2005) There is currently limited evidence on hormonal influence on $CD8^+$ regulatory cells. Data for E2 administration showed to increase $CD8^+$ $CD122^+$ cell levels in EAE.(Seifert et al., 2017) Meanwhile, progesterone seems to indirectly influence $CD8^+$ Tregs as an upstream regulator of heme oxygenase 1 (*Hmox1*). In murine decidua, only Hmox1 sustained by progesterone promoted $CD8^+$ Treg production, but not progesterone alone. (Solano et al., 2015)

The hormonal influence on maternal-fetal immune crosstalk has to be considered in regard to PABC. While direct effects of progesterone and estrogen on HR^+ tumors seem clear, the hormone-mediated immune modulation by regulatory cells suggests an indirect effect on all breast malignancies, regardless of HR status.

2.4. Regulatory T Cell Subsets and Cancer

In light of the previously described mechanisms, it seems prudent to consider the maternal immune adaption with regard to the risk and severity of PABC. Frequencies of some regulatory cell subsets already demonstrated adverse outcomes in various cancers, including mammary. (Shang et al., 2015)

Particularly the role of Tregs in cancer has been well established. Although a few studies suggest a beneficial role in some tumors, in breast cancer high Treg levels are overwhelmingly associated with negative outcomes. (Jørgensen et al.,

2019) One study also showed that high prevalence of FoxP3^{+} tumor infiltrating lymphocytes was not only associated with higher grade and positive nodal status but also with young age.(Liu et al., 2014) Beyond the previously mentioned mechanism by which Tregs induce immune tolerance, two additional cell contact dependent pathways should not be left unmentioned. The cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) receptor serves as a negative regulator of T cell responses in mice and humans. This was established through a model in CTLA-4 deficient mice, leading to a downregulation of CD80 and CD86 on dendritic cells and subsequent loss of immunosuppression. (Jørgensen et al., 2019) CD80/CD86 directly influences the immunomodulatory enzyme indoleamine 2.3-dioxygenase (IDO), which catabolizes tryptophan leading to immunosuppression. (Fallarino et al., 2003) Furthermore, Tregs show an upregulation of PD-1. which interacts with multiple other cell types. By negative modulation of T effector cells, PD-1 overexpression can lead to increased tumorigenesis, invasiveness and immune evasion. (Gianchecchi & Fierabracci, 2018)

The functionally similar subset of Tr1 cells has also been identified in various malignancies. Studies evaluating tumor progression and Tr1 frequency all showed a positive correlation.(Roncarolo et al., 2018)

In regard to their produced cytokines, Th1 and Th2 cells seem to be easily attributed to anti- and pro-tumerogenic phenotypes. While Th2 cells produce IL-4. Il-5. IL-10 and IL-13. all considered immunosuppressive, Th1 cells produce potent inducers of immune responses such as IFN-γ, TNF-α and IL-2. In particular, Th1 cells seem to play a prominent role in tumor immunity. Through their cytokines, they are potent inducers of M1 macrophages. Th2 cells, on the other hand, may inhibit Th1 function via IL-10 and promote M2 macrophage differentiation.(Burkholder et al., 2014) Much more uncertain is Th17 function. Studies examining the main Th17 cytokine IL-17 showed both positive and negative associations dependent on the tumor type. One study for breast cancer suggested a pro-tumorigenic action. (Chang, 2019)

Although the role of CD8+ Tregs in humans remains unknown, studies in mice showed an inverse relationship between CD8+ CD122+ cell count and tumor survival. (Villarreal et al., 2017) Furthermore, removal of CD8+ Tregs increases

tumor-specific T cells and tumor infiltration of functional effector/memory T cells in melanoma bearing mice. (L.-X. Wang et al., 2010).

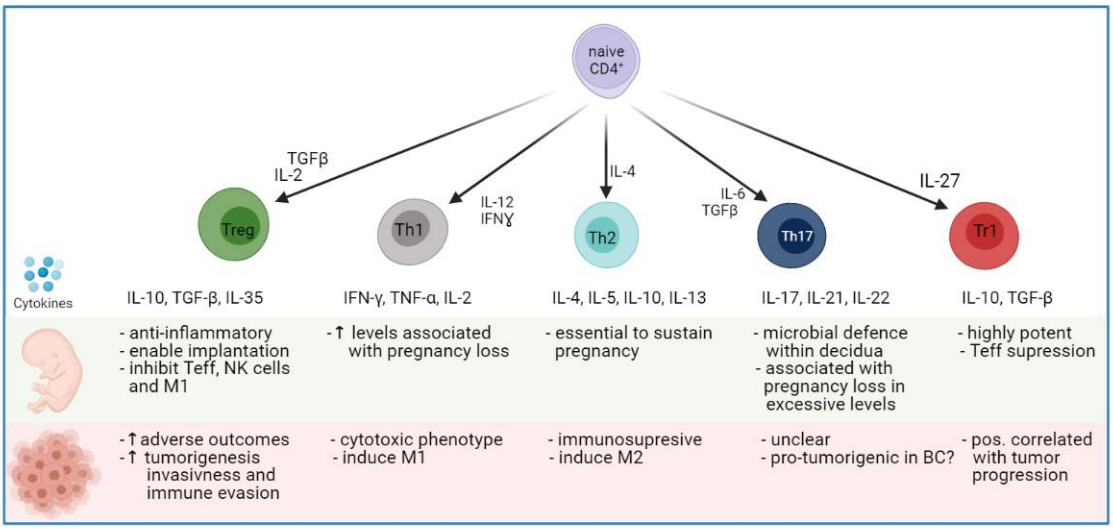

Figure 1: **Overview of regulatory CD4⁺ T cell subsets**
The common progenitor of all CD4⁺ regulatory cells are naïve CD4⁺ cells. Upon induction by cytokines, they mature into their effective state. Through the production of cytokines, they may influence pregnancy as well as tumor cells and/or the tumor microenvironment. (generated with biorender.com)

Opposing roles of regulatory cells in pregnancy and cancer, which are summarized in figure 1, prompt the conclusion of adverse outcomes in pregnant women experiencing malignant diseases. While clinical data in humans supports this hypothesis, knowledge of underlying immunologic mechanisms remains sparse. Therefore, establishment of a PABC mouse model in order to perform in-depth phenotyping of regulatory cells in pregnancy is a first step in shedding light on gestational immune tolerance and PABC.

3. Material and Methods

Table 1: **Antibodies, Reagents, Chemical, Software**

Antibodies

Target Antigen	Fluorochrome	Clone	Dilution	Source
Extracellular:				
CD3	PE-Cy7	145-2C11	1:200	BioLegend
CD4	AF700	GK15	1:100	BioLegend
CD8	BUV737	53-6.7	1:200	BD Horizon
CD25	BV605	PC61	1:100	BioLegend
CD44	PE	IM7	1:200	BD Pharmigen
CD45.2[+]	BV650	104	1:100	BioLegend
CD49b	BUV496	HMa2	1:200	BD OptiBuild
CD62L	BV711	MEL-14	1:100	BioLegend
CD122	BUV395	TM- β1	1:100	BD OptiBuild
Fixable viability dye eFluor™	V500	N/A	1:500	Invitrogen
Lag3	BV421/Pac Blue	C9B7W	1:50	BioLegend
Tim3	PE-Texas Red	5D12	1:100	BD Horizon
TruStain fcX (anti-mouse CD16/32)	N/A	93	N/A	BioLegend
Intracellular:				
CD45	APC-Cy7	30-F11	1:200	BD Pharmigen
FoxP3	PerCP-cy5.5	FJK-16s	1:100	Invitrogen
GATA3	FITC	L50-823	1:100	BD Pharmigen
RORyt	BV785	Q31-378	1:100	BD Horizon
T-bet	APC	4B10	1:100	BioLegend

[+] intraocular injection 10 min before elimination

Chemical, Reagents, etc.

Substance	Source	ID#
Collagenase A	Roche	10103586001
DMEM Medium	PAN Biotech	8811120
DNAse	Sigma	
F-12 Nutrient Mix Medium	Gibco	21765-029
Fetal bovine serum	Gibco	10082-147
Formalin		
FoxP3/Transcription Factor Fixation/Permeabilization	Invitroge	00-5521-00

L-Glutamine 200mM	Gibco	25030-024
Normal rat serum	Jackson Immuno Research	012-000-120
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco	14190-094
Penicillin/Streptomycin	Gibco	15070-063
Permeabilization Buffer 10x	Invitrogen	00-8333-56
Ix RBC Lysis Buffer	Invitrogen	00-4333-57
RNAlater™ Stabilization Solution	Invitrogen	AM7021
Trypan Blue Stain (0.4%)	Gibco	15250-061
Trypsin	Thermo Fischer Scientific	25300054

Software

Product	Source
FACSDiva	Becton Dickinson (BD), Franklin Lakes, USA
FlowJo Version 10.8.0	FlowJo LLC., Ashland, USA
Graphpad Prism Version 9.0	GraphPad Software Inc., La Jolla, USA

Animal Model

Specimen	Line#	Origin	Cat#
Mouse: C57BL/6J	6060	Charles River Laboratories	027
Balb/c	4882	Animal Facility UKE	028

Other

Tool	Source	ID#
Slide gauge	Gedore red	R94420021
FACSymphony A3	BD, Franklin Lakes, USA	

3.1. Animals

Female 8-10 week old C57BL/6J_2019 mice were acquired from Charles River Laboratories and accommodated in IVC cages at the local research animal facility throughout the experiment. Upon mating with male Balb/c bucks from the animal facility of the University Medical center Hamburg Eppendorf, a visible vaginal plug on the next morning was considered gestational day 0.5. Progression of pregnancy was monitored by daily weighing. Constant room temperature (20-24°C), relative humidity (40-70%) and a 12-hour light/dark circle were maintained and the animals received food and water ad libitum. All experiments were performed in special consideration of the 3R (replace, reduce, refine) model and in accordance with the animal ethics approval given by the State Authority of Hamburg (N20/055).

3.2. Statistical Analysis

The acquired data was analyzed with GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, USA). After screening for outliners, a Shapiro Wilk normality test was performed. With the majority of data showing parametric properties, Gaussian distribution was assumed for all further analyses. Based on two independent variables a two-way ANOVA was used for statistical testing.

3.3. Cell Lines

In consideration of the designated mouse line, I conducted a comprehensive literature review on PubMed with the search words “C57BL/6” AND “breast cancer; OR “C57BL/6” AND “mammary” AND “tumor” OR “carcinoma”. Thus, several potential cell lines were identified. Two of them, Py8119 and E0771, were selected upon their originating mouse line and properties such as metastatic potential and receptor status. While E0771 tumors were derived from spontaneous tumors in C57BL/6J mice, transgenic MMTV-PyMY (mouse mammary tumor virus promoter driven Polyoma middle T-antigen) C57BL/6 mice were used for Py8119 tumor generation. ((ATCC); Ewens et al., 2005; Le Naour et al., 2020; Pulaski & Ostrand-Rosenberg, 2001; Stagg et al., 2010; Steenbrugge et al., 2019). Both selected cell lines were gratefully received from Prof. Dr. Dr. Sonja Loges, German Cancer Research Center, University Medical Center Mannheim.

Table 2: **Characteristics of potential malignant cell lines in regard to key tumor features.**

Cell Line	Species	Strain	Metastatic Potential	Receptor Status
E0771	Mouse	C57BL/6	high	Controversial
Py8119	Mouse	C57BL/6	low	Triple-negative
Py230	Mouse	C57BL/6	low	HR-, HER2+
4T1	Mouse	Balb/c	high	Triple-negative

3.4. Cell Cultures

Cells were stored in liquid nitrogen prior to use. After thawing the frozen vials in a water bath, cells were transferred to a falcon tube and centrifuged at 1000rpm for five minutes at room temperature. The supernatant was discarded and the pellet suspended in the respective medium. The suspension was then transferred to a 75cm^2 flask and grown in an incubator set to 37° Celsius(C) and 5% CO_2. Growth medium was changed regularly and passage did not exceed more than eight cycles to ensure cell line stability. For cell line Py8119 F-12 Nut Mix Medium with 25ml fetal bovine serum (FBS) and 5 ml Penicillin/Streptomycin (Pen/Strep) and for line E0771 DMEM, 50ml of FBS, 5ml of Pen/Strep and 5 ml of L-Gultamine were used.

For cell harvesting the medium was removed via a suction pipette and 3ml trypsin were applied and incubated for 5 min. Trypsination was halted by adding 6ml of the corresponding medium. The flask content was transferred to a tube and centrifuged for 5min at 1000rpm and 21°C. Subsequently, the supernatant was discarded and the pellet was titurated in PBS for injection or growth medium for further incubation. Using a Neubauer counting chamber, cells were diluted in Trypan Blue (1:100), counted and calculated according to further use (Injection 0.5x10^6 cells).

3.5. Experimental Layout

3.5.1. Part I: Survival

To validate the model in regard to viability, tumor morbidity as well as mortality, I conducted a survival study consisting of both cell lines and a control group. Pregnant mice were generated by mating female C57BL/6 mice with Balb/c bucks in a 1:2 mating. The next morning a vaginal plug check was used to determine pregnancy. If a plug was visible, gestational day 0.5 was assumed. Virgin and pregnant mice were then randomly allocated to each group.

For the injection of $0.5x10^6$ tumor cells in 200ml PBS, mice were placed under brief CO_2 anesthesia. The cells were orthotopically injected in mammary fat pads 4 or 5. Body, food and water weight measures were acquired daily. In combination with a body conditioning score, the well-being of the animals was ensured throughout the entire experiment. To assess tumor growth and size, the injection region was palpated every other day and tumor nodes were measured using a sliding gauge. The volume was calculated via a well-established formula for mammary tumors in rodents, Volume = $\frac{width^2 \times length}{2}$. (Faustino-Rocha et al., 2013)

In alignment to the animal experiment proposal, a maximum life span of 28 days after tumor injection was implemented. Additionally, elimination criteria were defined: body weight reduction >20% in virgin and weight stagnation in pregnant mice, gross behavioral changes (apathetic behavior, stereotypies), coordination deficit, dyspnea, bloody diarrhea, dietary cessation, tumor ulceration, or a tumor volume of $>1500mm^3$. Elimination was conducted by CO_2 exposure and subsequent cervical dislocation.

