Dissertation

Submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

Presented by

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Born in: Shanghai, P.R. China

Oral-examination:

“From bench to bedside”

Role of tumour-specific regulatory T cells in breast cancer and exploration of their therapeutic modulation

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ACKNOWLEDGEMENT

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this dissertation possible and because of whom my graduate experience has been one that I will cherish forever.

My deepest gratitude is to my doctor father **PD. Dr. Philipp Beckhove.** I am greatly indebted to him for his intellectual supervision, constant support and endless patience. I have been amazingly fortunate to learn from his profound knowledge which broadened my view on immunology and his constructive criticism which developed my scientific way of thinking. I also thank him for providing a highly motivational working environment which helped me to overcome many crises and to be able to confidently face tough situations. Each line of this thesis is expressing my special gratitude to him.

I also feel very honoured to express my sincere appreciation to **Prof. Dr. Volker Schirrmacher** who researched from his heart and dedicated his life to NDV-mediated cancer therapy. I am deeply grateful to him for his insightful and thought-provoking comments at different stages of my research.

I would like to acknowledge the excellent cooperation with **Dr. Florian Schütz** and **Dr. Natalija Inzkirweli** from Universitäts-Frauenklinik Heidelberg. Also, I would like to express my special respects to the volunteering breast cancer patients.

My very special thank goes to a friend worthy of being cherished my whole life, **Maria Xydia**. It is thankfulness not only for her carefully reading and commenting on countless revisions of this manuscript, but also for sharing the ups and downs during my graduate period.

I want to thank **Irmtraud Williams** for correcting the grammar of my thesis.

Although it has been long told that there must be a supportive team behind a personal success, I felt my true luckiness only when I experienced it. Zuerst möchte ich unseren exzellenten Technikern,. **Mariana Bucur** und **Simone Jünger** danken. Dank Marianas unschätzbar sorgfältiger Zuarbeit konnten meine Laborversuche so effizient und präzise durchgeführt werden. Und es war Simones Hilfe, die den Abschluss dieses Projektes erst ermöglichte. Ich danke ihnen für ihr immerwährendes Verständnis und die wertvolle Unterstützung. Auch ihre Nachsicht für mein sehr lückenhaftes Deutsch möchte ich nicht unerwähnt lassen.

I would like to acknowledge the help I received from the colleagues from our former group **D010.** To **Dr. Carmen Choi**, I thank her for showing me how patient a supervisor should be in front of green hands who are naïve but eager for success. To **Dr. Katrin Ehlert,** I thank her

for influencing me with the particular interest in breast cancer immunotherapy. To my dear friend **Dr. Yvonne Ziouta,** who impressed me from the very beginning by her strict self-management and meticulous attitude towards science, I'm very much obliged to her every endeavour on helping me complete this dissertation.

Our current PhD student team is composed by many vigorous young scientists, **Kim Pietsch**, **Tobias Seibel**, **Andreas Bonertz, Christina Pfirschke and Maria Xydia**. I thank them for assisting in the collection of the fruitful discussions and the enjoyments they brought me on every group seminar, coffee break and Thursday social night.

Many girl friends have helped me stay sane through these difficult years. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value the friendship from specially **Ni Jing, Tang Wannan**, **Ulrike Quitsch and Johanna Eberhard.** Also, I thank them for a sweet memory they gave me on my bridal shower night.

There are many practical and master students who joined our lab temporally and left unforgettable tracks in my graduated study. I want to give a special mention to **Helge Felber** for the much necessary assistance he has provided me.

I would also like to acknowledge the members of **Treg Group** for numerous discussions in "Treg journal club" every Monday morning which helped me improve my knowledge in this area. They are **Dr. Nina Oberle**, **Eva-Maria Weiß**, **Angelika Schmidt**, **Dr. Garbi Natalio Garcia**, **Janie Suffner**, **Dr. Marie Kühnle** and **Lys Guibride.**

And I would not be reaching this far without the unyielding support of the love of my life, the rock of my family, and the unsung hero of this dissertation, my husband **Andreas Fischer**. Thank him for the much needed trust and inspiration he extended.

This is also a great opportunity to express my heartfelt gratitude to **my family in-law**. I feel so lucky to join this big and warm German family which is filled up with constant love and laughter.

Last but not least, most especially to my parents, 亲爱的**爸爸妈妈**, 我最需要感谢的人就是您们，谢谢您们给了我生命，并教导我成长！感谢你们给予我的无止尽的爱. 妈妈, 谢谢你为我付出了我此生都难以回报的爱. My debt to them is beyond measure. I would like to dedicate this dissertation to my dear Mama.

CONTENTS

1 ABBREVIATION INDEX

^{3}H-thymidine	Tritium labelled thymidine
αCD3	Anti-cluster of differentiation 3
°C	Degree Celsius
aa	Amino acids
A	Alanin
Ab	Antibody
ADI	Adoptive Immunotherapy
Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
B	Belgien
BAGE	B melanoma antigen gene
BM	Bone marrow
BMTC	Bone marrow T cell(s)
BSA	Bovine serum albumin
BT	Bone marrow T cell(s)
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CEA	Carcinoembryonal antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTX	Cyclophosphamide
cm	Central memory

c.p.m	Counts per minute
Cy	Cyanin
D	Germany
d	Day
DC	Dendritic cell(s)
ddH_2O	Double distilled water
depl.	Depletion
DKFZ	Deutsches Krebsforschungszentrum (German Cancer Research Center)
DMSO	Dimethylsulfoxid
Ebi3	Epstein-Barr virus-induced gene 3
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linke immunosorbent spot
et al.	et alii
E:T	Effector cells : Target cells
FACS	Fluorescence-activated cell sorter (flow cytometry)
FasL	Fas ligand
FCS	Fetal calf serum
F_c	Fragment constant (or crystallisable)
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein 3
FSC	Forward scattered
g	Gram(s)
g	9,81m/s^2

G	Glycin
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte and monocyte colony stimulating factor
GMP	Good manufactoring practice
H	Histidin
hr	Hour(s)
HA	Hemagglutinin
HD	Healthy donor(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her2/neu	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
I	Isoleucin
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
int	Intermediate
iTreg	Induced Treg
IU	International units
K	Lysin
l	Liter(s)
L	Leucin
L	Ligand
LPS	Lipopolysaccharid
m	Months

M	Molar (mol/l)
mAb	Monoclonal antibody(ies)
MaCa	Mammalian carcinoma
MACS	Magnetic-activated cell sorting
MAGE3	Melanoma-associated antigen 3
MHC	Major histocompatibility complex
min	Minute(s)
ml	Mililiter
mTC	Memory T cell(s)
µl	Microliter
Muc-1(TR)/(100)	Mucin 1 (tandem reapeat)/(leader sequence)
N	Asparagin
NaCl	Sodium chloride
NK	Natural killer
NL	Niederland
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
n.s.	Not significant
n.t.	Not tested
nTreg	Natural T regulatory cells
o/n	Over night
p1-100	Amino acid 1-100
PB	Peripheral blood
PBMC	Peripheral blood mononulear cell(s)
PBS	Phosphate-buffered saline
PBTC	Peripherla blood T cell(s)
PD	Progressive disease

PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PT	Peripheral blood T cell(s)
Q	Glutamin
R	Arginin
rpm	Rounds per minute
RPMI	Roosvelt Park Memorial Institute
RT	Room temperature
S	Serin
SD	Standard deviation
SD	Stable disease
SEB	Staphylococcus Enterotoxin B
SEM	Standard error of the mean
SSC	Side scatter
T	Threonin
TA	Tumour-antigen
TAA	Tumour-associated antigen
TC	T cell(s)
Tcon	Conventional T cell(s)
TCR	T cell receptor
TdR	Thymine-desoxy-ribose
Teff	Effector T cell(s)
TGF	Transforming growth factor
Th	Helper T cell
TIL	Tumour infiltrating lymphocytes

™	Trademark
TNF	Tumour necrosis factor
Treg	Regulatory T cell(s)
Tresp	Responder T cell(s)
TuLy	Tumor lysate
U	Units
USA	United States of America
V	Valin
VEGF	Vascular endothelial growth factor
vs.	Versus
v/v	Volume per volume
W	Tryptophan
W	Watt
WHO	World Health Organization
w/o	Without
w/v	Weight per volume
µCi	Microcurie
µg	Microgram(s)
µl	Microliter(s)
Y	Tyrosin

2 ABSTRACT

This thesis characterizes the extent of regulatory T cell–mediated immune suppression in breast cancer and explores the possibilities for its potential therapeutic modulation.

Based on initial results obtained from a pilot clinical study of adoptive transfer of autologous bone marrow derived T cells in advanced metastatic breast cancer patients, we observed that T-cell unresponsiveness in 53% of study patients was associated with an existing immune suppression exerted by Treg. Further IFN-γ secretion assays with primary breast cancer samples revealed that T-cell activation, proliferation as well as CTL responses upon tumour antigen stimulation were hampered when Treg were present.

Flow cytometric data displayed that in primary breast cancer patients the bone marrow (BM) contained significantly lower frequencies of Treg than the peripheral blood (PB) ($p<0.001$), which may at least partially explain the improved functional potential of BM-derived memory T cells. Different migratory capacities of Treg in the BM and the PB were observed regarding breast cancer profiled chemokine receptors CCR2, CCR4 and CCR5, which might be responsible for this scenario.

By removing Treg through CD25 targeting, the pre-existing T-cell anti-tumour immunity was evoked remarkably in both BM and PB, especially in otherwise less responding PB, as determined by IFN-γ ELISpot assay with polyvalent tumour antigens (MCF7 and KS24.22) as well as with defined tumour associated antigens (polypeptides).

This rescuing effect in the PB provides an opportunity of utilizing PB instead of limited BM as a source for adoptive T cell transfer immunotherapy. As a pioneer exploitation, a large-scale Treg-depletion in a leukapheresis sample obtained from a metastatic breast cancer patient was operated with CliniMACS system under clinical-grade conditions (GMP). A sufficient depletion of Treg, a high viability of isolated cells and a satisfying recovery of total cells were documented.

To exploit immunotherapy regarding eliminating Treg, an oral metronomic cyclophosphamide (CTX) treatment (50 mg/day) was performed to selectively deplete Treg *in vivo*. This pilot clinical trial was applied on 13 advanced metastatic breast cancer patients for 3 months. Clinical responses was observed in 64% of study patients accompanied by a rapid and effective reduction of Treg on Day14 ($p=0.005$) and by an augmentation of spontaneous anti-tumour T-cell immunity on Day70.

Last but not least, in this thesis we found that Treg existing in breast cancer patients exhibit breast tumour antigen specificity, which was evidenced indirectly and directly. MCF7- or KS24.22-specific IFN-γ secretion was abrogated when Treg were added back in ELISpot assay. The production of immunosuppressive cytokine IL-10 was increased only when Treg encountered breast tumour antigens measured by ELISA and intracellular staining experiments. By applying a novelly designed Treg Specificity Assay, we detected a broad spectrum of tumour antigens recognized by Treg in at least 47% of tested breast cancer patients with Mammaglobin A as the most frequent one (85%).

3 ZUSAMMENFASSUNG

Die vorliegende Arbeit charakterisiert das Ausmaß der über regulatorische T Zellen (Treg) vermittelten Immunsuppression im Mammakarzinom und erforscht potentielle Möglichkeiten zur therapeutischen Modulation.

Nach ersten Erkenntnissen einer klinischen Pilotstudie des adoptiven Transfers von autologen T Zellen aus dem Knochenmark in Patientinnen mit metastasiertem Brustkrebs, wurde beobachtet, dass das Fehlen einer T-Zell-Reaktivität in 53% der Studienpatientinnen mit einer existierenden Immunsuppression assoziiert war, die auf Treg zurückzuführen war. Weitere IFN-γ Sekretionstests mit klinischem Material vom primären Mammakarzinom zeigten, dass T-Zell-Aktivierung, Proliferation und zytotoxische Immunantwort nach Stimulation mit Tumorantigen unterdrückt wurden, wenn Treg präsent waren.

Durchflusszytometrische Untersuchungen von primäroperierten Brustkrebspatientinnen demonstrierten, dass das Knochenmark (KM) signifikant niedrigere Treg-Frequenzen als das periphere Blut (PB) aufwies ($p<0,001$); dies kann zumindest teilweise die verbesserte Funktion der Gedächtnis-T-Zellen im KM erklären. In Treg aus KM und PB wurde unterschiedliches migratorisches Potential für die Mammakarzinom-relevanten Chemokinrezeptoren CCR2, CCR4 und CCR5 beobachtet, das möglicherweise für die obengenannte Beobachtung verantwortlich ist.

Durch die Depletion von Treg über CD25-Targeting wurde die bereits existierende T-Zell Anti-Tumor Immunität in beiden Kompartimenten, KM und PB, bemerkenswert unterstützt; dies war im sonst nicht reaktiven PB besonders auffällig, wie IFN-γ ELISpot Tests mit polyvalenten Tumorantigenen (MCF7 und KS24.22) sowie mit definierten tumorassoziierten Antigenen (Polypeptide) demonstrierten.

Diese Rettungsaktion im PB ermöglicht den Einsatz von PB anstelle von limitiertem KM als Quelle für die adoptive T-Zell-Immuntherapie. In einem Pionieransatz wurden aus einem Leukapherese-Produkt einer metastasierten Brustkrebspatientin die Treg mit Hilfe des CliniMACS Systems nach guter Herstellungspraxis (GMP) in großem Umfang depletiert. Ausreichende Treg-Depletion, hohe Vitalität der isolierten Zellen und zufriedenstellende Ausbeute an Gesamtzellen wurden dokumentiert.

Auf diesen auf Treg-Depletion basierenden immuntherapeutischen Ansatz wurde näher eingegangen, indem Cyclophosphamid (CTX) metronomisch verabreicht wurde (50mg/Tag), um die Treg selektiv *in vivo* zu depletieren. An dieser klinischen Pilotstudie nahmen 13 Patientinnen mit metastasiertem Mammakarzinom für drei Monate teil. Klinische Antworten wurden in 64% der Studienpatienten beobachtet, die von einer schnellen und effektiven Reduktion der Treg an Tag 14 ($p=0,005$) und von einer Steigerung der Anti-Tumor T-Zell-Reaktivität an Tag 70 begleitet waren.

Nicht zuletzt wurde in der vorliegenden Arbeit gezeigt, dass Treg, die in Brustkrebspatientinnen existieren, eine Spezifität für Brustkrebsantigen aufweisen; dies wurde indirekt und direkt nachgewiesen. Die MCF7 oder KS24.22 spezifische IFN-γ Sekretion

wurde inhibiert sobald Treg im ELISpot Test zurückaddiert wurden. Die Produktion vom immunsuppressiven Zytokin IL-10 war nur dann erhöht, wenn Treg auf Brustkrebsantigen trafen, wie ELISA Tests und intrazelluläre Färbungen belegen. In einem neu entwickelten Treg Spezifikationstest wurde ein breites Spektrum an Tumorantigenen detektiert, das von Treg von mindestens 47% der untersuchten Brustkrebspatientinnen erkannt wurde; Mammaglobin A war dabei das am häufigsten erkannte Antigen (85%).

4 INTRODUCTION

4.1 T cells in tumour immunity

Worldwide, cancer is in the top 5 leading causes of death (WHO source). Breast cancer is the second most common type of cancer and the fifth most common cause of cancer death. However, without our immune defence system in identifying and eliminating nascent tumour cells the incidence of cancer would be much greater. This is the concept so-called tumour immunology proposed by Paul Ehrlich in 1909 and further advanced as immune-surveillance by Lewis Thomas and Frank MacFarlane Burnet.

T cells play a crucial role in tumour immunity. With the development of immunohistochemical and flow cytometric techniques, immunologists have elucidated that the major tumour infiltrating lymphocytes (TIL) are CD3 T cells, which can be further stratified to CD8 cytotoxic T lymphocytes (CTL) and CD4 help cells, including Th1, Th2, and most recently discovered regulatory T cells (Treg).

CTL destroy tumour cells via a triggering of apoptosis by means of cytotoxic granule exocytosis, such as perforin and granzyme B, and/or the Fas/FasL receptor-mediated pathway (1) (2) (3). The presence or absence of CTL in the tumour-draining lymph nodes predicts the disease-free survival in breast cancer, which is more accurate than tumour stage and nodal status (4).

The role of tumour-specific CD4 T cells in the host anti-tumour response is an area of considerable debate (5) (6) (7). Th1 response is associated with CTL activation and is considered to be beneficial for anti-tumour immunity, while Th2 cells facilitate antibody production by B cells and polarize immunity away from a beneficially cell-mediated anti-tumour response (8) (9). CD4 Treg are found to be accumulated in many human carcinomas and are known as tumour guardians. We will have a detailed look on Treg subset in chapter 4.2.

In the studies of human cancers including breast carcinoma, large interest and effort have been given to detect tumour-reactive memory T cells in the patients, since their responses might be required for the durable prevention of tumour recurrence and metastasis following the surgery. And one elemental characteristic of a T-cell response is its magnitude. In this regard, the frequencies of antigen-specific T cells are commonly evaluated. Breast cancer is characterized by high numbers of recurrences that are due to an early dissemination of tumour cells. A predominant organ for breast tumour dissemination and maintenance is the bone marrow (BM) (10). Accordantly, our group has previously observed and reported

higher frequencies of tumour-specific memory T cells in the BM than the peripheral blood (PB) of primary breast cancer patients (11) (12) (13).

Another fundamental parameter for the magnitude of T-cell response is the expression of specific effector functions, such as interferon-γ (IFN-γ) production which is a hallmark of Th1 lymphocytes, as well as CD8 T cells upon activation. IFN-γ secreted by CTL is critical for tumour elimination. IFN-γ can directly act on different tumour cells to facilitate apoptosis (14; 15; 16). It can also regulate angiogenesis and promote vasculature destruction leading to tumour necrosis (17; 18).

4.2 Regulatory T cells in tumour immunity

4.2.1 History of Treg

Historically, Treg were initially hypothesized as suppressive T cells in early 1971 by Gershon and Kondo (19) (20). Five years later, a negative correlation between tumour immunity and regulatory T cell was first proposed by Sehon and his colleagues (21). After another five years, North and his colleagues evidenced the existent tumour-suppressor T cells with $CD4^+CD25^+$ phenotype (22; 23; 24). Thus, the study of regulatory T cells in tumour immunity has been established. Unfortunately, subsequent studies were hindered due to an extensive scepticism as well as lack of specific molecular markers and difficulties in their isolation and culture. The renaissance of these regulatory cells came decades later attributed to two major investigations: 1) in 1995, Sakaguchi and his colleagues discovered that interleukin-2 (IL-2) receptor α-chain, CD25, could serve as a phenotypic marker for $CD4^+$ suppressor T cells. Transfer of CD4 T cells that had been depleted of the $CD25^+$ population into congenitally athymic mice induced autoimmune diseases while transfer of intact populations of CD4 T cells did not (25); 2) in 2001, the autoimmune Scurfy mice and a human immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) patient were found to have mutations in Foxp3 gene. Foxp3, forkhead box P3, is a member of the forkhead/winged-helix family of DNA transcription factor (26) (27) (28). In 2003 Foxp3 was reported as the master transcriptional regulator for naturally occurring Treg cells (nTreg) (29) (30). Recent studies have acknowledged Treg to be instrumental in the prevention of autoimmune disease, such as type I diabetes (31), the limitation of chronic inflammatory disease, such as inflammatory bowel disease (IBD) (32). However, on the down-side, Treg also appear to be a detrimental factor in the generation of host-versus-tumour immunity via suppression of tumour-specific effector T-cell responses and development of immune tolerance to neoplastic cells (33).

4.2.2 Origin and phenotype of Treg

Naturally occurring CD4 Treg originate in the thymus by high-affinity interaction of T cell receptor (TCR) with self-peptide/MHC expressed in the thymic stromal cells. nTreg constitutively express high level of IL-2 receptor α-chain, CD25, and other surface markers, such as glucocorticoid-induced tumour necrosis factor receptor (GITR) (34) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (35; 36). Foxp3 is a master gene, which controls nTreg development and function, and is currently the most reliable molecular marker for nTreg.

Induced regulatory T cells (iTreg) are generated in the periphery. Their phenotype resembles nTreg. However, to distinguish the function and the particularly relative importance of these two Treg populations in experimental animals as well as in humans has been elusively difficult. In addition, naive T cells in humans readily express Foxp3 upon TCR stimulation, although the expression is generally much lower and more transient than nTreg (37; 38).

4.2.3 Lineages of Treg

CD4 Treg are conceptually divided into three populations. Thymus-derived nTreg are the classic Treg with $CD4^+CD25^+FOXP3^+$ phenotype; iTreg can be further divided into two subsets: Tr1 and Th3 cells. CD4 Treg secreting IL-10, called Tr1 cells, are induced *in vitro* by antigenic stimulation of naive T cells in the presence of IL-10 (39). Antigen-specific TGF-β secreting Treg, called Th3 cells, are induced in the context of orally administered protein antigens (40; 41). Regarding the expression of Foxp3, at least some Th3 cells appear to be TGF-β-induced $Foxp3^+$ cells, while Tr1 cells do not express Foxp3. However, the properties of Tr1 cells *in vitro* are very similar to those of nTreg. For example, upon antigenic stimulation they display an activated cell surface phenotype, exhibit hypoproliferation, exert cell-cell contact dependent suppression, and scarcely produce IL-2. Therefore, some immunologists do not consider Tr1 cells as a distinct lineage but rather a certain state of nTreg. Actually, in this classification, the cytokine and suppressive modes are not mutually exclusive and they often overlap (42).

4.2.4 Suppression mechanisms of Treg

To understand how Treg exert their suppressive function has its therapeutic importance via elucidating potential targets. From the observations to date, the various suppression mechanisms utilized by Treg fall into four basic “modes of action”. They are the suppressions

through a) inhibitory cytokines, b) cytolysis, c) metabolic disruption and d) modulation of dendritic cell (DC) maturation and/or function (43).

Through inhibitory cytokines: IL-10 and TGF-β are no doubt immunological suppressors utilized by Treg. *In vitro* data showed that in a human tumour microenvironment, IL-10 is required for a cell-cell contact independent suppression mechanism (44). TGF-β is reported to be produced by Treg in order to limit anti-tumour immunity, for instance, in head and neck squamous-cell carcinoma (45), in follicular lymphoma (46) and in lung cancer (47). Whether IL-10 and TGF-β work in a synergetic way is not clear yet. However, IL-10 was found to be utilized by Treg alone (48) and with TGF-b (49).

Recently, a novel inhibitory cytokine, IL-35, has been described that is constitutively secreted by mouse $Foxp3^+$ nTreg but not by resting or activated effector CD4 T cells. IL-35 is a new member of IL-12 family and is formed by a pairing of Epstein-Barr virus-induced gene 3 (Ebi3) and p35 (also known as IL2α). $Ebi3^{-/-}$ and $IL12\alpha^{-/-}$ Treg had markedly reduced regulatory activity *in vitro*, failed to control homeostatic proliferation of effector T cells and resolved IBD *in vivo*. Therefore, IL-35 is required for maximal suppressive activity of Treg. Moreover, IL-35 is sufficient to confer a regulatory capacity on naive T cells (50).

Through cytolysis: Besides NK cells and CTL, few circulating human CD4 T cells can express granzyme A and B. Tr1 cells generated from CD4 T lymphocytes by antibodies CD3/CD28 were reported to be capable of expressing granzyme B and of killing target cells in a perforin-dependent, but MHC/TCR- independent, manner (51).

Through metabolic disruption: This idea was proposed long time ago that Treg might starve Teff through deprivation of local IL-2 due to their bright CD25 expression (52) (53).

Through targeting DC: By using intravital microscopy, Tang Q. and his colleagues first showed direct interaction between Treg and DC *in vivo*, and this interaction was considered to attenuate Teff activation by DC (54) (55). Treg can modulate DC through CTLA-4 to CD80/CD86 pathway. Consequently, they induce DC to express IDO, a potent enzyme inducing apoptosis in Teff cells (56).

4.2.5 Treg in cancer

Naturally occurring Treg constitute 5-6% of overall CD4 T cell population and bear a TCR repertoire for self-antigens (57). Thus, they are thought to be one likely source of tumour infiltrating Treg, as most of tumour-antigens are unaltered self-antigens (58). nTreg mediate immune suppression through cell-cell contact mechanism (59).

Also, iTreg have been identified in cancer patients, for instance, a human gastric cancer (60). These Treg induced by MHC-peptide stimulation secrete a large amount of IL-10 and/or TGF-β, and suppress immune responses through a cytokine-dependent mechanism (61). In human cancers, increased numbers of Treg accumulating in several types of solid tumour have been observed by using initially $CD4^+CD25^+$ Treg markers and recently with or without a combination of the more specific marker Foxp3.

Besides the high amount of tumour infiltrating Treg, elevated percentages of Treg among total T cell pool are often reported in various cancers, including breast carcinoma (62). The augmentation of Treg in the periphery was also reported to be correlated with a poor prognosis in ovarian cancer patients (63)

How tumour-associated Treg are recruited to the tumour microenvironment is under intensive study. To date, the only observation of chemokine-mediated attraction was revealed in ovarian cancer. Treg govern CCR4 on their surface in order to be response to its ligand CCL22 which is secreted by tumour-associated macrophages (63).

In addition to Treg recruitment, Treg expansion might be led by encountering tumour antigens at tumour sites. A third alternative would be a conversion of naive and/or effector cells into Treg by TGF-β and/or IL-10 cytokine secreted by tumour-associated cells, immature myeloid DC or immature myeloid suppressor cells etc at the tumour sites (64) (65).

Even though it is conceivable, whether tumour antigen specific Treg exist in human cancer or not remains largely unknown. To date, compelling positive evidence was only shown by the group of Rong-Fu Wang. Two Treg colons were isolated from melanoma TIL, LAGE-1-specific (a homolog of testis-specific antigen NY-ESO-1) and ARTC1-specific Treg. Importantly, these clones retain the suppression to peptide-specific CTL function (66; 67).

4.3 Immunotherapy in breast cancer

Much has been learned about the potential of the immune system to control cancer. Subsequently, immunotherapy is under vigorous development. There are two major approaches to immunotherapy for cancer: active specific immunotherapy (cancer-vaccine) and passive immunotherapy (antibody therapy and T-cell adoptive transfer therapy). An integration of immune therapies with standard treatments for cancer represents a modern modality as well as a challenge.

In order to achieve a clinically effective response, there are three requirements: a sufficient number of lymphocytes with avid tumour-recognition must be introduced into the tumour-

bearing host; these lymphocytes have to successfully migrate and extravasate to the tumour-site; and not least, those TIL must have appropriate effector mechanisms to destroy cancer cells (68).

4.3.1 Cancer vaccine in breast cancer

Cancer vaccine aims to stimulate the adaptive arm of the immune system directly *in vivo*. It has a variety of modality, such as tumour-associated antigen based vaccine, DC vaccine, DNA vaccine etc. Apart from the vaccine construction, to define an appropriate tumour-antigen is essential for developing a potentially effective vaccine. So far, more than 100 peptides have been identified. Some are derived from proteins which are the products of unique mutations (tumour-specific antigen), but most of them are derived from normal proteins that are differentially expressed by tumour cells (tumour-associated antigen). This list is still growing (69) (70). Particularly in breast cancer, Table 1 lists a number of defined tumour antigens which have been tested as potential targets for cancer vaccine. Many tumour antigens used in breast-cancer immunotherapy are expressed on normal tissues but overexpressed or mutated on tumour cells, such as Her2/neu, Muc1, p53, CEA, hTERT and carbohydrate antigens. Some of these antigens are universal tumour-antigen, such as hTERT, as they are broadly expressed by most tumours.

Preclinical testing of vaccines based on Her2/neu (71), Muc-1 (72) and CEA (73) in rodents showed tumour regression without any autoimmunity. However, it has been more than one decade that moving breast cancer vaccine from animal models to clinical trials only achieved limited or marginally clinical outcomes (74). There are two possible reasons. First, most immunotherapy is tested in advanced patients with progressive and refractory tumours after all conventional therapies have failed. Late stage tumours have already built up immunosuppressive environment in favour of their growth, such as suppressive factor TGF-b (75), soluble Fas ligand (76) or suppressive enzyme, IDO (77) (78). Under certain circumstances, those patients are often reported to be associated with a decrease in the number as well as in the function of peripheral blood lymphocytes and dendritic cells (79), which would dampen cancer vaccine in many means.

Another reason is brought along with increasing understanding of regulatory T cells. The elevated number of functional Treg in cancer patients is believed to be a big obstacle to immunotherapy. More recent evidence implies that the current cancer vaccine strategy might provoke Treg activation and expansion. For example, one important tumour vaccine strategy is using engineered DC (80), namely fully matured or polarized DC. It was well accepted in the past that in order to obtain therapeutic efficacy fully matured DC have to be

carefully manipulated, since immatured and semi-matured DC were evidenced to induce regulatory T cells in human studies (81) (82) (83). However, more recent observations in a transgenic mouse model suggested matured DC can also activate and expand autologous antigen-specific Treg (84).

Table 1. Antigens targeted in the experimental production of vaccines against breast cancer.

Tumour Antigen	Description	Relevance to breast cancer	Ref.
Her2/neu	• 185-kD receptor belonging to the epidermal growth factor receptor family; • It has both extracellular and intracellular domains.	• Gene is amplified or protein is overexpressed in 20-40% of patients with breast cancer. • Associated with poor grognosis	(85)
Mucin 1 (Muc-1)	• Membrane-associated glycoprotein expressed in ductal tissues of breast, pancreas, airway, and gastrointestinal tract; • Involved in protection of mucous membranes and signal transduction	• Overexpressed by more than 70% of cancers; • Abberrantly glycosylated in malignant cells	(86)
Mammaglobin A	• 10-kD glycoprotein of unknown function; • Expressed almost exclusively in breast epithelium	• Overexpressed in 80% of primary and metastatic breast cancers	(87)
P53	• Normal p53 contributes to DNA repair and, when DNA damage is irreparable, programmed cell death (apoptosis).	• Mutated in 20% of breast cancers	(88)
Carcinoembryonic antigen (CEA)	• Glycoprotein involved with cell adhesion; • Production normally restricted to fetal development	• Expressed in carcinomas of the colon, rectum, breast, lung, pancreas and gastrointestinal tract	(89)
hTERT	• The catalytic protein component of telomerase, which protects telomeric ends of chromosomes from degradation during successive rounds of cell division.	• Continued production of telomerase beyond the time of normal cellular senescence allows unlimited cell divisons to occur; • Nearly all human ceancer cells express hTERT.	(90)
Cancer-testis antigens (NY-ESO-1, MAGE, BAGE and GAGE)	• Highly immunogenic proteins expressed exclusively in normal germ cells of the testis and embryonic ovaries and in some cancers	• mRNA expression detected in 24% (NY-ESO-1), 8% (MAGE), 2% (BAGE) and 8% (GAGE) of patients with breast cancer	(91)

4.3.2 Passive immunotherapy in breast cancer

Passive immunotherapy of breast cancer includes immunotherapy with antibodies or T cells. The most clinically effective monoclonal antibody administrated against breast cancer is Trastuzumab (Herpcetin) (92). However, this treatment is restricted to Her2-positive cancer patients. More broad usages of antibody therapy are ongoing. Lots of efforts are made to produce antibodies not only against known tumour antigens but also to the molecules produced by tumours to promote their own survival, for example, Bevacizumab (Avastin). Bevacizumab is a recombinant humanised monoclonal antibody developed against vascular endothelial growth factor (VEGF). It binds to soluble VEGF preventing receptor binding and inhibiting endothelial cell proliferation and vessel formation. Bevacizumab is the first anti-angiogenetic treatment approved by the American Food and Drug Administration in the first-line treatment of metastatic colorectal cancer. It has shown preliminary evidence of efficacy for breast cancer (93).

Of all immune cells T cells are most often affected. Leading to a prolonged deficiency of T cells has important clinical consequences. Accordingly, strategies to improve and recover the function of T cells should have a direct impact on reducing the morbidity and mortality of many cancers. Adoptive transfer of antigen-specific T lymphocytes is a powerful therapy, which has been successfully used for the treatment of virus-associated malignancies, such as Epstein-Barr virus-positive post transplant lymphoproliferative disease (94).

The source of adoptively transferred T cells can be: 1) *in vitro* expanded CTL clones after several rounds of tumour antigen stimulation; 2) TCR engineered T cells, which have not yet been transferred into the clinical studies; and 3) short term *ex vivo* activated autologous T cells.

The clinical trials of using CTL clones for the treatment of patients with metastatic melanoma were well studied in Rosenberg's group. They used repetitive tumour-antigen stimulation for a relatively long term culture of CTL clones in order to achieve a sizable number of CTL for transfer. Also, a high amount of IL-2 was accompanied for the treatment. Even though the T-cell clones used for therapy were highly avid and exhibited potent tumour lysis *in vitro*, they did not persist after infusion, and the clinical response was observed at average of 15% (95) (96).

Regarding the possible explanations of the limited clinical outcomes, except the ones being mentioned above, the terminal differentiation of transferred tumour-reactive CD8 T cells appears to be another hindrance. The phenotype of late stage effector CD8 T cells in a progressive differentiation includes a loss of lymph node homing receptor CD62L; a loss of co-stimulatory molecules CD27 and CD28; and a loss of survival cytokine IL-7 receptor CCR7.

Although these phenotypes are strongly associated with effective function *in vitro*, they are evidenced to limit the survival and the effective function of the transferred T cells *in vivo* (97) (96) (98).

Alternatively, the strategy of adoptively transferring short term (72 hr) *ex vivo* activated autologous T cells into patients with metastatic breast cancer showed 47% of biological response from our phase I study (99). This supports the notion that early effector CD8 T cells exhibit better anti-tumour effects *in vivo* (100).

Besides a modulation of the status of transferred T cells, to hurdle the suppressive environment in hosts is becoming more and more essential for enhancing the efficacy of passive immunotherapy. The marked improvement of adoptive T-cell transfer in lymphopaneic host provided indirect evidence of the importance of Treg elimination before adoptive T-cell transfer (101) (102). Our previous evaluation of clinical non-responders also suggested that the existent of regulatory T cells have immunosuppressive impacts on both priming phase in the culture and proliferation phase *in vivo* after transfer (99).

4.4 Objectives of this thesis

This thesis is based on the context of our phase I clinical study of adoptive transfer of autologous bone marrow derived T cells in advanced metastatic breast cancer patients. The observations of T-cell unresponsiveness in 53% of study patients in this study led us to an investigation of Treg in their impact on type-I T-cell anti-tumour immunity. To meet this objective this thesis addresses five separate questions:

- What are the frequencies of Treg in the peripheral blood and bone marrow of patients with breast cancer?
- What is the extent of the immune suppression exerted by Treg in breast cancer?
- What is the suppressive mechanism utilized by Treg in breast cancer?
- Whether do Treg possess antigen specificity towards breast tumour?
- How can we develop an appropriate modality of immunotherapy for breast cancer in order to conquer the obstacle generated by Treg?

