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Platelet-expressed immune checkpoint regulator GITRL in breast cancer

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Zhou, Yanjun

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Dekan: Professor Dr. B. Pichler

1. Berichterstatter: Professor Dr. H. R. Salih
2. Berichterstatter: Professor Dr. R. Handgretinger

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My parents

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ADP	Adenosine diphosphate
APC	Allophycocyanin
AUC	Area under the ROC curve
BCR	B cell receptor
BSA	Bovine serum albumin
BV	Brilliant violet
CD	Cluster of differentiation
CFB	Cell FACS buffer
CI	Confidence interval
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTLA-4	Cytotoxic T lymphocyte–associated antigen-4
Cy-5	Cyanine-5
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GITR	Glucocorticoid-induced TNFR-related protein
GITRL	Glucocorticoid-induced TNFR-related ligand
GP	Glycoprotein
HD	Healthy donor
HER-2	Human epidermal growth factor receptor-2
hIgG	Human IgG
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cell
IFN-γ	Interferon-γ
IgG	Immunoglobulin
IHC	Immunohistochemistry
IL-6	Interleukin-6
mAb	monoclonal antibody
MHC	Major histocompatibility complex
mIgG	Mouse IgG

MK	Megakaryocyte
mRNA	Messenger ribonucleic acid
NK	Natural Killer
OR	Odd ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death-ligand-1
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PFB	Platelet FACS buffer
pGITRL	Platelet-derived GITRL
PR	Progesterone receptor
PRP	Platelet-rich plasma
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROC	Receiver operating characteristic
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SDF-1	Stromal-derived factor-1
SSC	Sideward scatter
TCIPA	Tumor cell induced platelet aggregation
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
TNFSF	TNF superfamily
TNM	Tumor Node Metastasis
TEP	Tumor-educated platelet
TPO	Thrombopoietin
Treg	Regulatory T cell
TSP-1	Thrombospondin-1

1 Introduction

1.1 Breast cancer

Breast cancer is the leading cause of cancer-related death in females comprising 11.6% of total cases.[1] With the improvement of screening programs, surgery, chemotherapy, hormone therapy, and the introduction of monoclonal antibody (mAb), the overall survival and progression-free survival of breast cancer patients have been significantly increased.[2] Although the majority of patients are diagnosed early and treated successfully, in almost 30% of patients who have initially been diagnosed with early-stage breast cancer, metastasis eventually occurs which then is associated with poor outcome.[3] So far, there are no precise and selective clinical parameters which identify patients with a high risk of metastasis. This would be even more important since there are no therapies available to cure patients with advanced breast cancer.

1.1.1 Prognostic parameters in breast cancer

The Tumor Node Metastasis (TNM) staging system is the (most) basic method to determine the breast cancer stage. It is used to estimate the disease prognosis based on the tumor characteristics in a given patient. The TNM staging system for breast cancer is shown in **Table 1**.[4]

However, the TNM classification is not a good predictor of clinical outcome. With the rise of molecular biology techniques, breast cancer is now classified into four main subtypes including Luminal A, Luminal B, human epidermal growth factor receptor-2 enriched (HER-2 enriched), and basal-like subtypes. Based on immunohistochemistry (IHC) results regarding overexpression of the HER-2 protein, IHC 0 and IHC 1 positive are considered as HER-2 negative, IHC 2 positive as equivocal, and IHC 3 positive as HER-2 positive.[5] Fluorescence in situ hybridization is used to further investigate the *HER-2* gene amplification upon equivocal results.[6] Breast cancer patients are classified as estrogen receptor

(ER) or progesterone receptor (PR) positive when at least 1 percent of cancer cells are stained positive for ER or PR. Besides the predictive and prognostic values of using these breast cancer subtypes, Ki-67 and histological G grading also are used as further determinants of prognosis.

Table 1: TNM staging system for breast cancer[4,7]

T1	tumor $\leqslant$ 20 mm
T2	20 mm $<$ tumor $\leqslant$ 50 mm
T3	tumor $>$ 50 mm
T4	any size with direct invasion to the chest wall and/or the skin
pN0	no regional lymph node metastases
pN1	micrometastases, or metastases in 1-3 axillary lymph nodes
pN2	metastases in 4-9 axillary lymph nodes
pN3	metastases in $\geqslant$ 10 axillary lymph nodes
M0	no evidence of distant metastases by clinical or radiographic means
M1	radiographic or clinical evidence of distant metastases and/or histologically proven metastases $>$ 0.2 mm

The histological G grade was established by pathologists according to the Nottingham modification of the Scarff-Bloom-Richardson grading system and is now used in addition to TNM staging.[8] G2 grading indicates moderately differentiated tumors that grow at an intermediate speed and have an intermediate risk to spread, whereas patients with G3 have tumors that are poorly differentiated and thus have a higher probability to quickly grow and disseminate. The level of Ki-67, a nuclear nonhistone protein expressed in G1, S, G2, and mitosis phases of the cell cycle, but not in the G0 phase, also is a prognostic marker and predictor of recurrence in breast cancer (proliferation index). Higher Ki-67 expression is associated with a higher risk of relapse and worse survival in early breast cancer patients.[9] Based on the molecular characteristics of breast cancer, cases are classified into four main subtypes shown in **Table 2**. However,

all the prognostic parameters currently employed do not serve to predict disease progression well.