Upon elimination, the liver, lung, sentinel lymph node, and a part of the tumor were dissected and placed in formalin. After 24 hours the samples were transferred to ethanol and later fixated in paraffin. Additionally, a smaller part of the tumor was placed in RNAlater and stored at -20°C for further analysis. The serum was centrifuged for 20min at 13000rpm and 4°C. Plasma portions of various volumes were then stored at -20°C as well.

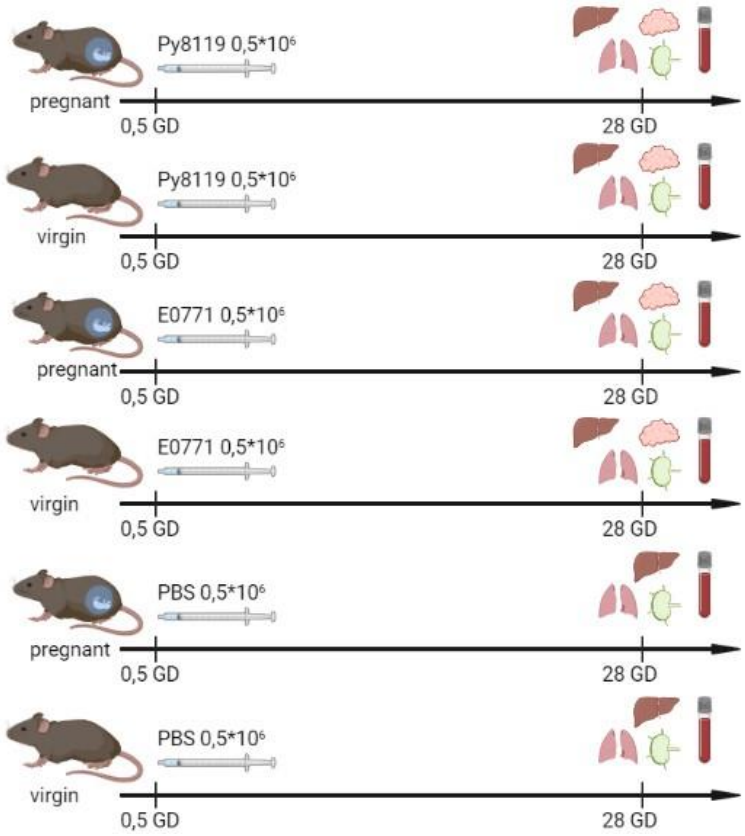

Figure 2: **Experimental layout of part I (survival)**
As a first step, a survival study was conducted to test the model's viability. C57BL/6J females were mated with Balb/c males. The morning of the vaginal plug appearance is considered gestational day 0.5. If termination criteria were not met before, animals were eliminated on day 28. After elimination, several organs were harvested. Liver, lung, and sentinel lymph node, as well as a part of the tumor were fixated in paraffin. Additionally, a smaller part of the tumor was stored in RNAlater. Serum was centrifuged and the plasma stored.
(generated with biorender.com)

3.5.2. Part II: Kinetic analyses of cellular subsets

In the next step, I aimed to acquire data on trajectories of T cell subpopulations throughout pregnancy. Therefore, another experiment was conducted with read outs at gd7.5 and gd14.5. Additionally, the data was used to undermine findings for tumor morbidity and burden from the first trial. Tumor injections were performed with the same technique as previously described, with the only difference being a switch from CO_2 anesthesia to Isoflurane for facilitated animal handling. Mice were then randomly allocated to either gd7.5 or gd14.5 groups.

Upon elimination, the aforementioned organs were harvested in addition to fetal and placental samples on gd14.5. After weighing, placentas were fixated in formalin and fetuses in Bouin solution. On gd7.5. on the other hand, implantations were counted but no pregnancy associated tissues were acquired.

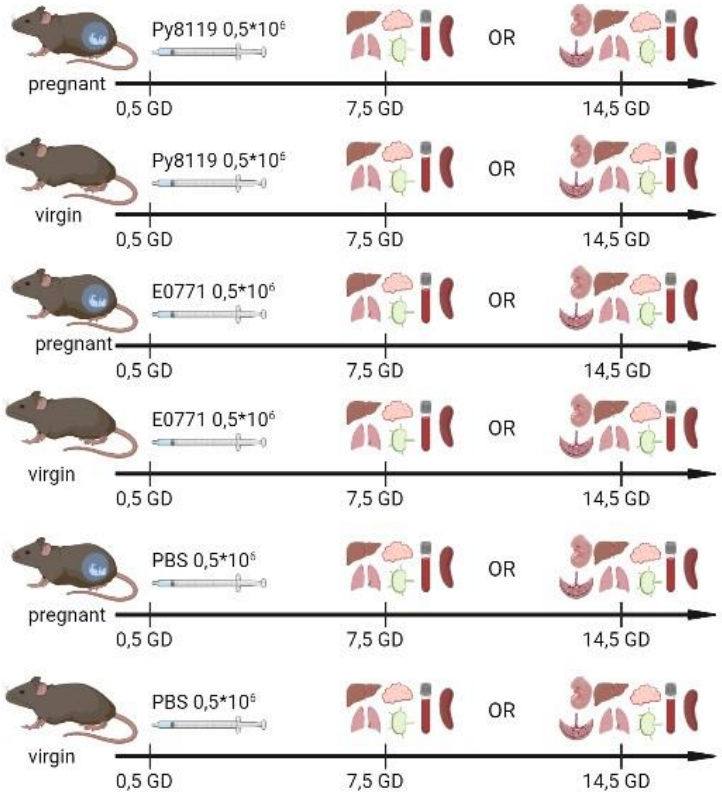

Figure 3: **Experimental layout of part II (kinetics)**
Animal model for the establishment of T-cell kinetics during pregnancy. C57BL/6J females were mated with Balb/c males. The morning of the vaginal plug appearance is considered gestational day 0.5. Mice were randomly allocated to the gd7.5 or gd14.5 group. Tissue samples were collected in the same way as previously described. Furthermore, the uterus was inspected for implantations on gd7.5 and fetuses and placentas fixated on gd14.5. (generated with biorender.com)

3.6. Tissue Preparation and Cell Staining

3.6.1. Tissue Preparation

Tissue samples of the sentinel lymph node, spleen and tumor were obtained on the day of staining and stored continuously on ice in PBS (spleen, lymph node) or medium (tumor).

After the tumor was fragmented with a scissors, it was placed in a 50ml tube with 15ml of disaggregation solution consisting of Collagenase A in growth medium (0.2 mg Collagenase A/ml medium). It was incubated at 37°C for one hour on a

rotating incubator. The supernatant was then discarded and 15 ml of disaggregation solution added for additional 30 minutes(min) of incubation. Another 30min incubation cycle was done after adding 10ml of DNAse solution (0.015mgDNAse/ml PBS). Then the sample was run through a 70µm sieve, suspended with PBS to 40ml, centrifuged for 5min at 450rpm, and again suspended with 1ml PBS after the supernatant was discarded. If the sample seemed macroscopically hemorrhagic, it was additionally lyzed with 5ml of Red Blood Cell Lysis Buffer for 5min. Lysis was stopped by adding 30ml of PBS and the samples then spun down and suspended as previously described.

The Spleen was prepared by processing through a 40µl sieve with consecutive lysis, centrifugation and resuspension according to the tumor protocol. The same process was used for the lymph node, except for lysis, which was not required.

After dilution in trypan blue (1:100), cells were counted using a Neubauer counting chamber. Each vial was filled with $1x10^6$ Cells.

3.6.2. Cell Staining and Flow Cytometry

In the first step, the cells were washed with 1ml of PBS and centrifuged at 450rpm for 8min. Subsequently, the supernatant was discarded and the samples were incubated for 20min at 4°C with TruStain fcX (anti-mouse CD16/32; 1:200) and normal rat serum (1:100) to block nonspecific binding. Afterwards, the extracellular antibody-mix, as well as Dead/Live staining (Fixable viability dye eFluor$^{TM)}$ were added. The samples were then incubated for 30min at 4°C. After incubation, another washing cycle was performed.

For fixation of extracellular antibody binding, 900µl of FoxP3/Transcription Factor Fixation/Permeabilization (1:4) were added while vortexing and incubated for 20min at 4°C. The samples were then washed three times with Permeabilization Buffer 10x (1:10 with distilled H_20). The extracellular antibody-mix was added and incubated for 30min at 4°C. The samples were washed again with Permeabilization Buffer 10x before adding 200µl of PBS for further analysis.

Flow cytometry data was acquired with a BD FACSymphony A3 cytometer and FACSDiva software. For further analyses, FlowJo Software version 10.8.0 was used.

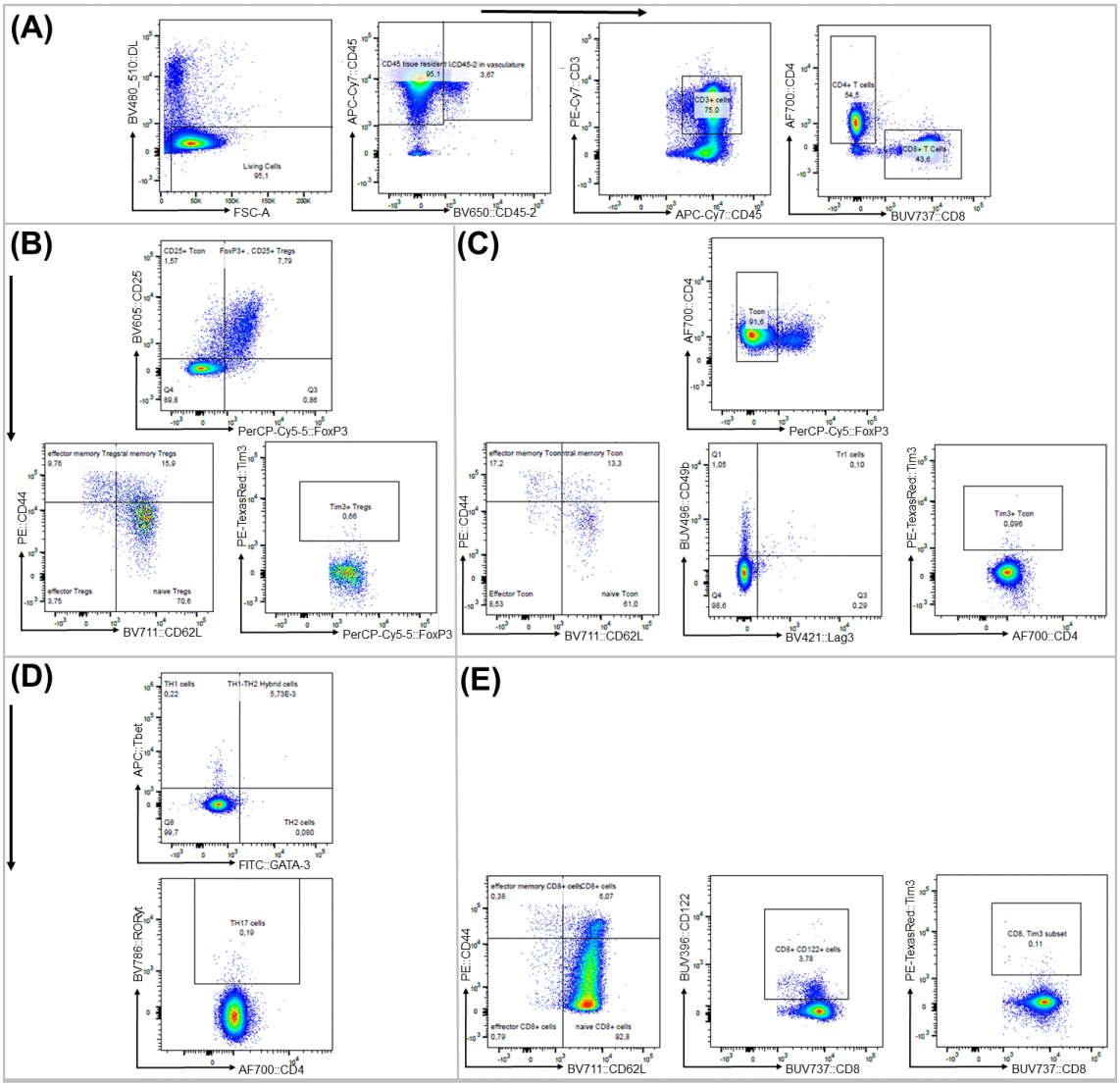

Figure 4: **Gating strategy**
The data was analyzed via the depicted gating strategy using FlowJo software. **(A)** Living cells were first selected through a D/L marker. By injecting a CD45-2 antibody before elimination, tissue resident cells can be distinguished from circulatory cells. To stain for specific lymphocytes further markers, e.g. CD3 and CD4 vs. CD8 are then applied. The clusters represent subpopulations of immune cells, (B) Tregs, **(C)** Tcon, **(D)** T helper cells and **(E)** CD8 cells. Cluster (E)'s parent population are $CD8^+$ cells. All other subpopulations share $CD4^+$ cells as a parent. While in **(A)** the hierarchy of parent populations is from left to right, in **(B)**, **(C)**, **(D)** and **(E)** it is from top to bottom.