5 MATERIALS AND METHODS

5.1 Materials

5.1.1 Clinical and healthy donor samples

Peripheral blood and bone marrow of breast cancer patients	Gynaecological Hospital, University of Heidelberg, Heidelberg, Germany
Peripheral blood and bone marrow of Healthy donors	Witzens-Harig M., Clinic of Internal Medicine, Department of Haematology, University of Heidelberg, Heidelberg, Germany
Leukocyte concentrate/ buffy coat	Blood donation centre, Heidelberg
Leukapheresis	Clinic of Internal Medicine, Department of Haematology, University of Heidelberg, Heidelberg, Germany

5.1.2 Flow cytometric antibodies

Specificity	Species	Isotype	Conjugate	Clone	Company
CD3	Mouse	IgG_{2a}	PE-Cy5	HIT3a	BD Pharmingen, D
CD3	Mouse	IgG_{1k}	PE	UCHT1	BD Pharmingen, D
CD4	Mouse	IgG_1	FITC	RPA-T4	BD Pharmingen, D
CD4	Mouse	IgG_{1k}	PerCP-Cy5.5	SK3	BD Pharmingen, D
CD25	Mouse	IgG_{2b}	PE	4E3	Miltenyi Biotech, D
IL-10	Rat	IgG_1	PE	JES3-9D7	BD Pharmingen, D
Foxp3	Rat	IgG_{2a}	APC	PCH101	eBioscience, USA
Foxp3	Mouse	IgG_1	APC	236A/E	eBioscience, USA
CCR2	Mouse	IgG_{2b}	-	48607	R&D System, D
CCR4	Mouse	IgG_{1k}	-	1G1	BD Pharmingen, D
CCR5	Mouse	IgG_{2ak}	-	2D7	BD Pharmingen, D
CCR6	Mouse	IgG_{1k}	-	11A9	BD Pharmingen, D
Mouse IgG/M	Goat	Ig	FITC	polyclonal	BD Pharmingen, D

5.1.3 Flow cytometric kit and supplements

BD Cytofix/Cytoperm™ solution	BD Pharmingen, Germany
BD GolgiStop™ protein transport inhibitor	BD Pharmingen, Germany
BD Perm/Wash™ solution	BD Pharmingen, Germany
Dulbecco's phosphate buffered saline	PAA Laboratories, Cölbe, Germany
Fixation/Permeabilisation Concentrate	eBioscience, San Diego, USA
Fixation/Permeabilisation Diluent	eBioscience, San Diego, USA
Permeabilisation Buffer	eBioscience, San Diego, USA
Rat-Serum	eBioscience, San Diego, USA

5.1.4 Cell cultivation media and supplements

DMEM	PAA Laboratories, Cölbe, Germany
Fetal Calf Serum (FCS)	Biochrom, Berlin, Germany
HEPES	Biochrom, Berlin, Germany
Human AB-Serum	Sigma, Deisenhofen, Germany
Geneticin (G418)	Life Technologies, Karlsruhe, Germany
Gentamycin	Biochrom, Berlin, Germany
Penicillin / Streptomycin	Biochrom, Berlin, Germany
RPMI 1640 with glutamine	PAA Laboratories, Cölbe, Germany
X-VIVO 20 (serum-free)	BioWhittaker, Vervier, Belgium
Recombinant GM-CSF	Essex Pharma, München, Germany
Recombinant human IL-2	Chiron, Ratingen, Germany
Recombinant human IL-4	PromoCell, Heidelberg, Germany

Recombinant human IL-7	PromoCell, Heidelberg, Germany
Recombinant human IL-15	PromoCell, Heidelberg, Germany

5.1.5 Kits, beads and supplements

5.1.5.1 Cell isolation kits, beads and supplements

CliniMACS® CD25 Reagents	Miltenyi Biotech, Bergisch Gladbach, Germany
CliniMACS® PBS/EDTA Buffer	Miltenyi Biotech, Bergisch Gladbach, Germany
mCD56 IgG_1, C218	Beckman Coulter, Germany
Dynabeads© Pan Mouse IgG	Invitrogen, Karlsruhe, Germany
Dynabeads© M-450 CD3	Invitrogen, Karlsruhe, Germany
Dynabeads© M-450 CD19	Invitrogen, Karlsruhe, Germany
Dynal© T cell Negative Isolation Kit	Invitrogen, Karlsruhe, Germany
IFN-γ Secretion Assay - Detection Kit (PE), human	Miltenyi Biotech, Bergisch Gladbach, Germany
MACS© $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human	Miltenyi Biotech, Bergisch Gladbach, Germany

5.1.5.2 ELISpot kits and supplements

Maus-anti-Human-IFN-γ	Mabtech, Hamburg, Germany
Maus-anti-Human-IFN-γ biotin	Mabtech, Hamburg, Germany
AP Conjugate-Substrate Kit	Bio-Rad, München, Germany
Staphylococcus Enterotoxin B	Sigma Aldrich, Deisenhofen, Germany
Streptavidin-Alkaline Phosphatase	MABTECH AB, Hamburg, Germany

5.1.5.3 ELISA kits

Quantikine Human IL-10 Immunoassay	R&D systems, Wiesbaden, Germany

5.1.5.4 Cell culture kits and beads

Dynabeads© Anti-Human-CD3/CD28 T cell Expander	Invitrogen, Karlsruhe, Germany
Treg Suppression Inspector Kit	Miltenyi Biotech, Bergisch Gladbach, Germany

5.1.6 Established cell lines

KS24.22	Gückel B., Department of Gynaecology and Obstetrics, University of Tübingen, Tübingen, Germany
MCF7	ATCC, # HTB-22, Rockville, USA
U937	ATCC, # CRL-1593.2, Rockville, USA

5.1.7 Polypeptides

CEA (p569-618)	DKFZ (V270), Heidelberg, Germany
EGFR (p479-528)	DKFZ (V270), Heidelberg, Germany
Her2/neu (p351-384)	DKFZ (V270), Heidelberg, Germany
Heparanase 1 (p1-50)	DKFZ (V270), Heidelberg, Germany
Heparanase 2 (p163-212)	DKFZ (V270), Heidelberg, Germany
Human immunoglobulin (Endobulin©)	Baxter, Frankfurt, Germany
hTERT (p958-1007)	DKFZ (V270), Heidelberg, Germany
Mam 1 (p4-56)	DKFZ (V270), Heidelberg, Germany

Mam 2 (p41-92)	DKFZ (V270), Heidelberg, Germany
MAGE 3 (p271-314)	DKFZ (V270), Heidelberg, Germany
Muc-1-100 (p1-100)	DKFZ (V270), Heidelberg, Germany
Muc-1-20 $(p137\text{-}157)_5$	DKFZ (V270), Heidelberg, Germany
p53 (p118-167)	DKFZ (V270), Heidelberg, Germany
Survivin (p93-142)	DKFZ (V270), Heidelberg, Germany

5.1.8 Chemicals

^{3}H thymidine	Amersham, Braunschweig, Germany
Aqua ad injectabilia	B Braun AG, Melsungen, Germany
Betaplate Scint	Perkin Elmer, Wellesley, USA
Biocoll Separating Solution	Biochrom, Berlin, Germany
Endoxan 50 mg	Baxter Oncology GmbH
DMSO	Merck, Darmstadt, Germany
EDTA	Biochrom, Berlin, Germany
Ethanol Absolute	Riedl-de-Haen, Seelze, Germany
Sprüh Kleber Super	Tetenal AG, Norderstedt, Germany
Trypan Blue	Serva, Heidelberg, Germany
Tween 20	Gerbu Biotechnik, Gaiberg, Germany

5.1.9 Consumables

Cell Culture Test Plates (flat bottom: 6-, 24-, 48- and 96-well; round-bottom: 96-well)	TPP, Trasadingen, Switzerland

CliniMACS® Tubing set	Miltenyi Biotech, Bergisch Gladbach, Germany
Combitips	Eppendorf, Hamburg, Germany
Cover Slips (24 x 50 mm)	R. Langenbrinck, Teningen, Germany
Cryotubes (1.8 ml)	Corning B.V., Schipol-Rijk, Netherlands
Disposable pipettes	Renner, Darmstadt, Germany
Disposable scalpels	PfM AG, Köln, Germany
Disposable syringes (1-50 ml)	BD Pharmingen, Heidelberg, Germany
ELISpot-plates	Millipore, Eschborn, Germany
FACS-tubes	Greiner, Frickenhausen, Germany
Parafilm	American National Can Company, USA
Petri Dishes (tissue culture-treated)	Biochrom, Berlin, Germany
Pipette tips (2, 20, 100, 200, 1000 µl)	Gilson, Bad Camberg, Germany
Printed Filtermate A (90 x 120 mm)	Wallac, Turku, Finland
Safe-Lock Reaction tubes (0.5 ml, 1 ml)	Ependorf, Hamburg, Germany
Sample bag for 1450 MicroBeta	Perkin Elmer, Wellesley, USA
Sterile-filter (0.22 µm)	Millipore, Molskeim, France
Tubes (15 ml, 50 ml)	Biochrom, Berlin, Germany
Tubes with Filter Leucosep© (50 ml)	Greiner, Frickenhausen, Germany
TPP tissue culture flasks (T75)	Sigma Aldrich, Deisenhofen, Germany

5.1.10 Equipments

autoMACS™ Separator	Miltenyi Biotech, Bergisch Gladbach, Germany
Biological Safety Cabinet	SterilGARD Hood Baker, Stanford, USA

CliniMACS® Plus Instrument	Miltenyi Biotech, Bergisch Gladbach, Germany
Centrifuge (Minifuge T)	Heraeus, Hanau, Germany
DOS-compatible Intel P4 computer	edo GmbH, Hockenheim, Germany
ELISA-reader	Labsystems, Helsinki, Finland
ELISpot-Microscope Axioplan 2 Imaging	Zeiss, Oberkochen, Germany
FACS Calibur	Becton Dickinson, Heidelberg, Germany
FACS Canto II	Becton Dickinson, Heidelberg, Germany
Freezer (-20°C)	Liebherr, Ochsenhausen, Germany
Freezer (-80°C) Bio Freeze©	Forma Scientific, USA
Glass Pipettes	Hirschmann, Eberstadt, Germany
Glassware	Schott, Mainz, Germany
Heat sealer (1295-012)	Perkin Elmer, Wellesley, USA
Incubator	Nuaire, Plymouth, USA
Liquid Scintillation Counter (1450 MicroBeta©)	Perkin Elmer, Wellesley, USA
MacBook Pro	Apple Macintosh, Cork, Ireland
Magnetic Particle Concentrator (6 x 15 ml tubes, 6 x 2 ml tubes)	Dynal, Hamburg, Germany
MACS® MultiStand	Miltenyi Biotech, Bergisch Gladbach, Germany
Micropipettes (2-1000 µl)	Gilson, Bad Camberg, Germany
Milli-Q Water Purification Device	Millipore, Eschborn, Germany
Multichannel Pipettes (8, 12 channels)	Rainin, Leiden, Netherlands
Neubauer counting chamber (0.1 mm)	Brand, Wertheim, Germany
OctoMACS™ separator	Miltenyi Biotech, Bergisch Gladbach, Germany

Pipetboy	Brand, Wertheim, Germany
Phase-contrast microscope	Zeiss, Göttingen, Germany
Refrigerator	Liebherr, Ochsenhausen, Germany
Roller-mixer RM5	Karl Hecht GmbH, Sondheim, Germany
Spectrophotometer Titertek Multiscan Plus	Labsystems, Helsinki, Finland
Tabletop centrifuge	Heraeus, Hanau, Germany
Tomtec Harvester Mach 3	Perkin Elmer, Wellesley, USA
Vortexer (Reax 2000)	Heidolph, Schwabach, Germany
Water bath (SW21)	Julabo, Seelbach, Germany

5.1.11 Softwares

CELLQuest Pro© (4.02)	Becton Dickinson, Heidelberg, Germany
FACS Diva Software	Becton Dickinson, Heidelberg, Germany
FlowJo FACScan Software (6.2)	Tree Star, San Carlo, USA
Graphpad Prism 5	GraphPad Software Inc., San Diego, USA
KS-ELISpot	Zeiss, Göttingen, Germany
Mac OS X	Apple Macintosh, Cork, Ireland
Microsoft© Windows XP	Microsoft, Redmond, USA
Microsoft© Excel 2007	Microsoft, Redmond, USA
Microsoft© Word 2007	Microsoft, Redmond, USA

5.2 Methods

5.2.1 Sample Collection

Each sample was obtained upon approval by the informed participant. The protocol was approved by the Ethical Committee of the University of Heidelberg. Clinical bone marrow samples were aspirated from the anterior iliac crest immediately after surgery, while the participants were still anaesthetised. The bone marrow of healthy donors was obtained under local anaesthesia.

Leukapheresis was obtained by the department of haematology of clinic of internal medicine, University of Heidelberg. This procedure is co-operated with Cytonet Heidelberg GmbH.

5.2.2 Methods of cell cultivation

5.2.2.1 Solutions and buffers

Trypan Blue stain:

Trypan Blue	1.28 g
NaCl	8.5 g
ddH_2O (filtered sterile)	ad 1 l
NaN_3	C_{final} = 1% (v/v)

Freezing solution:

Human AB-serum	800 µl
RPMI 1640 (for T cells) or X-VIVO 20 (for dendritic cells)	100 µl
DMSO	100 µl

5.2.2.2 Media and supplements

Tumour cell line medium:

DMEM	50 ml
FCS	10% (v/v)
Penicillin / Streptomycin	1% (v/v)

TC medium, pH = 7.4:

RPMI 1640	50 ml
HEPES	10 mM
Human AB-serum	10% (v/v)
Penicillin	50 µg/ml
Streptomycin	50 µg/ml
Human recombinant IL-2	100 IU/ml
Human recombinant IL-4	60 IU/ml
(Human recombinant IL-7	20 ng/ml)
(Human recombinant IL-15	20 ng/ml)

DC medium, pH = 7.4:

X-VIVO 20	50 ml
GM-CSF	560 IU/ml
Human recombinant IL-4	1000 IU/ml

5.2.2.3 Cultivation of cells

All types of cells were cultivated in sterile incubators at 37°C with a CO_2-concentration of 5% (v/v). T cells and dendritic cells were cultivated in the above described media, whereas DC medium was added 2% (v/v) autologous plasma. The cell lines U937, KS24.22 and MCF7 were cultivated in tumour cell line medium.

5.2.2.4 Quantification of viable cells

A Neubauer counting chamber was applied for the quantification of viable cells. For this purpose, cell suspension was diluted 1:10 or 1:100 in Trypan Blue stain and injected into the chamber. The average number of cells in one of large 9 counting areas was calculated under a microscope, whereat blue coloured cells were excluded from calculations. These cells were regarded to be nonviable, as Trypan Blue can only traverse the damaged plasma membrane of nonviable cells. The determined average cell number was multiplied with the respective dilution factor and the chamber factor 10^4 for the quantification of cells per 1 ml of suspension volume.

5.2.2.5 Passage of adherent cell lines

When confluence was reached, adherent cells were passaged under sterile conditions. After the removal of cell culture medium, cells were washed with Dulbecco´s PBS and detached from the flask by adding an EDTA/trypsin-mixture. EDTA resolves cell-cell contacts through the deprivation of essential divalent cations and the protease trypsin truncates cellular adherence molecules that establish the attachment to the flask. The reaction was quenched with RPMI 1640 + 10% (v/v) FCS after 5 minutes of incubation at 37°C, as the proteolysis by trypsin is not restricted to adherence molecules but cytotoxic with increasing reaction time. The detached cells were spun down at 1400 rpm for 10 min, the pellet was resuspended in tumour cell line medium and finally distributed to 3 tissue culture flasks.

5.2.2.6 Storage and thawing of cells

In order to store cells for longer periods of time, these were spun down at 1400 rpm for 10 min and resuspended in 1 ml freezing solution. The suspension was transferred to a cryotube and kept on ice for 30 min, to secure a slow cooling before dropping temperature to -80°C. The slow cooling prevents the formation of detrimental ice crystals. For thawing, 10 ml medium was pre-heated to 37 C and added to the frozen cell suspension. This effected a rapid transition to the liquid phase and allowed a fast separation of cells from the freezing solution by spinning down (1400 rpm, 10 min) and resuspending in a new medium. The fast separation of cells from the freezing solution is crucial, as DMSO not only has cryo-protective properties but also is highly cytotoxic.

5.2.3 Methods of immunobiology

5.2.3.1 Isolation of mononuclear cells from human peripheral blood and bone marrow

For the isolation of mononuclear cells, peripheral blood and bone marrow were subjected to a Ficoll gradient density centrifugation. In detail, 15 ml Biocoll solution were added to a Ficoll (Leucosep©) tube. This was quick-spun to 1000 rpm, to make the solution pass the filter. Now, 15 ml RPMI 1640 were added on top of the filter and 20 ml peripheral blood were slowly mixed with the medium. For bone marrow, 15 ml Biocoll solution were firstly added to a 50 ml tube. 20 ml bone marrow were mixed with the 15 ml RPMI 1640 and very carefully layered on top of the 15 ml Biocoll solution. Filter-added Ficoll tubes were not used for the purification of mononuclear cells from bone marrow, as these regularly contained

solid particles which would have plugged the filter and interfered with the formation of an interphase. After centrifuging the prepared tubes at 2000 rpm with a disabled break for 20 min, the formed interphase of mononuclear cells between supernatant and Ficoll-Hypaque was collected. Subsequently, the cells were washed with RPMI 1640 and cultured in cytokine-free X-VIVO 20 for 2-16 hr at 37°C. The incubation was carried out in tissue culture-treated petri dishes, enabling the later enrichment of plastic-adherent monocytes by slowly washing off non-adherent cells. Dendritic cells were generated from $CD14^+$ monocytes through cultivating for 5-8 days in DC medium, whereas non-adherent cells were separately cultivated in TC medium.

5.2.3.2 Enrichment of dendritic cells

Dendritic cells were enriched from plastic-adherent monocytes by slowly washing off non-adherent cells and followed with 5-8 days culture in DC medium. Enriched DC were used for pilot clinical studies.

5.2.3.3 Purification of dendritic cells

Dendritic cells were generated from $CD14^+$ monocytes through cultivating for 5-8 days in DC medium. Dendritic cells were purified via immunomagnetic Dynabeads©. These are superparamagnetic polymer particles sized to 4.5 µm and coated with a monoclonal antibody. Using the affinity of the antibody for defined cell surface molecules, specific cell subsets can be targeted by their expression of a surface marker and therefore are selectively labelled with magnetic beads.

Dendritic cells were negatively selected through the labelling of $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ natural killer cells with respectively coated beads and by separating the beads from the supernatant in the magnetic field of a magnetic particle concentrator. Previously, Pan Mouse IgG Dynabeads© were coated with αCD56 mAb, to be applied to the cell suspension together with the αCD3-/αCD19-coated Dynabeads©, according to the manufacturer´s instructions.

5.2.3.4 Purification of T cells

The purification of T cells from cultivated non-adherent cells was carried out with the Dynal© T cell Negative Isolation Kit. The procedural recommendations of the manufacturer were followed thoroughly. The applied kit depleted all non-T cells from the suspension by labelling B cells, NK cells, monocytes and erythrocytes with magnetic beads that were separated from the supernatant.

5.2.3.5 Isolation of $CD4^+CD25^+$ T cells

MACS buffer, pH = 7.2:

Human AB-serum	500 µl
Dulbecco´s PBS	47 ml
EDTA	2 mM

Regarding research grade, $CD4^+CD25^+$ T cells were isolated from previously purified T cells by applying the MACS© $CD4^+CD25^+$ regulatory T cell Isolation Kit. The instructions of the manufacturer were respected apart from using MS columns for both separations and flushing all columns 2x with MACS buffer, to more efficiently elute labelled cells. In detail, $CD4^+$ T cells first were negatively selected followed by a positive selection of $CD4^+CD25^+$ T cells. No cell subset was discarded, as eluted $CD8^+$ T cells from the first separation were pooled with $CD4^+CD25^-$ T cells in the flow-through of the second separation. The pooled population was denoted as Treg-depleted Tcon, whereas the isolated $CD4^+CD25^+$ T cells were regarded as Treg enriched.

Regarding clinical grade, $CD4^+CD25^+$ T cells were isolated from leukapheresis by applying the CliniMACS® CD25 Reagent. The instructions of the manufacturer were respected. In detail, the CliniCD25 labelling was carried out at beads to cells ratio of 1:27, and 30 min incubation at RT. Cells then were washed with CliniMACS® PBS/EDTA Buffer 2x before running through a CliniMACS® Plus Instrument with Program 3.1 or Program 2.1.

5.2.4 Analytical methods of immunobiology

5.2.4.1 Solutions and buffers

Flow cytometry buffer:

Dulbecco's PBS	50 ml
FCS	2% (v/v)

ELISpot wash buffer:

Dulbecco's PBS	1 l
Tween 20	500 µl

ELISpot substrate solution (for 100 wells):

ddH_2O	10 ml
25x substrate buffer	400 µl
Substrate A	100 µl
Substrate B	100 µl

5% (v/v) AB-medium:

Human AB-serum	2.5 ml
RPMI 1640	ad 50 ml

10% (v/v) AB-medium:

Human AB-serum	5 ml
RPMI 1640	ad 50 ml

Protein/peptide-solutions:
lyophilisates of polypeptides were dissolved in Aqua ad injectabilia to final concentrations between 2 and 50 µg/µl

5.2.4.2 Flow cytometry

The phenotyping of cells was conducted via flow cytometric analysis. 5 x 10^5 mononuclear cells or 10^5 enriched T cells were required for each experiment. In the cases that a combination of intracellular staining of IL-10 was applied, BD GolgiStop™ protein transport inhibitor was added into PBMC stimulation culture for the last 10 hr. In any cases, the flow cytometric assay was started with the staining of surface antigens. Cells were washed by spinning down (whole protocol: 5500 rpm for 1 min) and resuspending in flow cytometry buffer. The entire subsequent procedure was conducted on ice. After washing, cells were spun down, resuspended in 50 µl Endobulin (2.5 µg/µl) and cell surface F_c-receptors were blocked by incubating for 15 min. A following surface staining of washed cells was carried out, resuspending the pellet in 50 µl of 1:10 diluted primary, fluorescence dye- or biotin-labelled antibody(ies) and incubating for 20 min. Unbound antibody was washed away and the pellet was resuspended in 100 µl flow cytometry buffer for later analysis. Given the incubation with primary un-labelled antibody(ies), the pellet was resuspended in 50 µl of 1:10 diluted fluorescence dye-labelled secondary antibody and incubated for further 20 min, before preparing for analysis.

To stain intranuclear Foxp3, a fixation and permeabilisation of cells was conducted by resuspending in 750 µl Fixation/Permeabilisation Buffer and incubating for 30 min. Afterwards, a blocking with 2% (v/v) Rat-Serum and an incubation in 50 µl 1:10 diluted αFoxp3 mAb was performed analogously to the surface staining, but using Permeabilisation Buffer instead of flow cytometry buffer for dilutions and washing steps.

To stain intracellular IL-10, thoroughly resuspend cells were treated with 100 µl of BD Cytofix/Cytoperm™ solution for 20 min at 4°C followed by 2x wash with BD Perm/Wash™ solution. After being thoroughly resuspended, the fixed/permeabilized cells were stained in 50 µl of BD Perm/Wash™ solution containing 1:50 diluted PE-conjugated αIL-10 antibody for 30 min at 4°C in the dark.

Finally, expression patterns were read out, collecting > 5 x 10^4 events with a FACS Calibur or FACS Canto II that ran CELLQuest Pro© and FACS Diva Software, respectively. The acquired data was analysed with FlowJo FACScan Software.

5.2.4.3 IFN-γ Secretion Assay

The IFN-γ secretion assay was designed for the detection and analysis of viable IFN-γ-secreting leukocytes, especially for the detection and isolation of antigen-specific T cells.

After a restimulation with specific antigen *in vitro* secretion of IFN-γ is induced. In this thesis, we applied this assay to evaluate the impact of Treg on type-I T-cell anti-tumour immunity. In detail, purified T cells derived from breast cancer patients were stimulated with MCF7 or U937 cell lysates presented by autologous DC for 16 hr in an incubator at 37°C. After coating all cells with IFN-γ antibody on ice for 5 min warm X-VIVO 20 were added to dilute cells to 10^6 cells/ml. IFN-γ capture was then carried out in a rotating incubator for 45 min at 37°C. Next, the secreted and retained IFN-γ on cell surface was labelled with PE-conjugated secondary Ab anit-IFN-γ. Meanwhile αCD3 and αCD4 Abs were applied in order to define the interest population. The PE positive cells can finally be targeted by anti-PE magnetic beads, and isolated through a column applied in a magnetic field.

5.2.4.4 IFN-γ ELISpot (enzyme-linked immunospot) assay

The ELISpot assay was designed, in order to detect the secretion of immunological relevant molecules on the single cell level and to determine the frequency of secreting cells within a population. Thereby, it employs the sandwich ELISA technique, visualising molecules that have bound to an immobilised antibody followed by an automated readout. In this study, a short-term (40 hr) ELISpot assay was applied, to quantify activated T cells upon antigen encounter. Therefore, the secretion of IFN-γ was analysed, as this cytokine is known to be primarily produced and secreted by activated $CD4^+$ and $CD8^+$ T cells.

Initially, the nitrocellulose membrane of a 96-well ELISpot-plate was coated with αIFN-γ mAb. In detail, 100 µl Dulbecco´s PBS mixed with 15 µg/ml αIFN-γ mAb were added to each well. The plate was vortexed gently and incubated at 37°C for 3 hr or at 4°C o/n. After coating, the supernatant was discarded and the plate was washed 4x with 200 µl ELISpot wash buffer. Subsequently, 200 µl 5% AB-medium/well were added followed by an incubation at 37°C for 30-45 min. This step saturated remaining membrane binding sites with human serum proteins to prevent the non-specific binding of secreted IFN-γ. Afterwards, the blocking solution was discarded, the plate was washed 3x and 100 µl X-VIVO 20/well were added.

Simultaneously, enriched dendritic cells from peripheral blood and bone marrow were pooled and pulsed with antigens. For that purpose, 10^4 dendritic cells/well were cultured o/n at 37°C in 200 µl X-VIVO 20 with 200 µg/ml antigen. The next day, 5 x 10^4 total T cells or Treg-depleted Tcon/well were distributed to the ELISpot-plate. Each T cell type was added in triplicate of per tested antigen. Now, antigen-pulsed dendritic cells were distributed to the plate in a DC:Tcon-ratio of 1:10 (5 x 10^3 dendritic cells/well) to a total reaction volume of 200 µl/well. The 2x quantity of required dendritic cells had been pulsed, due to a strong loss

of cells during the pulsing procedure. As controls, only antigen-pulsed dendritic cells and only T cells were added to the plate. The T cells were activated by the superantigen SEB and represented the positive control. Now, T cells and antigen-pulsed dentritic cells were cocultured at 37°C for 40 hr.

The secretion of IFN-γ by total T cells and Tcon in response to several different antigens/antigen-mixes was analysed. First, the reactivity against lysates from the allogenic tumour cell lines MCF7, KS24.22 and U937 was tested. The breast cancer cell line MCF7 is known to overexpress several breast cancer marker-proteins like the oestrogen receptors α and β. Comparably, the genetically modified breast cancer cell line KS24.22 overexpresses the breast cancer-associated proteins Muc-1 and Her2/neu. Further, this cell line was generated under GMP conditions and can therefore be used in clinical studies. In contrast, U937 is a promonocytic leukaemia cell line that was applied to assess the background-reactivity against common tumour antigens and the basic reactivity against alloantigens.

Apart from cell line antigens, dendritic cells were also pulsed with polypeptides that comprised single antigens from breast cancer-associated proteins. All peptides were designed with respect to reported epitopes in these sequences. In addition, the T cell response against the human immunoglobulin-mix Endobulin was determined, to assess the background T cell reactivity against human endogenous proteins.

After incubating for 40 hr, the supernatant of the ELISpot-plate either was kept for further analysis or discarded. The plate was washed 4x with 200 µl wash buffer and each well was incubated with 200 µl ddH_2O in order to lyse remaining cells. The plate was washed for 3 additional times and then 100 µl Dulbecco's PBS/well with 1 µg/ml biotin-labelled αIFN-γ mAb were added. Incubation at 37°C for 2 hr was succeeded by 4 washing steps and a further incubation at 37°C for 1.5 hr with 100 µl alkaline phosphatase-labelled streptavidin/well (1 µg/ml). Afterwards, the plate was washed 5x with washing buffer and 1x with Dulbecco´s PBS. The bound IFN-γ was finally visualised, applying the AP Conjugate-Substrate Kit and incubating for 20-50 min in the dark. The colourigenic reaction was stopped by washing 3x with ddH_2O and the membrane was dried for 72 hr in the dark. The quantity of IFN-γ spots was read out automatically with an Axioplan 2-microscope and the KS-ELISpot software.

5.2.4.5 Suppression assay

Two different types of suppression assay were performed in this thesis, both quantifying the suppression of T cell proliferation by Treg via the detection of incorporated ^{3}H-thymidine.

The assays differed in form of the provided stimulus, as in one case these were polyclonally stimulated, whereat an additional antigen-specific stimulation of Treg via antigen-pulsed dendritic cells was provided in the second type.

For the polyclonal suppression assay, 5 x 10^4 Tcon/well were added to a 96-well U-bottom plate. The Tcon were seeded without and in coculture with isolated Treg in ratios of 1:8, 1:4 and 1:2 (Treg:Tcon). Each approach was resuspended in 10% AB-medium and delivered in triplicate to a volume of 200 µl/well. Next, T cells were stimulated by adding CD3/CD28 T cell Expander beads to each well (Tcon:beads = 1:1) and incubating at 37°C for 72 hr. 1 µCi ^{3}H-thymidine/well was added for the last 18 hr.

Another polyclonal suppression assay was performed with Treg Suppression Inspector Kit which has been developed for the functional characterization of human Treg by *in vitro* suppression assays. The instructions of the manufacturer were respected. In detail, $CD4^+CD25^+$ Treg and $CD4^+$ responder T cells (Tresp) were seeded as outlined in Table 2 in a total volume of 210 ul X-VIVO. In this protocol, one MACSiBead Particle per cell (bead-to-cell ratio of 1:1) was used for stimulation. Triplicates were carried out. Cells were stimulated in an incubator at 37°C and 5% CO_2 for 4 days. 1 uCi ^{3}H-thymidine was added into each well for the last 16 hr.

Table 2: Number of responder T cell (Tresp), regulatory T cells (Treg) and Treg Suppression Inspector (MACSiBeads Particles) per well.

Ratio Tresp cells: Treg cells	Tresp cells	Treg cells	Treg Suppression Inspector (amount of MACSiBead Particles)
1:0	5×10^4	-	5×10^4
0:1	-	5×10^4	5×10^4
1:1	5×10^4	5×10^4	10×10^4
2:1	5×10^4	2.5×10^4	7.5×10^4
4:1	5×10^4	1.3×10^4	6.3×10^4
8:1	5×10^4	0.6×10^4	5.6×10^4
Control 1:0	5×10^4	-	-
Control 0:1	-	5×10^4	-
Total cells/ MACSiBeads	3×10^5	2×10^5	4×10^5
Total cells/ MACSiBeads for 1 assay (triplicates)	9×10^5	6×10^5	12×10^5

(http://www.miltenyibiotec.com/download/datasheets_en/1099/DS130-092-909.pdf)

For the antigen-specific suppression assay, Tcon and Treg were stimulated separately and in different ways. In detail, 5 x 10^4 Tcon/well were incubated with αCD3 mAb-coupled magnetic beads in a ratio of 1:1 at 37°C for 18 hr. At the same time, triplicates of 2.5 x 10^4 Treg/well were cocultured with control antigen- and breast cancer antigen-pulsed dendritic cells in a ratio of 5:1 (Treg:DC). After 18 hr, the beads were detached from the polyclonally stimulated Tcon by heavy pipetting and were separated from the cells with a magnetic particle concentrator. Subsequently, the activated Tcon were added 1:1 to the differently stimulated Treg and cocultured at 37°C for further 72 hr. The quantification of Tcon proliferation in all approaches was conducted analogously to the polyclonal suppression assay. In contrast to the polyclonal suppression assay that only gave information on the general, non-specific suppression of the isolated Treg population, this suppression assay determined the impact of antigen-specific activation on the extent of exerted suppression.

No matter which suppression assays were performed, the incorporation of radioactively labelled thymidine by proliferating T cells was stopped through transferring the plate to a -20°C freezer. The frozen plate was thawed at 37°C, frozen again and thawed, in order to promote cell lysis. This was followed by the harvesting of cells, implicating the lysis of residual T cells with ddH_2O and the transfer of nucleic acids from the supernatant of each well to a filter membrane. This membrane now exhibited spots of bound nucleic acid, comprising different quantities of incorporated ^{3}H-thymidine. The more proliferation had taken place in a well, the more ^{3}H-thymidine had been incorporated and was now bound to the filter membrane. Next, the membrane was dried in a microwave at 600 W for 2 min, added 5-7 ml of scintillation fluid and sealed in a plastic bag. Finally, the extent of proliferation was read out with a liquid scintillation counter, enabling the quantification of Treg suppression on the proliferation of Tcon in each approach.

5.2.4.6 IL-10 ELISA (enzyme-linked immunosorbent assay)

The ELISA was developed by Engvall et al. in 1971 as an immunoassay that quantified antigen concentrations without the use of radioactively labelled compounds. While the original protocol was based on a competitive binding of the tested antigen to an antibody, the "sandwich ELISA" applied in this study employed the non-competitive binding of the antigen to an immobilised antibody and its detection with a second antigen-specific, enzyme-labelled antibody. The enzyme converted a transparent substrate into a coloured product in proportion to the amount of bound antigen. Finally, the concentration of coloured product could be read out with an ELISA-reader.

The concentration of IL-10 in the supernatants of conducted ELISpot assays was determined by the application of the Quantikine Human IL-10 Immunoassay Kit, following the instructions of the manufacturer.

5.2.5 Statistical analyses

Three statistical techniques were applied in this thesis to pertain the interpretations of data. They are Student´s t-test, Wilcoxon signed-rank test and Fisher's exact test. Student´s t-test was performed by a paired two-tailed calculation if not indicated. A paired Student´s t-test was applied in a comparison in-between the data obtained from the same individual. A one-tailed Student´s t-test was applied for determining a statistical difference between the data obtained against breast cancer antigens and control antigens. A Fisher's exact test was performed by a two-tailed calculation when a statistical difference between percentages of tumour antigen-reactive patients without and with Treg depletion was analysed. Wilcoxon signed-rank test was performed when a distributional assumption was regardless in a comparison. A statistical significance was considered when a calculated p-value is not more than 0.05.

6 RESULTS

Part I Proof of principle -- Role of regulatory T cells on spontaneous anti-tumour T-cell responses in breast cancer

6.1 Pilot clinical study of adoptive T cell transfer immunotherapy implies an undesired suppressive impact from regulatory T cells

6.1.1 Study design

Based on our previous findings that breast cancer patients often harbour anti-tumour type-I memory T cells (mTC) in their bone marrow (BM) but less frequently in the peripheral blood (PB), and that *ex vivo* reactivated BM mTC with specific breast tumour antigens completely rejected autologous tumours in xenotransplanted mice, a pilot clinical study of adoptive T cell transfer immunotherapy (ADI) was initiated by our group in 2003.

This study was applied on metastasized breast cancer patients who had previously received standard cytostatic treatments, such as chemotherapy or hormone therapy and were in a palliative treatment situation. Patients with secondary malignancies, auto-immune diseases, renal failure or pregnancy were excluded.

Patients whose BM contained detectable tumour-antigen (TA) reactive mTC were recruited for ADI. The presence as well as the frequencies of BM residing mTC were determined by a short-term (40h) IFN-γ ELISpot assay as a short activation period does not allow the priming and effector function of naïve T cells. Established breast tumour cell lines, MCF7 and KS24.22, served as sources of tumour antigens, whereas U937, a promonocytic leukaemia cell line, was used as a source of irrelevant tumour antigens. T cells were stimulated with autologous dendritic cells (DC) which were pre-pulsed with cell lysates. The amount of reactive T cells which secreted IFN-γ in experimental wells can be measured in the number of spots. When significantly higher numbers were exhibited in the wells with an interest antigen than ones with its control antigen, this patient was considered as an immunological responder. And the number calculated from a subtraction of the mean value of triplicates from test wells to that from the control wells is referred as the frequency of TA-specific T cells in this thesis.

To treat the eligible patients, their BM cells were collected for an enrichment of T cells, and PB-derived monocytes (PBMC) were cultured for a generation of DC. The reactivation of

tumour-specific TC was performed by co-culturing BMTC with TA pre-pulsed DC for 72 hr. Then the therapeutic T cells were adoptively transferred into the patients intravenously.

6.1.2 Clinical monitoring

In total, 17 patients with metastasized breast cancer were treated. In order to monitor the immunological responses, blood samples were collected prior to the therapy (Day0), weekly during the first month after the transfer (Day7, 14, 21, Mon1) and three months after the transfer (Mon3). BM samples were collected one month and three months after the treatment (Mon1 and Mon3). IFN-γ ELISpot assay was conducted to investigate the frequencies of tumour-reactive PBTC and BMTC. IL-4 and IL-10 secretions and CD69 expression on T cells in activation cultures were evaluated in assessable patients by Dr. Katrin Ehlert, a former PhD student of our group, who initiated this project.

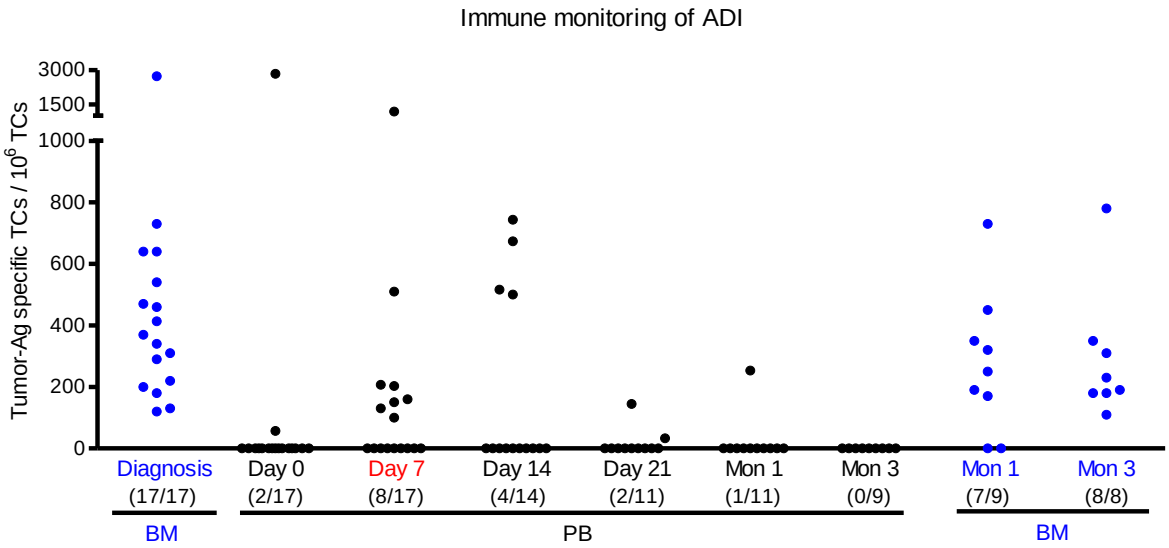


Figure 1. Immune monitoring of adoptive T cell transfer immunotherapy (ADI) in the frequencies of tumour-Ag specific type-I T cells. The frequencies of MCF7 or KS 24.22 –specific type-I T cells were measured in samples of PB (black) or BM (blue) obtained on days indicated below X-axis. Each dot represents a frequency that was identified in an individual patient. The frequencies of non-responders are depicted at X-axis. The numbers of responders to total analysed patients are displayed in parentheses.