Table 2: Molecule subtypes of breast cancer

Subtypes	Definition	Frequency	Features
Luminal A	ER positive and/or PR positive, HER-2 negative, and low Ki-67 (<14%)[10,11]	50–60%[10,11]	low mitotic activity, low histological grade, and good prognosis[10]
Luminal B	ER positive and/or PR positive/negative (PR<20%, positive Ki-67 ⩾ 14%) with HER-2 negative as well as ER positive and/or PR positive/negative (any PR positive and any Ki-67) and HER-2 positive[11,12]	15–20%[11]	a more aggressive phenotype with a higher histological grade and proliferative index and worse prognosis compared to Luminal A breast cancer[10]
HER-2 enriched	ER negative, PR negative, and HER-2 positive[12]	20–30%[13]	dysregulated cell proliferation and aggressive biological and clinical behavior[10]
Basal-like	ER negative, PR negative, HER-2, cytokeratin 5 and 6 positive and/or epidermal growth factor receptor positive[12]	10–15%[14]	shortest relapse-free and overall survival[15]

To identify better prognostic parameters in breast cancer, gene expression profiling for biomarkers associated with immunogenicity is increasingly

appreciated. Immunogenicity is the ability to induce a cell-mediated and/or humoral immune response and is relevant in malignancies for both, characterizing the tumor phenotype and evaluating therapeutic options.[16] Most subtypes of breast cancer are immunologically silent or known as so-called cold tumors, caused by low neoantigen burden and lacking tumor infiltrating lymphocytes.[16] A rare subtype of inflammatory breast cancer displays a strong expression of tumor antigens and an increased number of tumor infiltrating lymphocytes.[17] Moreover, increased levels of estrogens can obstruct interferon-γ (IFN-γ) signaling and major histocompatibility complex (MHC) II expression and promote immunosuppression and tolerance towards the “cold” tumor cells.[17,18] Breast cancer cells downregulate MHC I to escape from cytotoxic T lymphocyte-mediated lysis and acquire resistance towards apoptosis.[19,20]
The US Food and Drug Administration (FDA) recently approved atezolizumab as the first immunotherapy for metastatic triple-negative breast cancer with positive programmed cell death-ligand-1 (PD-L1) protein expression. Due to the modest response rates immunotherapy for breast cancer patients remains challenging and new targets are still needed.

1.2 Megakaryocytes

1.2.1 Maturation of megakaryocytes and generation of platelets

Megakaryocytes (MKs) are giant multinucleated cells ranging from 100-150 µm in diameter and comprise less than 1% of all hematopoietic cells.[21] MKs differentiate from bone marrow-residing hematopoietic stem cells (HSCs) in the endosteal niche under the influence of thrombopoietin (TPO) as observed by Kaushansky and colleagues.[22] TPO is also known as MK growth and development factor and is a glycoprotein (GP) hormone produced by the liver and kidney which stimulates the production and differentiation of MKs.[23] During differentiation and maturation, MKs bud off large numbers of platelets into the bloodstream, a process termed thrombopoiesis. The remaining MK cell bodies undergo apoptosis.[24]

Kiel and colleagues have described that there are two physiologically distinct HSC niches in the bone marrow: the endosteal (or osteoblastic) niche at the bone-marrow interface which contributes to stem cell quiescence, and the vascular niche around the specialized vascular endothelium which promotes HSC expansion.[25] MK precursors develop and migrate from the osteoblastic niche to the vascular niche. This journey is accompanied by the proliferation of MKs, deoxyribonucleic acid (DNA) replication without cell division (termed endomitosis), the formation of the demarcation membrane system (the plasma membrane of future platelets in MKs), proplatelet formation and ultimately release of platelets.[26] Several growth factors required for HSC maintenance are secreted by osteoblasts in the endosteal niche, including angiopoietin-1, TPO, and stromal-derived factor-1 (SDF-1).[27] Osteoclasts provide HSCs with proteolytic enzymes to mobilize them from the endosteal region by cleaving anchorage proteins such as SDF-1 and stem cell factor.[28] Avecilla and colleagues reported that the interaction of hematopoietic progenitors with the vascular niche, which is mediated by chemokines, allows the hematopoietic progenitors to relocate to a specific microenvironment for MK maturation and thrombopoiesis.[29] The mature MKs are located in vascular sinusoids which makes them the first cells to physically interact with cancer cells as they enter the bone marrow.

1.2.2 MKs and cancer

Little is known about the interaction of cancer cells and MKs, and data on the role of MKs in metastasis are conflicting. Walter and colleagues reported that an increasing number of MKs can be detected as a response to metastatic cells entering the bone and MKs display anti-metastatic effects.[30] In line, Zaslavsky and colleagues suggested that MKs in tumor-bearing mice endocytose circulating thrombospondin-1 (TSP-1) which results in TSP-1-enriched platelets as well as inhibited tumor angiogenesis and less metastasis.[31] Li and colleagues noted that MKs suppress tumor growth *in vitro* and *vivo*.[32] Expansion of MKs in the bone marrow upon stimulation with recombinant TPO leads to decreased bone metastasis and tumor burden after intracardiac inoculation of tumor cells.[32] Xu

and colleagues reported that the number of circulating megakaryocytes is associated with better survival in patients with advanced prostate cancer.[33]

In contrast to the results above, it was also reported that the numbers of pulmonary MKs were positively associated with the occurrence of lung metastases.[34] MKs can release bone matrix proteins to make the microenvironment in the bone marrow suitable for the colonization of metastatic cancer cells.[35]

MEG-01, a megakaryoblastic cell line, was established from the bone marrow of a patient with blast crisis of Philadelphia chromosome-positive chronic myelogenous leukemia.[36] MEG-01 cells are commonly used to explore the process of megakaryopoiesis, platelet biogenesis, and the biosynthetic mechanisms of proteins unique to the megakaryocytic lineage.[37] The limitations of the primary megakaryocyte cultures with the administration of TPO *in vitro* are mainly the low cell numbers available for a detailed study of cellular and molecular mechanisms.[38]

Valproic acid (VPA), a short-chain fatty acid and class I/II histone deacetylase inhibitor, is used as an anticonvulsant for the treatment of epilepsy and hematologic malignancies but was also found to induce differentiation of MEG-01 cells into MKs and platelet-like particles.[38,39] VPA enhances the proliferation of CD34 positive cells by phosphorylation of glycogen synthase kinase 3β and engraftment of CD34 positive cells by an increase in C-X-C Motif Chemokine Receptor-4 expression.[40]

1.3 Platelets

Platelets are enucleated and disc-shaped, ranging from 2-4 μm in diameter.[21] They are released by MKs into the bloodstream. The concentration of circulating platelets in the blood of healthy Caucasians ranges from 150,000 to 450,000/μl.[41]