3.6.3. Morbidity and Mortality

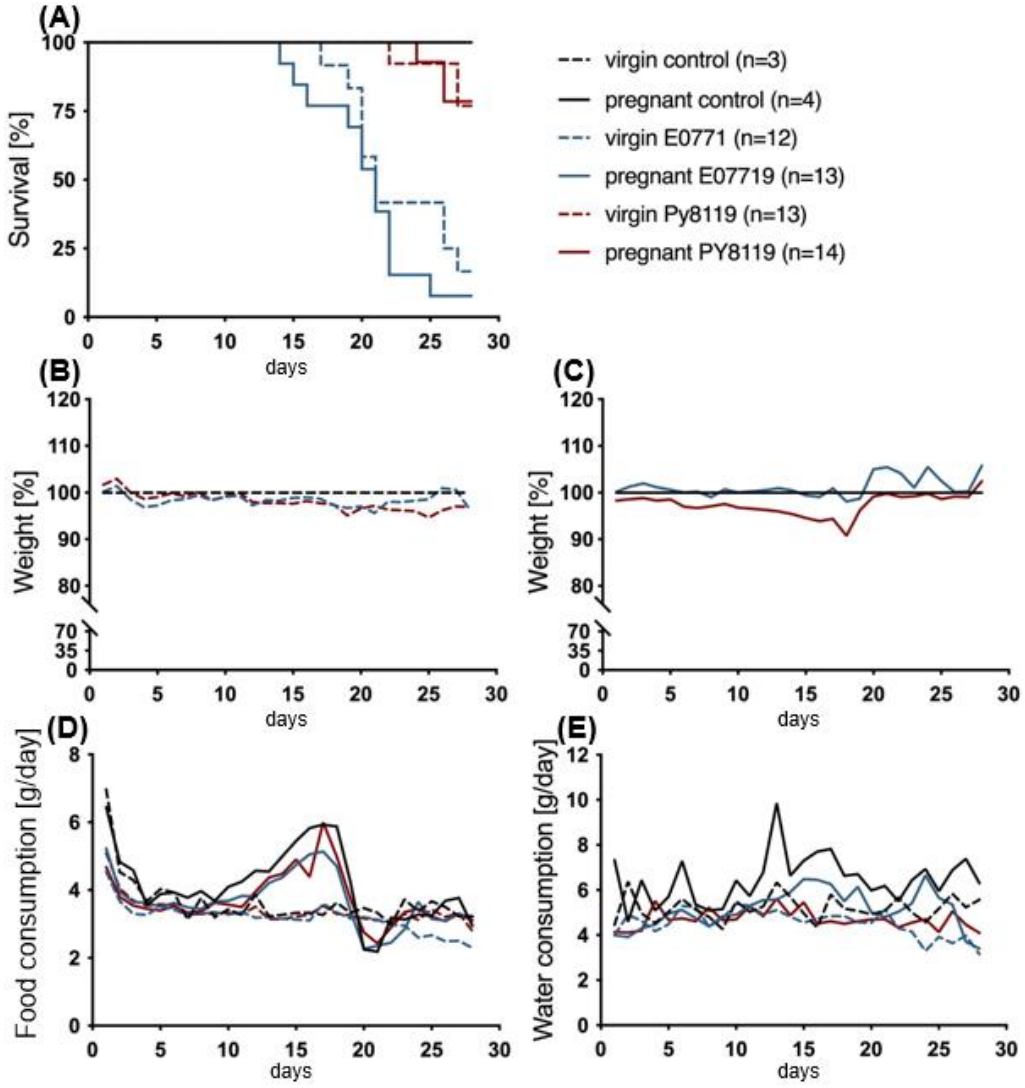

Figure 5: **Survival and morbidity**
(A) Mice with E0771 tumors showed a lower survival rate than Py8119. The majority of E0771 mice had to be sacrificed due to tumor maximum before day 28. In this group, pregnant mice had lower survival rates than virgin mice. For Py8119. the most common cause for elimination was reaching day 28. No controls were lost in the experiment's timespan. The weight development seemed independent of cancer in virgin **(B)** as well as pregnant **(C)** mice. The same was observed for food **(D)** and water **(E)** intake. Data expressed in (B), (C), (D) and (E) expressed as mean value.

Survival curves were different between the groups. (figure 5) While all controls reached day 28 in the Py8119 group 76.92% of virgin and 78.557% of pregnant mice did. For E0771 the numbers were 16.67% and 8.33%, respectively. In E0771-injected mice, the most common cause for elimination was maximum tumor size (1500mm^3). It was reached in 41.67% of virgin and 76.92% of pregnant

specimens. I also assessed for morbidity in tumor bearing mice. Measures to determine tumor cachexia were weight of the mice as well as food and water intake. For changes in weight, the mean of the controls was determined as 100%. More than 20% difference would have been considered a reason to sacrifice and more than 10% a reason for an intensified well-being surveillance of the mice. In both groups, no deviation of more than 10% in comparison to the controls occurred. In addition to the daily performed body condition scoring, which yielded no indications of health deficits due to tumor burden, these findings suggest general well-being. This assumption is further supported by unaltered eating and drinking behavior.

3.6.4. Tumor Characteristics

The day of tumor onset was defined as a palpable mass for which I observed earlier occurrence in pregnant mice. For E0771 tumors, the mean day of onset was day 7.58 after injection in virgin and gd7.54 in pregnant mice. The difference was more pronounced in Py8119 tumors with day 8.85 and gd7.43 respectively. Tumor volume differed between virgin and pregnant mice as well. While the volume of Py8119 tumors shows a clear gap between the groups widening over time, the volume curve for E0771 tumors seems to progress rapidly in size at first but then regresses. (see figure 6) This can be explained by the marked decrease in survival experienced in this cell line. Mice with tumors reaching a maximum size before gd28 had to be sacrificed. Therefore, tumors exceeding the maximum size did not yield further data points to the volume curve giving it the impression to decline.

The observed trend in differing tumor dimensions of virgin and pregnant tumors were verified by a significant difference in tumor weight. In both cell lines, pregnant tumors were heavier than virgin tumors with $p \leq 0.05$ for Py8119 and $p \leq 0.01$ for E0771.

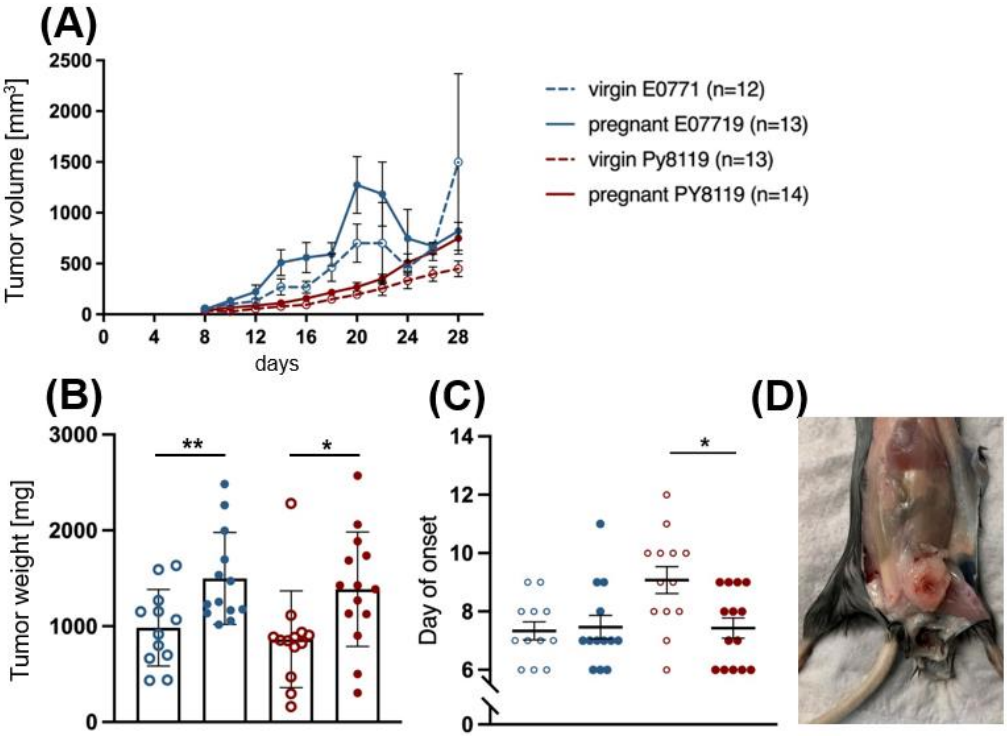

Figure 6: **Tumor characteristics**
(A) A clear gap in tumor volume between virgin and pregnant mice could be observed over time. The decrease in tumor volume of E0771 tumors can be explained by limited survival due to maximum tumor size in this cell line. **(B)** Tumor weight was significantly different in Py8119 tumors ($p \leq 0.05$) as well as E0771 ($p \leq 0.01$) tumors. **(C)** There was a significant difference in the day of onset between virgin and pregnant mice injected with Py8119 ($p \leq 0.05$). No difference was observed in E0771-injected mice. **(D)** Depicted is a tumor of the cell line Py8119 injected into a virgin mouse. The location of the injections correlated with mammary pads 4/5.
Data expressed as mean ±SD

3.6.5. Neonatal Outcome

Offspring count was different between the groups, with 8.25 pups in the control group, 7.21 for Py8119 and 9.4 for E0771. There was no significant difference in fetal weight between the groups (see figure 7). The duration of pregnancy was also unaffected in tumor mice (control=19.0 days; E0771=18.7 days, Py8119=18.83 days)

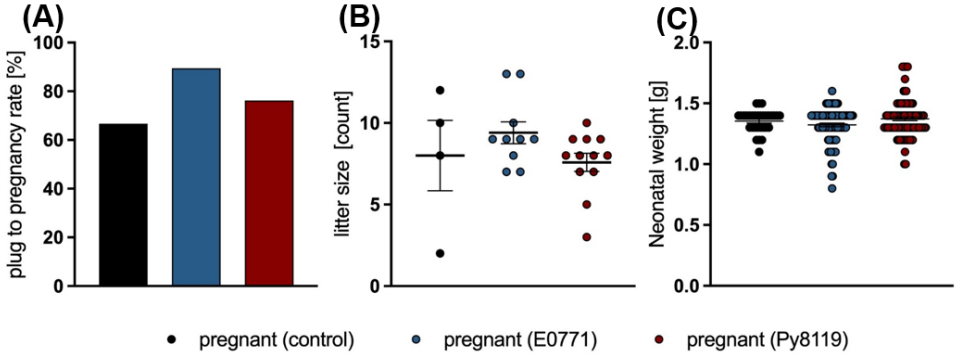

Figure 7: **Neonatal outcome**
C57BL/6J females which were mated with a Balb/c male overnight were checked every morning for a vaginal plug, which was defined as gd0.5. Depicted in **(A)** is the count of actual pregnancies, determined by weight gain, in comparison to the observed vaginal plugs. In regard to pregnancy outcome, neither litter size **(B)** nor neonatal weight **(C)** showed significant differences. Data expressed as mean and ±SD for (B) and (C)

Taken together, I was able to demonstrate heavier tumors in pregnant mice with slightly decreased survival and unaltered offspring. This data suggests that the model reproduces observed outcomes in humans and is therefore a viable option to study PABC in mice.

3.7. T Cell Kinetics

After establishment of the mouse model for PABC, the next step was to conduct a comprehensive phenotyping of the immune environment to assess the role of regulatory T cells. Therefore, I conducted flow cytometric analyses of tumor, lymph node and spleen tissue at two different time points (gd7.5; gd14.5) in order to account for immunity changes during the course of pregnancy. Additionally, I tried to validate my previous findings of morbidity, mortality, pregnancy outcomes and tumor characteristics.

3.7.1. Morbidity, Mortality and Pregnancy Outcomes

Similar to the previous experiment, no differences in weight development between the groups were observed. Furthermore, water and food intake of cancer bearing mice were unaltered as well. Neither in the gd7.5 nor gd14.5 group a mouse had to be sacrificed prematurely.
Pregnancy outcome was assessed based on the specimens gestational age upon elimination. For mice on gd7.5. only the count of implantations could be

counted. However, on gd14.5. fetal and placental weight were gathered. Neither yielded differences between virgin and pregnant mice. Additionally, the uterus was examined for abortions upon dissection. Abortion count was not significantly different between the tumor groups compared to the controls. (see figure 8) These findings support previous data suggesting that the offspring is unaffected by cancer development.