Considering the proportions of tumour-antigen responding patients, the best clinical effect was observed on Day7 (Figure 1). Before the therapy there were 11.8% (two out of 17) of study patients containing spontaneous tumour-reactive T cells in their blood, while one

week after the treatment, the proportion went up to 47.1% (eight out of 17) composed with seven *de novo* responders and one responding maintainer. Two weeks after ADI, we were still able to detect three responders who retained the immunological responses from Day7 and one additional *de novo* responder. The numbers of ADI responders were then declined to two, one and zero on Day21, Mon1 and Mon3, respectively. Interestingly, the maintenance of detected TA-specific T cells in the BM was persistent through the entire study time (Mon1: 77.8%, Mon3: 100%).

Regarding the immunological non-responders, Ehlert observed that the transferred T cells of those patients secreted increased type-II cytokines IL-4 and IL-10, exerted reduced proportions of early activated $CD4^+$ and $CD8^+$ T cells determined by CD69 expression and contained higher proportions of $CD25^{high}$ T cells (enriched in regulatory T cells) (99). These findings pointed to the possibility that regulatory T cells present in bone marrow T cell populations of some advanced breast cancer patients might inhibit the appropriate restimulation of pre-existing tumour reactive memory T cells.

Taken together, this study demonstrates a promising biological response, especially on Day7; and the existed immune suppression might hamper the non-responders from benefits of ADI. In order to enhance the clinical efficacy, conquering this immune suppression might not be evitable.

6.2 Frequencies of Treg are significantly lower in the bone marrow than the peripheral blood of patients with primary breast cancer

To further clarify our hypothesis that the present regulatory T cells impeded the occurrence of type-I T-cell anti-tumour immunity in immunological non-responders of the study, we started with quantifying the frequencies of Treg in breast cancer patients by multicolour flow cytometry. Despite of increasing observations showing the importance of $CD8^+$ Treg, we focused in this thesis on $CD4^+$ Treg which contribute a majority of suppressive Treg.

We defined Treg with cell surface markers CD3, CD4, CD25 and one intra-nuclear marker Foxp3. As shown in Figure 2, within a $CD3^+CD4^+$ T cell gate (Figure 2B), we further specified Treg subpopulation according to their double expressions of CD25 and Foxp3 (Figure 2C). The number in the upper-right quadrant of Figure 2C was used for quantifying frequencies of Treg. Hereafter Treg are referred to $CD4^+CD25^{high}Foxp3^+$ T cells.

PB and BM samples from a total of 34 primary breast cancer patients and 10 healthy donors were evaluated. Results are shown in Figure 3. The mean frequency of Treg among $CD4^+$ T

cells was found higher in the PB than the BM, which was counted for both healthy donors (PB: 4.32% ± 0.9% versus BM: 3.44% ± 1.04%) and patients (PB: 3.88% ± 1.49% versus BM: 2.48% ± 1.25%). The difference in patients but not healthy donors displayed a statistical significance ($p<0.001$). The comparisons between patients and healthy donors showed a significant decrease in the mean frequency of Treg in the BM of patients ($p=0.022$), but a statistically unaltered one in the PB.

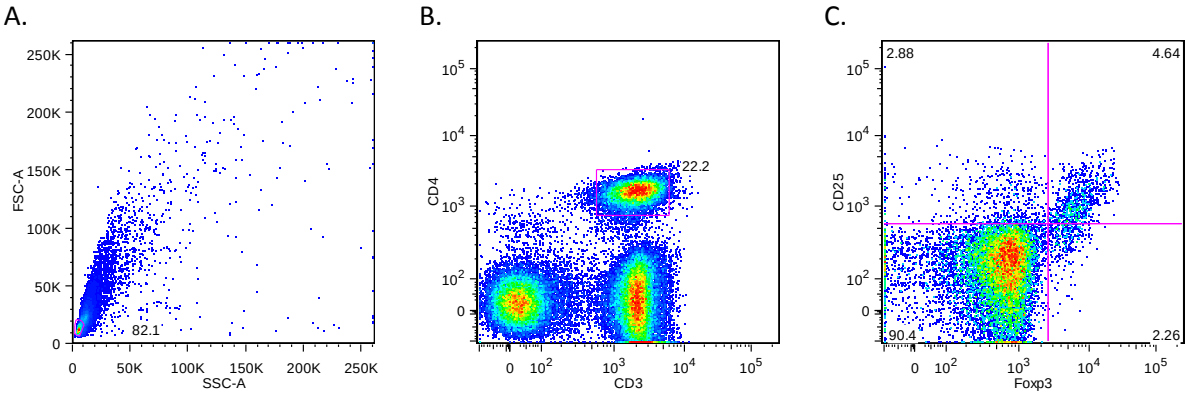


Figure 2. Definition of $CD4^{+}CD25^{high}Foxp3^{+}$ Treg. **A**, a lymphocyte gate was set according to the size (FSC) and the granularity (SSC) of all acquired cells. **B**, within the lymphocyte gate, $CD4^{+}$ T cells were selected according to CD3 and CD4 double expression. **C**, $CD4^{+}$ Treg were then quantified by $CD25^{high}$ and $Foxp3^{+}$ (the upper-right quadrant). Small numbers in the plots represent corresponding percentages.

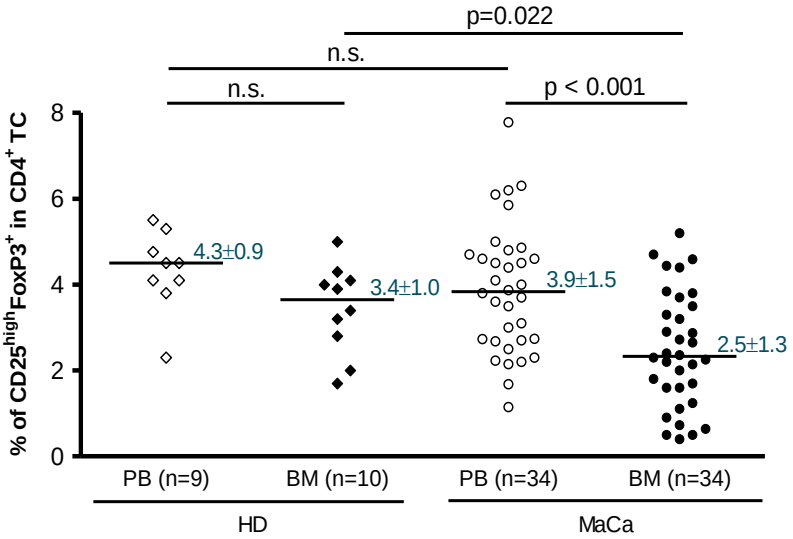


Figure3. Frequencies of Treg in the peripheral blood and the bone marrow of breast cancer patients and healthy donors. Each symbol represents an individual. Mean±SD is depicted in blue above each mean value bar in respective groups. Statistical analyses between interested groups are connected with horizontal lines above the plot. Paired two-tailed t-test was performed for comparisons between PB and BM; unpaired two-tailed t-test was performed for comparisons in-between MaCa and HD. n.s.: not significant.

6.3 Preliminary results from IFN-γ secretion assay enlightened a negative effect of regulatory T cells on anti-tumour type-I T-cell immunity

Next, we set out to determine the impact of Treg on anti-tumour T-cell responses in breast cancer patients. To start, we established a Treg depletion protocol with MACS© $CD4^+CD25^+$ Regulatory T Cell Isolation Kit. This protocol was conducted as illustrated in Figure 4. In order to achieve equal amounts of T cells required for a comparison with and without Treg fractions, two thirds of purified T cells were used for an accomplishment of Treg depletion procedure due to an inevitable loss of cells during additional purification steps. The depletion of $CD4^+CD25^+$ T cells was conducted by an initial isolation of $CD4^+$ T cells via negative selection (positive selection of $CD8^+$ T cells) followed by a positive selection of $CD25^+$ cells from the previously isolated $CD4^+$ T cells. The residual $CD4^+CD25^-$ cells were returned to the selected $CD8^+$ T cells from the first purification step and denoted as Tcon or "TC-". The un-touched T cell population is denoted as TC or "TC+". The sufficiency of depletion was confirmed by flow cytometry as shown by a representative example in Figure 4B. $CD25^{high}$ and $Foxp3^+$ cells were substantially reduced in TC- fraction compared to TC+ one, indicating this protocol is able to provide a successful depletion of Treg. Accordingly, the depleted $CD4^+CD25^+$ cells were largely enriched in "Treg" fraction as observed that more than 60% of these cells co-expressed Foxp3 and CD25.

IFN-γ catch assay was chosen to study the impact of Treg because of its allowance of specific isolation and expansion of viable T cells. First we compared the capacities of IFN-γ production of T cells in the presence or absence of Treg upon breast tumour antigen stimulation. Same amounts ($4x10^6$) of TC+ and TC- from one breast cancer patient were activated with MCF7 cell lysates presented by autologous DC for 16 hr. Then an IFN-γ catch assay was conducted. The numbers of captured cells, namely IFN-γ secreting cells were counted. Interestingly, 1.3-fold more cells were captured from TC- group ($1x10^6$) than from TC+ group ($0.76x10^5$) (Figure 5A), indicating that the present Treg have a suppressive impact on T-cell activation.

To evaluate which T-cell population was inhibited by Treg, we performed multicolour flow cytometric analyses. At a single cell level, we detected that the suppression was exerted on both $CD4^+$ and $CD8^+$ T-cell populations by Treg. As shown in Figure 5B, upon MCF7 stimulation the percentages of IFN-γ secreting $CD4^+$ T cells increased from 0.39% in TC+ group to 2.25% in TC- group (5.8x), and the percentages of IFN-γ secreting $CD8^+$ T cells increased from 0.23% in TC+ group to 2.59% in TC- group (11.7x). These data indicate that Treg of breast cancer patients have an inhibitory impact on both $CD4^+$ and $CD8^+$ T-cell activation in response to tumour antigens.

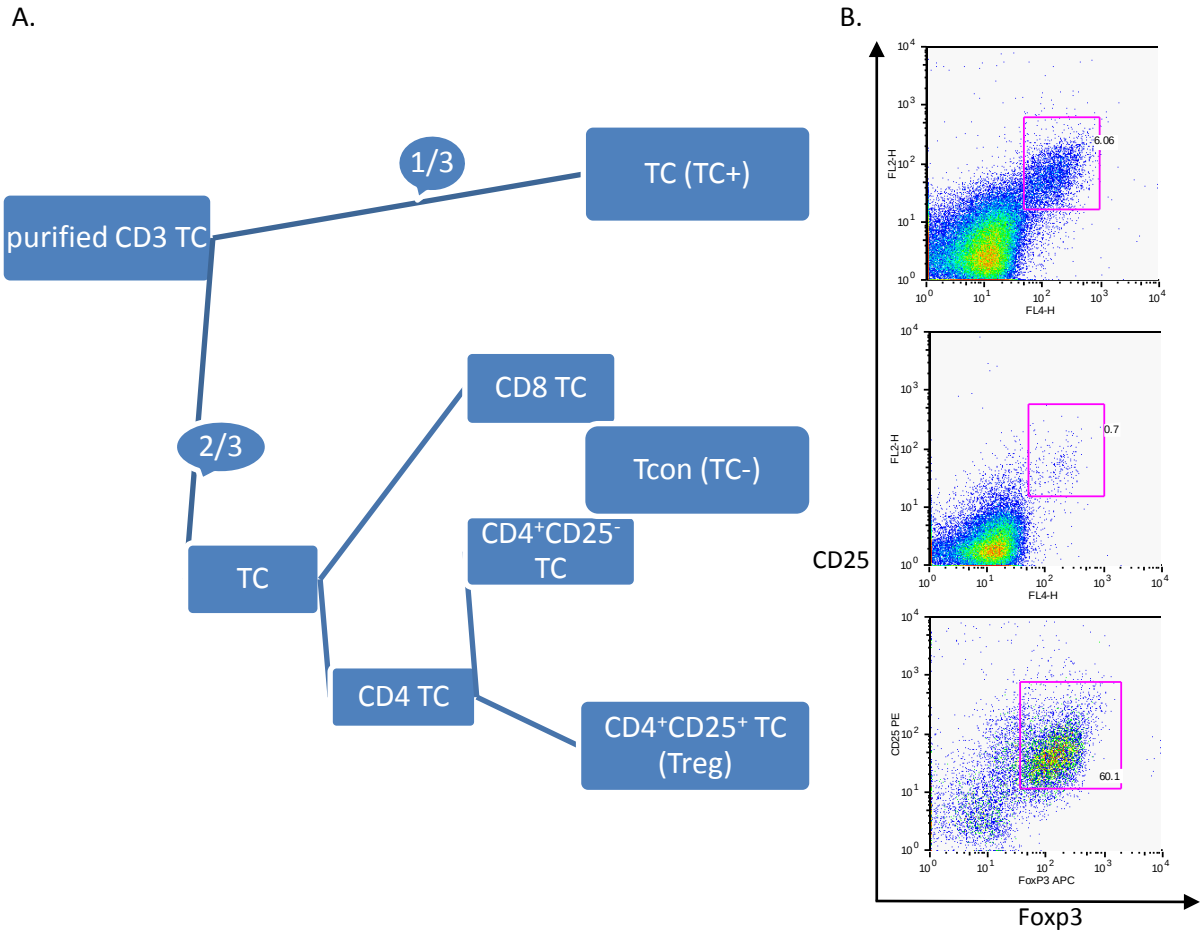
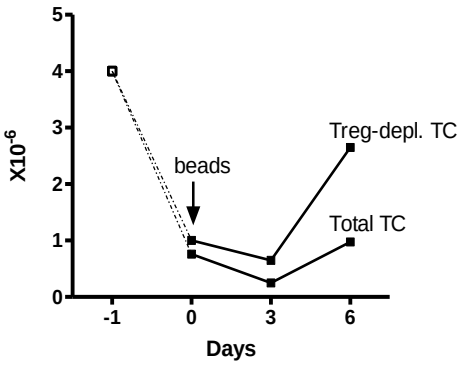


Figure 4**.** Schematic overview of Treg-depletion protocol. **A**, Purified TC are unevenly separated into two parts. One-third of TC containing un-touched Treg served as original TC+ population. Two thirds of TC was subjected to an isolation of Treg. This was achieved by a negatively selection of $CD4^+$ TC followed by a positive selection of $CD25^+$ TC. The residual $CD4^+CD25^-$ TC were pooled together with $CD8^+$ and denoted as Tcon or TC-. **B**, Flow cytometry analyses confirm the purity of Treg-depletion. Each plot shows the population designated at the left side. The cells were pre-gated on $CD3^+CD4^+$ TC, and are shown by Foxp3 staining versus CD25 staining. The pink boxes inside the plots indicate the Treg population. These results represent 3 independent experiments.

To understand whether this suppression was tumour specific, we repeated the experiment with an additional irrelevant tumour antigen (U937). Flow cytometric results are shown in Figure 5B. Similarly low percentages of IFN-γ secreting TC among $CD4^+$ and $CD8^+$ T cell populations were observed in response to both MCF7 and U937 prior to a conducted Treg depletion. However, a sharp difference was observed after eliminating the Treg. As described above, a dramatic restoration of type-I T-cell immunity was observed in response to MCF7, but not to U937 ($CD4^+$, TC+ versus TC-: 0.59% versus 0.0084%; $CD8^+$, TC+ versus TC-: 0.2% versus 0.66%). These data suggest that Treg exert a suppression through an antigen-specific manner in breast cancer patients.

A.



B.

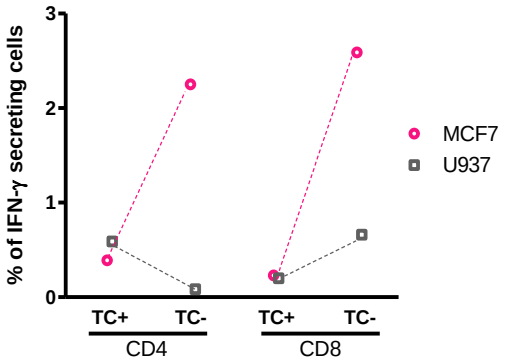


Figure 5. Exemplary results show the advantage of pre-Treg-depletion for IFN-γ secretion assay. Purified bone marrow T cells were stimulated with breast tumour antigens derived from MCF7 cell line or control tumour antigens from U937. 16h later an IFN-γ catch assay was performed. **A**, Treg-depletion benefited the expansion of IFN-γ-captured cells upon post-polyclonal stimulation. IFN-γ secreting cells were captured on Day0 and were immediately cultured with anti-CD3 & CD28 beads at a beads:cells ratio of 1:1. The absolute counts of cells were followed on indicated days. **B**, Enhancement of MCF7-specific type-I T cell proportions in both CD4 and CD8 subsets are found in Treg pre-depleted group but not in non-depleted one. The percentages of IFN-γ secreting T cells were determined by multicolour flow cytometric analyses.

Next, the impact of Treg on IFN-γ captured cells was evaluated regarding their proliferative ability. The captured MCF7-reactive T cells were stimulated with αCD3 and αCD28 mAb coated beads at beads to TC ratio of 1:1 in the cultivation with a concentration of $1x10^6$ TC/ml. Cell counts were followed before and after stimulation (Figure 5A). Compared to Day0, declines in cell counts were observed on Day3 in both TC+ and TC- originated groups, however, with a larger extent in TC+ group (% of decrease: TC+, 67% versus TC-, 35%); whilst, a vital expansion was obtained on Day6 in TC- group but not in TC+ group (compared to Day0: 2.7-fold and 1.3-fold increase, respectively). These findings suggest that Treg from breast cancer patients have a subsequent impact on polyclonal expansion of TA activated T cells.

This concept was further confirmed by a post-stimulation with tumour specific antigens. In detail, the captured KS24.22-reactive TC from TC+ and TC- groups, as well as the cells in flow through fraction, namely non-captured cells, of TC- group were re-stimulated with irradiated KS24.22 at a ratio of TC to tumour cell 5:1. Seven days later, a vigorous clonal expansion was observed in the wells of the TC obtained from TC- originated group (Figure 6A middle). The captured TC from TC+ group were able to recognize tumour antigens since several clusters were formed around tumour cells at beginning, but they failed to develop clonal expansions (Figure 6A top). Whilst the flow through TC from TC- group did not possess the specificity towards KS24.22 as they were found to be separated from tumour cells through the entire cultivating time (Figure 6A bottom), which also suggests an efficiency of this assay.

Collectively, these data demonstrate that besides an inhibitory effect on T-cell activation Treg in breast cancer patients have a subsequent impact on clonal expansion of Teff *in vitro*.

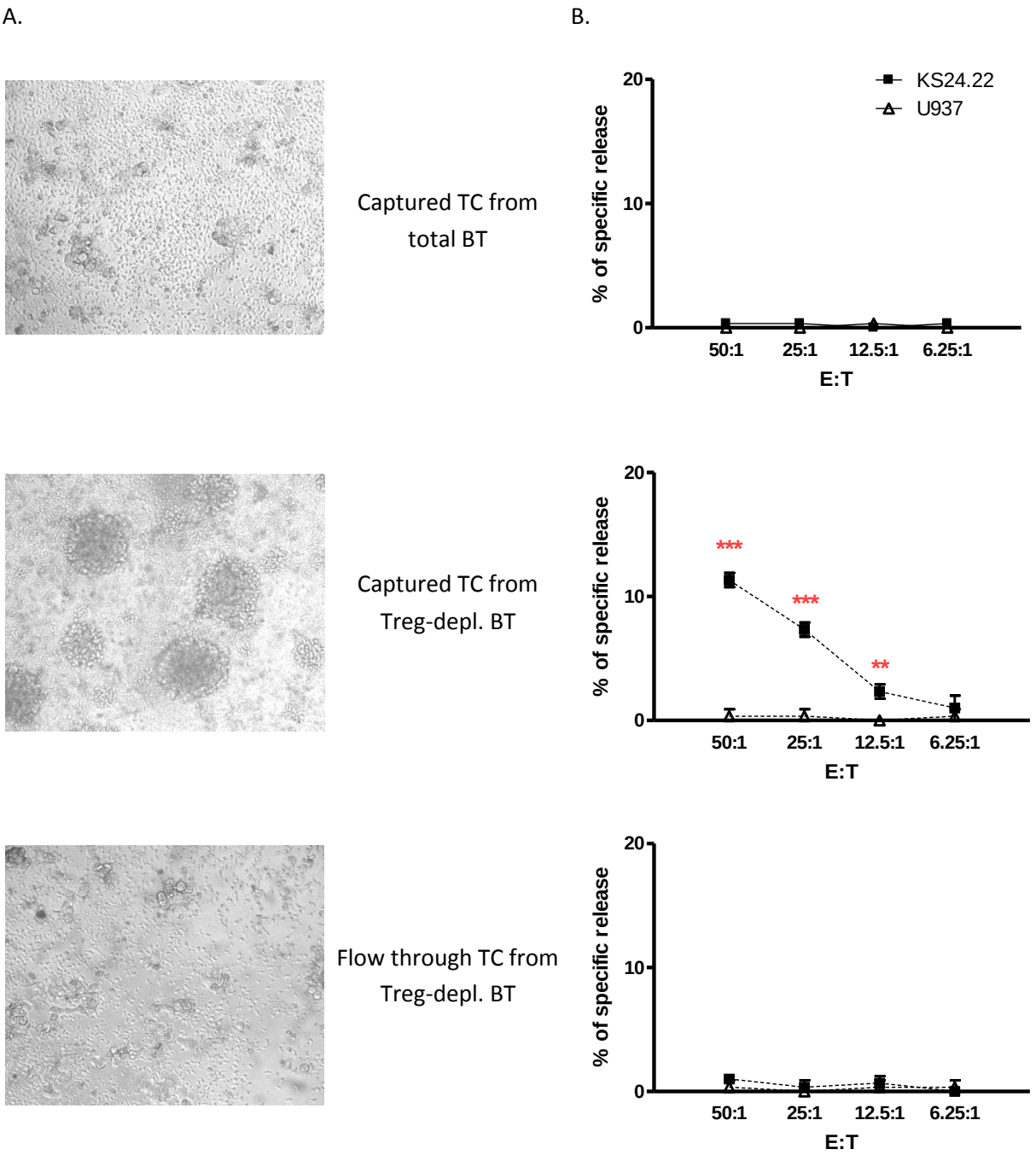


Figure 6. Exemplary results show the impact of Treg on clonal expansion and cytolytic activity of tumour-antigen specific T cells from IFN-γ secretion assay. The captured T cells from both TC+ and TC- groups, as well as flow-through T cells from TC- group were re-stimulated with irradiated KS24.22 at a ratio of 5:1. Different T-cell fractions are indicated in the middle panel. **A**, cell cultivations show clonal expansion capacity of different T-cell fractions observed 7 days after breast tumour antigen re-stimulation. **B**, ^{51}Cr release assay shows cytolytic activities of different T-cell fractions obtained 30 days after breast tumour antigen re-stimulation. E:T indicates the ratio of effect cells to target cells. Bars; standard deviation of triplicates.

Moreover, this antigen-driven clonal expansion also implies an impact of Treg on immune recall. To confirm this concept, ^{51}Cr release assay was carried out with those KS24.22 re-stimulated cells 30 days later. KS24.22 and U937 were used as target cells. Results are shown in Figure 6B. A tumour-specific cytotoxicity was remarkably elicited from TC- originated group with high significances observed from three different ratios of effector to target cell ($p\text{-value}_{50:1} = 2x10^{-5}$; $p\text{-value}_{25:1} = 1x10^{-4}$; $p\text{-value}_{1.25:1} = 2x10^{-3}$). In a sharp contrast, none of the compared samples exhibited tumour-specific killing. These data evidence a long term suppression of Treg on Teff function, which is likely imprinted into Teff during the priming phase.

On the other hand, our results demonstrate that by removing Treg from breast cancer patients, whose *in vivo* immunity was steadily established, their anti-tumour responses can be rescued.

6.4 Spontaneous anti-tumour type-I T-cell responses are suppressed by Treg in breast cancer patients

Being encouraged by the results presented above, we then enlarged the sample size in order to have a solid conclusion of Treg impact on spontaneous anti-tumour T-cell responses of breast cancer patients. The same Treg-depletion procedure was performed as described above. IFN-γ ELISpot assay was performed as a read-out system. T-cell responses in the presence or absence of Treg were evaluated against polyvalent breast tumour antigens derived from MCF7 and KS24.22 (Result 6.4.1), as well as against defined tumour-associated antigens derived from synthetic polypeptides (Result 6.4.2)

6.4.1 Treg suppress type-I T-cell responses against polyvalent breast tumour antigens derived from MCF7 and KS24.22 cell lines.

Taking an advantage that the cell lysates of breast tumour cell lines cover a wide spectrum of tumour antigens, we started this part of study with MCF7 and KS24.22 cell lysates as the source of breast tumour antigens; meanwhile, U937 was served as an irrelative control. Exemplary results of IFN-γ ELISpot are shown in Figure 7. In this patient, prior to a Treg depletion, TC immunity was lacking in the PB, while a weak reactivity was observed in response to KS24.22 but not to MCF7 in the BM. However, with a Treg deprivation, the breast tumour-specific T-cell immunity was unmasked dramatically in both PB and BM in response to both KS24.22 and MCF7 (see the p-values in the figure).

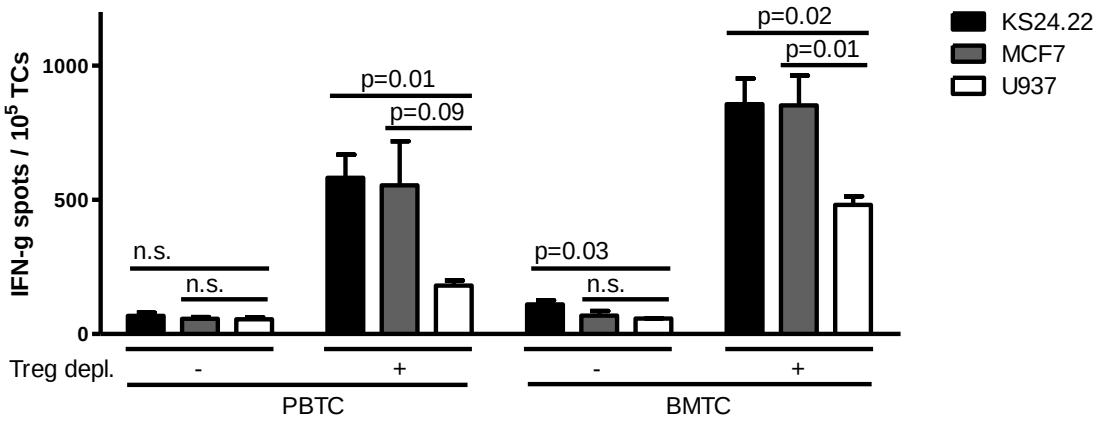


Figure 7. Impact of Treg on type-I T-cell responses. Primary data of an exemplary IFN-γ ELISpot assay. Peripheral blood (PBTC) and bone marrow (BMTC) T cells were stimulated with breast tumour antigens derived from KS24.22 (black columns) or MCF7 (gray columns) cell lines, and with control tumour antigens from U937 (white columns). The frequencies of IFN-γ secreting T cells were determined by mean values of three wells per antigen. Without and with a performed Treg-depletion are indicated as Treg depl. – and +, respectively. Statistical differences were calculated by unpaired two-tailed t-test. Significant p-values are depicted above small lines which connect the compared groups. n.s.: not significant.

In order to evaluate an overall effect of Treg-depletion in breast cancers, we analyzed the differences in the frequency of IFN-γ secreting T cells without and with a conducted Treg-depletion in each studied group. Accumulative data are shown in Figure 8A (PB) and 8B (BM). Regarding MCF7-reactive TC the mean frequencies increased 2.6-fold in the PB (141.9/10^5 versus 371/10^5 TC; n=26) and 2.3-fold in the BM (167.1/10^5 versus 387.2/10^5 TC; n=24). Similarly, the mean frequencies of KS24.22-reactive TC augmented 2.3 and 2.6 times in the PB (209.9/10^5 versus 484.2/10^5; n=20) and the BM (229.2/10^5 versus 596.2/10^5; n=21), respectively. All these effects displayed statistical significances (see p values in the figures).

More importantly, these effects appeared to be breast cancer specific as no significant differences were detected in response to the irrelevant tumour antigens (PB: 120/10^5 versus 200/10^5, n=31; BM: 133.9/10^5 versus 227.6/10^5, n=27) in despite of a few individuals exhibiting higher responsiveness after applying a Treg-depletion. To confirm it, we analysed the tumour specific responsiveness with altogether 31 PB and 27 BM samples of primary breast cancer patients (Figure 8C). Without a Treg-depletion, anti-KS24.22 T-cell immunity was detectable in the PB but with a relatively low extent, whereas it was absent in the BM. MCF7-specific reactivity was not detectable, neither in the PB nor in the BM. However, the absent or weakly existing T cell responses were profoundly recovered by applying a Treg depletion (see p values in the figures).

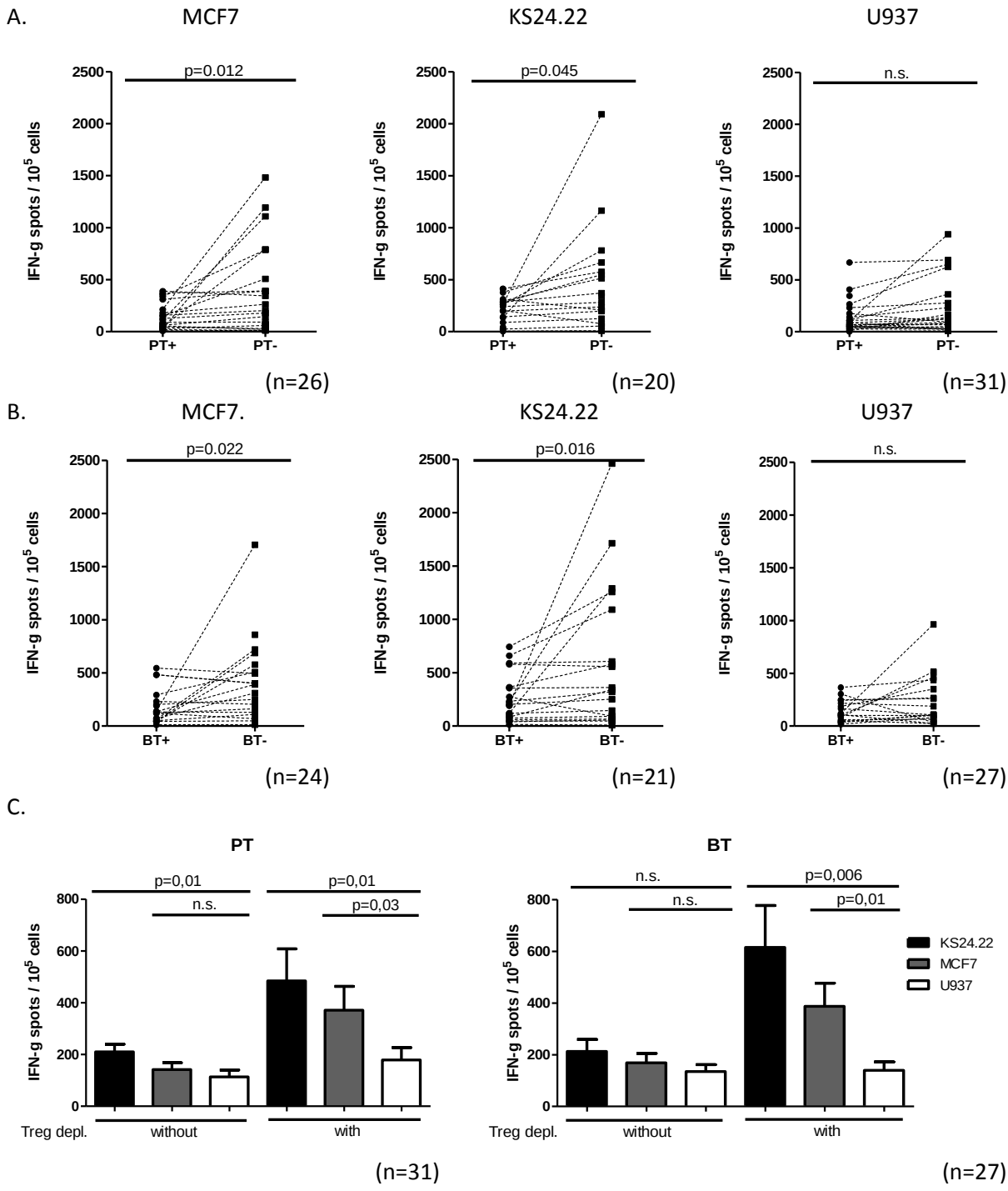


Figure 8. Treg-depletion leads to a specific increase of reactive T-cell frequencies against breast tumour antigens. **A**, PBTC and **B**, BMTC were stimulated with MCF7 and KS24.22 and control U937 from the left to the right, respectively. Frequencies of reactive TC were determined by IFN-γ ELISpot analyses. Each symbol indicates a mean value of triplicates. Dash lines connect corresponding frequencies within individuals before (+) and after (-) Treg-depletion. Statistical differences were calculated with unpaired two-tailed t-test. **C**, Treg-depletion has antigen specific effect in breast cancer patients. Frequencies of responding PBTC (left) and BMTC (right) were cumulated, and values are depicted as columns with SEM bars. KS24.22: black; MCF7: gray; U937: white. P-values indicate the statistic differences within the interested groups as combined by short lines. Unpaired one-tailed student t-test was performed. n.s.: not significant.

Collectively, these data substantially confirmed our previous finding that Treg existing in breast cancer patients suppress spontaneous anti-tumour type-I TC immunity.

Also, these data imply that the inhibitory function of Treg requires appropriate tumour-antigen activation. To confirm this concept, we calculated the frequencies of antigen-specific T cells by subtracting background ones (U937) from interested values (MCF7 or KS24.22) within those immunological responders, and normalized all non-responders to the value of 0. Accumulative data are shown in Figure 9. Within PBTC, MCF7-specific frequency was dramatically unmarked by a Treg depletion (10x, p=0.022). Similar significance was also displayed regarding KS24.22-specific frequency (5x, p=0.03). Within BMTC, the influence was relatively less as a 3-fold and 4-fold induction in MCF7 (p=0.02) and KS24.22-specific frequencies were detected, respectively.

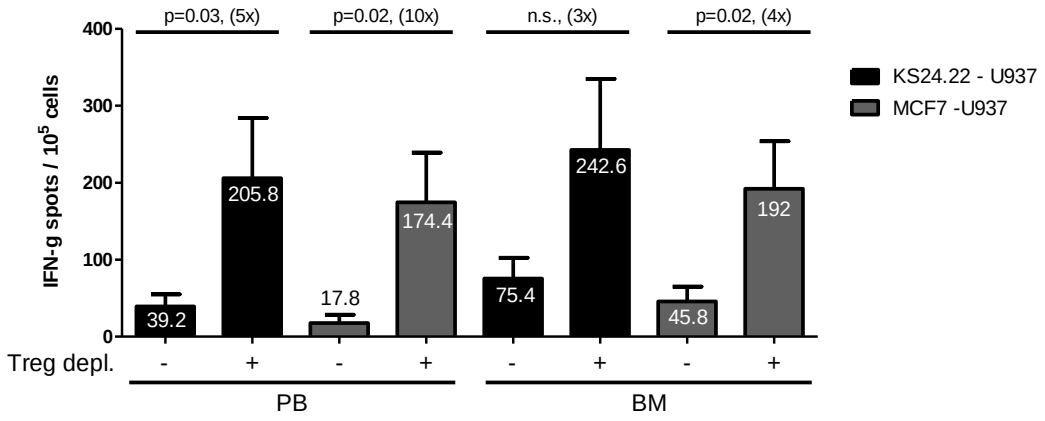


Figure 9. Treg-depletion unmasks anti-breast-cancer specific responses in both peripheral blood and bone marrow. **A**, the specific frequencies against breast tumour-antigens were determined by subtractions of mean values of U937 from KS24.22 (black columns) and from MCF7 (gray columns). The performance of Treg-depletion was indicated as –: not conducted; +: conducted. Bars represent SEM. Unpaired two-tailed p-values together with increased folds are depicted above short lines which connect compared groups.