1.3.1 Role of platelets in hemostasis and thrombosis

Platelets play a critical role in hemostasis and thrombosis. Osler first described white thrombi as the result of the hemostatic functions of platelets in 1873. After vascular injury, platelets are exposed to coagulation factors, platelet surface receptors, and negatively charged cell surface.[42] Platelets immediately interact with blood vessels via GPIb-IX-V and Von-Willebrand Factor. Platelets adhere to subendothelial collagen of blood vessels through platelet-specific collagen receptor GPVI and integrin α2β2. Upon activation, platelets undergo a shape change and degranulation to halt bleeding. During activation, platelets release three kinds of granules: alpha granules (200-500 nm in diameter) containing adhesive molecules, such as fibronectin and fibrinogen, coagulation factors, chemokines, and growth factors; lysosomal granules (175-250 nm in diameter) which contain proteases and glycosidase, such as cathepsin and collagenase; and dense granules (250 nm in diameter) containing platelet agonists, such as adenosine diphosphate (ADP), adenosine triphosphate and serotonin.[43]

1.3.2 Platelets and cancer

Besides their role in regulating hemostasis and thrombosis, an association between platelets and cancer has been observed by Trousseau in the 1860s. He discovered that unexpected thrombophlebitis might indicate an occult carcinoma, and this phenomenon was subsequently termed Trousseau's syndrome. He diagnosed the same condition in himself and died of gastric cancer shortly after.[44] Since then, the role of platelets in cancer progression and metastasis has been increasingly recognized.[45] Higher platelet counts or thrombocytosis (≥ 400×10^9/L) correlate with poor prognosis in various malignant entities. The depletion of platelets in animal models reduces metastasis in both xenograft and syngeneic tumor models in a wide range of cancer entities.[46] Yet, the interaction between platelets and cancer cells has not been fully elucidated.

Cancer cells can not only activate platelets in the bloodstream via direct interaction or by soluble mediators like ADP, but also mediate tumor cell induced platelet aggregation (TCIPA), protecting the tumor cells from shear forces and mediating endothelial adhesion through the expression of molecules such as

integrin and P-selectin (CD62P).[47,48] P-selectin is upregulated upon platelet activation, further enhancing the adhesive properties of the tumor cell platelet embolus. After TCIPA, the ability of platelets to secrete pro-angiogenic factors, including vascular endothelial growth factor, is enhanced.[49] Moreover, platelet-derived factors such as transforming growth factor-β (TGF-β) stimulate tumor cell proliferation, convey a mesenchymal-like state to tumor cells, and promote cancer metastasis.[50] Additionally, platelets have been found to mediate tumor cell immune evasion. It was demonstrated that tumor necrosis factor (TNF) superfamily (TNFSF) and TNF receptor superfamily (TNFRSF) molecules including receptor activator of nuclear factor kappa-B ligand (RANKL) and glucocorticoid-induced TNFR-related ligand (GITRL) play a major role in platelet-mediated immune evasion.[51,52]

Reciprocally, tumors can secrete granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor which may stimulate megakaryopoiesis and thrombopoiesis.[53] Tumor-derived interleukin-6 (IL-6) enhances the production of hepatic TPO, resulting in elevated platelet counts.[54] TPO can also be directly produced and released by ovarian and hepatocellular cancer cells.[55,56] Platelets from tumor patients are also referred to as tumor-educated platelets (TEPs) due to their molecular and biochemical changes in mRNA, microRNA, proteins, and exosomes.[57,58] TEPs from patients with tumors of different entities, including lung cancer, glioblastoma, and multiple myeloma, are distinct from healthy individuals and patients with noncancerous diseases.[59,60] TEPs enhance the proliferation, metastasis, and chemotherapy resistance of tumors, and the RNA and protein profiles of tumor-educated platelets can provide specific information on the occurrence and molecular characteristics of cancers.[58] The underlying mechanism of altered RNA and protein profiles in tumor-educated platelets may be due to modulations in megakaryopoiesis, resulting in the transfer of altered RNA and protein content into platelets, changes of RNA splicing and protein synthesis in platelets, and differential platelet aging.[61-63]

1.4 Immune system

The human body is constantly exposed to pathogens and spontaneously transformed cells. A large number of specialized cells and molecules - which together form the immune system - are involved in battling these threats.

1.4.1 Cellular and humoral immunity

The immune system is classically subdivided into cellular and humoral immunity. The cellular part is represented by leukocytes that arise in the bone marrow from pluripotent HSCs. In a first step, multipotent progenitor cells with only limited differentiation potential develop which give rise to common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cells. CMPs differentiate into monocytes, macrophages, dendritic cells (DCs), granulocytes, and mast cells, while CLPs differentiate into B cells, T cells, and Natural Killer (NK) cells. The humoral part of the immune system, in turn, consists of the complement system, acute phase proteins, numerous cytokines, and antibodies.[64-66] A further division of the immune system assigns the cellular and humoral components to innate and adaptive immunity.

1.4.2 Innate immunity

The innate immune system serves as the first line of defense to pathogens, including anatomical barriers, such as the skin and blood-brain barrier, and the cellular and humoral components that can respond to the pathogens within minutes to hours. For example, the complement system, an important humoral component of the innate immune system, can promote the ability of phagocytic cells to remove microorganisms and damaged cells, mediate inflammation, and attack the cell membrane of pathogens. The cellular parts of the innate immune system, especially macrophages and granulocytes, enhance phagocytosis and production of cytokines and chemokines, resulting in recruiting the immune effectors. Macrophages and DCs, also known as antigen-presenting cells, can

collect and express the antigen bound to MHC molecules on their surface to activate adaptive immunity. Activation and effector functions of NK cells are regulated by NK cell receptors' repertoire. NK cells can recognize the abnormal cells via NK cell receptors.[67] Although NK cells were classified to the innate immune system for a long time, more and more scientific findings indicate that NK cells have adaptive immune features, including antigen-specific expansion and differentiation into long-life memory subsets.[68]