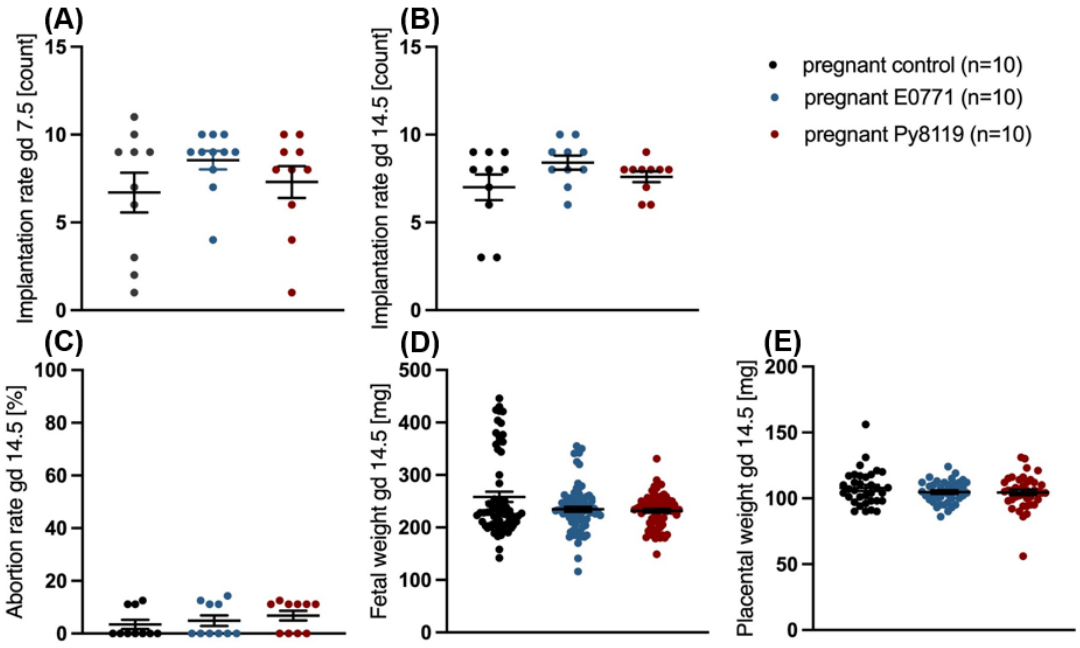

Figure 8: **Pregnancy outcome gd7.5 and gd14.5**
Both cell lines showed similar implantation rates on gd7.5 **(A)** and gd14.5 **(B)** compared to controls without tumor injection. **(C)** The abortion rate obtained on gd14.5 and expressed as percentage of total implantations was also unaffected by tumor injection. Similarly, no changes on fetal **(D)** or placental weight **(E)**could be observed. Data expressed as mean ±SD

3.7.2. Tumor Characteristics on gd7.5 and gd14.5

The tumor measures in the second experiment were conducted as previously described for the survival experiment. Tumor volume increased from gd7.5 to gd14.5. While the difference in volume increase between virgin and pregnant mice injected with Py8119 was minimal, E0771-injected mice had a more pronounced gaping between the two groups. These findings are consistent with previous findings. This effect can also be attributed to the more aggressive phenotype of E0771 tumors. Similarly, tumor weight showed an increase for both cell lines from gd7.5 to gd14.5. Additionally, the same pattern as in tumor

volume was observed for virgin-pregnant trajectories between the cell lines. Py8119 tumors again showed minimal weight difference on day 14.5 while E0771 did when virgin and pregnant specimens were compared. This trend did not reach significance. Nevertheless, when combined with the data of the survival experiment, tumor trajectories showed to match previous growth rates. In combination with pregnancy outcome, the data for tumor progression and weight further endorse the newly established model as viable to investigate PABC.

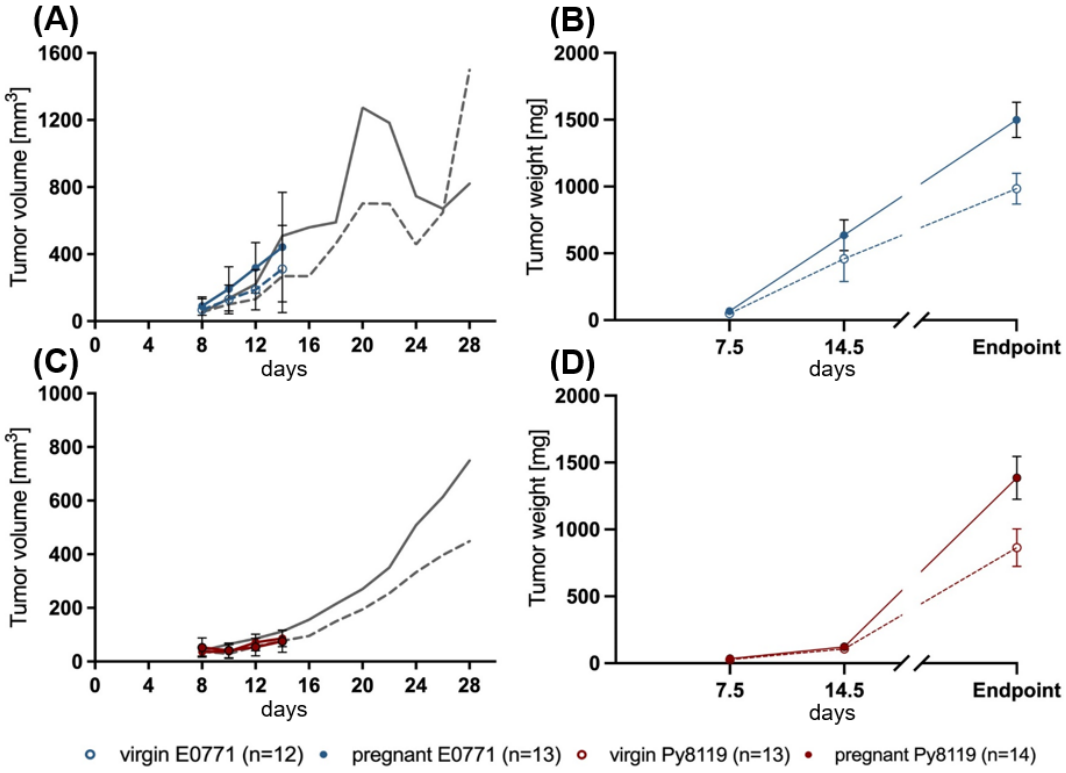

Figure 9: **Tumor characteristics on gd7.5 and gd14.5**
The volume growth curves obtained from the second experiment match with trajectories observed during the survival experiments. Depicted in grey are the correlating curves from the survival experiment merged with acquired E0771 **(A)** or Py8119 **(C)** data in color. **(B)** While tumors of the E0771 cell line show gaping between virgin and pregnant mice early on, **(D)** Py8119 tumors show minimal difference between gd7.5 and gd14.5 which increases with more time elapsed.
Data expressed as mean ±SD

3.7.3. Flow Cytometry of T Cells

3.7.3.1.CD4+ T cell

Besides the previously described regulatory subsets of $CD4^+$ cells (see figure 1), I additionally examined levels of conventional $CD4^+$ cells (Tcon). First established for $CD8^+$ cells but now widely acknowledged for Tcons as well, is the classification into naïve ($CD44^-$, $CD62L^+$), central memory ($CD44^+$ $CD62L^+$), effector ($CD44^-$, $CD62L^-$) and effector memory ($CD44^+$, $CD62L^-$) Tcons. Except for the latter ones, the data for these subsets did not yield any significant results. Only effector memory Tcons were significantly increased in the spleen of pregnant mice on gd14.5 (control $p \leq 0.05$; E0771 $p \leq 0.0001$; Py8119 $p \leq 0.0001$). Nevertheless, there was no difference between the control and tumor groups. (data not shown)

Table 3: CD4+ subset levels; parent population in parenthesis
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

	$CD4^+$ ($CD3^+$)		**Treg** ($CD4^+$)		**Th1** ($CD4^+$)		**Th2** ($CD4^+$)		**Th17** ($CD4^+$ $Tbet^-$ $GATA3^-$)		**Tr1** ($CD4^+$ $FoxP3^-$)	
	NP	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**
Control												
Spleen gd7.5												
Mean	53.23	52.97	5.4	7.62	0.51	0.46	0.14	0.29	0.01	0.17	0.08	0.1
SD	2.94	4.21	1.22	2.16	0.29	0.19	0.09	0.12	0.05	0.1	0.03	0.05
Significance												
Spleen gd14.5												
Mean	52.34	52.08	5.92	9.42	1.02	0.63	0.22	0.16	0.05	0.09	0.07	0.1
SD	2.85	6.63	1.25	2.16	0.95	0.37	0.18	0.09	0.03	0.06	0.04	0.04
Significance												
LN gd7.5												
Mean	54.07	51.97	7.11	6.81	0.14	0.23	0.07	0.18	0.1	0.15	0.15	0.1
SD	6.73	3.61	2.78	1.72	0.07	0.21	0.06	0.1	0.06	0.07	0.13	0.05
Significance												
LN gd14.5												
Mean	53.45	54.98	6.43	7.51	0.19	0.14	0.11	0.12	0.12	0.13	0.13	0.19
SD	3.43	10.9	1.29	2.37	0.13	0.07	0.08	0.06	0.06	0.06	0.05	0.11
Significance												
E0771												
Spleen gd7.5												
Mean	54.06	54.73	5.92	7.77	0.9	0.91	0.23	0.2	0.07	0.11	0.06	0.14
SD	2.9	0.97	1.68	1.88	0.91	0.96	0.12	0.12	0.03	0.06	0.04	0.08
Significance												
Spleen gd14.5												
Median	55.14	55.39	7.22	8.81	0.45	1.15	0.17	0.34	0.03	0.09	0.07	0.16
SD	2.56	6.84	3.18	4.83	0.23	1.09	0.11	0.32	0.02	0.1	0.04	0.1
Significance											*	*
LN gd7.5												
Mean	52.57	52.69	6.14	7.2	0.1	0.19	0.25	0.11	0.09	0.13	0.15	0.14
SD	4.38	4.07	1.38	1.51	0.07	0.12	0.2	0.09	0.04	0.07	0.04	0.05
Significance												
LN gd14.5												
Mean	51.28	51.61	7.89	8.96	0.15	0.42	0.11	0.29	0.07	0.08	0.11	0.27
SD	2.42	1.77	1.9	2.84	0.14	0.33	0.06	0.19	0.04	0.06	0.04	0.23
Significance					*	*	**	**				

Tumor gd14.5												
Mean	36.92	28.72	29.78	25.18	1.69	0.95	1.47	0.8	0.14	0.15	0.36	0.12
SD	8.6	3.72	12.23	10.91	1.72	0.78	1.17	0.46	0.16	0.15	0.17	0.09
Significance											***	***
Py8119												
Spleen gd7.5												
Mean	52.55	52.95	4.77	6.45	0.94	0.8	0.2	0.44	0.06	0.15	0.07	0.10
SD	2.53	2.75	2.09	3.43	0.67	0.5	0.06	0.22	0.02	0.1	0.05	0.08
Significance							**	**				
Spleen gd14.5												
Mean	54.72	54.96	6.93	10.46	0.21	0.54	0.29	0.23	0.09	0.14	0.07	0.08
SD	4.21	5.92	2.03	1.95	0.06	0.35	0.22	0.14	0.07	0.08	0.03	0.03
Significance												
LN gd7.5												
Mean	52.07	52.89	6.32	7.19	0.19	0.29	0.13	0.13	0.09	0.16	0.16	0.17
SD	4.27	5.66	0.98	1.66	0.11	0.19	0.1	0.11	0.05	0.08	0.1	0.11
Significance												
LN gd14.5												
Mean	52.52	50.85	6.77	7.57	0.16	0.15	0.13	0.09	0.12	0.16	0.09	0.14
SD	1.72	4.35	0.74	2.05	0.13	0.1	0.07	0.03	0.06	0.08	0.03	0.06
Significance												
Tumor gd14.5												
Mean	44.8	40.85	23.36	18.58	1.21	1.82	2.55	1.42	0.06	0.13	0.07	0.07
SD	14.14	12.01	3.58	7.66	1.28	1.87	1.93	1.08	0.03	0.07	0.04	0.07
Significance												

Tregs

Besides the expression of CD4 and their master regulator gene FoxP3. Tregs were defined by co-expression of IL-2 receptor subunit alpha (CD25). Furthermore, subpopulations of Tregs such as effector memory ($CD44^+$, $CD62L^-$), central memory ($CD44^+$, $CD62L^+$), naïve ($CD44^-$, $CD62L^+$) and highly potent $Tim3^+$ Tregs were quantified. Interestingly, I was also able to identify a distinct Treg subpopulation double negative for CD44. CD62L, here termed effector Tregs. While this nomenclature has been established for $CD8^+$ and $CD4^+$cells, Treg classification is usually limited to the previously mentioned subtypes because CD44 is commonly expressed by Tregs. (Liu et al., 2009)
Pregnancy associated non-significant increases in splenic $CD4^+$ $FoxP3^+$ $CD25^+$ Tregs were noted in all pregnant groups regardless of tumor status.
No differences in Treg levels within the tumor tissue or the tumor draining LN were observed between the groups. (see table 3) However, two subpopulations of Tregs, namely effector Tregs and $Tim3^+$ Tregs yielded significant results. These cells occurred more frequently in the spleen and tumor (effector Tregs; figure 10) and lymph node ($Tim3^+$ Tregs; figure 11) of pregnant mice experiencing cancer.