Surprisingly, the frequencies of breast tumour specific T cells in the PB approached that in the BM after conducting a Treg-depletion. In particular, the mean frequencies of spontaneous KS24.22-reactive T cells between PB and BM were narrowed down from 48% to 15%. Even better situation was observed regarding MCF7-specific responses, as the difference was greatly reduced from 61% to 9%. Given the evidence from our pilot study that the more tumour-reactive T cells which were transferred into patients, the better chance for them to achieve anti-tumour responses afterwards, this finding, therefore, has a profound meaning in improving ADI project as a large amount of T cells which contain

tumour-reactive ones are able to be generated from a leukaphoresis instead of limited BM source. Consequently, more patients would be benefited from this immunotherapy.

Next, we evaluated the impact of Treg on the proportions of immunological responders. (Figure 10). Without depleting Treg from the PBTC, there was 26.1% (6 out of 23) of MCF7 responders, and 35% (7 out of 20) of KS24.22 responders. When the Treg were eliminated from the samples, the proportion of MCF7 and KS24.22 responders significantly increased to 60.9% (14 out of 23 patients, p=0.036) and 76.5% (13 out of 17 patients, p=0.020), respectively. In other words, Treg-depletion revealed minimally 34.8% of patients who naturally contained tumour-reactive mTC in their blood.

In the BM compartment, Treg-depletion induced substantial but not significant proportions of responders. Without and with a Treg removal, the proportions of MCF7 responders went up from 31.8% (7 of 22) to 50% (10 of 20), while KS24.22 responders increased from 45.5% (10 of 22) to 68.4% (13 of 19). The less benefit of Treg-depletion found in the bone marrow of breast cancer patients might be due to the more pre-existing functional TA-specific mTC compared to the periphery compartment.

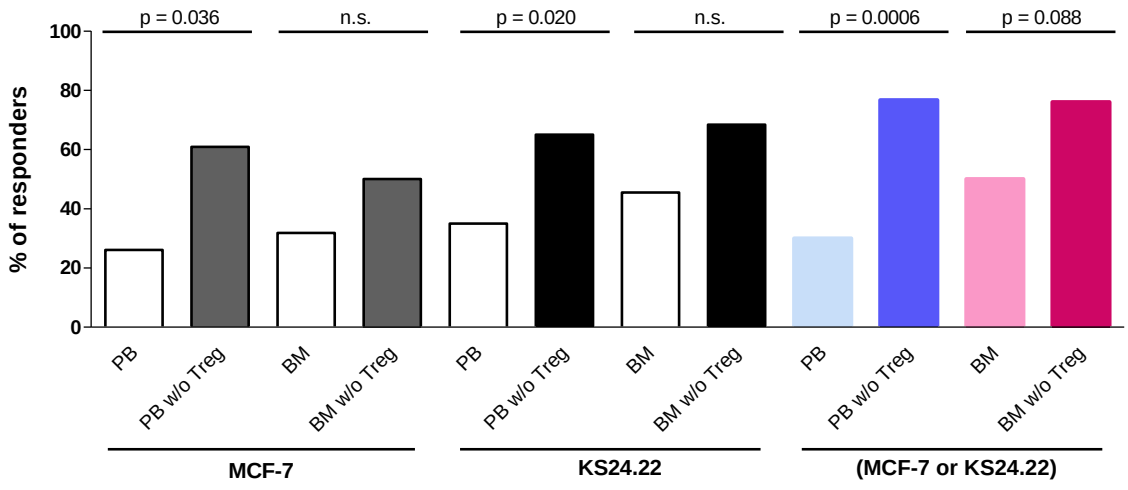


Figure 10. The beneficial effect of Treg-depletion in the proportion of tumour-antigen responding patients. The percentages of responding patients who exerted specific responses against breast tumour antigen (MCF7 and KS24.22) were compared before (white) and after (MCF7: gray; KS24.22: black) Treg-depletion. Last 4 columns show accumulated results of patients who responded either to MCF7 or KS24.22 (light blue and light pink: before Treg-depletion; blue and pink: after Treg-depletion). P-values indicate the statistical difference performed by Fisher's exact test. n.s.: not significant.

As the spectrums of tumour-antigens carried in MCF7 and KS24.22 not only have overlaps but also are compensative, we pooled the results from both studies in order to evaluate a general impact of Treg in breast cancer. To this end, patients who responded to at least one of the tumour antigens (either MCF7 or KS24.22, or both) were considered as immunological responders. Results are shown in Figure 10 (the last 4 columns). A highly significant benefit of Treg elimination from PBTC ($p=0.0006$) was obtained; whilst a marginal significance was displayed in the analysis of BMTC ($p=0.088$). Interestingly, the percentages of breast tumour responders were enhanced to the same level after depleting Treg from periphery (76.7%) and bone marrow (76%).

Taken all, these data demonstrate that spontaneous type-I TC immunity against polyvalent tumour antigens are inhibited by Treg in the patients with primary breast cancers. This suppression is likely triggered in an antigen-specific manner.

6.4.2 Treg suppress type-I T-cell responses against tumour-associated antigens derived from synthetic polypeptides

The so far presented findings are studied with polyvalent breast tumour antigens derived from allogeneic cell lines (MCF7 and KS24.22). It is interesting as well as important to clarify the impact of Treg on T-cell responsiveness against breast tumour-associated antigens (TAAs). To this aim, an appropriate activation for Treg which enables them to exert inhibitory function was considered. We elaborated 50-100mer synthetic polypeptides which flank HLA-A*0201 binding motifs and cover several potential $CD4^+$ epitopes. 12 well characterized HLA-A*0201-restricted nonameric peptides were selected from four breast cancer specific tumour antigens and six common cancer tumour-associated antigens (Table 3). A polyvalent human immunoglobulin served as control antigen. IFN-γ ELISpot assay was performed as a readout system.

An exemplary result obtained from PBTC of one breast cancer patient is shown in Figure 11. Among 12 tested polypeptides, T cell reactivities were found towards three and seven TAAs before and after a Treg-depletion, respectively, indicating a Treg-depletion benefit. Of note, in this patient, the Treg-depletion did not only favour to anti-tumour immunity, as the pre-existing hTERT and MAGE responses were abolished after applying it. This is possibly due to concomitantly depriving current effector T cells through their highly expressed CD25 marker. Nevertheless, these data showed that the responsiveness against four TAAs was suppressed by the existing Treg in this assessed patient.

Table 3. Synthetic polypeptides designed from tumour-associated antigens HLA-A*0201-restricted nonamers.

TAA and control	abbreviation	start position of nonamer in amino acid sequence	HLA-A*0201 – restricted-peptide sequence	Designed polypeptide position in amino acid sequence
Heparanase	HpaI	16	LLLGPLGPL	1-50
Heparanase	HpaII	183	DLIFGLNAL	163-212
Mammaglobin	Mam1	4	LMVLMLAAL	4-56
Mammaglobin	Mam2	66 83	FLNQTDETL LIYDSSLCDL	41-92
Her2/neu	Her2/neu	369	KIFGSLAFL	351-384
Mucin-1 tandem repeat	M20	9	STAPPAHGV	137-157
Mucin-1 signal sequence	M100	12	LLLLTVLTV	1-100
Epidermal growth factor receptor	EGFR	479	KLFGTSGQKT	479-528
p53	p53	149	STPPPGTRV	118-167
Melanoma asscociated antigen-3	MAGE3	271	FLWGPRALV	271-314
Human telomerase	hTERT	988	YLQVNSLQTV	958-1007
Survivin	Survivin	96	LMLGEFLKL	93-142
Carcinoembryonic Antigen	CEA	571	CGIQNSVSA	569-618
Human immunoglobulin	IgG / control	-	-	40-89

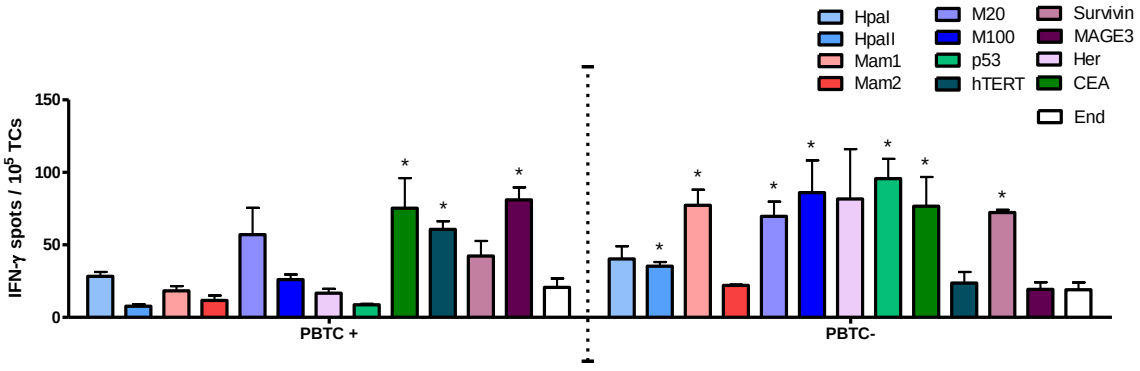


Figure 11. Treg impact on type-I TC reactivity against a spectrum of tumour associated antigens (TAA). An exemplary result shows the IFN-γ productions of PBTC before (PBTC+) and after Treg-depletion (PBTC-) against 12 tested polypeptides (represented in different colours) and control IgG (white) presented by autologous DC. Columns and bars represent mean value of triplicates and SEM. Asterisks indicate where the statistical significances ($p<0.05$) are obtained when compared to the values of control IgG. Pared two-tailed t-test was performed.

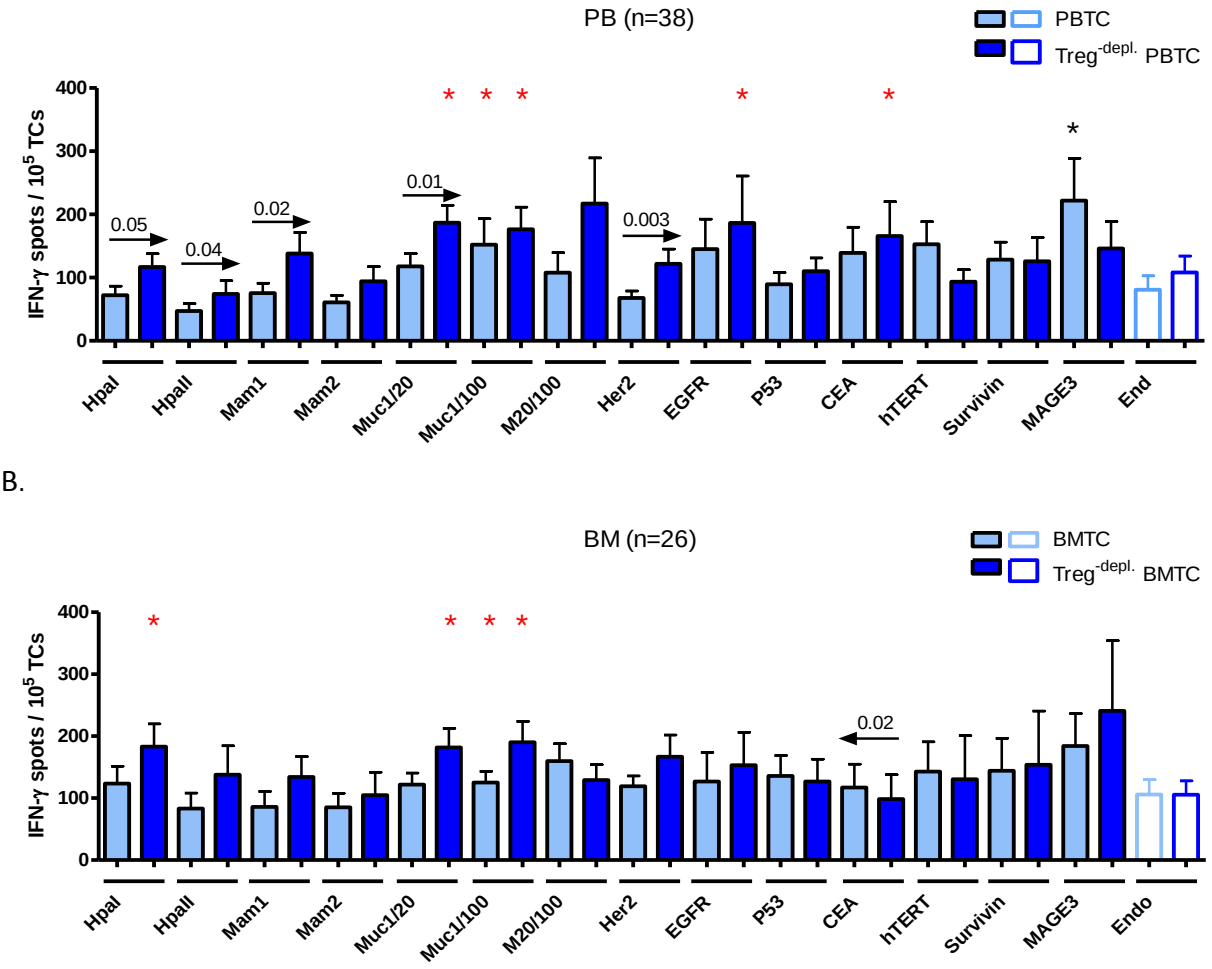


Figure 12. Impact of Treg on the frequencies of type-I T cell in response to polypeptides. Frequencies of polypeptide responding T cells were determined by IFN-γ ELISpot analyses. Light blue columns: without Treg-depletion; dark blue columns: with Treg-depletion. Bars:SEM. The studied polypeptides are depicted under the respective columns. Control IgG (Endo) was used as irrelevant antigens shown with open columns. Asterisk indicates a statistical difference of $p<0.05$ comparing the values of indicated polypeptides and respective controls (*: paired two-tailed t-test; *: unpaired two-tailed t-test). A significant difference within a studied polypeptide is depicted with an arrow and p-value above (2-tailed paired student t-test). The experiments were performed with peripheral blood T cells (PB) shown in **A**, and with bone marrow T cells (BM) shown in **B** from a total of 41 patients.

To have an overview, results from 38 PB and 26 BM samples of primary breast cancers were evaluated as shown in Figure 12. With PBTC, the benefits of Treg-depletion were observed in different extents with almost all studied polypeptides except MAGE3. In particular, the significant inductions were achieved against five tumour antigens: HpaI, HpaII, Mam1, Muc1-20 and Her2/neu. Regarding the TAA-specific responsiveness, Treg-depletion elicited significantly higher IFN-γ productions of PBTC in response to Muc1-20, Muc1-100, EGFR and CEA than to control IgG (see asterisks in the figure). Of note, this approach reversed the significant response towards MAGE3. As we speculated above, this loss likely implies a

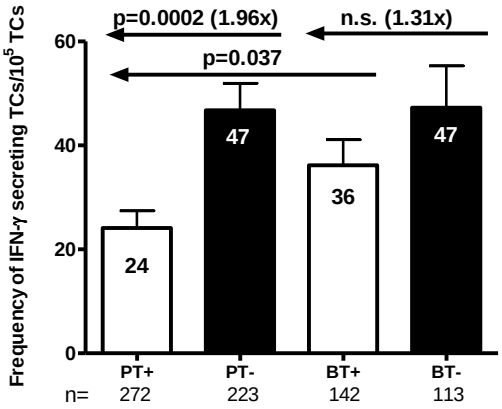
general on-going occurrence of MAGE3 Teff which were undesirably depleted through CD25 targeting.

In contrast to PBTC, Treg withdrawal from BMTC did not trigger any significant augmentations in TC immunity against TAAs, rather a significant reduction of CEA-responsiveness. However, compared to the values of controls, TAA-specific responses against HpaI, Muc1-20 and Muc1-100 were significantly favoured from the Treg-depletion protocol (see asterisks in the figure).

Of note, the Muc1-100-reactivities in both PBTC and BMTC were pre-established prior to a conducted Treg-depletion, and remained with an increasing degree after applying it.

So far, these data indicate that Treg suppression on TAA-responsiveness exist in both PB and BM. And this impact is stronger and broader in the PB than the BM.

A.



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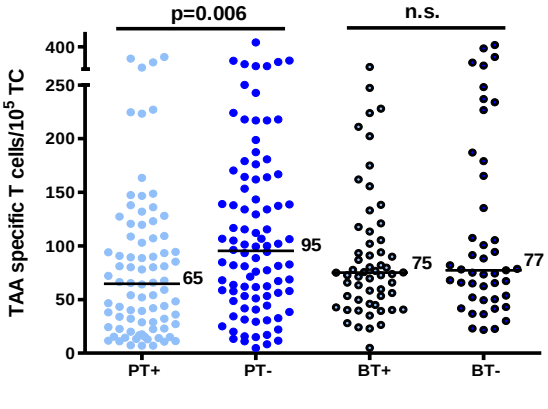


Figure 13. The same anti-tumour T-cell reactivity was obtained by PBTC and BMTC after a performed Treg depletion. **A**, Accumulated data of mixed polypeptides are gathered from in total 41 studied breast cancer patients. The mean frequencies of peripheral blood T cells (PT) and bone marrow T cells (BT) in the presence (+, white) and absence (-, black) of Treg are depicted as columns with SEM bars. Numbers depicted inside columns indicate the mean values. Numbers (n) of studied samples of each group are listed below respective columns. Statistical analyses were performed between the groups indicated by arrows with p-values above. n.s.: not significant. Numbers in parenthesis indicate increasing folds. **B**, Accumulated data of mixed polypeptides are gathered from only immunological responders. Each dot represents one individual value. Small bars inside groups represent median frequencies of T cells among TAA-recognizing breast cancer patients. Paired two-tailed t-test was performed.

In order to evaluate the accordance of using polypeptides with polyvalent antigens derived from cell line lysates, the frequencies of TAA-specific TC were accumulated from all tested samples from in total 41 breast cancer patients. Results are shown in Figure 13A. A highly

significant unmasking effect from Treg deprivation was revealed in the blood (p=0.0002). The mean frequencies boosted from 24 to 47 per 10^5 PBTC (1.96x). Although the influence in the bone marrow was not statistically significant, the mean frequencies of reactive BMTC were also increased from 36 to 47 per 10^5 BMTC (1.3x). Thus, in line with the results obtained with polyvalent breast tumour antigens, these data show that a Treg depletion is able to result in a replenishment of otherwise significant lower (p=0.037) spontaneous T-cell immunity in the PB and to a level as high as in the BM afterwards.

In consistence with this, when a similar comparison was preformed only within immunological responders, a pronounced increase in the mean value of responding T cells was observed in the blood (p=0.006), but not in the bone marrow (Figure 13B). Besides the mean frequencies, the median of TAA-specific PBTC also showed a substantial increase by 1.5-fold (PT+ versus PT-: 65/10^5 versus 95/10^5). Whilst the medians were relatively stable concerning BMTC (BT+ versus BT-: 75/10^5 versus 77/10^5).

These observations demonstrate that spontaneous T-cell responses towards tumour-associated antigens are suppressed by Treg in the blood and the bone marrow of primary breast cancer patients, especially in the blood.

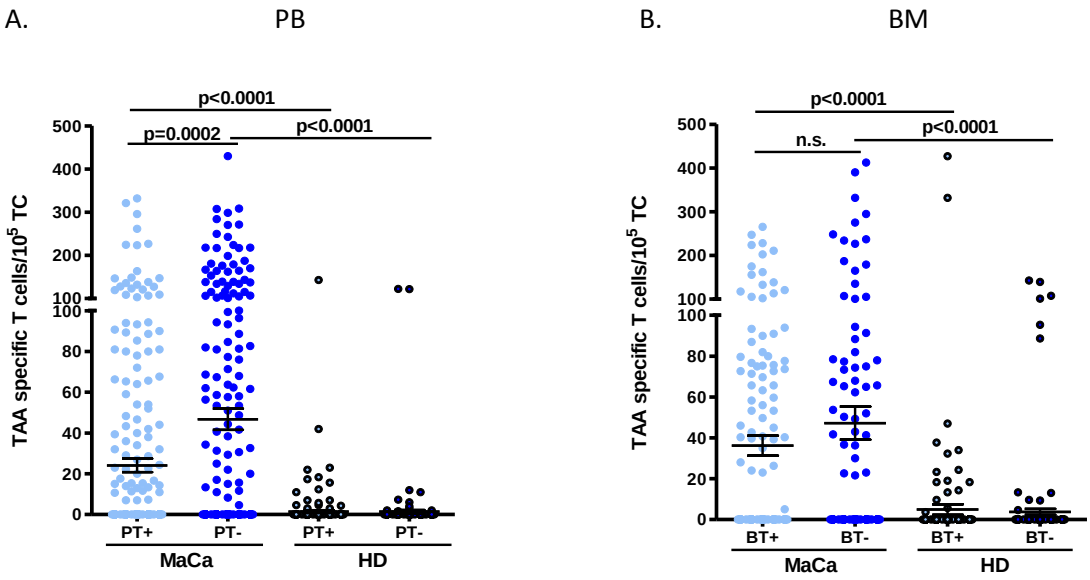


Figure 14. Comparisons of spontaneous T-cell reactivity against tumour-associated antigens in patients and healthy donors. **A**, cumulative data of frequencies of TAA-recognized T cells in the blood. Paired two-tailed t-test analyses were performed to compare the differences within breast cancer patients (MaCa) and within healthy donors (HD) before (+) and after (-) a performed Treg-depletion. Unpaired two-tailed t-test was performed to analyse the differences between breast cancer patients and healthy donors with a same treatment. Bars: mean±SEM. **B**, the same analyses as A were performed with bone marrow T cells.

Further, we evaluated whether this observed T-cell reactivity against designed polypeptides was specific to cancer patients. To this end, we conducted the same experiments with healthy donor (HD) derived T cells in the presence or absence of Treg. Altogether, 28 PB and 25 BM samples of HD were measured. Accumulative results are shown in Figure 14A (PB) and 14B (BM). A few of HD exhibited cross responses to TAA polypeptides with some of them even displayed exorbitant values. However, compared to breast cancer patients, the mean values of frequencies regarding TAA-reactive T-cells in HD was highly significantly low (see p-values in the figure), which is counted for both PBTC and BMTC, and also for both with and without Treg presence. These observations imply a potential relevance of utilizing these polypeptides for diagnostics, tumour vaccine design, and predicting T-cell immune responsiveness in breast cancer patients.

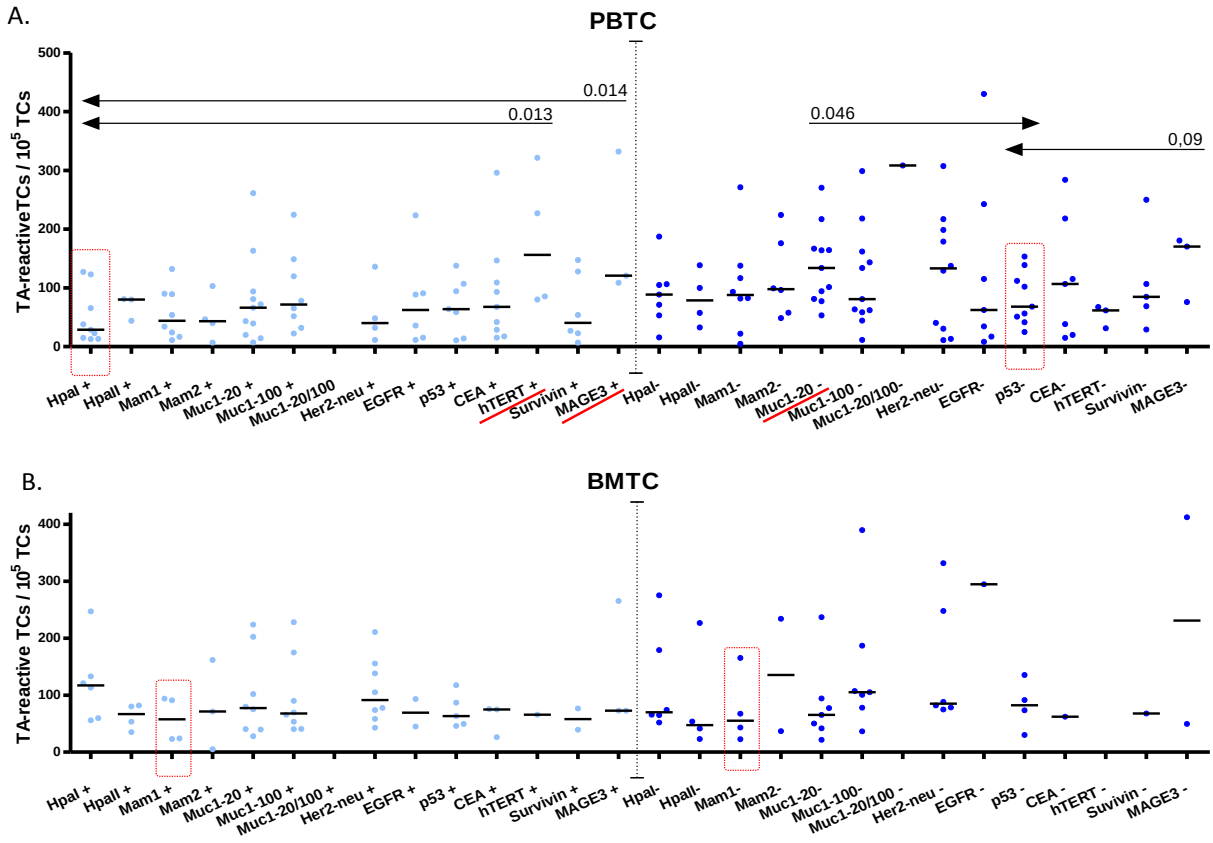


Figure 15. With or without Treg presence T cells show different patterns of tumour-associated antigen recognition in the peripheral blood (**A**) and the bone marrow (**B**). The frequencies of TAA-specific responding T cells were determined by IFN-γ ELISpot assay. Data from only immunological responders are shown here. Each dot represents an individual test. Light blue dots showed on left side and dark blue dots showed on right side represent the frequencies before (+) and after (-) a performed Treg-depletion, respectively. Bars indicate median values. To have a relative comparison, a lowest and most reliable value was chosen (red dash box) to be a comparative object for the other polypeptides in each section. P-values above arrows indicate statistical differences calculated with unpaired two-tailed t-test.

Next, we were interested in defining the spectrum of T-cell TAA-responsiveness exerted by Treg in breast cancer patients. To this end, we analyzed frequencies of TAA-specific T cells only from immunological responders, and compared the mean values of accumulated data to the lowest one displayed among interested T-cell groups. Results are shown in Figure 15 (A: PT, B: BT). The value of HpaI was designated as the reference for the study of PBTC without a conducted Treg-depletion. Compared to this, the frequencies of hTERT and MAGE3-specific T cells exhibited substantial elevations ($p=0.013$ and 0.014, respectively). After depleting Treg, the lowest frequency of TAA-reactive T cells was observed against p53. And in comparison to this, the frequencies of Muc1-20 and MAGE3-reactive T cells showed a significant ($p=0.046$) and a marginally significant ($p=0.09$) induction, respectively. These findings might have a potential clinical implication regarding peptide vaccine as different spectrums of TAAs should be considered according to whether a Treg depletion is preconditioned in breast cancer patients or not.

In the BM, the values obtained from Mam1 tests were designated as comparative objects in the groups of without and with a performed Treg-depletion. Under this condition, we did not observe any superior antigeneic tumour antigens according to statistical analyses.

Last, the impact of Treg on proportions of immunological responders was evaluated (Figure 16). In general, Treg-depletion-induced enhancements were detected in response to nine and five out of 13 studied polypeptides in the PB and BM, respectively. The significant effect appeared regarding Her2/neu in the PB ($p=0.03$), indicating a strong inhibition of Treg to T-cell reactivity against Her2/neu in breast cancer patients.

In a conclusion, these data demonstrate that type-I T-cell immunity against various tumour-associated antigens is suppressed by Treg in patients with primary breast cancer.

6.5 The suppressive regulatory T cells in breast cancer patients display tumour antigen specificity

So far we have shown the existence of Treg in breast cancer patients as well as their negative modulation capacity on anti-tumour type-I TC immunity. However, it remains unclear whether a tumour antigen-specific manner is utilized by Treg to exert this suppressive activity. Although the findings reported above have indirectly evidenced this conception, we are going to address this intriguing question with more direct evidence in this chapter.

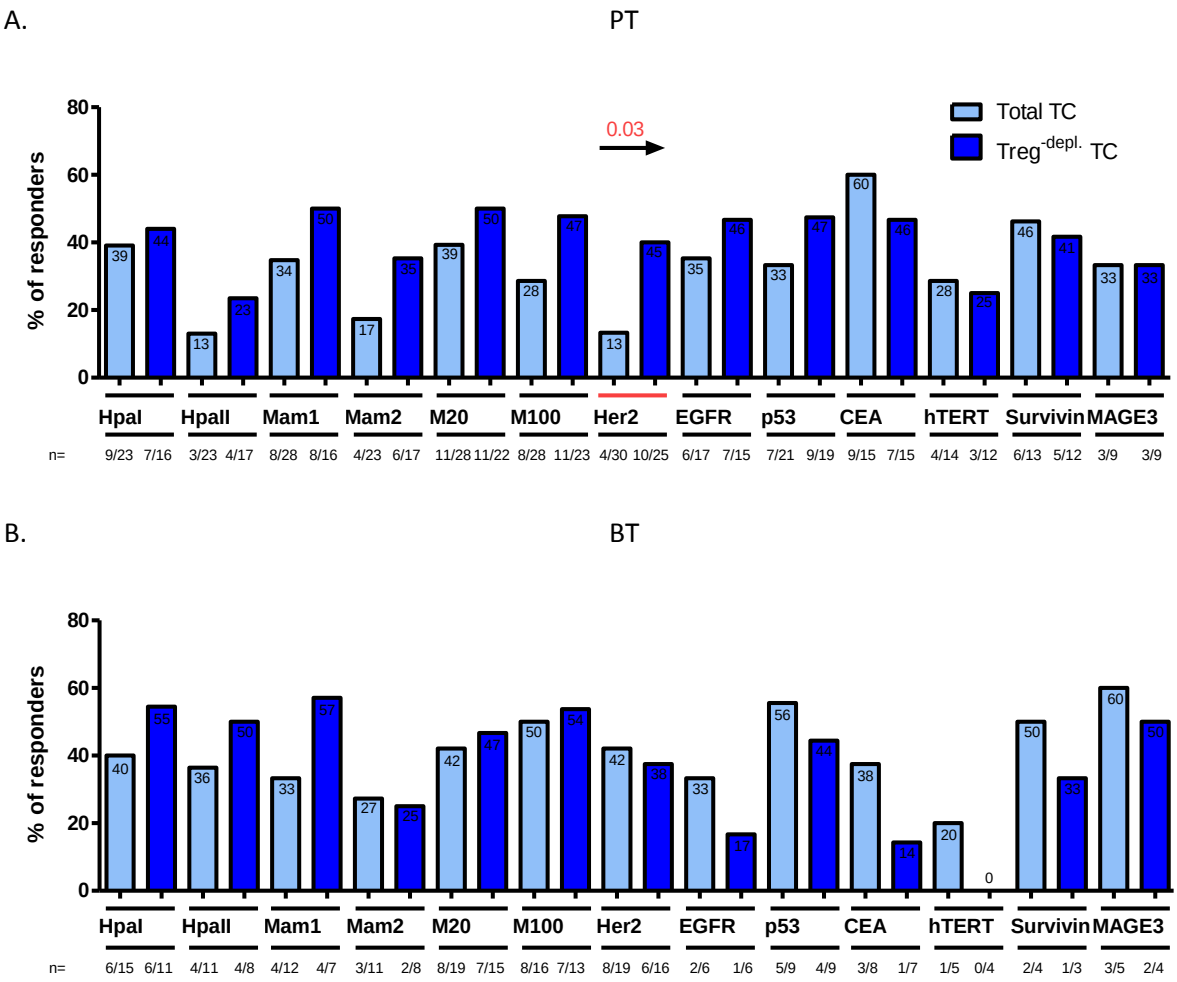


Figure 16. The impact of Treg on proportions of TAA-specific responders in breast cancer patients. Anti-tumour reactivity in the PB (**A**) and the BM (**B**) were tested against 13 different TAAs (indicated beneath X-axis) by IFN-γ ELISpot assay with (light blue) and without (dark blue) Treg presence. Columns represent the percentages (indicated in the column) of immunological responders among all tested patients. The exact numbers of responding patients to total analysed ones are listed below. Statistical differences within one studied antigen are calculated by Fisher's exact test.

6.5.1 Confirmation of the suppressive capacity of Treg enriched from an established protocol.

First of all, to identify the antigen specificity of Treg in breast cancer patients, a prerequisite is to confirm the purity of our so-called Treg, including the phenotypic and functional purities. In the previous report of this thesis (Figure 4B), we have shown that Treg are rather enriched than purified from a Treg-depletion approach as the phenotypic purity is constantly

around 60% of total isolated "Treg" fraction determined by co-expressions of Foxp3 and CD25. (Little note: to avoid confusion, even though there is a contamination with 40% of other $CD4^+$ TC, the term "Treg" is still used for denoting this population.)

However, a more important concern is whether these 60% of Treg are sufficient to exert suppression? To testify this, we performed a polyclonal suppression assay and Treg-adding-back IFN-γ ELISpot assay.

Treg are known to be hypo-proliferative and effectively suppress proliferation of Tcon upon a polyclonal stimulation. In this regard, we first assessed the functional purity of Treg with a standard proliferation assay performed as following: Tcon and Treg were co-cultured with titrated ratios of Tcon:Treg from 1:0, 1:1, 2:1, 4:1 to 8:1, and were polyclonally stimulated by αCD3 and αCD28 mAb coated magnetic beads for three days. During the last 18 hr ^{3}H thymidine was provided for being incorporated into proliferating T cells. The amounts of incorporated radioactivity were used for quantifying the extent of proliferation.

A representative result obtained with PBTC from a primary breast cancer patient is shown in Figure 17. An undetectable proliferation of Treg alone and a vigorous amplification of Tcon were observed upon a polyclonal stimulation as expected. The latter proliferation was inhibited in the presence of Treg. The intensity of inhibition was correlated to the proportion of Treg in the co-culture. Significant suppression of Treg was displayed at Tcon:Treg ratio of 1:1 ($p=0.004$) and 2:1 ($p=0.005$). Even though there was no statistical difference shown at higher ratios (4:1 and 8:1), substantial reductions in the amplification of Tcon were sustained. Hence, these data demonstrate that 60% Treg isolated from a Treg-depletion protocol are sufficient to inhibit polyclonal proliferation of Tcon *in vitro*.

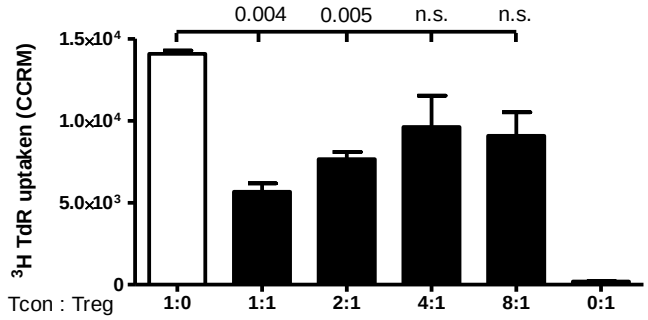


Figure 17. Treg suppress the proliferation of Tcon upon polyclonal stimulation. Primary data from PBTC of a breast cancer patient are shown here. White bars: Tcon only. Black bars: Tcon in co-culture with autologous Treg at different ratios as indicated. Cell cultures were polyclonally stimulated and incubated for 72h. The proliferations of Tcon were quantified by the values of incorporated ^{3}H-thymidine. Statistical analyses were performed between co-cultures and Tcon alone. P-values are calculated by paired two-tailed t-test. This result represents 3 independent experiments.

Next, we evaluated the functional purity of Treg by its suppression on cytokine secretion. Previously, we have shown that a withdrawal of Treg can rescue type-I cytokine secretion in PBTC and BMTC upon tumour-antigen stimulation. In order to obtain more direct evidence, we utilized the same IFN-γ ELISpot assay, and compared the outcomes with TC- population in the absence and re-presence of autologous Treg which were added back at different ratios.