1.4.3 Adaptive immunity

A fundamental feature of adaptive immunity is the ability to distinguish foreign (antigens) from self. Adaptive immune responses consist of cell-mediated immune responses and antibody responses which are carried out by T and B cells expressing variable antigen receptors with high specificity. The T cell receptor (TCR) gene segments, including V, D, and J, are combined through site-specific recombination during T cell development in the thymus, resulting in the diversity of TCRs. There is no antigen-driven somatic hypermutation employed in TCR diversification leading to the low affinity of TCRs. By contrast, numerous cell adhesion mechanisms and co-receptors substantially increase the affinity of T cells. T cells undergo both positive selection and negative selection in the thymus. The immature T cells which respond to foreign peptides presented by self MHC proteins survive during positive selection while those strongly reacting with self-peptides complexed with self MHC proteins undergo apoptosis. T cells with receptors strongly reacting with self-antigens are eliminated during negative selection, termed central tolerance, or actively kept suppressed after they leave the thymus by regulatory T cells (Tregs), T-cell anergy, cell-extrinsic tolerogenic signals, and peripheral clonal deletion. During the positive selection, T cells with receptors recognizing MHC-I molecules are selected to become cytotoxic cells while T cells with receptors recognizing MHC-II molecules are selected to become helper cells. T cells are activated into effector T cells in peripheral lymphoid organs by engaging with antigenic peptide and MHC in the presence of co-stimulatory signals on the surface of antigen-presenting cells.[69] Cytotoxic CD8

positive T cells recognize peptides derived from intracellular antigens presented via MHC-I and then induce the lysis of infected and transformed cells. CD4 positive T cells recognize peptides derived from extracellular proteins presented by MHC-II on antigen-presenting cells.[70] CD4 positive T cells then have numerous immunoregulatory functions including activation of B cells. Together with the co-stimulatory signals from T cells the activation of the B cells is also controlled by the B cell receptor (BCR). Upon activation, B cells differentiate into antibody-producing plasma cells and memory B cells. Antibodies are important components of humoral adaptive immunity.[71]

Another feature of adaptive immunity is the long-term memory of a given pathogen or related structures. Memory B and T cells enable a rapid and highly specific immune response upon renewed contact with the same pathogen.

1.4.4 Tumor immune evasion

Although the immune system utilizes multiple methods to eliminate transformed cells, cancer cells often take advantage of immune tolerance and suppressive mechanisms to escape from immune surveillance. To understand the dual elimination and protection effects of immunity on cancer, the cancer immunoediting model was established which is based on numerous scientific findings. This model assumes three main phases in cancer immune surveillance: elimination, equilibrium, and escape.[72] The transformed cells are killed by both innate immunity and adaptive immunity during the elimination phase, but only a few malignant cells with non-immunogenic or resistant phenotype survive. Under the selective pressure caused by anti-tumor therapies or immune surveillance, the surviving cells develop with various mutations that further enhance resistance to immune detection during the equilibrium phase. Tumor cells continue to proliferate and expand in an uncontrolled manner leading to cancer progression and metastasis in the escaping phase.[72]

Tumor cells exploit various mechanisms to escape from the immune system, including modulation of antigen-presenting cells, effector cells, and tumor cells. Inhibitory molecules in the tumor microenvironment like TGF-β and IL-6 impede

the maturation and activation of antigen-presenting cells and increase proliferation of regulatory antigen-presenting cells and Tregs.[73] Tumor cells produce CD95 ligand to activate the CD95 on T cells leading to enhanced T cell apoptosis and ultimately cancer invasiveness.[74] The selective pressure of cytotoxic agents or immune surveillance promotes the resistance to apoptosis in cancer cells.[75] Alterations in MHC I expression disturb T-cell activation and recognition.[76]

1.4.5 Immune checkpoint molecules

Immune tolerance is also regulated by co-stimulatory and co-inhibitory molecules, termed immune checkpoint molecules. The latter can regulate the immune response of T, NK, and myeloid cells.[77,78] To date, important and drugable co-inhibitory molecules for T cells include cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) while the co-stimulatory molecules on T cells include CD28/CD80/CD86 and GITR/GITRL. Based on an increasing understanding of the underlying mechanisms, the strategy to therapeutically block inhibitory immune checkpoint molecules was introduced in cancer patients to dampen immune suppression. Immune checkpoint blockade abrogates suppression of T cell activation and eliminates T cell inhibitory signals, which empowers tumor-reactive T cells to perform an effective antitumor response. Immune checkpoint blocking antibodies including ipilimumab, which targets CTLA-4, and pembrolizumab and nivolumab, which target PD-1, have been approved by the FDA for the treatment of meanwhile various cancer entities, with ipilimumab being the first immune checkpoint inhibitor that was clinically approved. During the early phase of T cell activation, CTLA-4 is physiologically upregulated on the T cell membrane to maintain normal immunologic homeostasis by inhibiting T cell function through the induction of the cell cycle arrest and the prevention of co-stimulation by binding competitively CD28 for its ligands, B7-1 and B7-2 (CD80/86).[79-81] Ipilimumab improved the median overall survival from 6.4 months to 10 months in advanced melanoma patients.[82]

Upon interaction with PD-L1 and PD-L2, PD-1 inhibits the function of effector T cells by suppression of kinase signaling pathways which lead to T cell activation at a variety of stages of the immune response.[83-85] Anti-PD-1 (nivolumab, pembrolizumab, pidilizumab) and anti-PD-L1 antibodies (atezolizumab, durvalumab, BMS-936559, and MSB0010718C) have achieved long-lasting responses with limited toxicity in different cancer entities.[86,87]

However, successful responses to immune checkpoint blockade are currently limited to a minority of patients and indications. Various co-stimulatory and co-inhibitory molecules as potential immune checkpoint targets are still under intensive investigation. Meanwhile, clinical parameters of immune or tumor characteristics in patients are lacking to accurately predict response to immunotherapeutics.[88,89] Biomarkers for the evaluation of the efficacy of immune checkpoint blockade and disease outcome are thus urgently needed.

1.4.6 TNFSF/TNFRSF

As mentioned above, several members of the TNFSF/TNFRSF, such as GITR/GITRL, serve as immune checkpoints and are evaluated as targets for immunotherapy. The TNFSF/TNFRSF members include 19 ligands and 29 receptors which are involved in the control of various cellular processes like embryonic development, tissue-homeostatic processes, and immune functions. Some TNFSF/TNFRSF members can induce apoptosis and other forms of cell death, others exhibit pro-inflammatory properties partially via activation of nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathways.