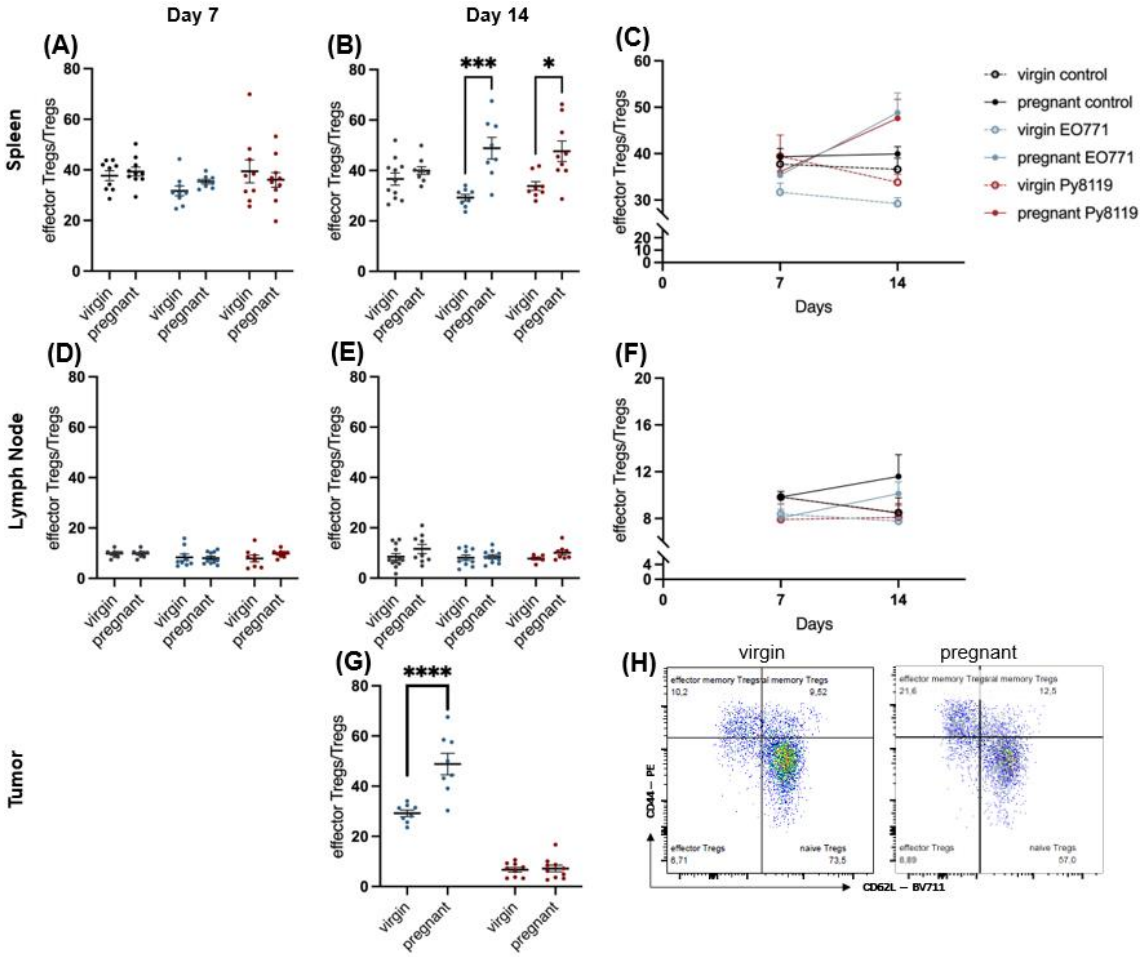

Figure 10: **Effector Treg levels on gd7.5 and gd14.5**
Neither splenic nor lymph node levels of $CD4^+$ $FoxP3^+$ $CD25^+$ $CD44^-$ $CD62L^-$ effector Tregs yielded significant differences between the groups on gd7.5. **(A, D)** On the other hand, on gd14.5. significantly higher levels of splenic effector Tregs were observed in pregnant tumor mice compared to the virgin counterparts **(B)** This increase becomes especially clear when looking at the cell trajectories **(C)** Unlike the spleen, the LN did not yield significant results in cell levels or trajectories. **(E, F)** The tumor, on the other hand, only showed significant results for one cell line, namely E0771. **(G) (H)** Depicts the applied gating strategy for this subset.
Data expressed as mean [%] ±SEM with each dot representing one specimen. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

In regard to $CD8^+$ and $CD4^+$cell nomenclature, I termed the identified double negative population of Tregs effector Tregs. Although they represent a small fraction of Treg subsets, significant differences in the tumor of E0771 mice and spleen of both tumor groups may indicate a distinct phenotype. Furthermore, I detected an increase of effector Tregs from gd7.5 to gd14.5 in the spleens of pregnant tumor mice, while levels in virgin and pregnant control mice remained steady. Analysis of the tumor draining lymph node did not show any differences.

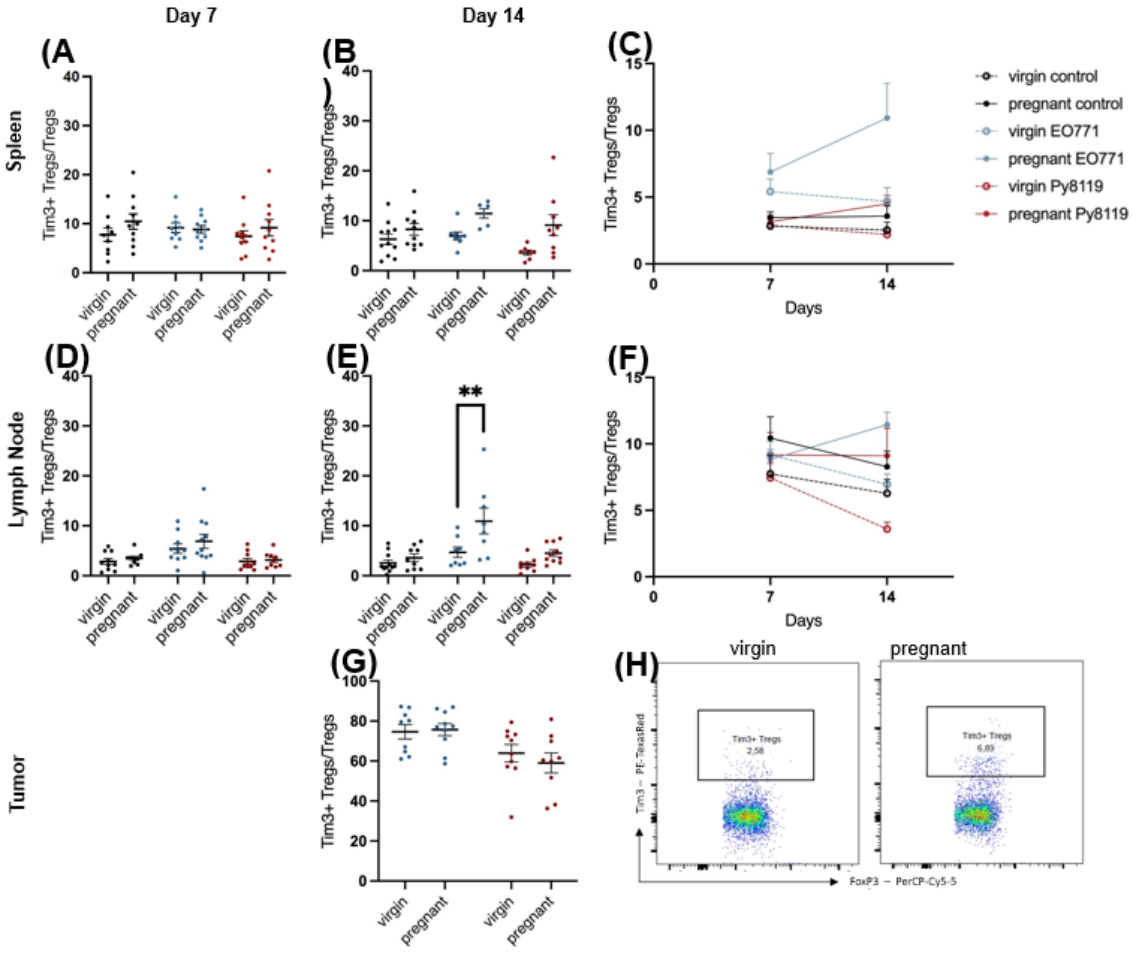

Figure 11: **Tim3⁺ Treg levels on gd7.5 and gd14.5**
Similarly, to effector Tregs, CD4⁺ FoxP3⁺ CD25⁺ Tim3⁺ Tregs only showed significant differences (LN) or trends (spleen) between the groups on gd14.5 **(B, E)** Meanwhile, there were no differences on gd7.5 as well as in the tumor **(A, D, G)** Trajectories of this population were also notably different in LN and Spleen for mice injected with E0771 tumor cells **(C, F) (H)** Depicts the applied gating strategy for this subset.
Data expressed as mean [%] ±SEM with each dot representing one specimen. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001

Tim3⁺ Tregs represent a highly potent subpopulation of Tregs and are therefore of interest in PABC. On gd7.5 there were no significant differences in any organ. Though on gd14.5 a trend of increased Tim3⁺ Tregs in the spleen of tumor mice (E0771 p=0.4606; Py8119 p=0.0770) and a significant increase in the lymph node of E0771-injected mice were detected (p≤0.01). Interestingly, the trajectory of Tim3⁺ Tregs in the spleen of pregnant E0771 mice shows a prominent increase from gd7.5 to gd14.5. The tumor did not yield any differences of cell levels.

T Helper Cells & Tr1 Cells

Pregnancy is considered a Th2 dominant state in contrary to an otherwise Th1 dominant state. Consistent with this theory, I was able to observe a minor trend to decreased Th1/Th2 ratios in pregnant controls compared to virgin mice in the spleen and lymph node. Results for tumor bearing mice were mixed without any significant changes between the groups at both time points and all three organs. Interestingly, Th17 cells showed a trend to increase in pregnant mice in the spleen as well as the lymph node. Within the breast cancer groups, only Py8119 tumors followed this trend in contrast to equal levels in E0771-injected specimens. Nevertheless, Th17 cells did not differ between the controls and tumor groups, suggesting an insignificant role in PABC.

Flow cytometric analysis of Tr1 cells yielded very low cell counts, making it difficult to draw reliable conclusions for this subset. While the spleen showed a significant increase of Tr1 cells on gd14.5 in the pregnant E0771 group ($p \leq 0.05$), the opposite was observed in the tumor itself ($p \leq 0.001$). Besides, no significant differences were detected. (see table 3)

3.7.3.2.CD8+ T cells

$CD8^+$ cells are commonly known as cytotoxic effector cells. Similar to $CD4^+$ Tcons, they were assigned to distinct subsets based on cellular markers with naïve ($CD44^-$, $CD62L^+$), central memory ($CD44^+$ $CD62L^+$), effector ($CD44^-$, $CD62L^-$) and effector memory ($CD44^+$, $CD62L^-$) phenotypes. While effector $CD8^+$ cells seem unaltered by malignancy, memory phenotypes were increased in some cases. Central memory cells were significantly elevated on gd14.5 in the lymph node of tumor bearing pregnant mice. On the hand, effector memory cells were significantly higher in the spleen of pregnant mice on gd14.5. In both instances, the other wo organs did not show any differences. Effector $CD8^+$ cells were neither influenced by pregnancy nor the tumor. Naïve $CD8^+$ cells, the reservoir for the previous subsets, only differed on gd14.5 in the spleen of Py8119 and the lymph node of E0771 pregnant mice showing lower levels. (see table 4)

Table 4: CD8+ subset levels; parent population in parenthesis
* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001

	CD8⁺ (CD3⁺)		**CD8⁺ Eff.** (CD8⁺)		**CD8⁺ EM** (CD8⁺)		**CD8⁺ CM** (CD8⁺)		**CD8⁺ N** (CD8⁺)	
	NP	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**	**NP**	**P**
Control										
Spleen gd7.5										
Mean	37.82	38.47	2.75	2.61	1.5	2.34	13.32	15.9	82	78.42
SD	4.10	5.03	1.49	1.44	0.33	1.47	2.31	5.4	2.25	6.7
Significance										
Spleen gd14.5										
Mean	41.72	36.27	1.88	2.77	1.46	2.81	13.73	16.4	82.1	77.98
SD	5.22	6.33	0.8	1.92	0.35	1.07	2.23	4.1	1.49	4.38
Significance										
LN gd7.5										
Mean	42.54	44.72	2.55	1.76	0.94	0.76	9.28	8.51	83.93	88.12
SD	6.76	3.53	1.94	1.6	0.55	0.30	3.5	2.21	7.71	1.76
Significance										
LN gd14.5										
Mean	44.05	41.75	1.08	2.58	0.59	0.73	8.63	9.96	89.72	85.36
SD	3.55	3.8	0.39	2.19	0.19	0.39	2.15	1.94	2.28	4.97
Significance										
E0771										
Spleen gd7.5										
Mean	38.22	36.78	2.03	3.46	2.28	2.61	14.96	17.08	79.95	76.85
SD	2.18	1.41	1.85	1.56	1.37	0.9	3.76	1.68	6.33	2.97
Significance										
Spleen gd14.5										
Mean	37.8	33.53	1.58	6.85	1.41	4.55	13.68	13.33	82.86	75.64
SD	2.08	5.78	1.15	2.92	0.65	1.52	2.99	3.24	4.99	2.59
Significance					***	***				
LN gd7.5										
Mean	42.74	42.98	3.53	2.25	1.37	0.68	9.95	9.28	85.15	87.74
SD	3.72	2.62	3.01	1.36	1.02	0.29	3.22	2.04	5.24	2.77
Significance										
LN gd14.5										
Mean	41.85	42.13	0.87	1.36	1.08	0.93	9.69	14.14	88.57	82.95
SD	1.87	9.06	0.22	1.06	0.5	0.53	0.9	3.49	0.84	5.52
Significance							**	**	*	*
Tumor gd14.5										
Mean	37.6	29.5	1.3	1.65	82.14	72.99	12.94	18.41	1.92	1.58
SD	8.93	18.31	1.07	1.28	9.58	21.04	6.58	11.86	1.46	0.98
Significance										
Py8119										
Spleen gd7.5										
Mean	39.51	38.32	4.74	3.8	1.71	2.04	12.71	14.92	80.94	79.7
SD	1.61	2.55	4.27	2.55	0.98	0.88	3.46	2.48	4.51	3.85
Significance										
Spleen gd14.5										
Mean	38.14	35.5	2.09	7.13	1.07	4.4	13.29	16.9	83.36	68.58
SD	3.52	5.19	1.03	8.53	0.3	2.14	2.86	6.18	2.86	11.57
Significance					****	****			****	****
LN gd7.5										
Mean	44.03	43.49	5.19	5.38	0.81	1.18	9.04	9.03	84.61	84.41
SE of Diff.	4.61	4.86	4.89	4.53	0.35	0.91	1.65	1.96	5.7	5.51
Significance										
LN gd14.5										
Mean	44.73	44.02	0.75	0.9	0.44	0.64	8.73	11.92	90.08	86.27
SD	2.87	2.97	0.39	0.61	0.07	0.18	2.32	1.91	2.33	1.99
Significance							*	*		
Tumor gd14.5										
Mean	22.81	34.68	1.16	0.79	76.65	83.04	19.92	14.72	2.26	0.97
SD	10.02	15.94	0.85	0.41	11.19	8.96	10.65	8.61	1.74	0.48
Significance										