An exemplary result of a primary breast cancer patient is shown in Figure 18. Without the presence of Treg, KS24.22-specific responsiveness was found in both PBTC (p=0.002) and BMTC (p=0-009). However, they were substantially abrogated by returning autologous Treg at TC- to Treg ratios of 2:1 and 4:1. The only restored KS24.22-specific reactivity in this experiment was observed in the BM (p=0.05) when Treg proportion was reduced to a ratio of TC:Treg 8:1.

Accordantly, the extent of Treg suppression on T-cell responsiveness again KS24.22 was dramatically reduced in the co-cultivations at TC-:Treg ratio of 2:1 and 4:1, which was counted for both PBTC and BMTC. This inhibitory effect appeared to be Treg dose dependent as IFN-γ productions were gradually restored determined by mean values of tumour-reactive T cells and eventually statistically vanished at a ratio of 8:1 (see p-values in the figure). Markedly, U937 background IFN-γ secretion was not inhibited by adding back Treg, suggesting an antigen specificity of Treg in breast cancer patients.

Collectively, these findings confirm the functional purity of Treg isolated from a Treg depletion protocol.

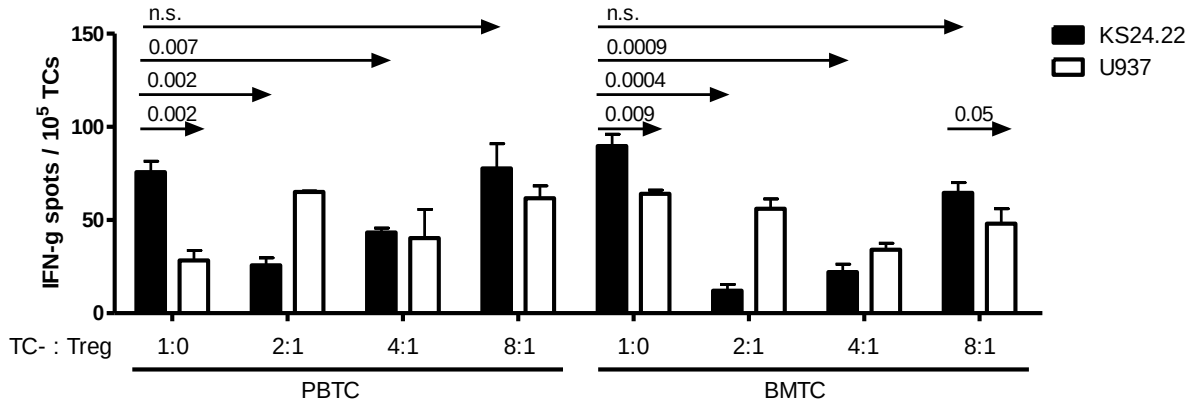


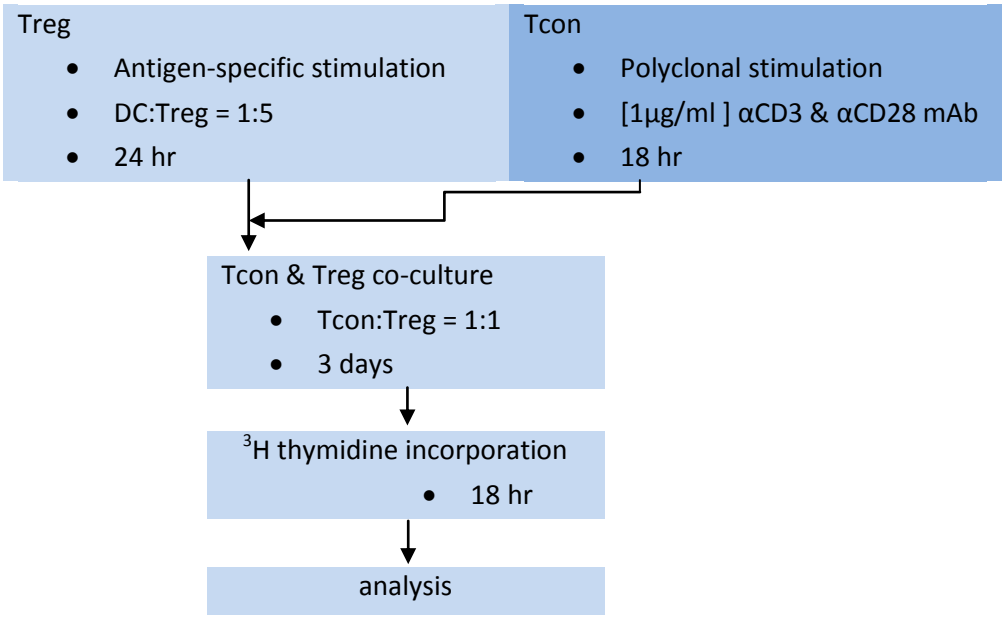
Figure 18. Added back Treg suppress autologous type-I T-cell response against breast tumour antigens. Columns represent the frequencies of KS24.22 (black) and U937 (white) reactive T cells with PBTC and BMTC in the absence of Treg (TC-) and in the presence of Treg with different ratios as indicated below X-axis. Statistical analyses were calculated between interested groups showed by arrows with p-values above. Paried two-tailed t-test was performed. n.s.: not significant. This exemplary result represents 3 independent experiments.

6.5.2 Treg from breast cancer patients recognize tumour-specific antigens

6.5.2.1 Experiment design

It is documented that Treg require TCR triggering to become functionally suppressive, but once activated they suppress function of Tcon blindly. According to this notion, we designed a novel approach for examining the existence of breast-tumour-specific Treg. As shown with a schematic in Figure 19, this method was modified from a T-cell proliferation assay. In detail, Treg were activated with breast tumour antigens presented by autologous DC for 24 hr. Meanwhile Tcon received polyclonal stimulation from αCD3 and αCD28 mAb coated wells. Afterwards, pre-activated Tcon were given into Treg stimulation cultivation at a ratio of 1:1 for three days to allow a full exertion of suppression. The last 18h ^{3}H thymidine was provided into the co-cultivations. The degree of Tcon proliferation was quantified by the amount of incorporated ^{3}H thymidine. We named this experiment "Treg specificity assay".

Figure 19. Schematic of Treg specificity assay.

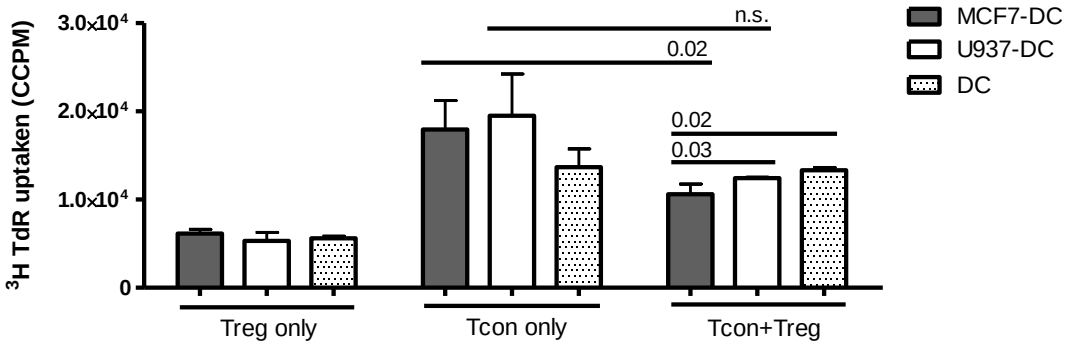


6.5.2.2 Treg recognizes polyvalent tumour antigens derived from MCF7 or KS24.22 cell lines

To start, we assessed the specificities of Treg towards a large spectrum of breast tumour antigens provided in MCF7 and KS24.22 cell line lysates. And in order to testify the reliability of newly designed protocol, we performed several rigid control experiments. 1) The proliferative extents of Treg alone with different antigen stimulations were evaluated. 2)

Since Treg are known to be generated according to self-antigen recognition, we also measured the spontaneous proliferation of Treg possibly triggered by autologous DC on which endogenous self-antigens were presented. 3) To further prove the genuine inhibition of Treg to the proliferations of Tcon, the ^{3}H thymidine incorporations in the wells of activated Tcon with and without a presence of Treg were examined.

A.



B.

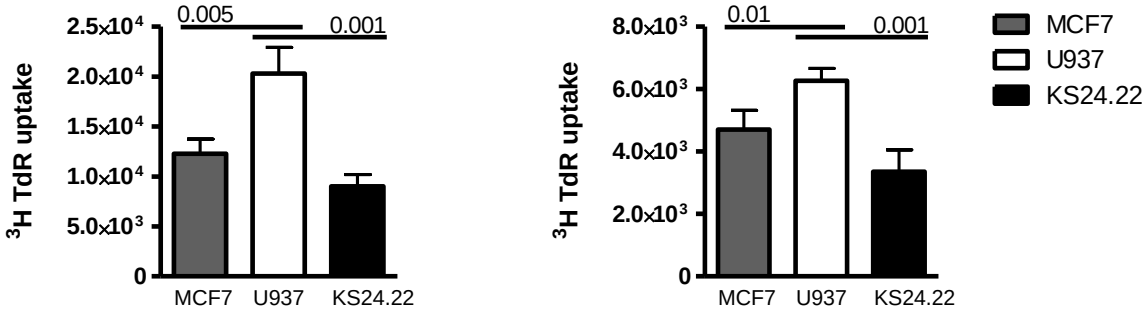


Figure 20. Preliminary data show a feasibility of Treg specificity assay. **A**, Approach controls. Treg received activation with MCF7 (gray column) and U937 (white column) pulsed DC, and non-pulsed DC (shadowed column) for 24h. Tcon were stimulated with polyclonal αCD3 and αCD28 mAbs for 18h. Afterwards, Tcon were added into Treg cultivation at ratio of 1:1 for three days (Tcon+Treg). Meanwhile, two control experimental settings were performed. Treg only: without Tcon co-cultivation; Tcon only: pre-activated Tcon were further cultivated with different models of pulsed DC but without Treg presence. The amounts of T-cell proliferation were measured by ^{3}H thymidine incorporation and shown by columns with SEM bars. Statistical analyses were performed between the interested groups connected by short bars with p-values above. n.s.: not significant. **B**, Primary data show that Treg from both PB and BM possess breast TA-specificity. Polyclonally stimulated Tcon were co-cultivated with Treg that were pre-stimulated with antigens from MCF7, KS24.22 (black column) and U937.

Exemplary results are shown in Figure 20A. First of all, the lowest proliferation levels were observed from Treg alone groups with no detectable differences in between different tumour-antigen stimulations and autologous DC stimulation, which dispelled the potentials of antigen-driven proliferations of Treg. Second, the highest proliferations were attested from polyclonal activated Tcon in the absence of Treg. And according to statistical analyses there were no influence of different models of antigen stimulations carried out by autologous DC. Third, with a co-cultivation of MCF7–activated Treg, a vital polyclonal proliferation of Tcon was significantly suppressed (p=0.02). And importantly, this effect was not observed with irrelevant tumour antigen (U937) or self antigen activated Treg. Hence, these data demonstrate a reliability of this newly designed protocol.

Furthermore, regarding the co-cultivated groups, a MCF7-mediated suppression was observed (MCF versus U937: p=0.03; MCF7 versus self-antigen: p=0.02), suggesting an existence of breast tumour-specific Treg in this patient. In line with this, the data from a representative example suggested that breast tumour specificity of Treg can be found in both PB and BM (Figure 20B).

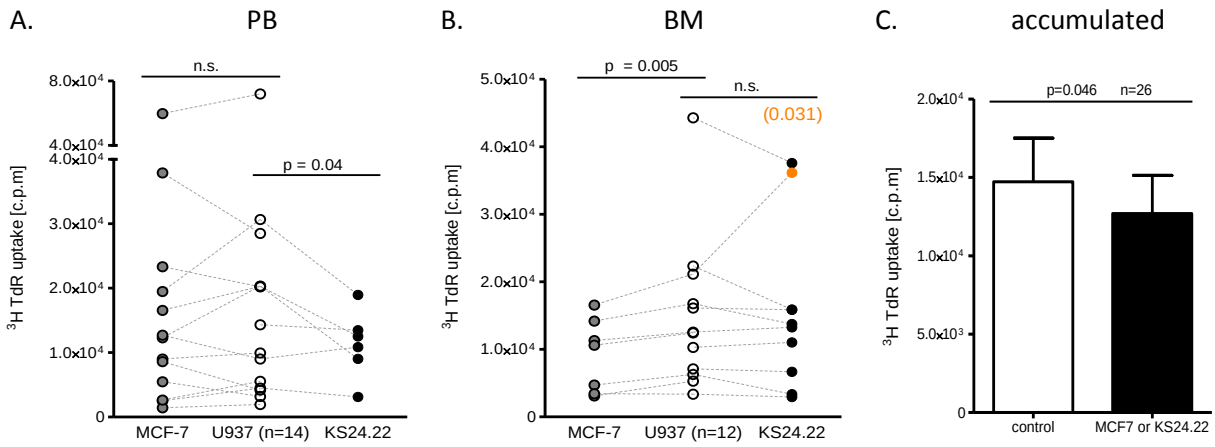


Figure 21. Treg possess antigen specificity towards polyvalent tumour antigens in breast cancer patients. Treg specificity assay were performed with purified Treg derived from peripheral blood (**A**) and bone marrow (**B**). Breast tumour antigens were provided by MCF7 (gray) or KS24.22 (black) and control tumour antigens were from U937 (open). Each circle represents one measurement. Dash lines connect values within one patient. P-values indicate significant differences between compared groups connected with small bars. n.s.: not significant. Paired one-tailed t-test was performed in A and B. In B, by excluding a strong influence from one patient (orange dot), the suppression on Tcon proliferation by KS24.22-stimulated Treg was significant (p-value showed in orange). **C**, Accumulated data of 26 breast cancer patients. When suppression existed in one patient no matter in the PB or BM and no matter elicited by MCF7 or KS24.22, the lowest value was considered in this analysis. P-value was calculated by unpaired two-tailed t-test.

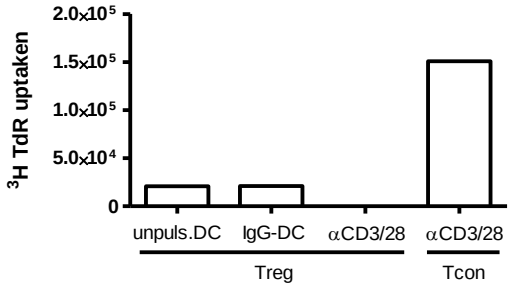
In an overview, results of Treg specificity assay from 14 PB and 12 BM samples of total 26 breast cancer patients were collected as shown in Figure 21A and B. In the peripheral blood, Treg pre-activated with KS24.22 antigens exerted a significant (p=0.04) and homogeneous suppression on polyclonal proliferation of Tcon. In contrast, the existence of MCF7-recognizing suppressive Treg is rather heterogeneous in the blood. Regarding BM samples, apart from one outlier patient (indicated in orange), both MCF7 and KS24.22-specific Treg elicited significant suppression on polyclonal proliferation of Tcon (p=0.005, p=0.031, respectively). By further pooling the results from different sources of breast tumour antigens (MCF7 or KS24.22) and different sources of Treg (PB derived Treg and BM derived Treg), we observed a significantly (p=0.046) lower proliferation of Tcon in the analysis with breast tumour-antigen stimulated Treg than that with respective control U937 stimulated ones (Figure 21C). Therefore, we substantiate that Treg possess polyvalent antigen specificities in breast cancer patients.

6.5.2.3 Treg exhibit multifarious tumour antigen recognition in breast cancer patients

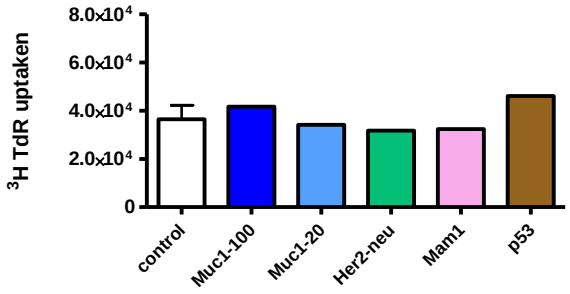
Next, we sought out to define the spectrum of tumour antigens which can be recognized by Treg in breast cancer patients by utilizing synthetic polypeptides.

First of all, we tested the feasibility of applying our designed Treg specificity assay with polypeptides. Figure 22 shows a series of testing experiments from the blood sample of one breast cancer patient. In A we tested the proliferative capacity of Treg upon different stimulation conditions, in detail, with autologous DC, with IgG pulsed DC and with αCD3 and αCD28 mAb. As expected, none of them triggered Treg expansion. Purified Tcon exhibited a vigorous proliferation in response to a polyclonal stimulation. In B, the responses of Treg against individual polypeptides are shown. A similarity of proliferative behaviours was observed including the control IgG. Thus, a hypo-proliferative characteristic of Treg upon stimulations with different peptides is confirmed. In C, we showed an example of Treg specificity assay performed with different polypeptides. Interestingly, polypeptide pre-activated Treg suppressed the proliferations of polyclonally stimulated Tcon in different extents with significant effects in studied groups of Her2/neu (p=0.01) and Mam1 (p=0.06) (Figure 22C). Of note, Mucin-derived antigens did no alter the outcomes; whilst p53 drove a significant higher proliferation (p=0.008). This interesting phenomenon implies that in the blood of this patient there was: i) a presence of Her2/neu and Mam1-specific Treg, ii) an absence of Mucin-recognizing Treg and iii) a presence of p53-reactive Teff with a possibly concomitant absence of p53-responsive Treg. Altogether, we claim this approach can be applied for investigating the existence of TAA-specific Treg.

A. control experiment



B. Treg only



C. Treg and Tcon co-culture

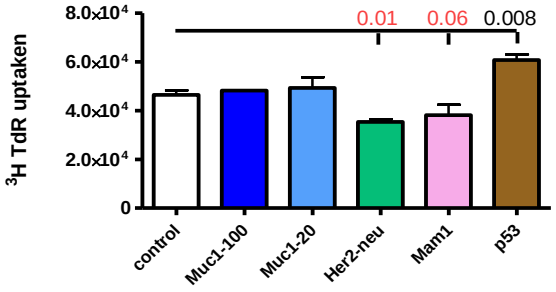


Figure 22. Preliminary data show a feasibility of utilizing polypeptides for Treg stimulation in Treg specificity assay. Columns represent the extents of proliferations measured by ^{3}H thymidine incorporations. **A**, Control tests confirm hypo- and hyper- proliferative activity of Treg and Tcon, respectively, upon different stimulations. Stimuli are indicated blow each bar. **B**, The proliferation of Treg are not induced by TAAs or IgG. Treg were cultivated with different polypeptides (colourful columns) and IgG (control, white column) pulsed autologous DC. **C**, The suppressions on polyclonal proliferation of Tcon are TAA-driven. In this particular patient, suppressions are significantly elicited by Her2/neu and Mam1-actiated Treg. P-values are shown above lines which connect the values of interested group and IgG control. Statistical differences were analysed by paired two-tailed t-test.

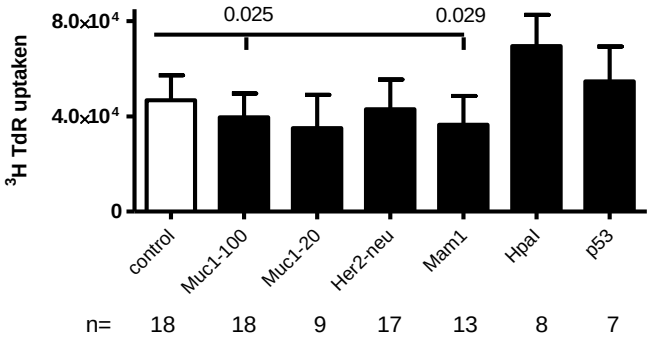
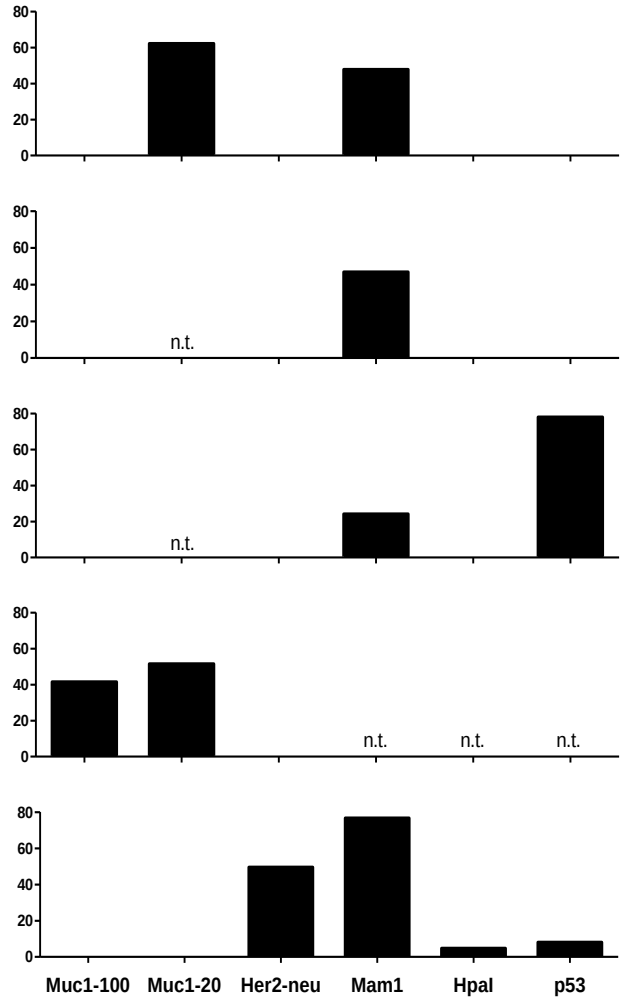
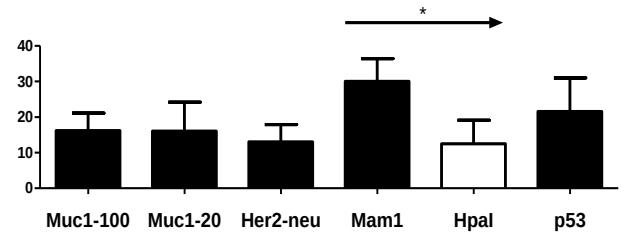


Figure 23. Treg in breast cancer patients possess diverse antigen specificities. Accumulated results of Treg antigen specificity assay performed with 18 blood samples of breast cancer patients. Columns: ^{3}H thymidine incorporation. Bars: SEM. Treg were activated with different TAA (black) and IgG control (white). P-values were calculated by paired two-tailed t-test. Exact numbers (n) of patients assessed for each TAA are indicated below.

A.% of inhibition



B.% of inhibition



C.

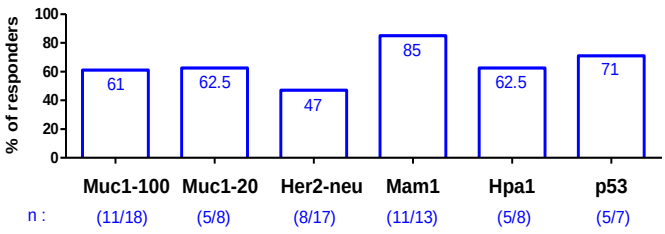


Figure 24. Antigen specificities of Treg in breast cancer patients possess a diverse and individual repertoire. **A**, Five randomly picked measurements show a highly individual pattern of antigen specificity of Treg existing in breast cancer patients. Columns represent percentages of inhibited proliferation of Tcon analysed by comparing the values of thymidine incorporation between TAA groups and IgG control ones. n.t.: not tested. **B**, Accumulated results of inhibition percentages pooled from PB and BM samples of 17 breast cancer patients. Take the least inhibited value, namely, HpaI (white), a significant higher inhibition was observed in Mam1- stimulated Treg group. P-value was calculated by unpaired two-tailed t-test. **C**, Diverse proportions of breast cancer patients in regarding to antigen specificity of Treg. Columns represent the percentages (depicted in each column) of patients who contain Treg specificity towards indicated tumour antigens. The exact numbers (n) of responders to total assessed patients are listed in parenthesis.

In an overall (Figure 23) from altogether 18 blood samples of breast cancer patients, Muc1-100 and Mam1-activated Treg were found to exert significant suppressions on Tcon proliferation compared to the control (p=0.025 and 0.029, respectively), suggesting a general existence of functional Muc1-100 and Mam1-specific Treg in the peripheral blood of breast cancer patients.

To further estimate if the spectrum of antigens recognized by individual patient might be largely different or overlapping, we calculated the ratio of proliferation inhibition by analysing the thymidine incorporation values between TAA groups and IgG control groups of each tested antigen in every patient. Surprisingly, the pattern is highly diverse. The multifariousness is not only counted for various antigen recognitions, but also for their differently inhibitory degrees as shown in Figure 24A with five randomly picked experiments.

Concerning the suppressive extent (Figure 24B), Mam1-recognized Treg exerted more than 30% inhibitory activity. This was substantially exceeding to the others, especially, when compared to the value of HpaI-driven Treg suppression (p=0.08). Moreover, Mam1 also displayed as the most frequent tumour-associated antigen recognized by Treg in breast cancer patients (85%) (Figure 24C).

In summary, we conclude from our data that Treg from breast cancer patients have preferential TAA-recognitions towards Muc1 and Mam1, and Mam1-recognized Treg trigger the strongest inhibitory activity. And in an individual level, the spectrum of tumour antigens recognized by Treg is rather broad.

6.5.3 IL-10 production of Treg is driven by tumour-specific antigens in breast cancer patients

Interleukin-10 (IL-10) is a major cytokine with pronounced immunomodulatory and immunosuppressive properties. It was reported that antigen-specific Tr1 cells were generated in the presence of IL-10, and, in turn, exerted suppression mostly through IL-10. For this reason, we tried to attest Treg specificity by analyzing an antigen-driven secretion of IL-10 in breast cancer patients.

To this end, we first carried out an IL-10 ELISA assay to evaluate the IL-10 secretion during TC activation. The supernatants of eight IFN-γ ELISpot experiments were collected from both with and without Treg-present cultures. The breast tumour antigen mediated IL-10 secretion was determined by subtracting mean value of IL-10 concentrations of test wells from the control cultures. Under these conditions, we observed an abrogation of IL-10 secretion after

Treg-depletion (Figure 25A, p=0.0195), which implies that secretion of IL-10 possibly from Treg is driven by specific breast tumour antigens.

In support of this, more direct evidence was obtained by assessing intracellular IL-10 production of purified Treg in response to different antigen stimulations. In detail, Treg were stimulated with breast cancer antigens derived from MCF7 and KS24.22, and control tumour antigens from U937 presented by autologous DC for 20 hr. Protein secretions were blocked by Brefeldin A for the last 12 hr. Afterwards, an intracellular staining of IL-10 and a staining for defining Treg population were performed. Data were acquired by flow cytometry. The percentages of IL-10 positive cells among Treg were then analyzed. An exemplary result representing three independent experiments is shown in Figure 25B. Upon MCF7 stimulation, the proportions of IL-10 producing Treg was four times more than that in the situation of U937 control (mean±SD; MCF7: 0.17±0.08%; U937: 0.04±0.03%). The difference is statistically significant (p=0.04). A similar effect was observed in response to KS24.22 (0.11±0.03%) with a less (2.4 times) but also significant (p=0.03) extent. Collectively, these data suggest that Treg in breast cancer patients possess antigen specificity determined by IL-10 production.

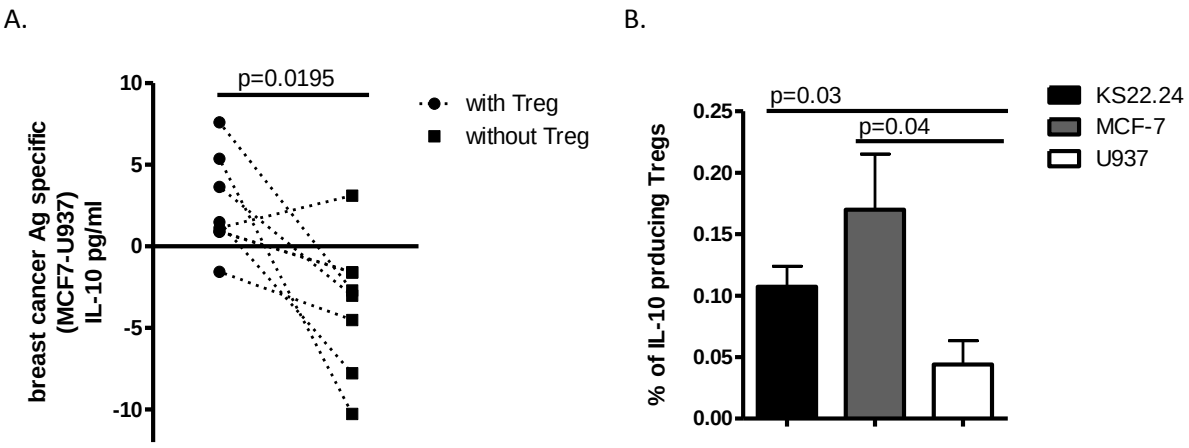


Figure 25. Treg possess breast tumour specificity suggested by measurements of IL-10 productions. **A**, Accumulated data of eight breast cancer patients show MCF7-dependent IL-10 secretion. The supernatants of ELISpot assay were collected after 40h, and measured for IL-10 concentration by ELISA assay. Each symbol represents a subtraction of IL-10 concentrations from the cultures stimulated with MCF7 to that with U937. Dash lines connect the values before (round) and after (square) a conducted Treg-depletion within one patient. P-value was calculated by an unpaired two-tailed t-test. **B**, A representative result shows percentages of IL-10 secreting Treg among total Treg upon different tumour antigen simulations (indicated in the plot) obtained from blood derived Treg of one breast cancer patient. These data represent three independent experiments. Columns: mean values of triplicates. Bars: SEM. P-values were calculated by unpaired two-tailed t-test between interested groups connected by short lines.

6.6 Expressions of chemokine receptors on polyclonally activated Treg

A high frequency of tumour-infiltrating Treg was reported in breast cancer, which was likely due to an environment of preferential recruitment created by tumour cells and tumour-associated cells. Previous study of our group revealed an interesting phenomenon in primary breast cancer patients that the higher numbers of tumour infiltrating Treg was associated with better type-I T-cell anti-tumour responses in the BM. According to the findings in this thesis, Treg retained in the BM were substantially less than the PB. We also reported that bone marrow served as a priming site for TC immunity upon blood borne antigen presented on immigrated DC. Based on these findings, we proposed a hypothesis that Treg were actively emigrating from bone marrow after encountering the tumour antigens, and then progressively accumulating in the tumour mass.

In support of this hypothesis, we analysed 13 adhesion molecules on Treg and found that the expressions of CCR2, CCR5 and CCR6 were higher in the BM of primary breast cancer patients than their counterpart PB and the BM of healthy donors, suggesting a potently migratory capacity of Treg obtained from the BM of breast cancer patients. In addition, CCR4 was reported to enable Treg to migrate into ovarian tumour. Whether these observations were resulted from activation (possibly occurred in the BM); and the comparisons of activation-induced expressions between Treg and Tcon were investigated in this thesis.

6.6.1 Treg from the peripheral blood of healthy donors are able to upregulate chemokine receptors upon a polyclonal stimulation

First, we evaluated whether Treg were able to respond activation in term of upregualting interested chemokine receptors. To this end, freshly isolated monocytes from healthy donors were cultured in the wells pre-coated with αCD3 mAb, or in parallel with αCD3 and αCD28 mAb, for 16 hr. In order to design a proper dose of stimuli, we titrated mAb with 4 different concentrations: [1μg/ml], [0.5μg/ml], [0.25μg/ml] and [0.125μg/ml]. As a readout system CCR2, CCR5, CCR6 expressions were performed with activated cells by flow cytometric assay. CD3, CD4, CD25 and Foxp3 were co-stained for specifying Treg.

The increases in the percentages of respective CCR expressions were found on both Treg and T conventional cells (Tcon) upon polyclonal TCR-mediated stimulation with different extents as shown in Figure 26. The most significant upregulations were observed with the strongest stimulation setting in all tests, namely [1μg/ml]. Accordingly, this concentration was applied hereafter for all the experiments. Of note, the existence of co-stimulatory molecule, CD28, hindered CCR2, CCR5 and CCR4 expressions on Treg but not (CCR2, CCR4) or less (CCR5) on

Tcon. Besides, by giving a less alteration observed concerning CCR6, it was not included in the rest of this study.

Nevertheless, these data demonstrate that Treg possessed the ability of upregulating certain chemokine receptors upon a polyclonal activation.

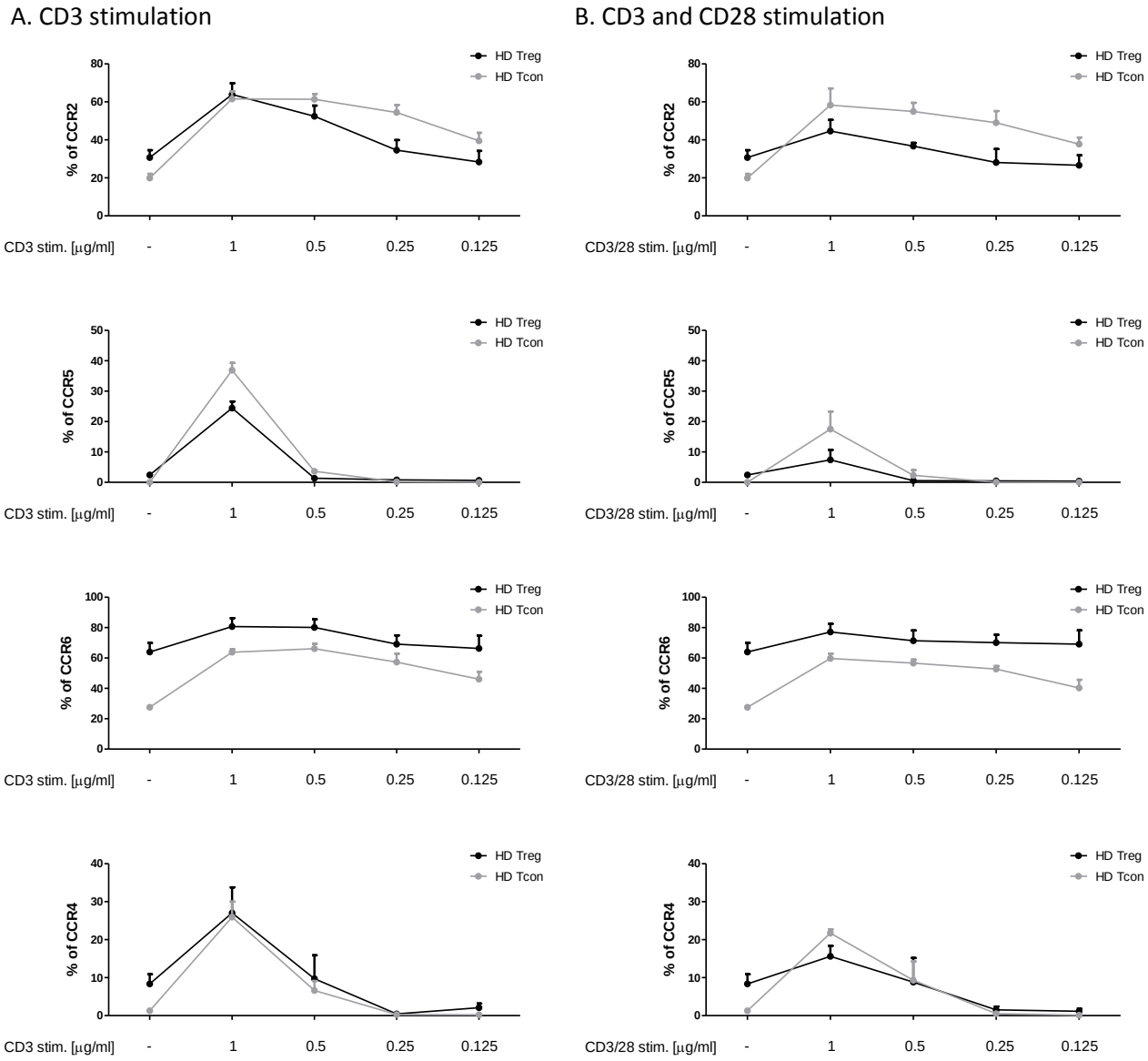


Figure 26. Treg and Tcon derived from healthy donor respond to polyclonal stimulation. Monocytes isolated from healthy donor peripheral blood were cultivated in αCD3 (**A**) or αCD3 and αCD28 (**B**) mAb bounded wells and control wells for 16h. Ab titrations were depicted below X-axis. Flow cytometry were performed afterwards with multicolour staining in order to identify the CCR expression on $CD4^+$ Treg and $CD4^+$ Tcon subpopulations. The assessed chemokine receptors are indicated beside Y-axis. Black lines: Treg; gray lines: Tcon. Bars: SEM.