The members of TNFSF share the TNF homology domain which binds to a cysteine-rich domain in the N-terminus of TNFRs.[90] For some of the TNFSF members, such as ectodysplasin A, the soluble form is the active form. However, for others, such as CD95 ligand, solubilization inhibits the physiological function. The members of the TNFSF are not only present on immune cells including NK cells, T cells, and DCs but also expressed on tumor cells, for example 4-1BB ligand on chronic lymphocytic leukemia and RANKL on breast cancer and colorectal carcinoma cells.[52,91]

The solubilization of TNFRSF members is generated by alternative splicing or proteolytic processing. The extracellular domains of TNFRSF members contain six cysteine residues involved in the formation of three disulfide bonds.[92] TNFRs, can be divided into two groups according to their structure: death receptors containing the death domain (DD) and non-death receptors without DD. Death receptors are characterized by a long alpha-helical fold, including TNFR1, Fas (CD95), death receptor 3, TNF-related apoptosis-inducing ligand receptor 1 and 2, and death receptor 6.[93] After the interaction of DD and DD-containing adaptor molecules, a membrane-proximal scaffolding complex is formed initiating caspase recruitment to activate and induce apoptosis.[93] Non-death receptors, e.g. CD40 and lymphotoxin receptor, either recruit TNFR-associated factors to activate non-death signaling pathways leading to cell survival, proliferation, and cytokine production.[94]

1.4.7 GITR/GITRL system

GITR, also known as activation-inducible TNFR family member or TNFRSF18, can be detected on effector CD4 positive and CD8 positive T cells at low levels and CD4 positive CD25 positive Tregs at high levels.[95,96] GITR is also expressed on macrophages and NK cells.[97,98] GITRL can be a trimer, monomer, or multimeric structure the formation of which results in the recruitment of different adaptor molecules. Expression of GITRL, detected in endothelial cells, macrophages, DCs, and platelets, can be upregulated by pro-inflammatory cytokines.[95,99] GITRL was found to be expressed in various primary solid tumors, primary leukemia cells, and tumor cell lines.[100] Levels of soluble GITRL are elevated in sera from cancer patients.[100] During maturation of megakaryocytes, GITRL is upregulated. This leads to GITRL expression in their platelets progeny.[51] Stimulation of human GITR by an agonistic antibody, by GITRL overexpression or upon application of soluble GITRL has been shown to eliminate the suppressive function of CD4 positive CD25 positive Tregs and to increase T cell proliferation and cytokine production. GITR can protect T cells from activation-induced cell death and may abrogate suppression of Tregs on

responder T cells in *Gitr*-/- mice.[101,102] DTA-1, an agonistic GITR antibody, eradicated established fibrosarcoma in *BALB/c* mice with minor adverse effects, including mild autoimmunity symptoms. Upon rechallenging with 10-fold higher tumor cell numbers, the tumor regressed and degenerated indicating the development of specific antitumor immunity. The number of IFN-γ secreting T cells was increased by DTA-1 treatment.[103] The potent antitumor effects of DTA-1 occurred upon a decrease of tumor-infiltrating and circulating Tregs.[96]

It was shown that GITR/GITRL impairs IFN-γ production and cytotoxicity of NK cells.[104] Cancer cells, which are coated by GITRL-expressing platelets and thus show a "GITRL pseudo expression", impair cytotoxicity of NK cells.[51] Taken together, GITR inhibits NK cell antitumor activity while it apparently acts as an activating receptor on T cells.[51,96] The role of GITR in antitumor immunity thus depends on the time point, cellular activity, level of an ongoing immune response, and the respective cell type.[105]

The role of GITR/GITRL in tumor progression and immune evasion stimulated further investigation for immunotherapeutic intervention in patients.

1.5 Aim of the thesis

GITRL is gaining more and more attention as a novel target for immune checkpoint therapy. The role of platelets in cancer progression and immune evasion is increasingly recognized, but surprisingly little is known concerning the role of platelet-derived GITRL (pGITRL) in the context of cancer. It is known that most subtypes of breast cancer are immunologically silent which makes immunotherapy for these patients particularly challenging, and new targets are needed for intervention. We here studied the expression of pGITRL and GITR on immune effector cells in breast cancer patients. So far, no comprehensive analysis concerning the relevance of pGITRL in the pathophysiology of breast cancer exist. In this thesis, we performed a systematic investigation of the association of pGITRL and clinical characteristics that reflect different disease states in breast cancer. Furthermore, the regulation of GITRL expression by breast cancer was studied in a model of megakaryopoiesis. It is already known

that pGITRL is upregulated upon platelet activation in the benign situation. But the influence of the tumor on pGITRL levels in breast cancer is not understood yet. We thus analyzed the modulation of pGITRL upon platelet activation in breast cancer. As it has already been described that GITRL was upregulated during megakaryopoiesis, we further explored the regulation of GITRL by soluble factors derived from breast cancer cells. Together, this thesis aims to gain a deeper insight into the role of pGITRL in breast cancer progression and to develop a rationale to further explore the GITR/GITRL axis as a target for immune checkpoint therapy.

2 Material and methods

2.1 Material

2.1.1 Equipment

Table 3: Equipment used in the project

Equipment	Manufacturer
BD LSRFortesa™ cell analyzer	BD, Heidelberg, Germany
BD FACSCanto™	BD, Heidelberg, Germany
Block heater	Neolab, Rittersteig, Germany
Cell counting Neubauer chamber	Brand, Werheim, Germany
Cell culture incubator Heraeus HERA Cell 240	Heraeus, Hanau, Germany
Cell culture incubator Nuaire	Nuaire, Caerphilly, UK
Cell culture incubator Thermo Scientific	Thermo, Waltham, Massachusetts, USA
Centrifuge Desktop	Biozym, Illinois, USA
Centrifuge Fresco 17	Eppendorf, Hamburg, Germany
Centrifuge Multifuge 3 S	Heraeus, Hanau, Germany
Centrifuge Multifuge 3 S-R	Heraeus, Hanau, Germany
Centrifuge Varifuge 3.0 L-R	Heraeus, Hanau, Germany
Clean bench Herasafe	Heraeus, Hanau, Germany
Refrigerator 4°C	Bosch, Gerlingen, Germany
Freezer -20°C	Bosch, Gerlingen, Germany
Freezer -80°C	Thermo, Waltham, Massachusetts, USA
Freezer -80°C	SANYO, Osaka, Japan
Glass Equipment	Schott, Mainz, Germany
Liquid nitrogen tank LS 4800	Taylor-Wharton, Theodore, AL, USA