CD8⁺ regulatory Cells

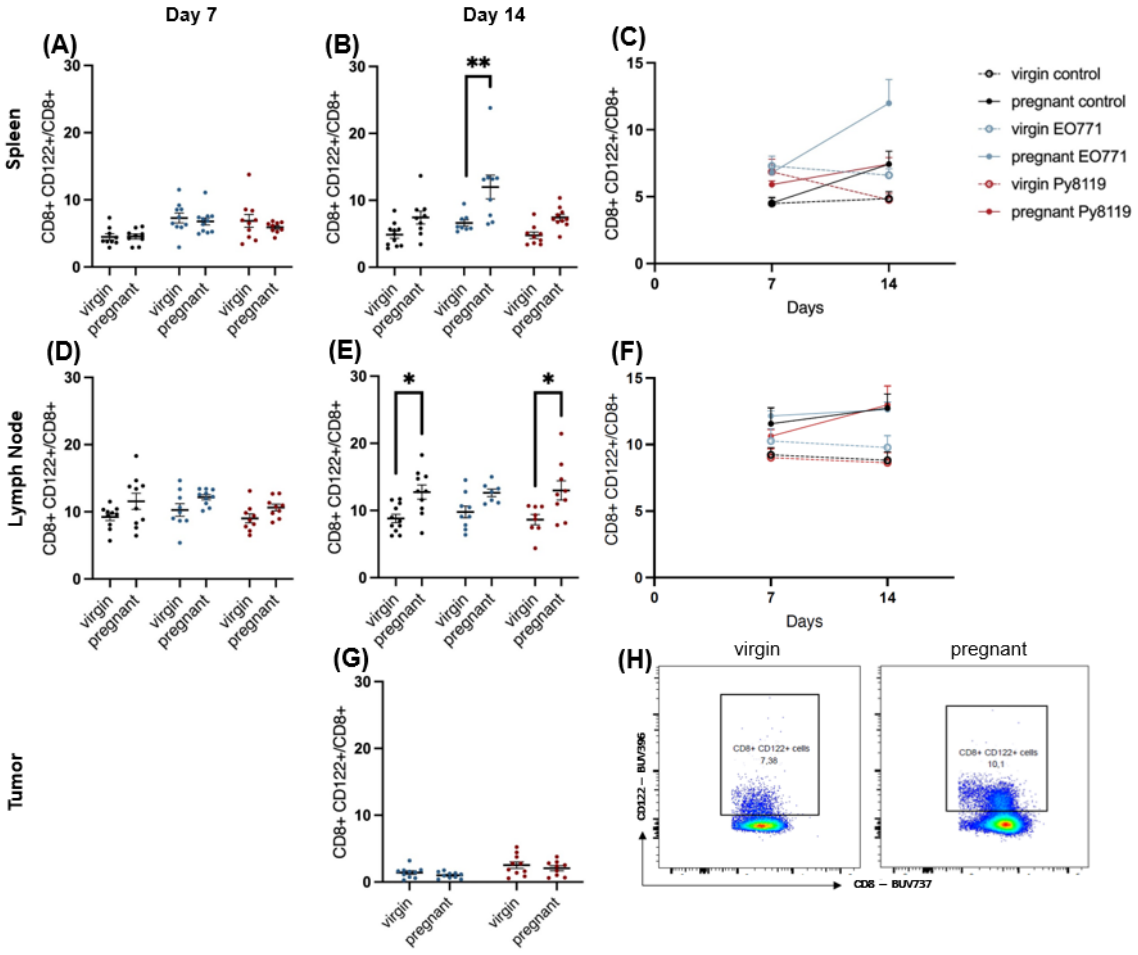

Figure 12: **CD8⁺ regulatory T cell levels on gd7.5 and gd14.5**
While no differences between the groups on gd7.5 or within the tumor were identified **(A, D, G)**, the data for gd14.5 provides a mixed picture of immune response. The spleen shows significant higher levels of CD8+ Tregs for E0771 but not for Py8119 or the controls. **(B)** On the other hand, results for the LN show the opposite **(E)** In regard to cell level trajectories, the increase of CD8+ Tregs in the spleen seems most pronounced, with little changes in the LN. **(C, F) (H)** Depicts the applied gating strategy for this subset.
Data expressed as mean [%] ±SEM with each dot representing one specimen. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001

A novel player in maternal-fetal immune crosstalk are CD8⁺ CD122⁺ regulatory cells. Although an IUGR model suggested beneficial effects of these cells, much of the mechanism behind it remains unknown. Additionally, evidence on their role in cancer is limited as well. Nevertheless, functions in both instances have been established and therefore deem further investigation necessary.

Through my analysis I was able to show an increase of $CD8^+$ regulatory cells in pregnancy. Significance was reached for the E0771 group on gd14.5 in the spleen and, in contrary to that for the control and the Py8119 group on gd14.5 in the lymph node. Levels in the tumor seemed unaltered.

3.7.3.3. Antitumor Impairment

By various mechanisms, regulatory T cells are capable of altering the function of effector cells. (Maeda et al., 2014) Through quantification of cellular markers, I therefore aimed to assess effector cell dysfunction which, in part, is caused by regulatory cells. For this purpose, I used Tim3 as marker for cellular dysfunction. Unlike as in Tregs, where Tim3 characterizes a highly potent phenotype, it proofed to be upregulated in exhausted and dysfunctional effector cells and might be a potential target for immunotherapies in order to restore endogenous antitumor activity by effector T cells.(Acharya et al., 2020; Sakuishi et al., 2010)

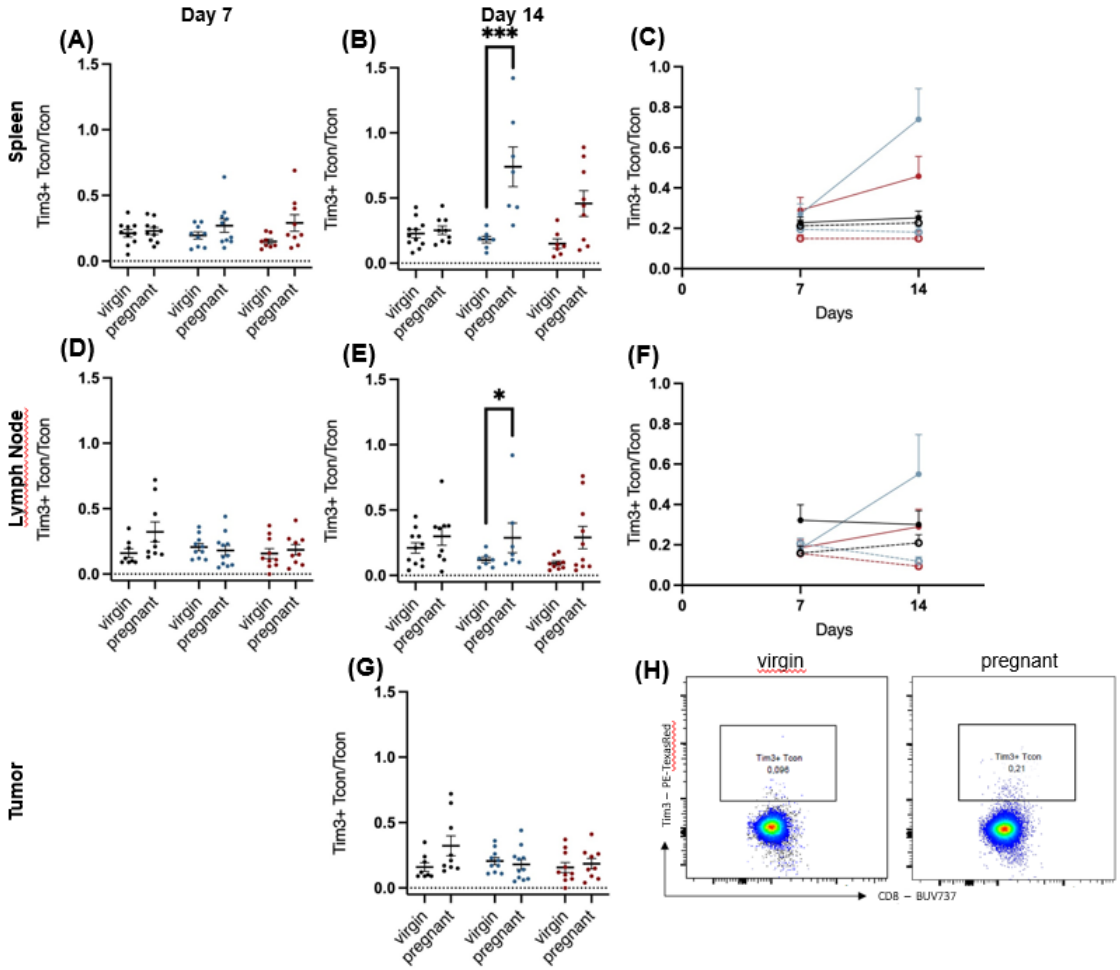

Figure 13: **CD4⁺ Tim3⁺ T cell levels on gd7.5 and gd14.5**
Consistent with other cell subpopulations, no difference was shown for gd7.5 data or within the tumor. **(A, D, G)** Nevertheless, both LN and spleen show significantly elevated levels of dysfunctional CD4⁺ cells on gd14.5 for E0771 mice. **(B, E)** Additionally, a sharp increase of these cells over time can be seen in tumor bearing mice, especially for E0771 **(C, F) (H)** Depicts the applied gating strategy for this subset.
Data expressed as mean [%] ±SEM with each dot representing one specimen. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001

Since Tim3⁺ is an important and abundant marker for CD4⁺ Tregs, the subset in figure 13 was gated in CD4⁺ FoxP3⁻ conventional T cells. By excluding FoxP3⁺ cells, it is possible to detect dysfunctional effector CD4⁺ T cells such as cytotoxic Th1 and Th17 cells. Although small cell counts were achieved, I observed a significant difference of CD4+ FoxP3- Tim+ cells in the spleen (p≤0.001) and lymph node (p≤0.05) of E0771-injected mice on gd14.5. Controls did not show any differences and specimens of the Py8119 group trends without significance. The tumor did not show any differences.

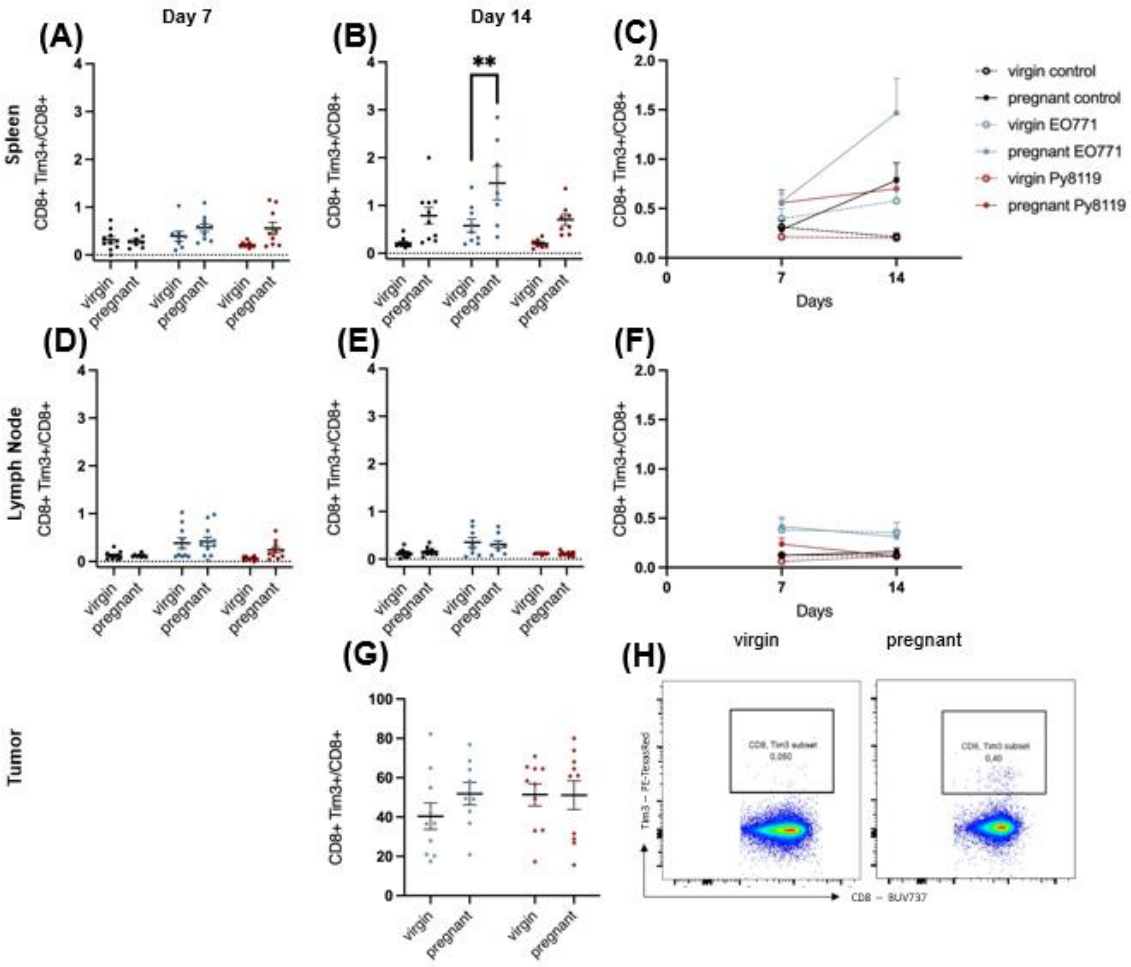

Figure 14: **CD8⁺ Tim3⁺ T cell levels on gd7.5 and gd14.5**
Since regulatory T cells often exert their effect through CD8⁺ cell inhibition, Tim3 is a helpful marker to identify exhausted cytotoxic CD8+ cells**.** In the spleen, a trend of elevated dysfunctional Tim3⁺ CD8+ T cells on gd7.5 developed to a significant difference for E0771 tumors by gd14.5. **(A, B)** Nevertheless, these findings were isolated to the spleen **(D, E, G)** Only a trend of increasing levels could be observed in E0771-injected mice within splenic tissue. **(F) (H)** Depicts the applied gating strategy for this subset.
Data expressed as mean [%] ±SEM with each dot representing one specimen. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001

Especially for CD8+ cells, Tim3 is a well-established exhaustion marker. Since Tregs are strong inhibitors of effector T cells through TGFβ, levels of Tim3 indirectly reflect regulatory cell function. Although the tumor and its draining LN did not show any significant differences, significance was achieved in the spleen. Minor trends on gd7.5 especially for Py8119. further developed by gd14.5 and reached significance (p ≤ 0.01) in E0771 bearing mice (see figure 14).