6.6.2 Evaluation of CCR2, CCR4 and CCR5 expression on Treg from breast cancer patients upon a polyclonal stimulation

Next we sought to evaluate chemokine receptor expressions on breast cancer derived Treg upon polyclonal activation. A same method as described above was performed.

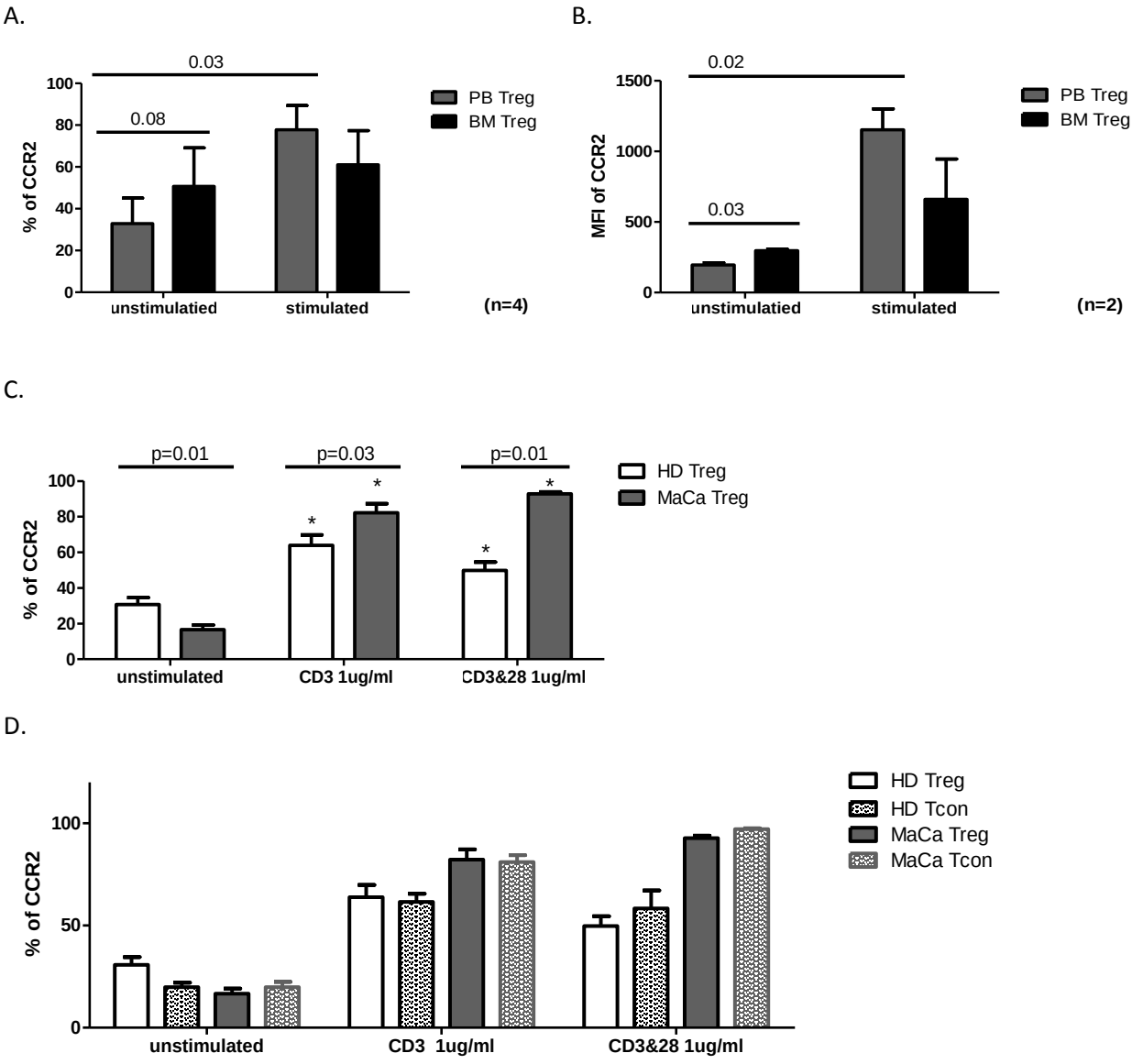


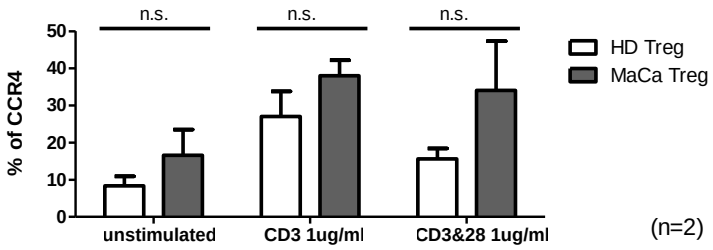
Figure 27. Upon polyclonal stimulation CCR2 were upregulated more potently on Treg from breast cancer than from healthy donors. **A**, percentage and **B**, MFI of CCR2 expression on MaCa PB Treg (gray bars) and BM Treg (black bars) without and with [1µg/ml] αCD3 mAb stimulation. **C**, comparison of percentage of CCR2 expression on HD PB Treg (white bars) to MaCa PB Treg without and with different polyclonal stimulations. Asterisks show $p<0.05$ when compared to the respectively unstimulated values. **D**, CCR2 induction capacity has no difference between Treg and Tcon. P-values were calculated with unpaired two-tailed t-test.

Results from CCR2 studies are shown in Figure 27. In an unstimulated groups, namely *ex vivo* situation, CCR2 expressing Treg were significantly higher in the BM than the PB (marginally, p=0.08) in regard to percentages and MFI (p=0.03), which is in accordance with Christopher's finding.

A given polyclonal activation allowed substantial inductions of CCR2 expressing Treg in the PB but not the BM in regard to proportion (p=0.03) as well as MFI (p=0.02). Perhaps the basal activation status in the BM dampened the effect (Figure 27A and B). When compared to healthy donors (Figure 27C), the response of Treg to activation in the blood was significantly potent in patients. A sharp reverse pattern was observed as the percentage of CCR2 positive PB Treg was originally found to be significant lower in patients than the healthy donors (p=0.01); whilst turned to be significantly higher post-activation (CD3 stimulation, p=0.03; CD3&CD28 stimulation: p=0.01). Certain response illuminates a ready-to-facilitate mode in breast cancer patients.

Further, we asked whether Treg owned better induction potency than Tcon. As shown in Figure 27D, it was not the case. Similar inductions in percentage of CCR2 expressing cells were observed with both Treg and Tcon in breast cancer patients. This was also true in regard to MFI analyses (data not shown).

A.



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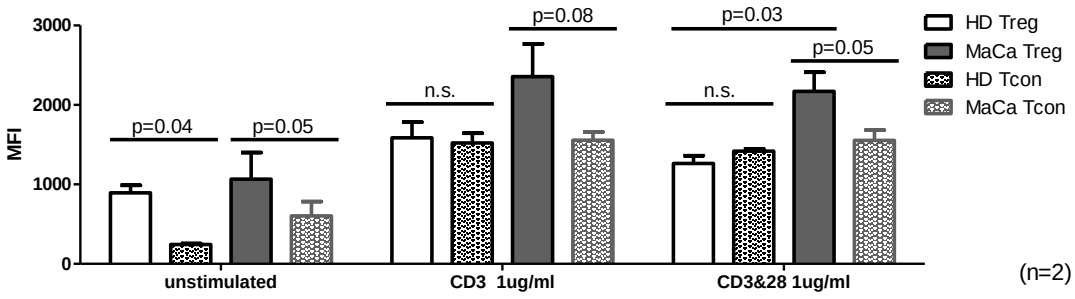
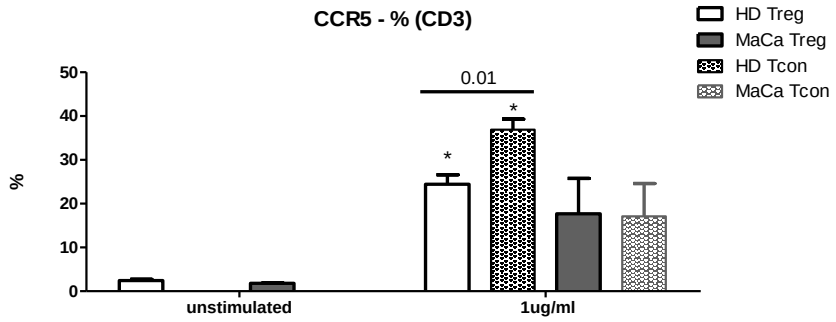


Figure 28. CCR4 is preferentially harnessed by Treg upon activation. **A**, percentages of CCR4 expression on PB Treg of HD and MaCa. HD Treg:white; MaCa Treg: gray. Different culture conditions are indicated. **B**, comparison of CCR4 expression in MFI on different T cell sources. Treg: solid; Tcon: patterned. Cultivation conditions are as indicated. P-values were calculated with unpaired two-tailed t-test. Compared groups are linked with horizontal lines.

Unlike CCR2, the *ex vivo* CCR4 expression was higher on PB Treg derived from breast cancer patients than that from healthy individuals (Figure 28A). This pattern was remained after polyclonal activations, even though both counterparts showed substantially elevated percentages of CCR4 expressing Treg. The lack of statistical significance was very likely due to the small size of patient samples (n=2). Of note, the hindered effect in healthy donors resulted from the presence of co-stimulatory molecules was not observed with breast cancer patients.

In the *ex vivo* situation, Treg from both HD and breast cancer patients exhibited significant higher MFI of CCR4 than Tcon (Figure 28B). Upon activation, the MFI level of CCR4 expressing Tcon kept up with that of Treg in healthy donors but remained significant lower in the breast cancer patients, which was counted for both given stimulation conditions. Interestingly, among all studied populations, Treg in breast cancer patients exhibited the most prevalent responsiveness to activation.

A.



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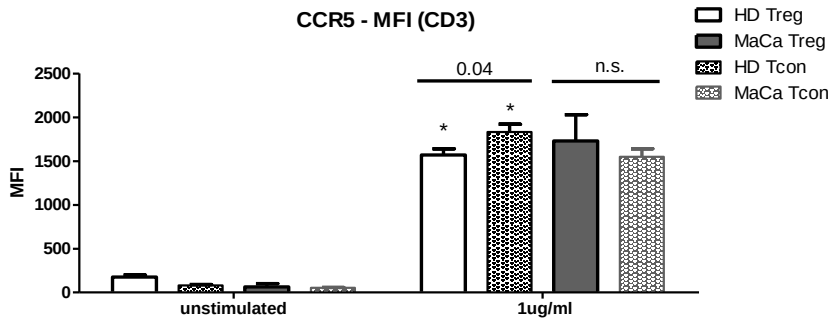


Figure 29, Upon polyclonal stimulation CCR5 expression is suppressed on Tcon by Treg with breast cancer. CCR5 expressions on different T cell sources without or with αCD3 stimulation are compared (**A**) in percentage and (**B**) in MFI. Treg: solid columns; Tcon: patterned columns. HD: white background; MaCa: gray background. Cultivation conditions are as indicated. P-values were calculated with unpaired two-tailed t-test. Compared groups are linked with horizontal lines. Asterisks show $p<0.05$ when compared to the respectively unstimulated values.

Concerning CCR5, upon stimulation, a substantial induction of CCR5 expressing Treg in regard to proportions and MFI were observed in both healthy individual and breast cancer patients (Figure 29). However, this effect did not result a preferential pattern towards Treg from breast cancer patients as observed with the other chemokine receptors. Further, this stimulation induced CCR5 expression benefited Tcon in healthy donors but not breast cancer patients counted for the analyses of percentage ($p=0.01$) and MFI ($p=0.04$).

Part II From bench to bedside -- Clinical exploitations of Treg manipulation in breast cancer

The observations in Part I of this thesis demonstrate that regulatory T cells play a crucially negative role in breast cancer and by removing them the T-cell anti-tumour reactivity can be rescued, especially, in the peripheral blood. These basic findings provide a principle of developing an immunotherapy of breast cancer by modulating existing Treg in the patients. Thus, our next challenge is: how to move “from bench to bedside”?

Immunotherapy regarding Treg depletion is currently being exploited in clinical trials, such as daclizumab, an αCD25 antibody; Ontak (Denileukin Diftitox), an IL-2 fusion toxin; αCD25 microbeads (magnetic cell sorting, MACS); an αCD25 immunotoxin (IT); or metronomic administration of low-dose cyclophosphamide which selectively eliminates Treg, and so on. However, the most effective method has not yet been defined.

In this thesis, a pre-clinical study of large-scale depletion of Treg from a leukapheresis and a pilot clinical study of metronomic low-dose cyclophosphamide therapy were applied on metastatic breast cancer patients. The outcomes are analysed and presented in Result 1.7 and Result 1.8, respectively.

6.7 Pre-clinical study of a large scale depletion of Treg from leukapheresis of a metastatic breast cancer patient

6.7.1 Transferring the protocol of Treg-depletion to a clinical grade under GMP guideline

Good Manufacturing Practice (GMP) is a term that is recognized worldwide for the control and management of manufacturing and quality control testing of foods, pharmaceutical products and medical devices.

According to the European Union’s GMP, all the manufacturing and testing equipments are required to be qualified, and all the operational methodologies and procedures (such as manufacturing, cleaning, and analytical testing) utilized in the drug manufacturing process are required to be validated.

In order to transfer the Treg depletion protocol to a clinical use, we did following changes according to the GMP guideline:

Agents: αCD25 microbeads from Miltenyi Biotec Company were successfully utilized in our bench experiments. The same Ab clone has been produced under the GMP guideline in the company. Also, clinical grade microbeads are available for coupling this GMP mAb, so called CliniCD25. Therefore, to transfer this agent to a clinical application is feasible.

Devices: For a therapeutic aim, CliniMACS is supplied for depleting Treg with CliniCD25 by the company.

Protocols: Basically the experiments are performed according to the manufacturer's instruction. However, optimizations need to be carried out. For example, a sufficient but not exaggerated ratio of CliniCD25 microbeads to cells needs to be determined. A suitable flow rate needs to be selected with CliniMACS machine.

6.7.2 Optimizing a CliniMACS protocol with a small-scale Treg-depletion on the autoMACS machine

To evaluate this clinically potential protocol, we started with validating the efficacy of applying CliniCD25 microbeads regarding the effect of immune-responsiveness subsequently. To this end, small amounts of blood samples (40ml/patient) of primary breast cancer patients were operated; TC and DC were generated and purified under a research grade. The protocol was adapted accordingly. In detail, a beads to cells ratio of 1:27 was carried out as suggested for positive selection, and an incubation was performed at room temperature for 30min. The cell separation was running with an autoMACS machine which carries the same column structure as CliniMACS but for a smaller sample size. The fastest flow rate (40ml/min) program was chosen as it allows retaining normal to high antigen expressing cells on the magnetic column.

Under these conditions, IFN-γ ELISpot assay was applied as a readout system. Results from one exemplary appliance are shown in Figure 30. After applying this protocol, we observed a significant ($p=0.04$) induction of IFN-γ secretion in response to KS24.22 but not to the control U937, which also resulted in a breast tumour specific response ($p=0.004$) in an otherwise absent situation. Most impressively, the benefit of applying this protocol was shown not only quantitatively but also qualitatively in an antigen specific way as illustrated in Figure 30B that higher amounts as well as more potent IFN-γ spots were appeared in KS24.22 stimulated wells afterwards. These data represent five independent experiments. Altogether, we conclude that CliniCD25 microbeads applied with the new protocol function as efficiently as the system with research grade.

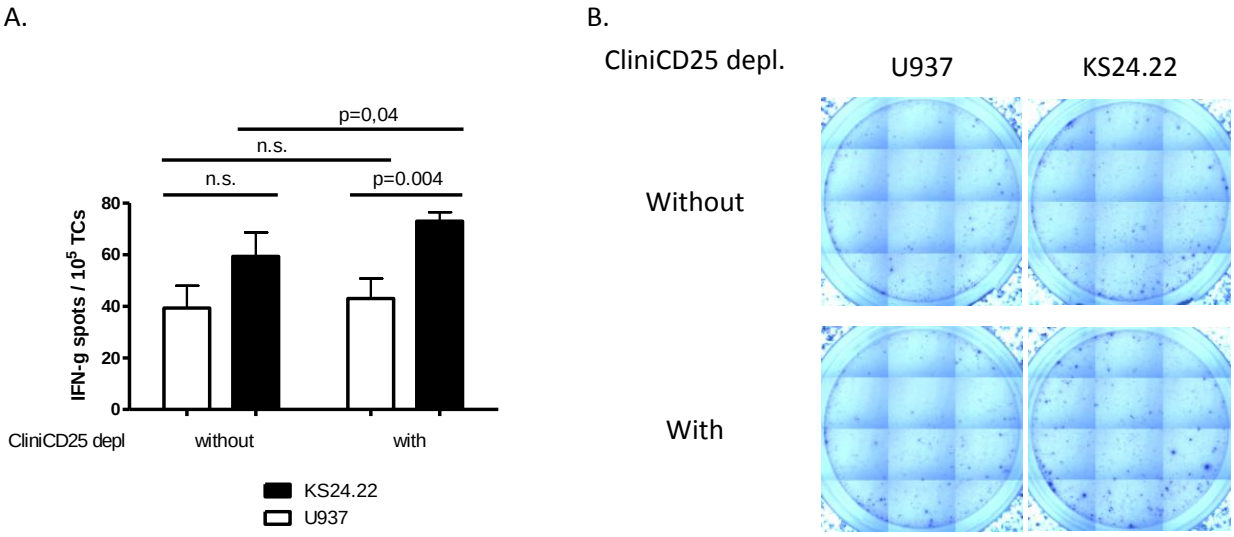


Figure 30. Performed CliniCD25 enhanced type-I T cell response against breast tumour antigen. **A**, An exemplary result of IFN-γ ELISpot assay from a primary breast cancer patient. The frequencies of KS24.22 (black) and control U937 (white) reactive PBTC were assessed without or with a CliniCD25 microbeads performance and depicted here by columns. Bars: SEM. Statistical differences between mean values were calculated with unpaired two-tailed t-test. P-values are indicated above small lines which connect compared groups. n.s.: not significant. **B**, corresponding original data show higher counts and more potent IFN-γ dots are observed in the well of responding to KS24.22 with a performed CliniCD25 depletion (right lower picture).

The presented experiments above were performed with TC and DC purified under research grade. In order to adapt it to GMP, DC purification was replaced with DC enrichment from adherent mononuclear cells as we used in the clinical study of adoptive T cell transfer immunotherapy of metastatic breast cancer patients discussed in Part I of this thesis.

Regarding the negative selection of TC, the available GMP agents are very costly. On the other hand, it has been reported that $CD4^+$ Treg depletion could be conducted directly with peripheral blood mononuclear cells (PBMC), which subsequently resulted in an enhanced anti-tumour immune response determined by IFN-γ ELISpot assay. Thus, we then tested a feasibility of utilizing PBMC as a substituent by evaluating the purity of Treg depletion with flow cytometry and anti-tumour effect with IFN-γ ELISpot assay. Results shown in Figure 31 are from one representative breast cancer patient out of eight tested ones. According to the phenotypic analyses, the frequencies of Treg among $CD4^+$ TC were substantially reduced (before: 3.15% versus after: 0.026%), suggesting a successful Treg-depletion performance directly with PBMC. Further, by applying IFN-γ ELISpot assay, a breast cancer specific benefit was confirmed with both PBMC and BMMC samples (see p-values in the figure).

In total, the proportions of immunological responders analysed with PBMC displayed a substantial increase (before: 37.5% versus after: 62.5%) after a performed CliniCD25

depletion (Figure 32), which was comparable to the outcome obtained previously, namely, with TC purified and Treg depleted under research grade (before: 38.5% versus after: 77%).

Collectively, these data demonstrate a feasibility of applying CliniCD25 with peripheral blood mononuclear cells in a small size by an autoMACS machine.

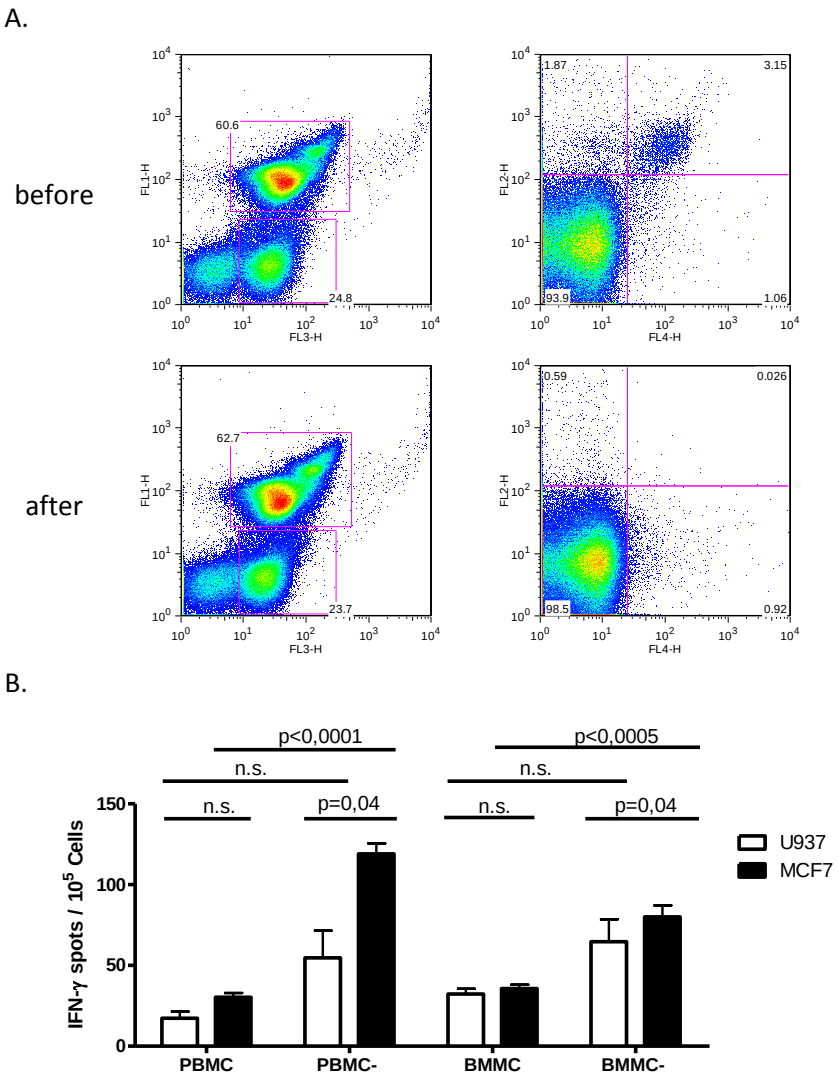


Figure 31. CliniCD25 depletion can be applied directly with PBMC. **A**, Purity of Treg-depletion directly from PBMC using CliniCD25 microbeads. Representative flow cytometric data show cell proportions before (upper row) and after (lower row) conducting a CliniCD25 depletion. Left panel: Y-axis versus X-axis shows CD4 versus CD3 staining. Pink squares in each plot gate $CD4^+$ and $CD8^+$ T cell populations at top and bottom, respectively. $CD4^+$ T cells were further analysed in right panel with CD25 versus Foxp3 (Y-axis versus X-axis) staining. Up-right quadrants indicate Treg population. Small numbers in the plots depict respective percentages. **B**, Treg-depletion directly from PBMC by CliniCD25 microbeads resulted in an enhancement of type-I T cell responses determined by IFN-γ ELISpot. Columns represent frequencies of MCF7 (black) and U937 (white) reactive TC. Bars: SEM. Statistical analyses were performed between interested groups connected by short lines. P-values were calculated with paired two-tailed t-test. n.s.: not significant.

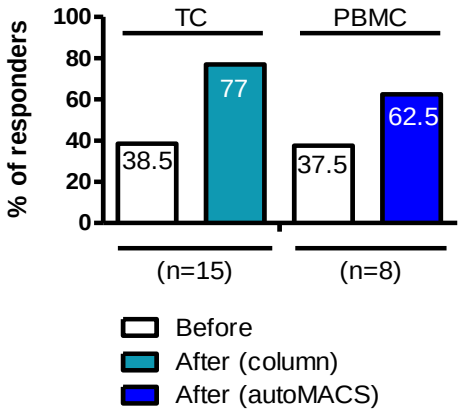


Figure 32. Comparable benefits of Treg-depletion are exhibited with pre-clinical procedure and research system in regard to percentages of immunological responders. Percentages of IFN-γ ELISpot positive patients are depicted with small numbers in representing bars. White bars: without treatment; light blue: Treg-depletion with research grade CD25 microbeads applied with purified TC; dark blue: Treg-depletion with CliniCD25 microbeads applied with PBMC. Numbers of tested samples in each group are shown at the bottom in parentheses.

In addition, we evaluated the correlation between input cell numbers and total cell recovery (% = output # / input #) with autoMACS machine. Interestingly, $1x10^7$ of input cell numbers were minimally required in order to exceed 85% of recovery. A higher yield was associated with a higher input (Figure 33). This side investigation might be useful for the people who are dealing with this device.

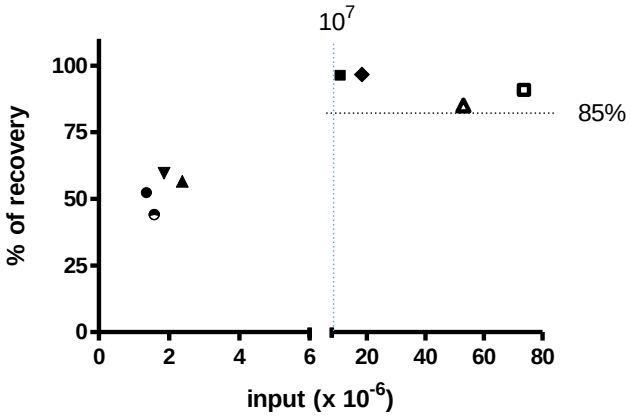


Figure 33. Correlation between input of cell numbers and recovery in percentages with autoMACS machine. Each symbol represents an independent experiment. X-axis: absolute input cell numbers. Y-axis: percentages of recovery calculated by dividing a sum of cell counts from depleted and flow through fractions to the cell counts of input. 4 symbols separated by dash lines at the up-right corner indicate that in order to reach higher than 85% recovery with autoMACS, at least $1x10^7$ input cells are required.

6.7.3 Adapting a large-scale Treg-depletion from a leukapheresis of one advanced breast cancer patient on the CliniMACS machine

The reproducibility observed in small-scale preparations prompted us to further develop a CliniMACS procedure suitable for large-scale application. In this regard, leukapheresis was operated. One metastasized breast cancer patient was recruited, whose spontaneous TC anti-tumour immunity could be detected in the peripheral blood and was sustained after Treg-depletion with a manual protocol (Figure 35). One hour running apheresis allowed us to collect $4.5x10^9$ white blood cells (WBC) from this donor.

CliniMACS provides two possible depletion programs according to the flow rate and customers' desire. Program 2.1, which utilizes a relatively low rate of 10 ml/min, is originally designed for purer negative selection; hereafter, we denote it as Depl. 2.1. Whilst program 3.1 is designed for an enrichment of labelling cells due to its higher flow rate of 20 ml/min; we named it Enri. 3.1. These two programs were conducted in parallel in order to compare and to establish the protocol. To this end, WBCs were first aliquoted evenly into two bags according to the weights, meaning that the assumed cell numbers for each program would be approximately $2.25x10^9$ (half of total numbers). Afterwards, the cell labelling procedure was performed as delineated in small scale system.

The evaluations concerned cell viability, recovery and phenotypic purity. Anti-tumour responses of desired cell fractions were assessed by IFN-γ ELISpot assay.

We observed that cell viabilities of all fractions were greatly above 90%. Table 4 enumerates cell count and its relative proportion in each fraction separated with different programs. Both programs showed similar yields of desired cell fractions (CD25 negative fractions) and not more than 7% systemic loss (waste bag).

Table 4. Enumerations of cell products separated by a large-scale CliniCD25 application with CliniMACS

	cell count ($x10^8$) /% of initiation				
	Initiation	post- CD25 pos. fraction	post- CD25 neg. fraction	Waste bag	Total Yield
Enri. 3.1	22.5 / 100	1.1 / 5.0	18.8 / 83.6	1.6 / 7.1	21.5 / 95.6
Depl. 2.1	22.5 / 100	5.75 / 25.6	18.4 / 81.8	1.3 / 5.6	25.4 / 112.9

Depl. 2.1 resulted 5-folds more cell counts in Treg containing fraction (CD25 positive fraction) than Enri. 3.1, implying an either insufficient Treg removal by Enri. 3.1 or an undesirable depletion of effector T cells by Depl. 2.1. Flow cytometric analyses ruled out the

first assumption as Treg populations determined by double expressions of CD25 and Foxp3 were substantially reduced by both programs (Figure 34) (before: 1.74%; Enri. 3.1: 0.09%; Depl. 2.1: 0.03%). However, considerable amounts of non-Treg were concomitantly removed observed by both programs (data not shown). Thus, further optimizations, such as beads to cell ratio, labelling duration are urged.

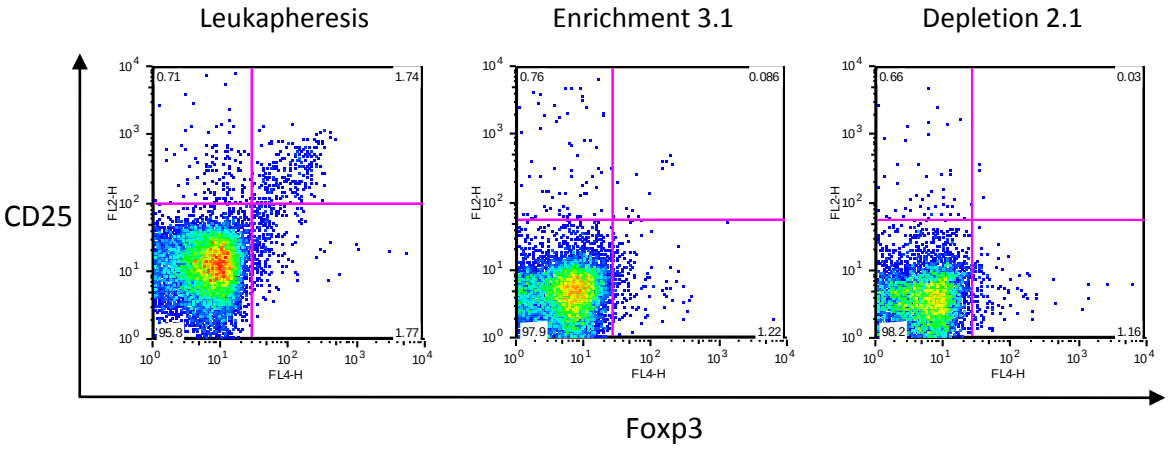


Figure 34. Flow cytometric evaluations of the purity of Treg depletion with a large scale CliniCD25 application with CliniMACS. Cells from leukapheresis, CD25 negative fractions from a performed Enri. 3.1 and Dep. 2.1 were stained with αCD3, αCD4, αCD25 and αFoxp3 mAb. CD25 versus Foxp3 expressions of the cells pre-gated with $CD4^+$ TC populations were shown here. Numbers displayed in each quadrant represent corresponding percentages.

In addition, we compared the sub-groups of T cells in proportion in CD25 negative fractions generated by Enri. 3.1 and Depl. 2.1 (Table 5). Concerning the absolute cell numbers, the latter program resulted in less $CD3^+$ T cells, but more $CD8^+$ T cells in the desired fraction with lower Treg contamination.

Table 5. Phenotypic analyses of T cells in leukapheresis products and in CD25 negative fractions generated by Enri. 3.1 and Depl. 2.1.

%	$CD3^+$ of total	$CD4^+$ of CD3	$CD25^+Foxp3^+$ of CD4	$CD8^+$ of CD3
Pre- separation	80.1	66.5	1.74	30.7
Post- CD25-neg. Fraction (Enri. 3.1/Depl. 2.1)	76.6 / 71.4	57.9 / 40.6	0.09 / 0.03	40.4 / 57

Nevertheless, the most valuable evaluation towards different procedures is whether the potency of anti-tumour response is enhanced within Treg eliminated population. To clarify this, we compared the results of IFN-γ ELISpot assays performed with diagnosis samples and leukapheresis in the presence or absence of Treg manipulated by a manual protocol or by different CliniMACS programs (Figure 35). Observations are the following: 1) without any treatment (depicted as – in the figure) we observed an overall decline of TC immunity from the diagnosis day to the leukapheresis taken date (one month interval) in despite of a remaining anti-tumour significances, suggesting the spontaneous anti-tumour responsiveness was weakening *in vivo*; 2) a manually applied Treg-depletion did not influence the outcome in diagnosis sample but gave rise to a loss of anti-tumour reactivity in leukapheresis; implying Treg may not play an essential role responsible for the declining T-cell immunity; 3) when comparing the results sourced from leukapheresis, lower frequencies of IFN-γ secreting T cells were detected with CliniMACS-manipulated samples, which evidenced directly that CliniMACS programs dampened the potencies of T-cell anti-tumour reactivity possibly due to excessive depletion of Teff; 4) not least, between the two CliniMACS programs, Enri. 3.1 exhibited better efficacy according to statistical analyses, suggesting that accelerating flow rate may facilitate less Teff retaining when running through the column.

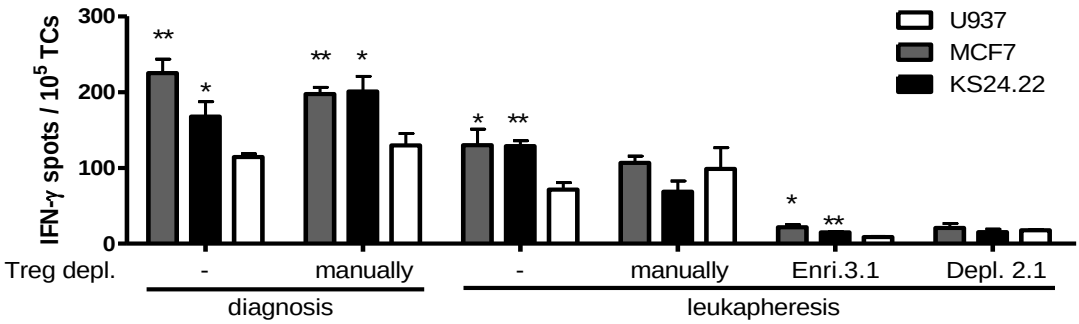


Figure 35, Evaluation of the efficacy of applying a large scale Treg-depletion with CliniMACS system. IFN-γ ELISpot assay was carried out with one metastatic breast cancer patient at two different time points: on diagnosis day and 1 month later when leukapheresis was achieved and CliniMACS system was applied. The frequencies of IFN-γ secreting TC in response to MCF7 (gray), KS24.22 (black) and irrelevant control U937 (white) were shown by columns. Bars: SEM. Treg depletion was performed in three different means as indicated. Statistical analyses were performed by unpaired two-tailed t-test. *, $p<0.05$; **, $p<0.005$.

Altogether, the data from this preliminary experiment confirmed a feasibility of modulating Treg in leukapheresis of advanced breast cancer patient under a clinical-grade condition by a sufficient depletion of Treg, a high viability of isolated cells and a remarkable recovery of total cell counts. However, the protocol needs to be further optimized and the therapeutic potential has to be validated.

6.8 Pilot clinical study of metronomic cyclophosphamide treatment of patients with advanced breast cancer

6.8.1 Study design

Cyclophosphamide (CTX) when administrated with a low amount and daily (metronomic) was reported to have a capacity of selectively eliminating Treg and consequently enhancing anti-tumour immunity in refractory cancer patients. However, the sole role of metronomic CTX in dampening Treg regarding proportions as well as functions has not yet been documented in clinical trials with breast cancer patients. In this regard, we performed a pilot clinical study of metronomic CTX treatment with advanced breast cancer patients who were under progressive disease. Also being interested in a long-term efficacy of this regimen, we followed up Treg percentages and type-I T-cell immune responses in the blood of study patients for three months.

Table 6. Patients Characteristics.

Median age (range)	60.3 (47-74)
ECOG performance status, median	1.4
Most common sites of metastases:	n=
Liver	5
Lung	9
Pleura	2
Bone	13
Soft tissue	5
Brain	3
LN	3
Prior Therapy:	n=
Surgery	13
Chemotherapy	13
Radiation	10
Bisphosphonat	9
Herceptin	3
Tam/aromatase inhibitors	7

(ECOG, Eastern Cooperative Oncology Group)

From October 2007 to July 2008, 13 breast cancer patients with metastatic disease were randomly enrolled in this study. These patients had previously received surgery and standard cytostatic treatments such as chemotherapy, endocrine and antibody therapy (Table 6) and were in a palliative treatment situation. Two patients failed to go through an entire treatment due to their strong tumour progressions (stopped at Day14 and Day56). One patient resigned inclusion after 28-days treatment with a partial regression of secondary

tumour (glioma tumour). Data from one patient except the one from Day0 was excluded due to her poorly clinical condition.