Magnetic stirrer MR3002	Neolab, Heidolph, Germany
Multichannel pipette	Eppendorf, Hamburg, Germany
Multistep pipette	Eppendorf, Hamburg, Germany
pH-meter Hydrus 100	Thermo, Schwerte, Germany
Pipetboy comfort	Integra Biosciences, Fernwald, Germany
Pipettes	Eppendorf, Hamburg, Germany
Table Scale AT261 Delta Range	Mettler Toledo, Columbus, Ohio, USA
Thermomixer compact	Eppendorf, Hamburg, Germany
Transmitted-light microscope, Axiovert 25	Zeiss, Jena, Germany
Tube roller	Start lab, Brussels, Belgium
Vacuum pump Vacusafe	Intera, Fernwald, Germany
Vortex-Genie 2	Heidolph, Zurich, Switzerland
Water Bath, W22	Störk-Tronic , Stuttgart, Germany

2.1.2 Consumables

Table 4: Consumables used in the project

Consumables	Company
5 ml Polystyrene round-bottom tube, 12×75 mm style	Corning Science Mexico S.A. de C.V. Reynosa, Mexico
Cell culture flasks (25 cm^2,75 cm^2)	Corning, New York, USA
Cell culture plates (6-,24-, 48-, 96-well)	Costar, New York, USA
Cryotubes (2 ml)	Thermo Scientific, Massachusetts, USA
Falcon tube (15 ml, 50 ml)	Greiner, Frickenhausen, Germany
Gloves	NitraTex, Staffordshire, United Kingdom
Microplate, 96 well, Polystyrene, V-bottom, Clear	Greiner Bio-One, Frickenhausen, Germany
Polypropylene-micro plate, 96 well, U-shape	Greiner Bio-One North America, Monroe, USA
Safe-lock Reaction tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg, Germany
SafeSeal-tips (10 µl, 200 µl, 1,000 µl)	Biozym, Oldendorf, Germany
Serological pipettes	Costar, New York, USA
Sterile filter (0.2 µm, 0.33 µm, 0.45 µm)	Merck, Darmstadt, Germany
Syringe, 10 ml, 50 ml	Henke-Sass, Wolf, Tuttlingen, Germany

2.1.3 Reagents, buffers, and medium

Table 5: Reagents used in the project

Reagents	Company
1x Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Paisley, UK
1x Dulbecco's phosphate-buffered saline (PBS)	Gibco, Paisley, UK
1x Roswell Park Memorial Institute (RPMI)-1640	Gibco, Paisley, UK
Ampuwa	Fresenius Kabi, Bad Homburg, Germany
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich, St. Louis, MO, USA
EDTA Solution	Cambrex, Rockland, ME, USA
Ethanol purity > 99.9% p.a.	SAV, Flintsbach am Inn, Germany
Fetal Calf Serum (FCS)	Gibco, Paisley, UK
Ficoll Solution	Biochrom AG, Berlin, Germany
Formaldehyde, 37% solution	AppliChem
L- Glutamine 200 mM	Lonza, Basel, Switzerland
Penicillin 10 000 U/ml Streptomycin 10 mg/ml (Pen/Strep)	Lonza, Basel, Switzerland
Sodium azide (NaN_3)	Sigma Aldrich, St. Louis, MO, USA
Sodium hydroxide (NaOH), 4 mol/L	Merck, Darmstadt, Germany
Sodium pyruvate solution	Lonza, Basel, Switzerland
Trypan Blue Stain 0.4%	Thermo Fisher, Waltham, MA, USA
Trypsin/EDTA	Sigma Aldrich, St. Louis, MO, USA
Türks Solution	Merck, Darmstadt, Germany

Table 6: Buffers used in the project

Buffer	Components
Cell FACS buffer (CFB)	0.01% w/v NaN_3 1% v/v FCS 99% v/v 1X PBS
Platelet FACS buffer (PFB)	99% PBS 1% fetal calf serum (FCS)

Table 7: Medium used in the project

medium	Components
Freezing medium	70% v/v RPMI-1640 20% v/v FCS 10% v/v DMSO

2.1.4 Antibodies and cytokines

Table 8: Staining agents used in this project

Staining agent	Supplier	Final concentration
Aqua fluorescent reactive dye	Invitrogen	1:1000
CD14-BV785	Biolegend	10 µg/ml
CD16- Allophycocyanin(APC)	eBioscience	10 µg/ml
CD19-FITC	BD Pharmingen/BD Biosciences	10 µg/ml
CD3-APC/fire	Biolegend	10 µg/ml
CD41a-PE-Cy-5	BD Pharmingen/BD Biosciences	1:5
CD4-Pacific Blue	Biolegend	10 µg/ml
CD56-Pecy7	Biolegend	10 µg/ml
CD62P-FITC	BD Pharmingen/BD Biosciences	1:5
CD61-FITC	BD Pharmingen/BD Biosciences	1:500
CD8-BV605	Biolegend	10 µg/ml
GITR	R&D	10 µg/ml
GITRL	R&D	20 µg/ml
Goat-anti-mouse-PE	Dako	10 µg/ml
hIgG	Sigma	2 µg/ml
hIgG2	Sigma	10 µg/ml
HLA-ABC-PE	BD Pharmingen/BD Biosciences	1:5
HLA-DR-BV650	Biolegend	10 µg/ml

2.1.5 Cell lines

Table 9: Description of cell lines and their culture conditions

Cell line	Host organism and description	Growth characteristics	Culture medium
MCF 7	Human, breast invasive ductal carcinoma	Adherent	DMEM 10% FCS 1% Pen/Strep
MDA-MB 231	Human, breast adenocarcinoma	Adherent	DMEM 10% FCS 1% Pen/Strep
MDA-MB 468	Human, breast adenocarcinoma	Adherent	DMEM 10% FCS 1% Pen/Strep
MEG-01	Human, megakaryoblast	Mixed, adherent and suspension	RPMI-1640 10% FCS 1% Pen/Strep
SK-BR-3	Human, breast adenocarcinoma	Adherent	RPMI-1640 10% FCS 1% Pen/Strep

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Freezing and thawing of cells

Cells were harvested during their exponential growth phase, counted and centrifuged (1500 rpm, 5 minutes). The supernatant was removed and 5×10^6 cells were resuspended in 1 ml freezing medium and transferred into cryotubes. The cells were immediately stored at - 80 °C for at least one day and transferred

into liquid nitrogen for long term storage. For thawing, frozen cells were quickly thawed in a water bath at 37 °C. The cell suspension was then immediately diluted in 10 ml of the respective prewarmed culture medium and centrifuged (300 g, 8 minutes) (for the respective media see **Table 9**). The supernatant was discarded and the cells were resuspended in 10 ml of their respective culture medium and seeded in a 75 cm^2 cell culture flask.