4. Discussion

4.1. Tumor Model Viability

To enable in depth immune phenotyping of regulatory cells it, was required to establish a mouse model having common features with human PABC outcomes and characteristics. Through the use of two cell lines with distinct HR profiles, Py8119 as a triple negative and E0771 as a likely HR^+ tumor, I was able to investigate the most common cancer subtype, HR^+, as well as the one having the worst outcome, triple negative. Although controversial, many studies favor a luminal B phenotype of the E0771 cell line because of a frequently reported $ER\beta^+$ and $ER\alpha^-$ status. (Le Naour et al., 2020) Nevertheless, beyond ER expression, further molecular characterization is required to establish PR and HER2 status. In regard to tumor burden, I was able to verify the current paradigm of E0771 as a more aggressive cell line. Compared to Py8119 it showed decreased survival and larger tumors. In a next step, assessment of metastatic potential would be needed to not only verify cell line characteristics but also to identify negative effects of pregnancy itself.

The chosen injection site enabled orthotropic tumor cell implantation into mammary tissue and, beyond that, facilitated animal handling which further ensures wellbeing of the mice through reduced stress. Unaltered pregnancy outcomes also make mechanic interference of the tumor in proximity to the uterus unlikely. Besides the positive aspects of the injection site, I observed occasional accidental intraperitoneal (i.p.) injections, which consequently led to reduced conditions of the mice, resulting in elimination. Therefore, isoflurane anesthesia was used in the second experiment to facilitate injections into the sub-cutaneous compartment. Subsequently, accidental i.p. injections dropped significantly.

The timespan of the survival experiment was chosen according to the animal ethics approval given by the State Authority of Hamburg. The limit of 28 days seemed appropriate for E0771 injection because maximum tumor size was reached in the majority of animals by then. However, in order to increase the chance of metastasis over time, a reduction of injected cells could be useful. A study using injections of $0{,}25 \times 10^6$ cells allowed mice to survive up to 50 days

leading to a high degree of tumor spread in various organs. (Ewens et al., 2005) Although Py8119-injected mice would be able to withstand longer timespans, due to slower tumor growth, metastatic spread has not been reported for this cell line, deeming longer trials unnecessary to investigate perigestational phenomena while putting animal well-being at risk. Time points for the second experiment were chosen for two main reasons. Gd7.5 represents an early state in murine pregnancy immunity and may therefore may be best to show differences in Treg levels. It has been well established that Tregs are main players enabling implantation. (Chen et al., 2013; Ghaebi et al., 2019; Jasper et al., 2006) Gd14.5 on the other hand, represents a mid-gestation time point with an abundancy of regulatory cells which provide the required tolerogenic state. (Ruocco et al., 2014) I did not acquire an end-gestational read out because, due to the parturition process, I suspect fundamental changes during this time period which, compared to the duration of pregnancy, is relatively short. (Gomez-Lopez et al., 2014) Furthermore, three time points would increase the number of mice used in this exploratory study colliding with 3R principles (reduce).

Nevertheless, based on the presented results, additional data of the postpartal period would be of interest in future studies. Johansson et al. reported the highest hazard ratios for 5-year and 10-year mortality in 0-6 months postpartal women. (Johansson et al., 2018) These fidnings may be explained by either prolonged exposure to a pro-tumor environment during pregnancy or by factors occuring after pregnancy. Besides the exentsively discussed role of progesterone and estrogene in breast cancer and their influence on regulatory cells, prolactin is another key pregnancy-associated hormone, which surges after delivery. Increased levels of circulating prolactin in premenopausal women have already been linked to increased breast cancer risk. (Tworoger et al., 2006) Prolactin also escalates bone metasis through the PRL receptor. (Sutherland et al., 2016) Unlike the two previously discussed hormones, prolactin decreases the suppressive function of Tregs, therefore acting through mechanisms independent of Treg function. In light of these alternative theories, additional data collection at a post delivery timepoint as well as hormone profiling may shed further light on this aspect.

In regard to clinical outcome, the experimental layout itself proved to produce a reliant model. Neither the virgin nor the pregnant group showed signs of tumor cachexia. Unaltered food and water intake further supports the absence of cachexia in consideration of the applied criteria (weight loss >10% compared to $\bar{x}$ control). These findings concur with human data. In a French cohort consisting of various cancer types, patients with breast cancer were less likely to develop cachexia. (Poisson et al., 2021) In terms of survival, there was only a minor difference in E0771-injected mice. Pregnant mice tended to be eliminated earlier compared with their virgin counterparts. Both Py8119 groups, which experienced no difference in survival, had significantly better outcomes than E0771 mice. At the time of elimination, both cell lines produced significantly heavier tumors in pregnant mice. Although the mean mass seemed similar between the cell lines, E0771 again proved to be the more aggressive cell line considering that the mass was reached in a shorter period of time. Surprisingly, the day of onset only differed between Py8119 but not for E0771 groups. A reason for this may lie in the method used to determine this parameter. The injection site was palpated with the index finger every day which yields highly subjective examiner-dependent results. Similar bias may affect volume measurements, which were assessed by using a sliding gauge. Nevertheless, there was clear gaping between pregnant and virgin mice in both tumor groups. While pregnant Py8119 mice show increasingly bigger tumors than virgin mice, the trajectory for E0771 tumors shows notable dips. They likely result from elimination of mice which reached maximum tumor size. Subsequently, these mice with particularly large tumors did not yield further data points and, in consequence, lowered the groups mean.

Although treatments for PABC may influence neonatal outcome, in humans the tumor itself is not reported to be of influence. A retrospective study in the United States showed no intrauterine growth restriction, congenital anomalies or intrauterine fetal demise associated with PABC. However, the authors did observe a greater risk for preterm birth and premature rupture of the membranes in cancer affected women. This may be accounted for by chemotherapy or prematurely induced labor for the sake of more aggressive treatment. Interestingly, the study also reported significantly increased

incidence of PABC in 1999-2012. further emphasizing the relevance of new treatment strategies based on the elucidation of pathomechanisms. (Shechter Maor et al., 2019)
In accordance to human data, the established mouse model did not show decreased neonatal outcomes. In both experiments, all measures were equivalent in control and tumor mice, validating the mouse model in this aspect as well. In addition to the clinical outcomes, fetuses and placentas were taken and fixated in the second experiment and could be used for further analysis.

Based on the findings of the survival experiment, which were confirmed and expanded by the second part, the newly established mouse model proved to be a reliable model for PABC, consistent with human outcomes. The model yielded larger and heavier tumors in pregnant mice. The survival of the E0771 cell line also differed between virgin and pregnant mice. Furthermore, offspring was unaffected by the malignancy.

4.2. Regulatory Cells in PABC

4.2.1. CD4+ Cells

Because of the aforementioned mechanisms of maternal-fetal crosstalk the tolerogenic immune environment foremost presents itself as a feasible theory of compromised anti-cancer immunity in pregnant women. Since many regulatory cells which have been proven as indispensable in pregnancy are $CD4^+$, I put the main emphasis of the study on this subclass.

Most conventional T cells, which were additionally assessed, did not yield significant differences between control and tumor groups. Only effector memory Tcons (T_{EM}) did increase in pregnancy, as already shown in humans. (Shah et al., 2017) However, an effect on tumor immunity is likely negligible since differences both in control and tumor groups were significant. Additionally, the common denominator of Tcons in pregnancy and cancer is believed to lie in the recognition of antigens, such as MUC-1, shared by pregnancy, especially placental and breast antigens, and the tumor. This overlap leads to generation of TAA specific Tcons during pregnancy which could therefore enable improved tumor immunity if a malignancy occurs. This may explain the lower lifetime risk

of multiparous women to develop breast cancer. (Krause et al., 2017)
Nonetheless, I believe that TAA specific memory T cells would not be beneficial to primigravid mice, as in this experiment, but could be of interest in multiparous animals.

Tregs have been well established as inducers of maternal immune tolerance. (Robertson et al., 2018) They exert their immunosuppressive function on various immune cell types through different mechanisms. For the maintenance of pregnancy, three aspects are particularly significant. They inhibit effector T cells via production of IL10. TGFβ and IL35, as depicted in figure 1. Additionally, they produce perforin and granzyme which damage effector T cell membranes leading to apoptosis. Within the innate immune system, they cause a switch from pro-inflammatory M1 macrophages to the anti-inflammatory M2 macrophages by decreased expression of CD86. The latter show decreased production of IL6 and TNF-α and decreased activation of NF-kB. Furthermore, Tregs inhibit natural killer (NK) cells through membrane bound TGFβ leading to down regulation of NKG2D receptors on the cell surface and decreased production of Interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α). The favorable downregulation of NK cells during implantation has adverse effects in malignancies and favors tumor cell survival. A part from the cellular effects of Tregs, they indirectly promote an anti-inflammatory environment by expression of CD39/CD73. which converts extracellular ATP to adenosine and AMP. (Romano et al., 2019)
In this study, Tregs were defined by the expression of CD4. FoxP3 and CD25. Two alternative theories suggest that CD25 is either necessary for FoxP3 induction or prevents cells already $FoxP3^{+}$ from apoptosis. (Lio & Hsieh, 2008; Tai et al., 2013) Either way, CD25 is considered necessary for active Tregs. Especially peripheral Tregs, derived from naive CD4+ cells, proved to be required for successful implantation as well as maintenance of pregnancy. Interestingly, a major population of these Tregs are allogen-specific deeming their function in tumor immunity questionable. (Samstein et al., 2012; Shima et al., 2015) Nevertheless, allogen-independent Tregs also expand during pregnancy, showing potent immunosuppressive activity. (Aluvihare et al., 2004) These properties and the previously discussed important functions in cancer and pregnancy put this cell type in the spotlight again. A study in humans also

reported TAA specific Tregs which negatively influence TAA specific conventional T cells, which might accelerate tumor growth during pregnancy. (Krause et al., 2017)
Indeed, results for both gd7.5 and gd14.5 did show a trend of increased Treg numbers during pregnancy in all groups. Systemic and tumor draining lymph node levels did not differ between control groups and the tumor-injected mice. In the tumor, the pregnancy-associated surge was not observed, which suggests a minor role of Tregs. Nevertheless, a broad definition of Tregs was chosen and further analysis by antigen-specificity or phenotype, e.g. ICOS, would be of interest.

Besides classical $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs, I also discovered significant differences in two distinct subpopulations. Tim3 commonly serves as an exhaustion marker. In Tregs however, $Tim3^+$ cells make up a highly potent subpopulation. One study identified these cells as strong suppressors of Th1 and Th17 cells, which are essential in anti-cancer immunity. (Gautron et al., 2014) $Tim3^+$ Tregs seem to be highly prevalent in the tumor but sparse in non-tumor tissue. (Sakuishi et al., 2013) An exception to this rule is the decidua where high levels of Tim3+ Tregs were recently identified, peaking in early pregnancy and diminishing to normal levels by end-gestation (Hu et al., 2020) In contrast to these findings, I was able to identify significant differences in the periphery, especially on gd14.5 but not in the tumor. This non-difference may stem from the already high prevalence of Tim3+ Tregs in the tumor, leaving little potential for pregnancy effects.
One subpopulation that has not been extensively studied are $CD44^-$ $CD62L^-$ Tregs, here termed effector Tregs. A study in breast cancer which determined mRNA expression of CD44 as well as FoxP3 found a negative correlation between CD44 levels and tumor recurrence, presence of necrosis, lymph-vascular invasion, grade 3 tumors, and aggressive phenotype. (Sanmartín et al., 2017) Based on this study, the results showing significantly higher levels in the spleens of pregnant specimens as well as the E0771 tumors, may be indicative for a tumorigenic role of effector Tregs in PABC. Higher cell levels in E0771 tumors are furthermore consistent with the aggressive phenotype of this cell line. Nevertheless, independently from each other, $CD44^+$ and $CD62L^+$ are

both considered markers for Tregs with a superior immunosuppressive phenotype, deeming the potential of a double negative cell type's influence on PABC questionable. (Lange et al., 2011; Liu et al., 2009)

An aspect which has not been investigated in this study, but should be considered in the future is not only the influence of Tregs on the tumor but vice versa. Tregs are capable of adapting to tissue hypoxia and the anaerobic metabolic state within the tumor microenvironment by cellular reprogramming. Hypoxia itself, which leads to a high expression of the transcriptional factor HIF, promotes the release of immunosuppressive cytokines. Additionally, it increases chemotaxis of Tregs likely via the HIF signaling pathway. Differentiation of new Tregs is further enhanced by increased FoxP3 as well as TGFβ expression through HIF signaling. Tumors also express PD-1, which increases FoxP3 expression in T cells. (Wang et al., 2018)
By these mechanisms tumors are capable of increasing Treg numbers even more, in an already Treg high state as pregnancy, altering the immunological environment in their favor.