Patients received oral CTX 50mg daily for three months. Before and fortnightly during the treatment, percentages of $CD4^+CD25^{high}Foxp3^+$ Treg among $CD4^+$ T cells in the peripheral blood were monitored by flow cytometry and spontaneous anti-tumour T-cell responses were evaluated by 40hr IFN-γ ELISpot assay with breast tumour antigens (MCF7 or KS24.22) or irrelevant control ones (U937) presented by autologous DC. The status of tumour was monitored via tumour markers (CA15-3 and CEA, or MRI-Scan) in the peripheral blood.

The treatment was well tolerated, as no severe side effects were observed, except one patient had anaemia (Hb 6.6 g/dl) and stopped therapy because of this. Grade I leukopenia was found in all treated patients.

6.8.2 The frequencies of Treg were significantly reduced at early study time points

In order to monitor Treg frequencies, flow cytometric analyses were performed with PBMC of enrolled patients prior and during the treatment. Treg population was specified with $CD3^+CD4^+CD25^{high}Foxp3^+$ as delineated in Part I of this thesis. The percentages of $CD25^{high}Foxp3^+$ Treg of overall $CD4^+$ T cells were evaluated as shown in Figure 36.

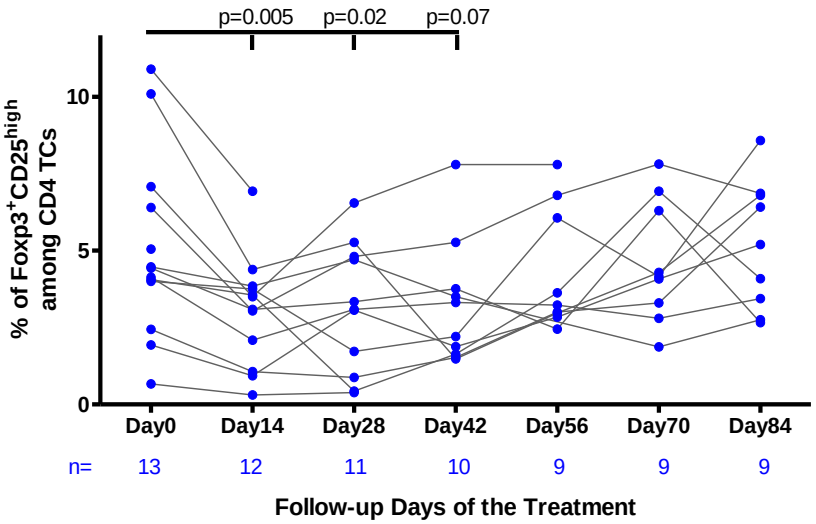


Figure 36. Metronomic cyclophosphamide treatment significantly reduces Treg proportion among $CD4^+$ T cells at early time points of this study. Percentages of Treg among $CD4^+$ T cells in the blood were determined by flow cytometric analyses. Each dot represents one patient. Lines connect Treg frequencies measured on indicated days within one patient. Numbers of assessed patients are depicted below X-axis in blue. Statistical differences between mean values on the respective days to Day0 were analysed with paired two-tailed t-test. Significant p-values are shown above corresponding days.

Prior to the treatment (Day0) the mean frequencies of Treg obtained in the blood of metastatic breast cancer patients were 5.1±3.0% (mean±SD), which is substantially higher than primary patients (3.9±1.5%; p=0.078) and healthy donors (4.3±0.9%; n.s.).

On Day14, a dramatic decrease of Treg frequencies was observed (Day0: 5.1±3.0% versus Day14: 3.0±1.8%; p=0.005). This metronomic CTX induced efficacy of Treg-elimination sustained significance on Day28 (3.1±2.1%; p=0.02), marginal significance on Day42 (3.2±2.0%; p=0.07), and then disappeared. These data suggest that metronomic CTX has a strong inhibitory impact on Treg population at early period of administration in metastatic breast cancer patients.

Regarding the late stage of study a gradual replenishment of Treg in mean frequency was observed with a value as high as Day0 obtained on Day84 (5.2±2.2%). Whether a prolonged regimen will convey Treg frequency to an even higher degree is obscure. We also found that the individual Treg percentages of assessable patients showed a less heterogeneous on Day84 (ranging from 2.7% to 8.6%) than Day0 (ranging from 0.7% to 10.9%). Whether metronomic CTX has Treg normalizing efficacy is not clear.

Day 0	Day 14	Fold of Decrease
0.67	0.31	2.2
1.93	0.93	2.1
2.44	1.07	2.3
4	3.75	1.1
4.06	3.56	1.1
4.14	2.09	2
4.43	3.09	1.4
4.47	3.85	1.2
6.4	3.04	2.1
7.08	3.5	2
10.1	4.39	2.3
10.9	6.93	1.6

Table 7, Metronomic CTX is more effective to patients who contain abnormal Treg frequencies in the blood. Percentages of Treg of individual patients on Day0 and Day14 are listed in the left and middle column, respectively. The frequencies not more than 6% or less than 3% are shadowed in light blue. The ratio of reduction in Treg frequencies from Day0 to Day14 were calculated and listed in the right column (Fold of Decrease). When the ratio is more than 1.5-fold, the cell is shadowed in blue.

It is worth mentioning that patients who contained abnormal Treg frequencies on Day0, either extremely high or low, were more susceptible to the treatment as evaluated by the ratio of Treg reduction from Day0 to Day14 (Table 7). In particular, when 1.5-fold alteration was set as a threshold, 100% patients whose Treg were either below 2.44% or above 6.4% of $CD4^+$ TC on Day0 had an average 2.1-fold reduction in Treg frequencies on Day14. While, the other four out of five assessable patients whose initial Treg belong to a normal range, referred to healthy donor situation (4.3%), showed almost unaltered Treg proportions on Day14 with an average of 1.3-fold decrease.

6.8.3 Metronomic cyclophosphamide treatment shows a long term immune efficacy

Next, we monitored the impact of metronomic CTX treatment on anti-tumour T-cell immunity by performing IFN-γ ELISpot assay with blood samples of enrolled patients before and fortnightly. Exemplary data are shown in Figure 37. A *de novo* spontaneous anti-tumour T-cell reactivity was revealed in response to both tested tumour-lysates (MCF7 versus U937: p=0.05; KS24.22 versus U937: p=0.001). Certain responsiveness was also detected on Day42 and Day70 according to statistical analyses, suggesting a persistent efficacy of this treatment.

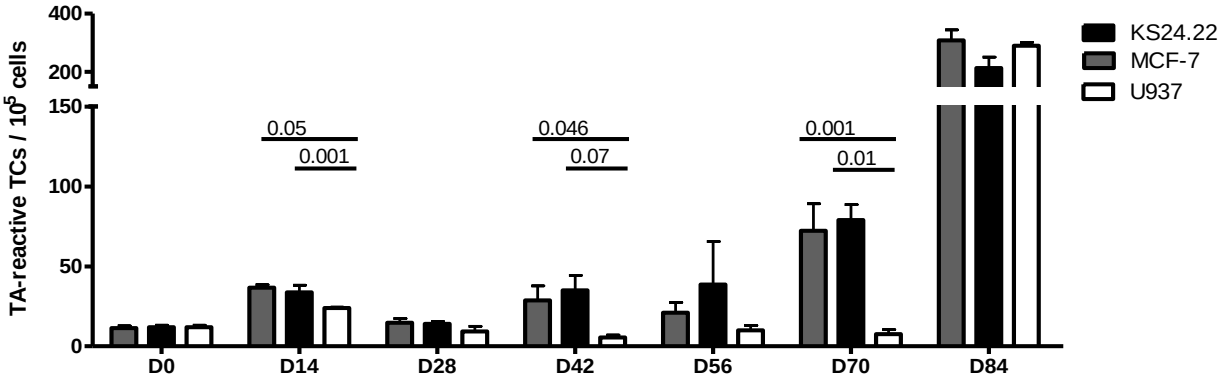
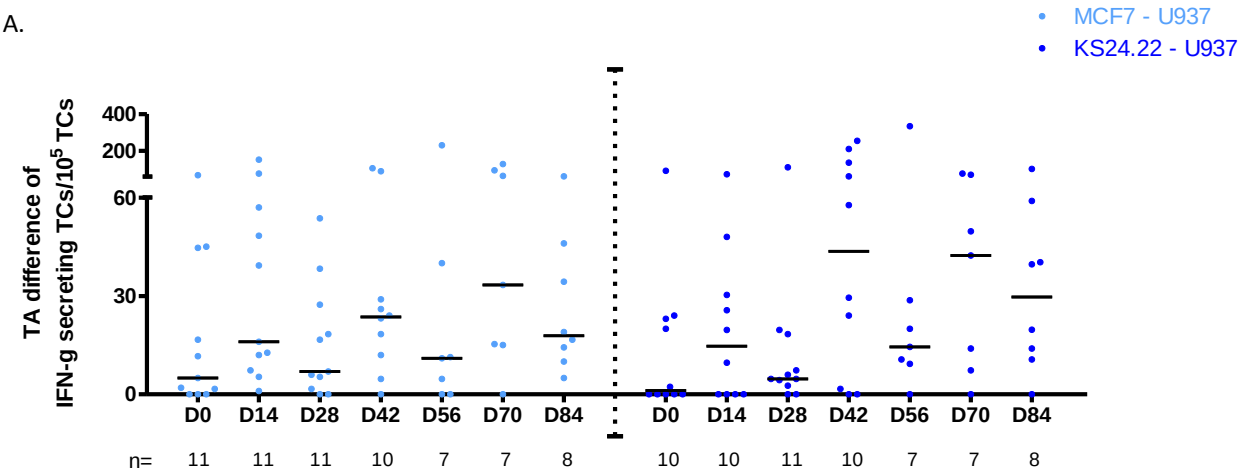


Figure 37, Exemplary patient data show type-I T cell immunity monitoring before and during the treatment. Frequencies of tumour reactive T cells were determined by IFN-γ ELISpot assay stimulated with breast cancer antigens KS24.22 (black) or MCF7 (grey) and control antigen U937 (white). Columns represent mean values of frequencies of IFN-γ secreting T cells from triplicate wells. Bars: SEM. Statistical analyses were performed with unpaired two-tailed t-test within interested groups connected by short lines. Numbers above the short lines indicate p-values.

To overview the effect of applied regimen on anti-tumour T-cell immunity, we accumulated the results of IFN-g ELISpot assay from all treated patients and analysed it with Wilcoxon signed-rank test suggested by statisticians as it is regardless of distributional assumptions. A value referring to a frequency of tumour-different TC was introduced by subtracting the numbers of U937-reactive TC from MCF7 or KS24.22-reactive ones regardless of a detectable immunological response (Figure 38A). Both MCF7 and KS24.22 responsiveness displayed a zigzag pattern in concern of the median values (small bars) accompanied with a gradual progressing.

A.



B.

p-value (CI)	Day 14	Day28	Day42	Day56	Day70	Day84
MCF7	0.25 (-16.0,61.8)	0.80 (-22.7,18.2)	0.44 (-14.7,46.2)	0.40 (-20.8,114.8)	0.03 (7.7,92.7)	0.48 (-28.0,46.0)
KS24.22	0.78 (-21.3,28.8)	0.95 (-33.5,43.7)	0.12 (-24.0,156.5)	0.17 (-6.2,174.4)	0.04 (0.77,3.0)	0.46 (-39.3,39.7)

C.

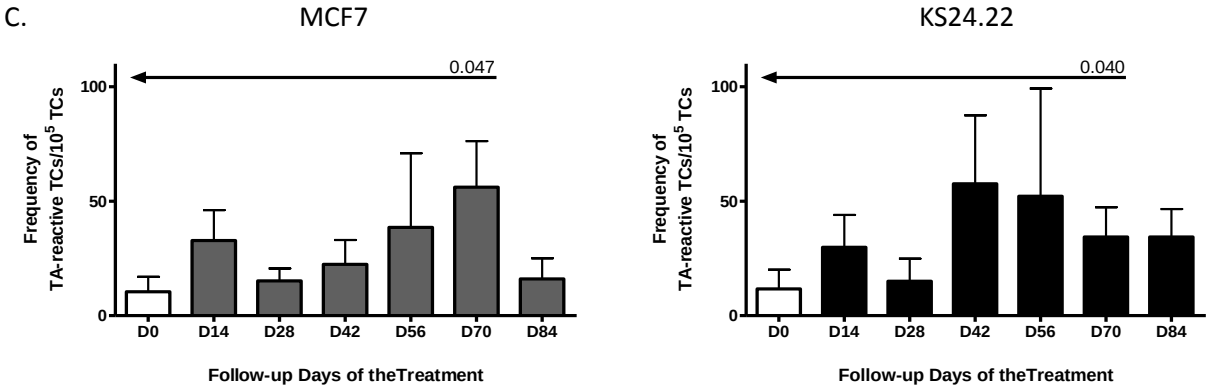


Figure 38. Day70 of the treatment display a statistical significance of anti-tumour T-cell immunity. **A**, Accumulated data of frequencies of tumour-different T cells calculated by subtracting the values of MCF7 (light blue dots) or KS24.22 (dark blue dots)-reactive T cells to that of U937-reactive ones. Each dot represents one patient. Negative results are depicted as 0 on X-axis. Short bars indicate median values of respective groups. Numbers (n) of assessed patients on respective days are shown below the plot. **B**, Statistical analyses performed with Wilcoxon signed-rank test. Based on the calculation in A, type-I T-cell immunities against MCF7 or KS24.22 on each studied day were evaluated with p-value and confidential interval (CI). Individual clinical follow-up days indicated on the top row were compared to Day0 for each antigen. Significances are shown in red. **C**, Statistical analyses performed with student t-test. Columns represent frequencies of MCF7 (left) and KS24.22 (right) responsive T cells accumulated from all study patients. Significant p-values were appeared on Day70 calculated by paired two-tailed t-test.

Based on these calculations, a Wilcoxon signed-rank test was performed. Results are listed in Figure 38B. Interestingly, statistical significances were obtained on Day70. It was counted for both MCF7 (p=0.03) and KS24.22 (p=0.04) responsiveness. In line with this, a student t-test with frequencies of tumour antigen-specific T cells also displayed statistical significances on Day70 (Figure 38C) in response to either MCF7 (p=0.047) or KS24.22 (p=0.040). These analyses provide conclusive evidence that this regimen has a long-term efficacy of enhancing anti-tumour T cell immunity in metastatic breast cancer patients.

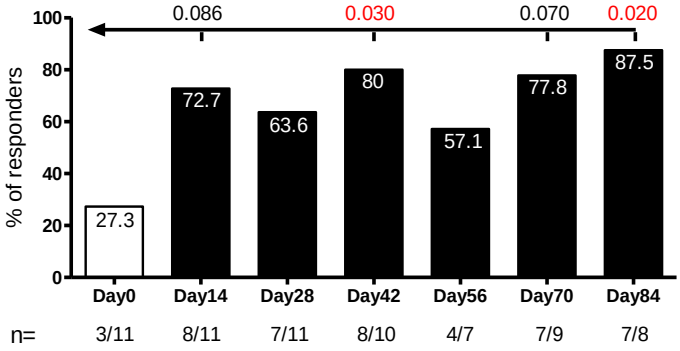


Figure 39. Percentages of immunological responders before and during metronomic CTX treatment. Numbers in the columns and columns themselves show percentages of responders assessed on indicated days. The absolute numbers of responders and total assessable patients are are displayed at the bottom with n= responder/total patients. The statistical analyses of each monitoring day comparing to Day0 were calculated by Fischer's exact test, and the p-values are shown on the respective days.

Next, we assessed the proportions of patients who exhibited spontaneous tumour-specific T-cell responses on each clinical monitoring day. Results are shown in Figure 39. Prior to the treatment there were 27.3% of responders. However, it increased dramatically (p=0.086) on Day14 (72.7%) and persisted higher than 57% through the entire study. Statistical effects were observed on Day42 (p=0.03) and Day84 (p=0.02). These data support the conclusion above that this regimen possesses a long-term clinical efficacy.

6.8.4 Clinical responses are correlated with T-cell immunity in the blood of study patients.

Serum concentrations of tumour marker CA15-3 and CEA are the signs of disease activity, as well as magnetic resonance imaging (MRI). In this regard, we followed the development of tumour markers' level in treated patients who were priory under progressive disease in order to evaluate the clinical efficacy of this study. Results are listed in Table 8. Promisingly,

there were seven out of 11 (63.6%) study patients showed stable disease after treatment, especially, when achieved by a sole application of metronomic CTX.

Pat	TM	Performance of Tumour Markers (TM) – Clinical monitoring Day							Disease Status		Frequencies of TAA-reactive TC (/10^5 PT)	
		0	14	28	42	56	70	84	Bef	Aft	Bef	Aft
1	CA15-3	48.1	-	-	-	-	-	-	PD	-	0	102.33
	CEA	1.3	-	-	-	-	-	-				
2[a]	MRI-Scan								PD	SD	0	40.33
4	CA15-3	152.9	-	-	-	496.9	-	-	PD	PD	45	18.33
	CEA	75.7	-	-	-	433.0	-	-				
5	CA15-3	90.8	-	69.4	-	-	-	-	PD	SD	0	111.33
	CEA	13.2	-	7.2	-	-	-	-				
6	CA15-3	358.9	-	386.8	-	368.6	-	388.5	PD	SD	0	61.33
	CEA	7.0	-	7.8	-	8.1	-	9.2				
7	CA15-3	1980.0	-	3656	-	-	-	-	PD	PD	0	0
	CEA	155.2	-	297.8	-	-	-	-				
8	CA15-3	526.1	-	-	-	-	-	1547	PD	PD	0	0
	CEA	178.4	-	-	-	-	-	185.8				
9	CA15-3	281.0	-	286.0	-	275.5	-	323.3	PD	SD	0	19.67
	CEA	1.2	-	1.2	-	1.4	-	0.9				
10[b]	CA15-3	239	-	1080	2480	2290	-	1760	PD	SD[b]	0	39.67
	CEA	5.9	-	56.3	89.6	85.5	-	78.4				
11	CA15-3	1978.0	-	-	2222.0	-	2768	-	PD	PD	0	16.67
	CEA	47.1	-	-	54.3	-	82.4	-				
12	CA15-3	503	-	576.6	-	470.3	-	558.2	PD	SD	92.67	14.00
	CEA	19.7	-	35.5	-	28.9	-	33.9				
13	CA15-3	13.9	-	-	-	-	-	19.6	PD	SD	11.67	42.33
	CEA	93.8	-	-	-	-	-	4.6				

Table 8. Correlation between clinical responses determined by tumour markers and disease status with frequencies of tumour-reactive T cells determined by IFN-γ ELISpot assay. TM: tumour markers. Pat: patient. PD: progressive disease; all the patients enrolled in this study were under PD. After initiation of this regimen, PD was determined by the extent of increase of TM in the blood, shown in red. SD: stable disease. Bef: before the treatment. Aft: the last time when the clinical monitoring performed corresponding to the wells shadowed in dark blue. -: performance was not done. a: Pat 2 was followed by MRI Scan. b: Pat 10 was considered as clinical SD determined by the observations of last two months.

In order to identify the effect of type-I T-cell potency to clinical responses, we compared the correlation of tumour status with frequencies of tumour-responding T cells (Table 8, Figure 41). Prior to the therapy, spontaneous T-cell tumour reactivity was not found in 72.7% (eight out of 11) of study patients. After the therapy, 100% of patients who showed disease stabilization (SD) were associated with a detectable T-cell responsiveness in the blood, suggesting that type-I T-cell immunity is necessary to achieve a clinical benefit. While, 50% (two of four) of patients who underwent progressive disease were found to contain T-cell anti-tumour immunity, however, with relatively low frequencies (blue dots in Figure 40). By setting a comparative threshold according to the highest frequency of tumour-reactive T cells obtained among PD patients, we observed that 87.5% (seven of eight) benefit of clinical responses were associated with higher than 18.33 tumour-specific T cells found in per $1x10^5$ circulating T cells.

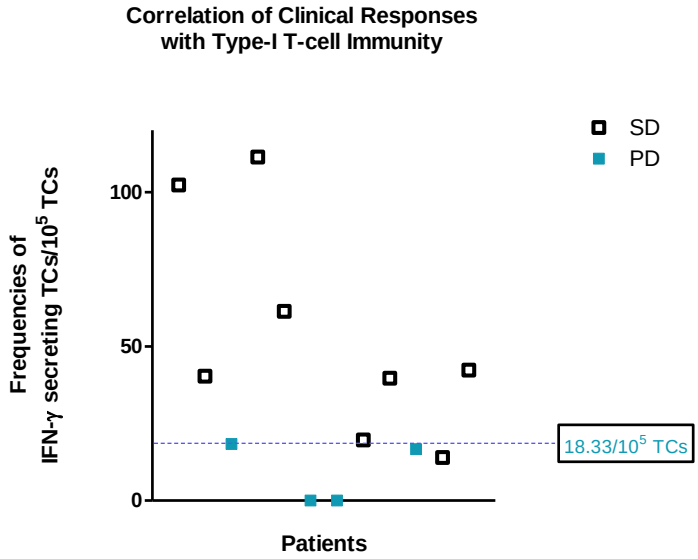


Figure 40, Correlation of clinical responses with frequencies of type-I T cells in treated patients. Each symbol represents one patient and scaled according to the frequencies of tumour-reactive T cells assessed in the blood after the treatment. Open squares represent patients with stable disease; blue ones represent patients with progressive disease. The highest frequency of tumour-responding T cells of PD patients is indicated by a dash line with absolute numbers shown in a box.

6.8.5 Correlation of Treg frequencies with immunological effects of study patients

We have reported a significant reduction in Treg frequency on Day14. And interestingly, we also observed a considerable group of treated patients who gained enhanced anti-tumour T-cell responses on Day14. For example, as the presented data in Figure 37, after 14 days therapy, this patient obtained a *de novo* anti-tumour response. Simultaneously, the frequency of circulating Treg within this patient was decreased from 4.1% on Day0 to 3.7% on Day14. Thus, it is interesting to elucidate whether there is a correlation between Treg frequency and T-cell anti-tumour reactivity with metronomic CTX dosed patients. Disappointingly, a significant improvement of tumour-specific IFN-γ production was not

found statistically on Day14 as shown by both Wilcoxon signed-rank and student t-test (Figure 38), which is likely due to an insufficient power of study.

Next, a long term effect was evaluated with mean percentages of Treg among $CD4^+$ T cells and mean frequencies of tumour-specific T cells (responding to either MCF7 or KS24.22) of all assessable patients on each clinical monitoring day. Results are shown in Figure 41. As discussed above, Treg frequencies (black line) displayed an early decline phase and a late rebound phase separating on Day42. Interestingly, a similar but reversed pattern was exhibited with frequencies of tumour-reactive T cells (blue line). By applying Pearson test, a negative correlation was found (r=-0.932, p=0.068) between Day42 and Day84.

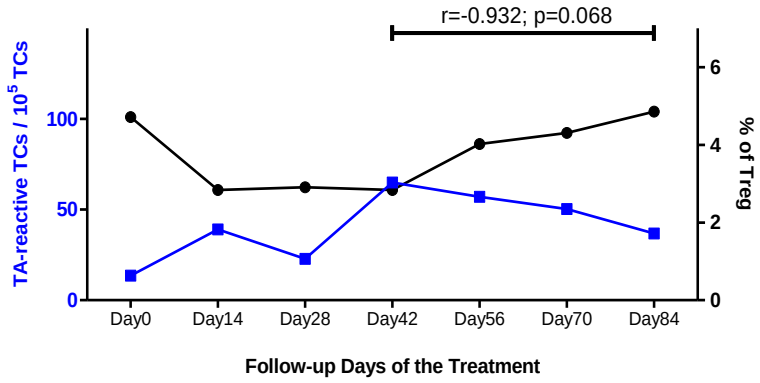


Figure 41. Correlation between frequencies of tumour antigen reactive T cells and proportions of Treg before and during the treatment. Blue line: frequencies of responding T cells and scaled according to the left Y-axis. Black line: percentages of $CD4^+$ Treg and scaled according to the right Y-axis. Negative correlation was found at Treg rebound phase calculated by Pearson test shown by r and p-values.

It is conceivable that the reduction of Treg from Day0 to Day14 allowed initial priming of Teff and consequently increased activated Teff in circulation as implied by the figure. The negative correlation at the late period of study is reminiscent of our previous finding with primary breast cancer patients that the presence of breast cancer specific Treg was associated with the absence of spontaneous T-cell immunity in the blood. However, given the evidence from this study that metronomic CTX treated patients obtained benefits such as profound anti-tumour reactivity on Day70 and significant increase in proportion of immunological responders on Day84, the being outnumbered Teff by Treg in the circulation might likely migrate to secondary lymph organs or even to the tumour site.

6.8.6 Replenished Treg at late study time points exhibited potent proliferative as well as suppressive characteristics

The above investigations led us to a discrepancy of metronomic CTX regimen on the late study time points (Day56, Day70 and Day80): a increasing Treg frequencies in the circulation were found to be associated with a declining tumour-antigen specific T cells, and associated with a significant enhanced anti-tumour responsiveness observed on Day70 and a highest proportion of immunologic responders (87.5%) observed on Day84. To understand this discrepancy, a fundamental question is whether CTX altered Treg characteristics including proliferative and suppressive abilities at late time points of this study.

First of all we examined the proliferative capacity of Treg by evaluating proliferation marker Ki67. Flow cytometric data were obtained and compared in between following groups: (a) study patients at late study time points (Day56: n=5, Day70: n=7 and Day80: n=7); (b) advanced patients who fit the same criteria of recruited patients (n=4) (note: data from this group are supposed to represent the proliferative status on Day0 of study patients); (c) primary breast cancer patients (n=7) and (d) healthy donors (n=5).

Results measured with $CD4^+$ Treg population are shown in Figure 42A. Regarding the median values, 11.9% of Treg from healthy individuals expressed Ki67, indicating a homeostatic proliferation. Compared to it, a slightly higher proportion (14.8%) of Ki67 expressing Treg were observed with primary breast cancer patients; and even higher value (22.45%) was found with advanced patients (Day0), suggesting a disease associated abnormal amplification of Treg occurring in breast cancer patients. The median values of Ki67 expressing Treg from metronomic CTX treated patients increased gradually starting from a lower value than Day0 on Day56 to a value approaching to Day0 on Day84 (Day56: 16.9%; Day70: 19.2%; Day84: 21.0%). According to the statistical analyses, Day84 exhibited significant differences in comparison to healthy donors (p=0.044) and primary patients (marginally, p=0.07). These data indicate that a vital proliferation capacity was gained by Treg of study patients at later period of this study, which provides a possible explanation for the observed rebounded frequencies of Treg.

In addition, the proliferative potencies of $CD4^+$ Tcon were analysed (Figure 42B). The median value of Ki67 expressions obtained from the group of advanced patients displayed the lowest level among all tested groups (1.32%), which implied a silence of T-cell immunity in metastatic breast cancer patients. Interestingly, study patients contained marked inductions of Ki67 expressing $CD4^+$ Tcon (Day56: 3.78%, Day70: 4.00%, Day84: 4.51%), especially, when compared to the group of prior to the treatment (see p-values in the figure), indicating a strong impact of metronomic CTX on $CD4^+$ Tcon proliferation. It is known that proliferation of Tcon is associated with IL-2 secretion and Treg maintenance and expansion is dependent

on the presence of IL-2. Therefore, we speculate that Treg replenishment at late study time points is consequently facilitated by Tcon amplification. Whether CTX provoked Tcon proliferation from initiating time or not will be interesting to validate. Moreover, this observed amplification of Tcon might also explain the restoring of anti-tumour T-cell immunity at the late study phase. To confirm this, experiments such as tetramer staining are required for clarifying the antigen specificities of proliferating $CD4^+$ Tcon.

Another very interesting phenomenon was observed by evaluating the $CD25^{high}Foxp3^+$ Treg in $CD8^+$ T cell population (Figure 42C). Relatively higher proportions of Ki67 expressing $CD8^+$ Treg were detected with study patients (Day56: 20.4%, Day70: 12.3%, Day84: 25.2%). Statistical significances appeared on Day84 when compared to HD (9.92%, p=0.011), primary (8.85%, p=0.008) and advanced breast cancer patients (6.35%, p=0.009). Whether that $CD8^+$ Treg represent an alternative suppressive mechanism induced by metronomic CTX treatment and whether they exerted suppression on anti-breast tumour T-cell immunity are not clear. Also, it has to be considered that even though the percentages of proliferating Treg in $CD4^+$ and $CD8^+$ Treg populations are comparable, $CD8^+$ Treg represent much smaller proportion in total $CD3^+$ T cells compared to $CD4^+$ Treg (data not shown). Nevertheless, it is worth to be further investigated.

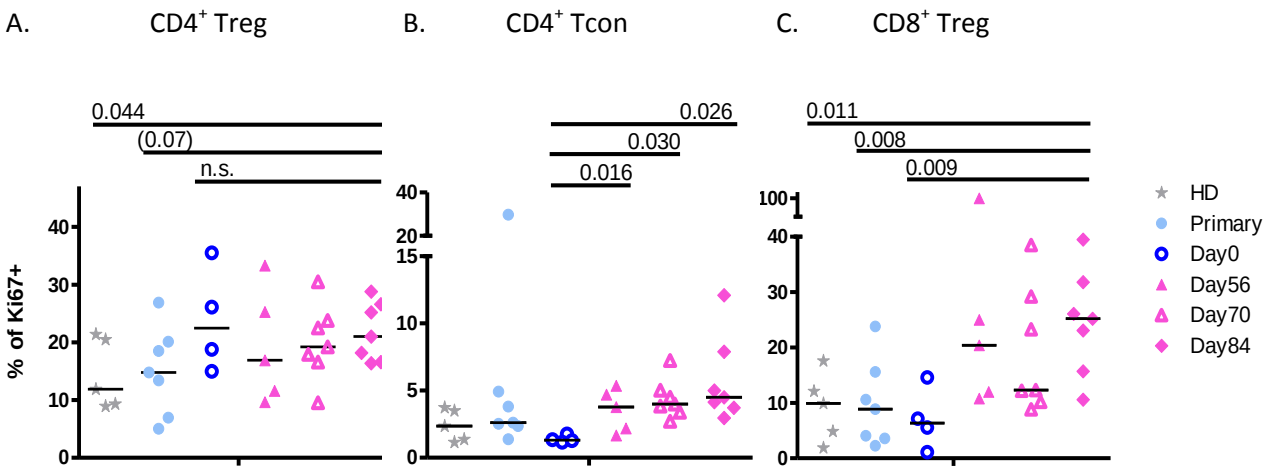


Figure 42. Percentages of Ki67 expression with $CD4^+$ Treg (**A**), $CD4^+$ Tcon (**B**) and $CD8^+$Treg (**C**). Each symbol represents individual measurement. Different groups are depicted on rightmost. Small bars indicate median values. P-values are calculated by unpaired two-tailed student t-test and shown on short lines connecting compared groups. n.s.: not significant.

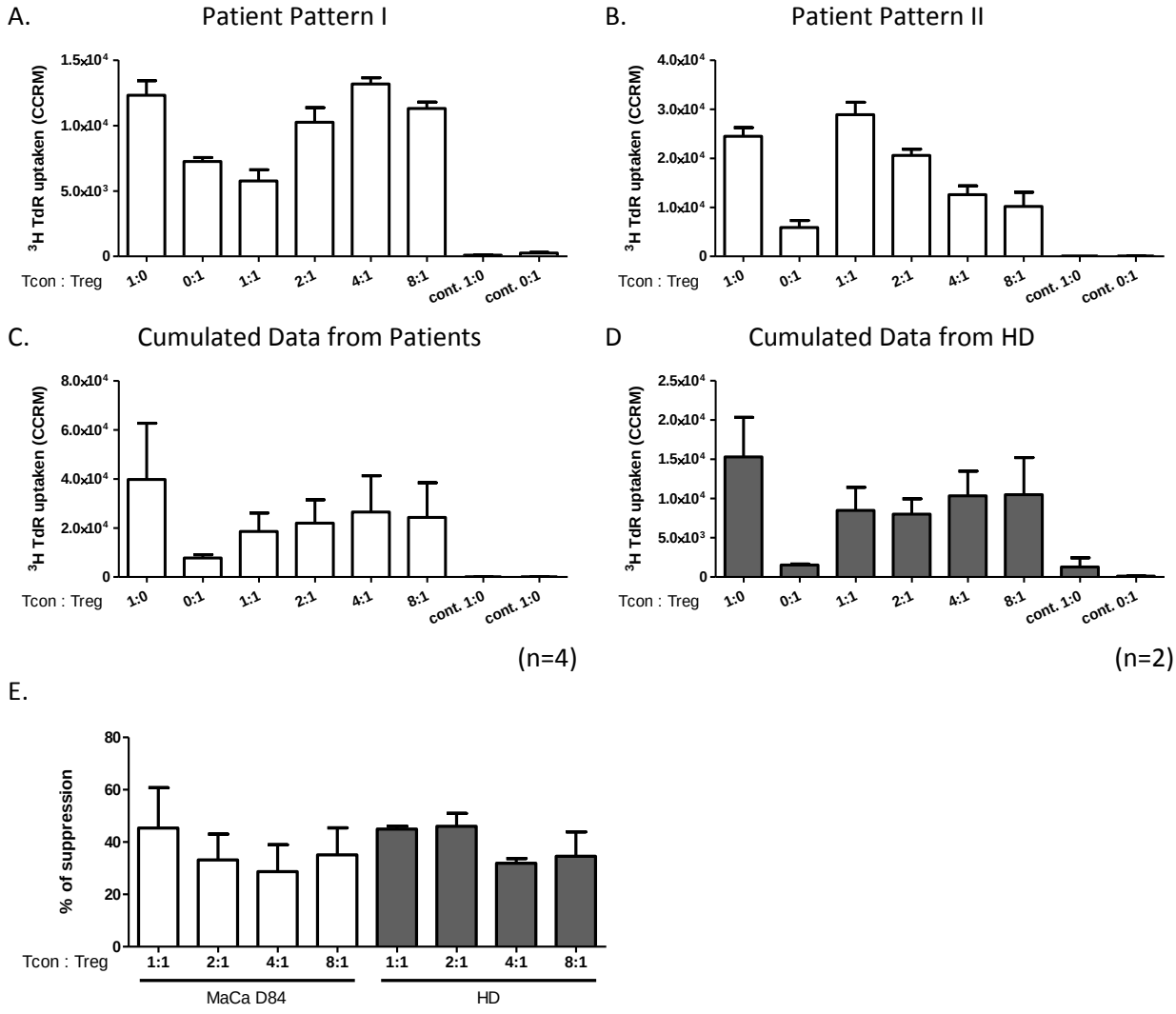


Figure 43. Suppressive capacity of Treg on $CD4^+$ Tcon proliferation with study patients on Day84 and healthy donors. Experiments were performed according to manufacturer instruction of Miltenyi Treg suppression inspector kit. Tcon to Treg ratios are indicated under each column. Columns in A-D represent values of Tcon proliferation determined by ^{3}H thymidine incorporation. Bars; SEM. **A** and **B** show different Treg characters after from two exemplary patients on Day84. **C** and **D**, Accumulative data show Treg suppressive capacity with 4 study patients on Day84 and 2 healthy donors. **E**, Treg in 84 days treated patients exert not altered suppressive capacity compared to healthy donors. Each column represents a inhibitory percentage of Tcon proliferation calculated as: (Tcon alone – Tcon:Treg) /Tcon alone x100%. White columns: study patients on Day84. Grey columns: healthy donors. Bars: SEM.

Next, we studied suppressive function of Treg isolated from four study patients on Day84 with standard Treg suppression assay. Surprisingly, we observed two completely opposite patterns (Figure 43A and B). Pattern A representing three of four patients showed that Treg sustained suppressive potency but concomitantly gained proliferative capacity. Pattern B obtained from the other one patient showed that Treg retained hypoproliferative characteristic but lost inhibitory ability simultaneously (note: this patient died one month after stopping dosing metronomic CTX). Then, we compared the accumulated data of study

patients (Figure 43C) to that of healthy donors (Figure 43D). Treg from both sources displayed similar hypoproliferation in response to a polyclonal stimulation and a similarly suppressive capacity on activated Tcon amplification. These findings were further confirmed by analyzing the extent of suppressed Tcon proliferation in the presence of Treg. Results are shown in Figure 43E. There were no statistical differences found in between study patients and HD. Collectively, these data demonstrate that rebounded Treg in study patients possess a suppressive potency. But whether it is tumour antigen specific and whether it is responsible for the observed declining tumour-reactive T cells at that phase are not clear. We assume that Treg antigen specificity assay may provide an answer.