2.2.1.2 Culture of cell lines and passaging

All cells were cultured at 37 °C, 5% CO_2, and 90% relative humidity. Cell numbers were determined using a Neubauer chamber and a Zeiss Axiovert 25 microscope. Tumor cells that were growing in suspension were passaged 2-3 times per week to keep the cells at a density of 0.5 - 1.0x10^6 cells/ml. The cell suspension was collected and transferred into a 15 ml Falcon tube and centrifuged (1500 rpm, 5 minutes). The supernatant was aspirated and the pellet was resuspended in fresh culture medium (for the respective media see **Table 9**). Cells were cultured at a ratio of 1:10 and moved to a new culture flask in a total of 10 ml medium. Adherent cells were split for 2-3 times per week to keep at a confluence level of approximately 30-70%. Adherent cells were passaged before reaching confluence using Trypsin-EDTA (1 ml per 75 cm^2 flask) for 5-10 minutes at 37°C. Then, 7 ml medium was added to stop the reaction. Cells were pelleted by centrifugation (1500 rpm, 5 minutes), the supernatant was removed and cells were resuspended in 10 ml of the respective culture medium. Cells were cultured at a ratio of 1:10 and moved to a new 75 cm^2 flask in a total of 10 ml medium.

2.2.2 Isolation of platelets

Blood samples of breast cancer patients and healthy donors were obtained after written informed consent following the Declaration of Helsinki. This study was approved by the Ethics Committee at the Medical Faculty of the Eberhard Karls University and the University Hospital Tübingen (reference no. 13/2007V). The

approval date was September 21st, 2017. Citrated blood from breast cancer patients and healthy donors who did not take any anticoagulants for at least 10 days before blood collection, was centrifuged (20 minutes, 120 g without brake) and the upper layer was harvested as platelet-rich plasma (PRP). Subsequently, the fixation of platelets was performed by incubation of PRP with 4% paraformaldehyde (PFA)-PBS (final concentration for PFA 2%) for 10 minutes at room temperature (RT) followed by two washing steps using PFB (1200 g,10 minutes). The fixed platelets were stored at 4°C for a maximum of one week before further analyses.

2.2.3 Treatment of MEG-01 cells with supernatant from breast cancer cell lines

MEG-01 cells were seeded at 0.3 million cells/ml in 12-well culture plates and incubated overnight in standard medium. Breast cancer cells were seeded at 1 million/ml respective culture medium and incubated for 24 hours. Then, supernatants from breast cancer cell lines was centrifuged and added to MEG-01 cells (final dilution 1:2). After culture for one day, MEG-01 cells were harvested and subsequently analyzed by flow cytometry.

2.2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by Ficoll/Bicoll density gradient centrifugation of peripheral blood. The heparinized blood was diluted in PBS at least twofold to a final volume of 175 ml. 15 ml of Ficoll solution were carefully floated with 35 ml of diluted blood suspension per reaction tube. Density gradient centrifugation was performed without brake (2000 rpm, 18 minutes). Cells in the interphase between Ficoll and plasma were collected and washed twice with PBS (1500 rpm, 5 minutes). For removal of residual platelets, cells were resuspended in PBS and centrifuged at 800 rpm for 10 minutes. Isolated PBMCs were resuspended in 30 ml of PBS and counted in a Neubauer chamber. PBMCs were then either cultured in the

standard medium at a density of 5×10^6 cells/ml or frozen at 2×10^7 cells/ml in the freezing medium.

2.2.5 Flow cytometry

For flow cytometric analyses of platelets, samples were blocked (20 minutes, RT) using PFB containing hIgG to prevent unspecific binding of staining reagents. Platelets were transferred into a 96-well polypropylene microplate and centrifuged (1000 g, 2 minutes). After removal of the supernatant, for indirect staining the samples were resuspended in either 50 µl staining solution of the specific mAb or an isotype control. After incubation (15 minutes, RT), the samples were washed twice with PFB. Then, the samples were resuspended in 50 µl of goat-anti-mouse-PE and incubated in the dark (13 minutes, RT). For staining with directly conjugated staining agents, samples were incubated with the respective antibody solution in the dark (13 minutes, RT). Staining agents were diluted in PFB containing hIgG. Analysis of samples was performed using a BD LSR Fortessa™.

For staining of tumor cells or PBMCs, samples were blocked (20 minutes, RT) using CFB containing hIgG to prevent unspecific binding of staining reagents. Cells were transferred into a 96-well polystyrene microplate and centrifuged (2000 rpm, 2 minutes). After removal of the supernatant, the samples were resuspended in 50 µl of the staining solution either consisting of the specific mAb or an isotype control. After incubation (25 minutes, RT), the samples were washed twice with CFB. For staining with directly conjugated staining agents, samples were incubated in dark (18 minutes, RT). All staining agents were diluted in CFB containing hIgG. Samples were incubated with aqua fluorescent reactive dye for the staining of dead cells for 30 minutes on ice. Subsequently, flow cytometric measurement was performed using a BD FACSCanto™ II cell analyzer or BD LSRFortessa™.

The expression of a specific molecule was analyzed as percent positive platelets or cells and was calculated as follows: “percent surface expression obtained with specific antibody” - “percent surface expression obtained with isotype control”. B

cells were characterized by CD19 positive, T cells by CD3 positive, and NK cells by CD56 positive, CD3 negative. Platelets were characterized as CD41a positive and CD62P negative (resting) or CD62P positive (activated).