Tr1 cells are a highly potent population of regulatory T cells. Though well known to correlate with progression of various malignancies, their functional role in pregnancy awaits to be fully elucidated. Defined by their distinct marker profile from Tregs, namely $CD4^+$ $FoxP3^-$ $Lag3^+$ and $CD49b^+$, they present only a small fraction of immune cells.(Gagliani et al., 2013) De facto, in this experiment levels of Tr1 cells were exceedingly low, deeming interpretations limited. Nevertheless, trends of increased levels in pregnant mice, particularly on gd14.5 and in the spleen, support the paradigm of an immunologically tolerant state during pregnancy. In contrary to that, levels of Tr1 cells were significantly lower in tumors of pregnant E0771-injected mice compared to their non-pregnant counterparts. This inverse proportion in tumor vs. lymph node and spleen did not occur in Py8119 specimens. Besides the possibility of an incidental finding biased by a very low cell count for Tr1 cells, tumor characteristics such as aggressiveness might also have an influence. Breast tumors are capable of generating an immunosuppressive environment, for instance through the production of extracellular adenosine (ADO). ADO may in

consequence favor the induction of Tr1 cells. (Mandapathil et al., 2021) Concentrations of ADO rise with progressing tissue damage and hypoxia, which could promote Tr1 differentiation. (Sitkovsky et al., 2008) Through this mechanism, high levels of Tr1 cells in virgin E0771 tumors may be explained. Nevertheless, the cause for the inverse levels of Tr1 cells in pregnant vs. virgin tumors I here observed remains unknown and future assessments should also assess the potential role of ADO as a modulator of Tr1 cells in PABC.
As observed in other cell populations, which showed non-reflecting or contradicting trends in different organs, as for Tr1 cells the significantly higher levels in the spleen vs. significantly lower levels in the tumor of E0771 pregnant mice on gd14.5, the method of tissue preparation, which differed between the organs, may also be reevaluated. Since $CD45^+$ cells only represent a minor portion of all $CD3^+$ cells in the tumor, enrichment of lymphocytes based on marker expression, e.g. CD45. would facilitate identification of small cell populations, like Tr1 cells. (Chihara et al., 2016) Additionally, the treatment with reagents such as collagenase or RBC lysis buffer may have also influenced the viability of lymphocytes. For instance, a study on the digestion of murine vaginal and uterine tissue showed decreased viable as well as $CD45^+$ cells in collagenase treated specimens compared to other methods. (Skulska et al., 2019) However, the applied digestion method is currently the most widely used one. (Leelatian et al., 2017)

Before the discovery of Tregs, the paradigm of pregnancy as a tolerogenic state was mainly based on the dichotomy of Th1/Th2 cells. (Wegmann et al., 1993) Although T helper cells moved out of the spotlight, they are still considered essential for sustainment of pregnancy. Together with their distinct function in tumor immunity, they may in theory also influence outcomes in PABC. It is assumed that a shift occurs after conception from a Th1 dominant pregestational state to a Th2 dominant gestational state. In this study, I was not able to reproduce this shift. Neither a difference in Th1/Th2 ratio occurred neither between virgin and pregnant nor tumor and healthy mice. Trajectories of Th1 and Th2 levels between gd7.5 and gd14.5 did not differ either. Notably, the significantly elevated levels of both Th1 and Th2 cells in the lymph node on gd14.5 seem to nullify themselves, because of an unaltered ratio. It is unlikely

that these findings are a sequela of the tumor, since the Th1/Th2 ratio is often reduced in cancerous tissue and the draining lymph node owning to the tumors suppressive immune escape mechanisms. (Ehi et al., 2008) A more likely explanation is a bias by the low cell counts of T helper cells. Without previous stimulation or sorting, these cells only occur in sparse numbers in healthy tissue. A different methodical approach may therefore be advisable in future studies.
Similarly, Th17 cells, in addition to the Th1/Th2 dichotomy, also accounted for a minor population of immune cells. However, current literature as well as equal levels for all tissues at both time points in this experiment do not suggest an influence of Th17 cells on PABC specific immunity.

In order to gain insights on the impact of regulatory cell function, I aimed to quantify the degree of effector cell dysfunction. For this purpose, $CD8^+$ cells as well as conventional $CD4^+$ cells were gated for their expression of Tim3. This transmembrane protein has several ligands including galectin 9. phasphatidylserine, HGMB1 and CAECAM1. through which it acts as a co-inhibitor and checkpoint receptor. (Wolf et al., 2020) Although first described on IFN-γ producing $CD4^+$ cells, it is now commonly used as a marker for exhausted $CD8^+$ cells. (Monney et al., 2002) One obstacle in applying Tim3 as a marker for dysfunctional $CD4^+$ cells is its frequent expression by cells, also positive for FoxP3. (Sakuishi et al., 2013) In order to exclude these cells, which have an immensely differing immune function, $CD4^+$ cells were also gated to be $FoxP3^-$. The remaining IFN-γ producing $CD4^+$ cells, such as Th1 and Th17 loose much of their cytotoxic capability upon Tim3 activation by one of its ligands due to constant antigen exposure. (Zhu et al., 2005) In this study, cancer-associated mechanisms showed to produce a trend of increased $Tim3^+$ conventional $CD4^+$ cells in pregnant tumor-bearing mice compared to their virgin and healthy counterparts, with a significant difference for E0771 tumors. Again, the stronger effect in the E0771 cell line could be attributed to its more aggressive phenotype.

The levels and trajectories of regulatory cells in PABC do not clearly indicate an unambiguous source of increased conventional T cell exhaustion in pregnant mice so far. However, these changes may prove that the immune system plays a salient role after all.

4.2.2. $CD8^+$ Cells

Cytotoxic $CD8^+$ cells, in synergy with the innate immune system, are the main phagocytic cells in tumor immunity. (Iwahori, 2020) In consideration of the heavy influence of regulatory cells, especially Tregs and Tr1 cells, on $CD8^+$ cells, there is a great interest in this population not only based on an insight in anti-tumor immunity in PABC, but also regulatory cell function.
Significant differences of multiple $CD8^+$ subpopulations where found in pregnant vs. virgin tumor-bearing mice varying by tissue, but having in common to only occur by gd14.5. While central memory $CD8^+$ cells where significantly increased in the lymph nodes of pregnant tumor mice, effector memory CD8+ cells reached significance in the spleen. Again, the tumor itself yielded no differences in the levels of either subpopulation. These findings are unsurprising considering that central memory $CD8^+$ cells reside in the lymph node, while effector memory $CD8^+$ cells can be found anywhere in the body. Functionally, these two subsets are also distinct. Central memory CD8+ cells have limited effector function, but serve as a reservoir in order to generate new effector memory and effector CD8+ cells. (Sallusto et al., 1999) Therefore, increased levels might be considered a sign of decreased conversion. Nevertheless, because effector memory CD8+ cell levels are increased in the spleen, this theory can be dismissed. The upregulation of effector memory and central memory CD8+ cells at the same time indicates a functioning adaptive tumor immunity. Without differences in the tumor, however, the effect on the PABC microenvironment by the increased cell levels remains uncertain.
The significant decreases in naïve $CD8^+$ cells can most likely be attributed to recruitment due to expansion of the effector phenotypes.

Another subset of regulatory cells which has not yet been well characterized in pregnancy as well as malignancies are $CD8^+$ regulatory cells. Because of the exploratory character of this study, examining these cells was supposed to shed further light on their status systemically as well as on tumor proximity. Unexpectedly, significant differences were found depending on the organ, having in common that they only occur by gd14.5. Py8119 as well as control pregnant mice had significantly higher numbers of these cell, in the lymph node, while E0771 pregnant mice reached significance in the spleen. Since control specimens also experienced this increase, the findings may be attributable to pregnancy itself but not cancer, which is further supported by equal levels in the tumor. The increase of $CD8^+$ Tregs in the lymph node was particularly unexpected, since the harvested node is not draining the uterus, where one might expect pregnancy-associated changes. Nevertheless, increases in different tissues, although incoherent, warrant further investigation on a possibly major function of these cells outside the uterine compartment.

Similarly to Tcons, $CD8^+$ cells increasingly express Tim3 upon exhaustion by persistent antigen exposure. (Fourcade et al., 2010) Unlike $Tim3^+$ $CD4^+$ cells, $CD8^+$ cells do not lose their capacity to produce IFN-γ, but rather their cytotoxic ability upon Tim3 expression. (Sawada et al., 2020) In regard to the significant role of $CD8^+$ cells in anti-tumor immunity, it seemed necessary do determine effector status in PABC as well. In contrast to $Tim3^+$ Tcons, levels for $CD8^+$ cells were not significantly different in most tissues. Only the spleen of E0771 pregnant mice on gd14.5 showed significantly increased levels of dysfunctional cytotoxic CD8 cells. Although systemic changes have been observed in cases of local immune stimulation, e.g. HBV, without correlations in tumor or tumor-draining tissues themselves, these findings cannot be easily attributed to direct effects by the malignancy. (Mohammadizad et al., 2019) Other mechanisms may have led to these discrepancies, which requires further investigation.

4.3. Alternative Theories

Beyond my theory of the regulatory T cell influence on PABC, other possibilities should be considered too. I would like to draw closer attention to two additional aspects. Firstly, other immune mechanism, which are critical in the cancer microenvironment, may be responsible for adverse outcomes in pregnancy. Especially innate immunity takes an important role within the cancer immunity. NK cells, for instance, are capable of reducing tumor formation, recurrence, and metastasis upon activation by IL-15. (Liu et al., 2012) Although human *ex vivo* data suggests unaltered NK defense against tumor cells during pregnancy, *in vivo* phenotyping may shed further light on this innate subset. (Le Gars et al., 2019) Another main cell type of innate tumor immunity are macrophages. In order to understand their function in malignancy, two subsets have to be distinguished. Classically derived M1 macrophages show cytotoxic activity and are associated with tumor regression and better outcomes. (Hachim et al., 2020) Tumor-associated macrophages (TAMs), on the other hand, frequently show a M2 phenotype and are associated with decreased survival. (Chen et al., 2011) Interestingly, TAMs are capable of Treg recruitment and trafficking into the tumor tissue via the chemokine CCL22. (Curiel et al., 2004) Pregnancy, as an Treg abundant state, may therefore augment an “anti-inflammatory spiral”. Investigating innate immunity appears to be of particular interest since $CD3^+$ T cells only accounted for a minor portion of all $CD45^+$ cells in the tumor and spleen. This suggests a major population of non T-cell immune cells in these tissues.

Secondly, the reason for adverse outcomes may not lie in immunity after all. Although I was able to show various differences in regulatory cell levels and much of the pregnancy-associated anti-tumor immunity awaits to be elucidated, other factors may contribute to decreased survival rates. A well-documented delay in PABC diagnosis may stem from both the patient’s and the doctor’s side. Women may either not recognize changes of the breast due to the tissue remodeling during pregnancy or attribute abnormal findings to it. Additionally, doctors must show a high level of suspicion in these patients because awareness may be limited due to young age or pregnancy as a distracting factor. Once a clinical suspicion is established ultrasound and fine needle biopsy are the gold standard of diagnosis in pregnancy with sensitivity and

negative predictive values of up to 100%. (Macdonald, 2020) Nevertheless, one older study, in which diagnostic standards during that time have to be taken into consideration, suggested a 1-2 month delay in PABC diagnosis. (Bonnier et al., 1997) This may explain the higher incidence of positive lymph nodes and larger tumors in pregnant women. However, a diagnostic delay may only partly contribute to decreased survival since a study matching for TNM (tumor, nodes, metastases) stage showed that women affected by PABC still presented with worse outcomes than their non-pregnant counterparts did. (Johansson et al., 2018) Through this study, diagnostic delay seems even more improbable as a main contributor to adverse outcomes in PABC since the mouse model did show significantly larger tumors regardless of diagnostics in humans.

5. Conclusion

I was able to conduct the first in depth phenotyping of regulatory immune cells in a newly established murine model of pregnancy-associated breast cancer. Through the inoculation of two cell lines with distinct features, I reproduced clinical outcomes comparable to humans. The model additionally enabled in-depth phenotyping of regulatory cell populations on two different time points. Compared to their virgin counterparts, pregnant mice had higher levels of the regulatory subsets ($Tim3^+$ Tregs, effector Tregs). Indicative for increased exhaustion in PABC dysfunctional CD4+ and CD8+ cells were also significantly elevated in some tissues on gd14.5. Apart from other possible theories on negative outcomes in PABC, these results support my hypothesis of an influence by the tolerogenic immune environment. However, these preliminary findings need to be validated and supplemented with additional experimental methods.

6. Zusammenfassung

Mit Hilfe zweier Zelllinien unterschiedlicher Charakteristika, konnte ich ein neues Mausmodell etablieren, das humanen klinischen Verläufen entspricht. Basierend darauf führte ich die erste tiefgreifende Untersuchung von regulatorischen T Zellen beim Schwangerschafts-assoziierten Brustkrebs (PABC) durch. Erhöhte Niveaus von regulatorischen ($Tim3^+$ Tregs, effector Tregs) sowie von dysfunktionalen Effektor Zellen weisen auf eine Rolle der schwangerschaftsbedingten immunmodulatorischen Vorgänge bei PABC hin. Weitere Studien, unter Verwendung zusätzlicher tierexperimenteller und molekularbiologischen Methoden, sind jedoch zur Validierung dieser ersten Ergebnisse erforderlich.

7. References

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9. Contributions

Data collected and analyzed for the survival experiment as well as clinical data for the kinetics experiment was generated in collaboration with Katharina Hecker. Parts of the results will also be published in her Master's thesis at Hamburg University titled:" The impact of γδ T cells on breast cancer progression during murine pregnancy"
Tissue harvesting, preparation and immune phenotyping was performed by myself.

10. Curriculum Vitae

Dr.med.univ. Lukas Steger

work	*10/2021-present*	**Anesthesiology Residency** University Medical Center Hamburg Eppendorf Center for Anesthesiology and Critical Care Medicine
	01-09/2021	**Research Fellow** University Medical Center Hamburg Eppendorf Department of Obstetrics and Prenatal Medicine Laboratory for Experimental Feto-Maternal Medicine
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	08/2012 – 01/2013	**EF Foreign Exchange** / Timberline High School Boise, Idaho, USA
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internships	07-08/2020	**Anesthesiology and Critical Care Medicine** Traunstein Hospital, Germany
	01-02/2020	**Traumatology and Emergency Medicine** University Hospital Zurich, Switzerland
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miscellaneous

United States Medical Licensing Examination Step 1. Step 2CK
Medical Didactics Seminar / Paracelsus Medical University
Emergency Medicine – EMT Course / Speaker / Paracelsus Medical University – Austrian Red Cross

11. Eidesstattliche Versicherung

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