7 DISCUSSION

The major aim of this thesis was to characterize the extent of regulatory T cell–mediated immune suppression in breast cancer and to explore the possibilities for its potential therapeutic modulation. Based on initial results obtained from a pilot clinical study of adoptive T cell transfer in advanced metastatic breast cancer patients, the subsequent observations of this project led us to the following major conclusions: 1) in primary breast cancer patients the peripheral blood contained significantly higher frequencies of Treg than the bone marrow, which may explain at least partially the improved functional potential of bone marrow-derived memory T cells, 2) removal of Treg evoked effective T cell tumour immunity in the peripheral blood, 3) Treg existing in breast cancer patients exhibited specificity for breast tumour-associated antigens and 4) using metronomic cyclophosphamide treatment to selectively deplete Treg *in vivo* was associated with clinical response in 64% of metastatic breast cancer patients.

Breast cancer is the most frequent form of cancer in women. Immunotherapy which utilizes the patient's own immune system to eradicate tumour cells offers a variety of advantages over conventional therapies, such as low toxicity, high specificity, and a continuous anti-tumour effect attributable to immunologic memory (103).

Current immunotherapy target tumour cells by tumour-associated antigen (TAA) -specific cytotoxic T lymphocytes (CTL) generated through dendritic cell (DC) vaccinations, *in vivo* expansion of TAA-specific $CD8^+$ CTL clones or adoptive transfer of TCR-transduced T cells. To date, adoptive T cell transfer is performed after an extensive *in vitro* expansion of CTL (104). One major obstacle of this procedure is a poor persistence of transferred T cells in the patients which is most likely due to an activation induced early T cell apoptosis resulting from a state of "terminal differentiation" (105; 106; 107; 108; 109), namely loss of the homing receptor CD62L (110), co-stimulatory molecules CD28 and CD27 (111) and an increase of pro-apoptotic molecules BID and CD95L (100; 112). In addition, this procedure does not encompass $CD4^+$ T helper cells which may provide a hitherto unsuspected important support during $CD8^+$ T cell survival, activation, effector function and memory formation (113; 114; 115; 116; 117; 118; 119; 120).

The adoptive T cell transfer study evaluated in this thesis was designed to overcome the caveat of T-cell exhaustion and lack of $CD4^+$ T-cell help by a short term (72h) reactivation of the complex repertoire in the bone marrow (BM) of tumour-antigen reactive $CD8^+$ and $CD4^+$ memory T cells. We also carried out a pre-selection of TAA-reactive patients, and used whole tumour cell lysate as a source of tumour antigens. In this pilot study 17 advanced breast

cancer patients including two containing tumour-reactive T cells in their blood before therapy were treated with breast tumour-antigen stimulated autologous BMTC. One week after initiation eight patients (47.1%) showed tumour-reactive T cells in the peripheral blood (immunological responders) (Figure 1).

The presence of tumour-reactive T cells in the blood of our patients mainly seven to 14 days after activation and their subsequent decrease (Figure 1) might be explained by circulating TAA-reactive T cells having re-entered lymphatic organs or immigrated into peripheral sites, as also suggested by the observation of CA15-3 reduction in two patients (99). In xenotransplant models of breast cancer (121; 122) and in murine melanoma (106) tumour-infiltration and –rejection by transferred tumour-reactive memory T cells were similarly observed after 7-10 days.

Alternatively, the transferred T cells might have been functionally inhibited by immune suppressive mechanisms, e.g. by regulatory T cells (Treg). Early studies by Robert North demonstrated that during tumour progression the initial development of concomitant immunity was followed, but finally subverted by the establishment of $CD4^+$ T-cell-mediated immune suppression (123).

We also cannot exclude the possibility that the transferred T cells might have undergone programmed cell death. The loss of T cells within the first two weeks after transfer had been described in clinical studies using repetitively stimulated and excessively expanded T cell lines (124; 125; 96). Similar observations were reported by other clinical trials in which TAA-specific T cells could be detected for only 14 days even with the additional infusions of IL-2 (126; 127). Since the T cells we used were not excessively stimulated, we assume that the latter hypothesis is less likely.

Regarding the possible causes which impeded the occurrence of T cell-mediated anti-tumour immunity in the other patients (non-responders), we observed that transferred T cells of these patients secreted increased type-II cytokines IL-4 and IL-10, exerted reduced proportions of early activated T cells determined by CD69 expression and contained higher proportions of $CD25^{high}$ T cells (enriched in Treg) (99). These findings pointed to the possibility that regulatory T cells present in bone marrow T cell populations of some advanced breast cancer patients might inhibit the appropriate restimulation of pre-existing tumour reactive memory T cells. In support of this hypothesis, previous clinical study showed that cytostatic, non-myeloablation preconditioned adoptive T cell transfer improved the clinical outcome in up to 50% of the patients most likely due to the pre-elimination of (chemotherapy-sensitive) Treg (128; 129). In murine models it was shown that the depletion of Treg enhanced spontaneous tumour immune protection and elicited concomitant immunity in tumour-bearing mice (130; 131). Accordingly, in our study we could show that

the depletion of Treg from BM T cell populations resulted in a significantly reduced secretion of IL-10 upon T cell stimulation with tumour antigens (Figure 25).

Direct evidence for the inhibitory role of regulatory T cells in breast tumour specific T-cell responses was firstly obtained by an IFN-γ catch assay (Figure 5 and Figure 6). The removal of Treg by MACS© $CD4^+CD25^+$ Regulatory T Cell Isolation Kit led to an increased secretion of IFN-γ by 5.8 times in $CD4^+$ TC and by 11.7 times in $CD8^+$ TC, in response to tumour-specific antigens. It also facilitated polyclonal proliferation of captured T cells to the double amount. In a long term impact, effector functions upon tumour-specific-antigen recall such as IFN-γ production and cytolytic activity persisted in T cells captured from an initially Treg-depleted group but not in T cells from a non-depleted group. These observations suggested that in breast cancer patients Treg exerted a negative effect on $CD4^+$ and $CD8^+$ T-cell activation, clone expansion, type-I T-cell effector function and CTL cytotoxicity.

This impact of Treg on anti-tumour immunity appeared to be superior in breast cancer patients' blood than in their bone marrow as implied by the following two observations. The previous study from our group suggested that tumour-reactive T cells were more frequently harboured in breast cancer patient BM (bone marrow) than in PB (peripheral blood) (121). In this pilot clinical study we now observed that the proportion of tumour responsiveness in the BM was persistent through the entire study (Diagnosis day: 100%; Mon 1: 78%; Mon 3: 100%), whereas that in the PB declined (Figure 1). We are aware that Treg might not be the only reason for this scenario. However, assuming they were, the less inhibitory environment in the BM than the PB might be due to either more suppressive Treg or higher counts of Treg circulating in the PB. The former assumption was ruled out by a suppression assay which showed that the proliferation of T conventional cells (Tcon) was suppressed in a comparable way by Treg from both PB and BM (data not shown). Similar inhibitory capacities of IFN-γ production was also observed with both PB and BM derived Treg by re-adding Treg to IFN-γ ELISpot experiments (Figure 18).

Indeed, the results from flow cytometric analyses strongly favoured the latter explanation, as in primary breast cancer patients the frequencies of Treg among the total $CD4^+$ T cell count were significantly ($p<0.001$) lower in the bone marrow (2.5±1.3%) than in the peripheral blood (3.9±1.5%) (Figure 3). A significant ($p=0.022$) reduction of Treg frequencies in breast cancer patient BM was further observed compared to healthy donor BM (3.4±1.0%). Thus, we provided here at least a partial explanation of the improved functional potential of bone marrow-residing memory T cells in breast cancer patients.

Naturally occurring Treg represent 5-10% of total $CD4^+$ T cells in the periphery of normal mice and humans (42). In malignant cancer patients, including breast cancer patients, Treg proportions are elevated in the circulating blood (132). Compared to those findings, the

frequencies of Treg in PB reported here are relatively low. In contrast of the knowledge on Treg in blood, very little is known about BM residing Treg regarding their numbers and immunosuppressive functions, neither with cancer patients nor with healthy donors. The limited reports to date are divergent. For example, Atanackovic D. and his colleagues could show that there were no differences between newly diagnosed myeloma patients and healthy donors in the percentages of Treg in both BM and PB (133). However, Zou Weiping and his co-workers claimed there were more than 25% phenotypic Treg in both normal human and murine bone marrows (134).

All those discrepancies could be due to the heterogeneity of recruited patients, various tumour entireties, or different stages of cancer. For example, Her2/neu positive patients were reported to contain higher Treg in the tumour site than Her2/neu negative ones. In patients with later stages of ovarian cancer, Treg preferentially accumulated in tumours but rarely entered draining lymph nodes (135). Similarly, we observed in this thesis that percentages of Treg were higher in the blood of advanced breast cancer patients (5.1±3.0%) than of primary cancer patients (3.9±1.5%) and healthy donors (4.3±0.9%).

More likely, the discrepancies resulted from different Treg markers used for specifying this population. To date, many markers associated with Treg have been identified, but some of them are also activation markers and are not specific for Treg cells. For example, the expression of CD25 on T cells has been a useful marker for Treg (25), but it is also known to be associated with activated $CD4^+$ T cells. Caution is advised when using CD25 for quantifying bone morrow-residing Treg as it is also unspecifically expressed on a large number of conventional $CD4^+$ and $CD8^+$ T cells in the bone marrow (133). Fontenot's group reported that CD25 was not necessarily obligatory for $Foxp3^+$ T cells which acted as immune suppressors (136). On the other hand, these observations were possibly due to the loose gates of CD25 expression for specifying Treg as suggested by Clare Baecher-Allan *et al* that only the highest level of CD25 expressing $CD4^+$ T cells, approximately representing 2-3% of total $CD4^+$ T cells, *in vitro* exhibited the properties that were identical to the $CD4^+CD25^+$ regulatory T cells initially characterized in mice (137). Besides, there was a direct functional link between the presence of $CD25^+$ Treg and $CD8^+$ T cell mediated tumour protection shown by adoptive transfer of tumour-specific CTL with or without Treg isolated by CD25 targeting (138; 139).

Even better marker for Treg than $CD25^{high}$ is Foxp3 at least in murine models because of its essential and sufficient role in mouse Treg development and function (140; 29). However, human Treg are less exclusively related to Foxp3 protein. For instance, the typical hypoproliferation of murine Treg following a T cell receptor (TCR)–mediated stimulation was altered in the human situation due to a slightly higher IL-2 production by the second isoform of the human Foxp3 protein which lacks exon2 (141). Human Foxp3 could be induced from

naïve or conventional T cells upon both polyclonal and antigen-specific activation (142), which did not occur in murine experiments (57; 30). Therefore, to ensure a relatively more reliable investigation we defined Treg by double expressions of Foxp3 and $CD25^{high}$ among $CD4^+$ T cell population.

According our study, the BM of primary breast cancer patients contained significantly lower frequencies of Treg than the PB. The reason for this could be a lack of migratory capacity of Treg towards BM or a reduced retention of BM for circulating Treg. But none of these hypotheses were possibly true as, firstly, it had been reported that upregulated CXCR4 expression on activated Treg enabled them to migrate to the bone marrow through its ligand CXCL12 elaborated by bone marrow stromal cells (134). The blockade of CXCR4 *in vivo* abrogated the otherwise occurring migration of activated $CD4^+CD25^+$ T cells to the BM (134). Therefore, it was unlikely that Treg would lack BM migratory capacity. Secondly, the clinical study of post-allogeneic stem cell transplantation of patients with multiple myeloma showed an exuberant regeneration of Treg inside the BM, suggesting that the BM is capable of providing an environment for retaining Treg as well as for fostering their expansion (133).

Thus, we proposed the third hypothesis that Treg in breast cancer patients actively emigrated from BM after encountering tumour antigens there, and were subsequently recruited to the tumour site. Several lines of evidence from our group as well as from others led us to formulate this hypothesis: i) BM was known to be able to serve as a site for naïve TAA-specific T-cell priming upon blood borne antigens presented on immigrated DC and as a preferred site for homeostatic proliferation of memory $CD8^+$ T cells (143; 144; 145; 11). These findings illustrate a possibility for Treg to encounter tumour antigens in the BM. Also, it is conceivable that immune suppressors such as Treg might actively leave BM because of the type-I T cell favoured environment. ii) Treg are known to modulate immune responses through selective migration and progressive accumulation at sites where regulation is required (146; 147). Thus, the emigrated Treg from BM were likely recruited to the tumour site where they could promote tumour growth. In support of this, our previous experiments showed that the percentages of Treg among breast tumour infiltrating lymphocytes was inversely correlated with the outcomes of IFN-γ ELISpot assay performed with BMTC (Michael Hillier, unpublished data, 2007). iii) The recruitment of Treg is most likely triggered by chemokine-chemokine receptor interactions which are also responsible for other T-cell trafficking. In healthy individuals, CCR4 expressing Treg in the periphery had been reported to represent a major subset of circulating $CD4^+$ T cells responding to CCL22, one of its two ligands (CCL17 being the other one) (148). A study of 104 ovarian cancer patients showed that Treg were constantly recruited to the tumour site via CCR4-CCL22 attraction (135). With our preliminary data, the expressions of chemokine receptors CCR2, CCR5 and CCR6 on Treg were found to be significantly higher in breast cancer patients' BM than in their PB

(Christopher Schnappauf unpublished 2008). This distinct pattern between BM and PB was not observed in healthy donors (Christopher Schnappauf unpublished 2008). Accordantly, the levels of the ligands for those chemokine receptors (CCL2, CCL5 and CCL20, respectively) were frequently reported to be elevated in mammary tumour cells or tumour associated cells (149; 150).

In order to testify this hypothesis, we needed to prove whether Treg could be activated by tumour antigens, and could consequently upregulate breast cancer profiled chemokine receptors in response to the tumour recruitment. In this study, following a polyclonal TCR-mediated activation the elevated proportions of CCR2, CCR5, CCR6 and CCR4 expressing Treg were able to be detected in a stimuli dose-dependent manner (Result 1.6). Regarding CCR2 and CCR4 expressions upon activation, higher responsiveness was obtained with Treg derived from breast cancer patients than those from healthy donors. The upregulation of CCR4 was predominant on activated Treg in breast cancer patients compared to Tcon, which is in line with others' findings in ovarian cancer as described above. Further definitive experiments are planned, such as applying breast tumour specific antigens instead of polyclonal antibodies for stimulation and confirming chemokine receptors responsible for Treg emigration from BM with a chemotaxis assay.

CCR2 and CCR5 are also expressed on other T cells which enable them to migrate to the tumour site (151; 152). However, this T-cell tumour infiltration may be beneficial or detrimental for tumour survival. For instance, CCL2 induced Th2 polarization which mediated humoral immunity and consequently suppressed anti-tumour activities (153). CCL5 was associated with a recruitment of $CD8^+$ T cells which presumably had cytotoxic activity against tumour (154). CCR5 was predominantly expressed on Th1-polarized T cells (155; 156), suggesting an anti-tumour potential. In this study, we observed an elevated expression of CCR2 but a suppressed one of CCR5 on Tcon in breast cancer patients compared to healthy donors. We deducted that that was due to different strategies of immune suppression exerted by activated Treg. Since CCL2-responding $CD4^+$ T cells would likely be polarized to Th2 cells or even be converted to Treg once inside tumour microenvironment (157), the control of CCR2 expression on Tcon seems not necessary; whilst CCR5 expressing Th1 cells are able to facilitate CTL function inside tumour, the control of CCR5 expression on Tcon might be inhibited by activated Treg. This inhibition could have possibly resulted from *in vitro* activated Treg, or be due to an inert responsiveness of Tcon due to long term suppression by Treg *in vivo*. Although further definitive experiments are required, these findings at least indicated an interesting immune suppressive capacity of Treg in breast cancer patients.

Next we set out to understand the extent of Treg-mediated immune suppression in breast cancer patients by comparing type-I T-cell tumour immunity with and without Treg

presence. In a study of 31 PB and 27 BM breast cancer samples, the removal of Treg uncovered 2.3 to 2.6 folds of T-cell responsiveness towards polyvalent breast tumour antigens with statistical significances (Figure 8A&B). Importantly, the same effect was not found with irrelevant controls (Figure 8A&B).

Further study with defined tumour associated antigens (TAA) confirmed Treg-depletion-mediated improvements in type-I T-cell anti-tumour immunity with statistical significances observed in response to HpaI, HpaII, Mam1, Muc1-20 and Her2/neu in the blood of 38 breast cancer patients (Figure 12A). In contrast to blood, in the bone marrow of 26 breast cancer patients the statistical significance was not achieved despite substantial inductions of TAA-reactivity observed in ten out of 13 groups (Figure 12B). This reduced effectiveness is likely due to pre-existing higher frequencies of functional memory T cells in the bone marrow than in the blood, making the beneficial impact of Treg depletion less obvious.

We are also aware of the disparity of Treg-depletion-mediated benefits exhibited by BMTC in response to polyvalent tumour antigens and to selected TAAs. The observed better outcomes displayed in the former situation implied the involvement of other TAAs than those chosen for this study in breast tumour immunity.

Regarding the frequencies of breast tumour-specific T cells, a dramatic unmasking-effect attributed to Treg-depletion was mutually supported by the evaluations of T-cell responsiveness towards polyvalent tumour antigens and mixed TAAs. The frequencies MCF7 and KS24.22-specific T cells were augmented by 5 and 10 times, respectively, in the peripheral blood, and by 3 and 4 times, respectively, in the bone marrow (Figure 9). Accumulative data of overall 13 studied TAAs revealed a highly significant ($p=0.0002$) induction (1.96-fold) in the frequencies of TAA-specific T cells in the blood and a 1.31-fold increase in the bone marrow (Figure 13).

It is worth mentioning that these findings are nicely in accordance with our acquired flow cytometric data. The higher spontaneous T-cell responses pre-existing in the BM, as well as the feebler Treg-depletion-induced benefits are well correlated with the lower Treg frequencies found in the BM. Whereas the relatively higher Treg pool circulating in the blood probably accounts for the stronger immune suppression.

The benefit of Treg-depletion in breast cancer patients could also be confirmed by the induction in proportions of immunological responders. About 35% patients obtained *de novo* tumour reactivity in their blood after Treg-depletion, which was accompanied by a highly significant impact ($p=0.0006$) (Figure 10). In particular, MCF7 and KS24.22 responders went up from 26.1% to 60.9% and from 35% to 65%, respectively. A similar effect was observed with an induction of Her2/neu responders analysed in the blood (from 13% to 45% $p=0.03$)

(Figure 16). As Her2/neu is known as one of the most prevalent breast tumour associated antigens, it is conceivable that the suppression of Her2/neu-reactivity is strongly modulated by breast tumour *in vivo* possibly through Treg as shown here.

On the whole, our data strongly demonstrated a profound immune suppression of Treg on spontaneous anti-tumour type-I T-cell responsiveness in breast cancer patients which was in accordance with a plethora of recent *in vitro* as well as *in vivo* studies with different other tumour entities that drew comparable conclusions (158; 159; 160; 161).

Our data also suggested that the tumour-tolerance could be broken by applying a CD25-mediated Treg-depletion in breast cancer patients, which implicates a clinical application. The possible downside of CD25 administration for Treg elimination *in vivo* would be an undesired onset of autoimmunity as reported in the murine model (162). However, according to the results of this study, it would be unlikely as the unspecific secretion of IFN-γ against irrelevant controls did not alter before and after applying CD25 mAb (Figure 8 and Figure 12), suggesting a specific effect of this approach. Similarly, the mean frequencies of TAA-reactive T cells in healthy donors were substantially lower than those in breast cancer patients ($p<0.0001$) (Figure 14) in both PB and BM whether Treg were depleted or not.

Another possible disadvantage of applying this approach would be a concurrent loss of $CD25^+$ Teff as discovered in both animal and human investigations (163; 164). In this study the type-I T-cell responsiveness towards MAGE3 was reversed possibly due to this reason (Figure 11 and Figure 12). To avoid it, further optimization has been tried out, such as reducing the amounts of α-CD25 Ab, adjusting the ratio of Ab to targets, or shortening the incubation time of Ab with cells.

This pilot clinical study of adoptive T cell transfer immunotherapy (ADI) in metastatic breast cancer patients clearly demonstrated that at least $7x10^3$ tumour-reactive T cells were needed in order to achieve an immunological response (99). However, with a limited BM source of not more than 100 ml per patient this becomes a challenge. In this thesis, CD25-mediated Treg-depletion restored the prior absent T-cell anti-tumour immunity especially in the peripheral blood of breast cancer patients, and importantly to a corresponding extent as in its counterpart bone marrow (Figure 9 and Figure 13). The profound meaning of this was instead of BM we could use leukapheresis which provides substantially larger counts of lymphocytes including tumour-reactive T cells for the potential improvements of ADI.

Based on this thought, as well as being prompted by the another report (165), we set about a larger-scale depletion of $CD25^+$ regulatory T cells by CliniMACS CD25 microbeads from a bone marrow metastatic patient leukapheresis. This pioneer exploitation proved the possibility of modulating Treg under clinical-grade condition by a sufficient depletion of Treg

(Figure 34), a high viability of isolated cells and a remarkable recovery of total cell counts (Table 4). However, the potential therapeutic use of this protocol was not convincing, as the T-cell tumour responses were not as potent as shown in the applied manual depletion protocol determined by an IFN-γ ELISpot assay (Figure 35), indicating a possible undesired loss of T effector cells by this approach. In fact, our flow cytometric data acquired from cells in the depleted fractions displayed a considerable loss of $CD25^{inter}$ T cells (data not shown). Another possible reason for this lesser responsiveness might be that tumour-specific T cells derived from an advanced breast cancer patient were more susceptible to a longer manipulation (CliniMACS: 12 hr) than a short one (manual depletion: 2 hr). Nonetheless, further optimization is required.

Currently, many other cancer immunotherapy approaches dealing with Treg elimination are under intensive investigations with in pre-/clinical trials. Also concerning CD25 as a Treg target, most studies in cancer patients used immunotoxin denileukin diftitox (Ontak), a fusion protein between IL-2 and diphtheria toxin. To date *in vivo* anti-tumour efficacy of Ontak displayed discrepant results, which were very likely due to different administrated amounts or schedules as well as different types of tumour (166; 167; 168; 169). In addition to targeting CD25, an agonistic antibody which is specific to CTLA-4 is under pre-/clinical investigations. CTLA-4 has long been thought to control the Treg activity (170), but not until recently Sakaguchi's group reported its superior role on suppressive ability of Treg with a conditional knockout system (171). The clinical responses of administrating CTLA-4-specific antibodies showed concomitant tumour regressions and severe but manageable autoimmune diseases (172; 173; 174). In contrast, a completely disappointing clinical result showing no detectable effects on decreasing numbers of suppressive Treg after CTLA-4 administration was reported by Rosenberg's group (175). Other immunologists suggested that the observed anti-tumour efficacy by applying CTLA-4-specific antibodies was a synergistic regulation rather than a direct suppression on Treg (176). Thus, the application of CTLA-4 in modulating Treg requires further critical investigations.

Alternatively, cyclophosphamide (CTX) emerged to be an effective drug of depleting Treg *in vivo.* Cyclophosphamide belongs to a group of cytotoxic alkylating nitrogen mustard compounds that mediates DNA crosslinking. Because of its selective cytotoxicity on lymphocytes without being myeloablative, high-dose CTX treatment was initially developed as a preconditioning regimen for allogeneic bone marrow transplantation to prevent graft-versus-host disease. Later, high-dose CTX treatment without bone marrow transplantation was used to treat severe autoimmune disorders for which multiple therapies were ineffective (177; 178). In contrast to such high-dose applications of CTX, with the primary aim of dampening ongoing immune responses in autoimmune patients, administration of low-dose CTX could, paradoxically, augment anti-tumour immune responses in a number of

murine tumour models (179; 180; 181) and in patients with metastatic melanoma (182; 183; 184). A simultaneous enhancement of CTL response as well as antibody production was accompanied by tumour control. In elderly breast cancer patients, uninterrupted daily low dosing CTX therapy, referred to as metronomic therapy, indicated an antiangiogenic effect as without concomitant CTX treatment, letrozole alone led to a relatively higher Ki67 and VEGF expression on the residual tumour histology (185). The correlation between CTX administration and Treg was initially suggested in 1974 reporting that a CTX regimen-induced anti-tumour efficacy was associated with a removal of T lymphocyte suppressors (186; 102), but the first evidence specifying $CD4^+CD25^+$ Treg was recently documented by Ghiringhelli F. *et al* in mice (187) as well as in late stage cancer patients (188).

Regarding breast cancer immunotherapy, a combined regimen with metronomic CTX was reported to have a prolonged clinical benefit and a relevance to the Her2/neu expressing level by Colleoni M.'s group (189; 190). However, its own effect on Treg alterations in their counts and function after administration was not stated. And a long term effect of metronomic CTX on type-I T-cell immunity has not yet been evaluated. To address these issues, we applied a small pilot study of metronomic cyclophosphamide (50 mg daily) treatment with 13 multi-metastatic breast cancer patients, and followed up Treg counts in circulation by flow cytometry and type-I blood T-cell responses by IFN-γ ELISpot assay every two weeks for three months.

This regimen was well tolerated. Severe side effects was not observed in any of the patients except one who suffered from anaemia (Hb 6.6 g/dl) and was withdrawn from the therapy because of this.

In this study, we reported that low-dose CTX treatment was correlated with declines of Treg counts up to 42 days after initiation with significant decreases observed on Day14 ($p=0.005$) and Day28 ($p=0.02$) (Figure 36). Conversely, the numbers of other lymphocytes were preserved (data not shown), suggesting a selective depletion of Treg *in vivo* by this strategy, which is in line with Ghiringhelli F.'s finding (188). The rapid and effective elimination of Treg observed on Day14 also implicated a preconditioning potential of low dose CTX for adoptive T cell transfer or DC vaccine immunotherapy.

According to the extents of declining Treg between Day0 and Day14, we were able to subgroup patients into a CTX susceptible and a non-susceptible type (Table 7). The patients belonging to the CTX susceptible group contained either higher (6.4% to 10.9%) or lower (0.67% to 2.4%) Treg frequencies on Day0 than healthy donors (average 4.3%). A possible interpretation is that Treg in those abnormal proportions were under vigorous self-renewal, which allowed them to be perfect targets for CTX (191; 192). Further investigation of the proliferative status of Treg on Day0 might be helpful to clarify this assumption.

This 3-months-follow-up study on Treg frequency allowed us to detect a gradual replenishment of the Treg pool in the late period of this treatment on an average level. By the last clinical monitoring day, the average of Treg percentages of all assessable patients rebounded back to the starting level (Day0: 5.1%, Day84: 5.2%) (Figure 36). Whether an extended administration will take the Treg frequency to an ever-growing rate was uncertain. It may be implied by the results of Ki67 expression assay in which we observed accordantly climbing percentages of under-proliferating $CD4^+$ Treg (Figure 42A). Whether there were other factors contributing to the replenishment of the Treg pool remains unclear. Meanwhile, a markedly proliferative potency of $CD4^+$ Tcon was obtained in a late study phase compared to Day0 (Day56, p=0.016; Day70, p=0.03; Day84, p=0.026) (Figure 42B). IL-2 secretion is known to be associated with T cell proliferation, and Treg maintenance and expansion crucially depend on the presence of IL-2 (193). Thus, the Treg replenishment was likely facilitated by Tcon amplification in the late phase of this study.

We also monitored the immunological responses in the blood of treated patients by performing IFN-γ ELISpot assay. Our results demonstrated that this regimen had a long-term clinical benefit as a significant enhancement in frequencies of tumour-reactive T cells were obtained on Day70, which was confirmed by two different statistical analyses (Wilcoxon signed-rank test, Figure 38B and student t-test, Figure 38C). The presumed T-cell immunity augmentation on Day14 did not appear in a statistically significant way possibly due to an insufficient power of study.

In addition, a persistent clinical efficacy was shown by the percentages of immunological responders. Starting with a dramatic induction on Day14 (Day0: 27.3%; Day14: 72.7%), the percentages of immunological responders sustained above 57% through the entire course of the study, and ended with 87.5% on Day84 showing a statistical significance (Figure 39).

Most importantly, of 11 patients assessable for tumour markers or MRI-Scan, we observed seven patients with a stabilization of the disease (SD) (64%), and four with a progression of the disease (PD) (36%) after the treatment. Moreover, this clinical response was well correlated with T-cell anti-tumour reactivity, as augmentations of tumour-reactive T cells in frequencies associated with 85.7% of SD patients (six out of seven); whilst all PD patients failed to reach 183 tumour-reactive T cells per 10^6 T cells in their blood (Table 8, Figure 40).

The isolated Treg from patients' blood in the late period of this study were found to possess suppressive ability on the polyclonal proliferation of $CD4^+$ Tcon (Figure 43E). This together with the observation of potent type-I T-cell responsiveness at this period suggested that replenished Treg changed their Ag-recognition; therefore the maintained suppressive capacity could not affect tumour-antigen specific response. To prove it, definitive experiments such as our designed Treg-specificity assay are warranted.

It is worth mentioning that Ki67 expression was significantly elevated on the $CD25^{high}Foxp3^{+}CD8^{+}$ T cell population three months after initiating metronomic CTX. $CD25^{high}Foxp3^{+}$ $CD8^{+}$ Treg have recently been detected at the tumour sites of different cancer patients (194; 195; 196; 197). However, in contrast to $CD4^{+}$ Treg, less is known about $CD25^{high}Foxp3^{+}$ $CD8^{+}$ Treg and their detrimental effects on immunotherapy. Unlike $CD4^{+}$ Treg which were naturally generated in the thymus and can be detected in the periphery, $CD8^{+}$ Treg cells were not detectable in the peripheral blood mononucleocytes (PBMC) of healthy donors or cancer patients, suggesting that $CD8^{+}$ Treg cells were antigen induced (194). Thus, our data might indicate an alternative Treg subset existing and an additional suppressive mechanism occurring in advanced breast cancer patients. Further experiments are required to clarify how metronomic CTX modulates $CD8^{+}$ Treg and what the impact of $CD8^{+}$ Treg on type-I T-cell immunity is.

These data suggest that this simple protocol described here is worth being tested along with specific immunotherapy aimed at enhancing T cell function in patients bearing advanced breast cancer.

In this study, we were also interested in antigen specificity of Treg in breast cancer patients. In contrast to the ever increasing evidence of the presence of $CD4^{+}CD25^{+}$ Treg in tumour-bearing hosts, very little is known about their tumour-antigen specificity. $CD4^{+}$ Treg are important in modulating self-tolerance as well as homeostasis following productive immunity; therefore, generation and maintenance of Treg have long been thought to require the presence of targeting antigens or tissues (198; 199). Treg were reported to display TCR repertoire as broad and as diverse as the other mature αβ-T cells with the tendency to focus on self-antigens (200; 201). Therefore, it is possible that Treg are specific for at least one subgroup of TAAs. Tumour-specific Treg could also be induced *de novo* as evidenced in HA-TCR transgenic mice with Rag2-/- background that did not obtain any natural Treg but developed into Treg when presented with HA-peptide under subimmunogenic conditions (202). Other origins of TAA-specific Treg were proposed such as a conversion from naïve or antigen-experienced $CD4^{+}$ T cells in the suppressive cytokine milieu at tumour sites (142; 203; 204). Thus, it is reasonable to assume that tumour evasion is mediated by tumour-antigen specific regulatory T cells. Several lines of observation in this thesis supported this concept: The removal of Treg specifically abrogated MCF7-mediated IL-10 secretion in an activation culture (Figure 25A). Giving isolated autologous Treg back into the ELISpot culture, the IFN-γ secretion was blocked in response to breast tumour derived antigens but not in the controls (Figure 18). And more directly, breast tumour antigen stimulated Treg produced significantly excessive intracellular IL-10 (Figure 25B).

Our knowledge of tumour-antigen specific Treg comes mainly from studies with antigen-specific TCR transgenic murine models (205; 206; 207; 208). Regarding human cancer specific Treg, Rong-Fu Wang and his co-workers contributed to the identification of two melanoma-infiltrating Treg, LAGE1 and ARTC1 -Treg (209; 66), which in association with CD25, GITR and Foxp3 expressions, suppressed the proliferation of naïve $CD4^+$ T cells and inhibited IL-2 secretion of $CD4^+$ effector T cells upon activation with tumour-specific ligands. Defining a tumour Treg clone certainly has a profound meaning in immunotherapy, but the immunologic methods they used, such as limiting dilution assay and single cell cultivation, are very time and effort–consuming, which limited their applications for a large screening aim in clinical exploitation such as diagnosis or prognosis. MHC-II tetramer technique could fill this request, but it is not the most economical approach. Thus, a need for cost and time - efficacy immunological method for detecting tumour-specific Treg is urged.

To date, human tumour specific Treg, either induced *de novo* or isolated from TILs, required cognate antigens for activation. Once activated, they were capable of suppressing responses in an antigen-nonspecific manner. Treg were well characterized to suppress the proliferation and cytokine production of TCR-stimulated conventional $CD4^+$ T cells (210; 211). Based on this knowledge, we proposed to identify TAA-specific Treg by measuring their inhibition capacity on proliferation of polyclonally activated Tcon after tumour-antigen stimulation (Figure 19). In detail, the protocol was designed as follows: purified Treg were activated with breast tumour or control antigens presented on autologous DC for 24 hr. Meanwhile Tcon received a polyclonal stimulation from αCD3 and αCD28 mAb coated wells. Afterwards, the pre-activated Tcon were given into the stimulated Treg' culture at a 1:1 ratio for 3 days to allow a full suppression exertion. The degree of Tcon proliferation was quantified by the amount of incorporated ^3H thymidine.

To ascertain the reliability of this novel approach, a set of rigid controls was primarily carried out with breast cancer patient samples. First, we compared the extents of Treg proliferations upon different stimuli: antigen pulsed DC (Figure 20A, Figure 22B), non-pulsed DC (Figure 20A, Figure 22A) and TCR-mediated polyclonal stimulation (Figure 22A). The hypoproliferations were observed from all tests, and importantly at a non-distinguishable level, which dispelled the potential or possibly uneven contributions from Treg itself in this approach. Second, the potently proliferative ability of Tcon in response to a polyclonal TCR-mediated activation was ensured (Figure 22A). Last, the degrees of Tcon proliferation with different manipulated DC were compared. Ag-pulsed DC induced a slightly higher proliferation of Tcon than non-pulsed DC, but not in a statistically significant way (Figure 20A). Collectively, we demonstrate this newly designed approach is reliable for examining Treg specificity.

Accumulative data from 14 PB and 12 BM samples of breast cancer patients showed that the proliferations of activated Tcon were significantly suppressed in the co-culture with MCF7- or KS24.22-stimulated Treg compared to the co-culture with control U937-stimulated Treg (Figure 21C), suggesting the immune suppression was mediated in a tumour-antigen specific manner.

Next, we used defined tumour-associated antigens for Treg activation. Surprisingly, the spectrum of TAAs recognized by Treg was highly diverse in breast cancer patients (Figure 24), which was reminiscent of our previous finding that the T-cell repertoire of breast TAA-recognition was greatly individual (212). Hence, we postulated that breast tumour might actively govern Treg according to its TAA expression status as well as to the ongoing anti-tumour T-cell responsiveness in order to dampen immune surveillance.

Despite of the multifarious pattern in individuals, an overall analysis of 18 blood samples revealed that Muc1-100 and Mam1 were prevalently recognized by Treg (Figure 23), which mutually support our IFN-γ ELISpot results that depletion of Treg significantly induced Mam1-responsiveness and Muc1-100 specific reactivity (Figure 12A). Moreover, Mam1 mediated Treg suppression (30%) was substantially exceeding the other tested TAAs and existed in more than 85% patients (Figure 24C). Mam1 is a polypeptide derived from Mammaglobin A which is an antigen expressed exclusively on breast epithelia (213), and overexpressed in 80% of primary and metastatic breast cancer patients (87). Capable of inducing a specific $CD8^+$ T cell response in breast cancer patients further endowed Mammaglobin A a very attractive target for immune involvement (214; 215; 216; 217; 218). Currently, a clinical phase I trial of Mammaglobin A vaccination based on full-length DNA is carrying out with stage IV breast cancer patients in Peter S. Goedegebuure's group (219). However, our results warned that manipulation of Mammaglobin A has to be deliberated carefully in order not to evoke suppressive function of Treg in breast cancer patients.

In contrast to Mam1, Her2/neu specific Treg exerted only 13% inhibition of Tcon proliferation which was the second lowest among all tested TAAs. It is interesting as only Her2/neu-responders significantly benefited from Treg-depletion approach determined by IFN-γ production (Figure 16A) as described above. This superficial discrepancy may implicate different modes of suppressive preference exerted by Her2/neu specific Treg on Teff: inhibiting cytokine production or clonal expansion. It is possibly related to the tumour stage as it is known that nature Treg suppressed the priming of naïve T cells in lymph nodes (220; 221), whereas in the later tumour stage Treg were found to be lacking in the tumour draining lymph nodes but accumulating at tumour site to suppress function of Teff (135).

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