2.2.6 Statistics

For the continuous variables, the D'Agostino-Pearson normality test was used to test the distribution of the variables before analysis of statistical differences. For two groups of continuous and unpaired variables, if the values differ from Gaussian distribution, Mann-Whitney U test was used. For continuous variables of more than two groups, if the values differ from Gaussian distribution, nonparametric test was used, while, if the values fit Gaussian distribution, one-way ANOVA test was used. For categorical data we used chi‑squared test or Fisher's exact test. Correlation of platelet activation and GITRL expression and Ki67 was analyzed using linear regression analysis. The predictive value of pGITRL was evaluated by examining the area under the receiver‑operator characteristic (ROC) curve with a confidence interval of 95%. For the comparison of the ROC curves, the DeLong method was employed.[106] For correlation studies of pGITRL and different clinical parameters Odds ratios (OR) were calculated. High pGITRL expression was defined as follows: pGITRL high = mean pGITRL (Healthy donor, HD) + 2 standard deviation (SD) pGITRL (HD). All statistical tests were considered significant when p was below 0.05.

3 Results

3.1 pGITRL and GITR on lymphocytes in breast cancer

3.1.1 Expression of pGITRL in platelets

When tumor cells leave the primary site and enter the blood circulation, they get immediately coated by platelets which promotes metastasis by multiple mechanisms. Among others, platelets mediate evasion from immune surveillance, e.g. of cytotoxic lymphocytes. Platelets reportedly express the immune checkpoint molecule GITRL which inhibits NK reactivity, but little is known about the expression and function of pGITRL in the context of solid tumors. We characterized levels of pGITRL in breast cancer patients using flow cytometry. Platelets were identified as subcellular, CD41a positive particles **(Figure 1)**.

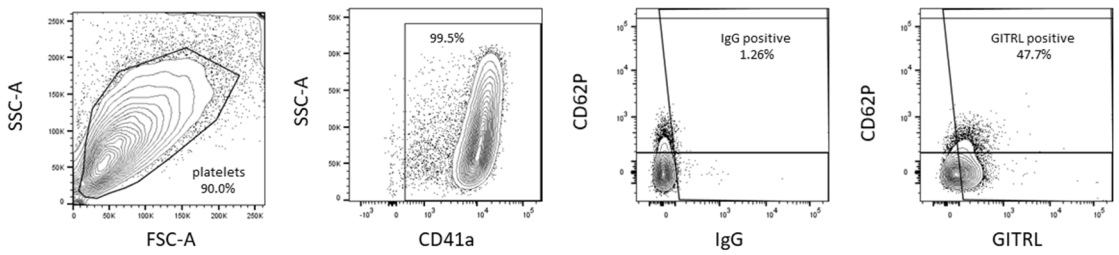


Figure 1: Gating strategy used to analyze GITRL expression on platelets *ex vivo*. Expression of GITRL, the platelet marker CD41a, and the platelet activation marker CD62P on the surface of platelets were determined by flow cytometry using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. Samples where co-stained with PE-Cy-5-conjugated CD41a mAb and by FITC-conjugated CD62P mAb.

While platelets of healthy individuals are known to express moderate levels of pGITRL, the levels on platelets from breast cancer patients were found to be significantly higher (p = 0.001, Mann-Whitney U test) (**Figure 2**).[51] The clinical characteristics of the breast cancer patients used in this study are presented in **Table 10**.

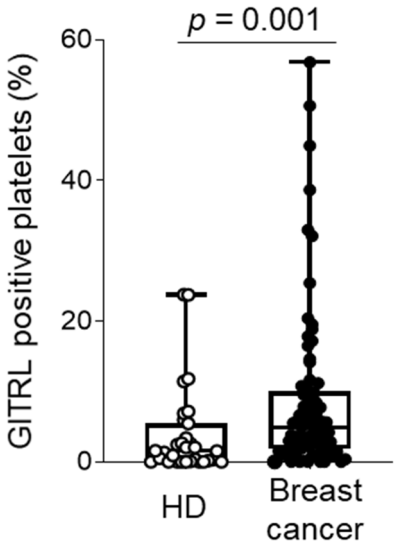


Figure 2: pGITRL levels in breast cancer patients. Platelets from HD and breast cancer patients were analyzed for surface expression of GITRL by flow cytometry. A summary of pGITRL levels obtained in a total of 31 HD (open dots) and 79 breast cancer patients (shaded dots) are depicted. Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of pGITRL levels. Whiskers indicate minimum and maximum of pGITRL levels. Mann-Whitney U test was used for statistical analysis. Statistically significant differences were defined by $p < 0.05$.

Table 10: Clinical characteristics of breast cancer patients in this project

Patient characteristics	**Total (**n=79)
Age	
Age in years, mean–yr. ± SD (range)	60.1 ± 13.5 (27 to 87)
Gender	
Female, n (%)	78 (98.7)
TNM classification, n (%)	
Tumor size	
T0	8 (10.1)
T1	25 (31.6)
T2	28 (35.4)
T3	11 (13.9)
T4	7 (8.9)
Node	
N0	43 (54.4)
N1	23 (29.1)
N2	10 (12.7)
N3	3 (3.8)
Metastasis	
M0	59 (74.7)
M1	20 (25.3)
Localization of primary tumor, n (%)	
Right	35 (44.3)
Left	44 (55.7)
Histological grading, n (%)	
G1	10 (7.9)
G2	33 (41.8)
G3	35 (44.3)
Unknown	1 (1.3)
Receptor status, n (%)	
ER positive	57 (72.2)
PR positive	52 (65.8)
HER-2 positive	48 (60.8)
Treatment, n (%)	
Adjuvant chemotherapy	20 (25.3)
Neoadjuvant chemotherapy	20 (25.3)
Adjuvant Endocrine therapy	17 (21.5)
Adjuvant radiation	18 (22.7)

3.1.2 GITR expression on B, T, and NK cells

Next, the expression of GITR, the cognate receptor of GITRL, on immune effector cells from seven breast cancer patients and 14 HD was comparatively analyzed. To this end, PBMCs were freshly isolated and characterized by flow cytometry **(Figure 3)**.

GITR expression on B cells from breast cancer patients was not significantly altered compared to HD (p = 0.29, unpaired t test) **(Figure 4a)**. GITR levels tended to be higher on T cells from breast cancer patients as compared to HD (p = 0.064, unpaired t test) **(Figure 4b),** whereas expression on NK cells was significantly enhanced in breast cancer patients (p = 0.036, unpaired t test) **(Figure 4c)**.

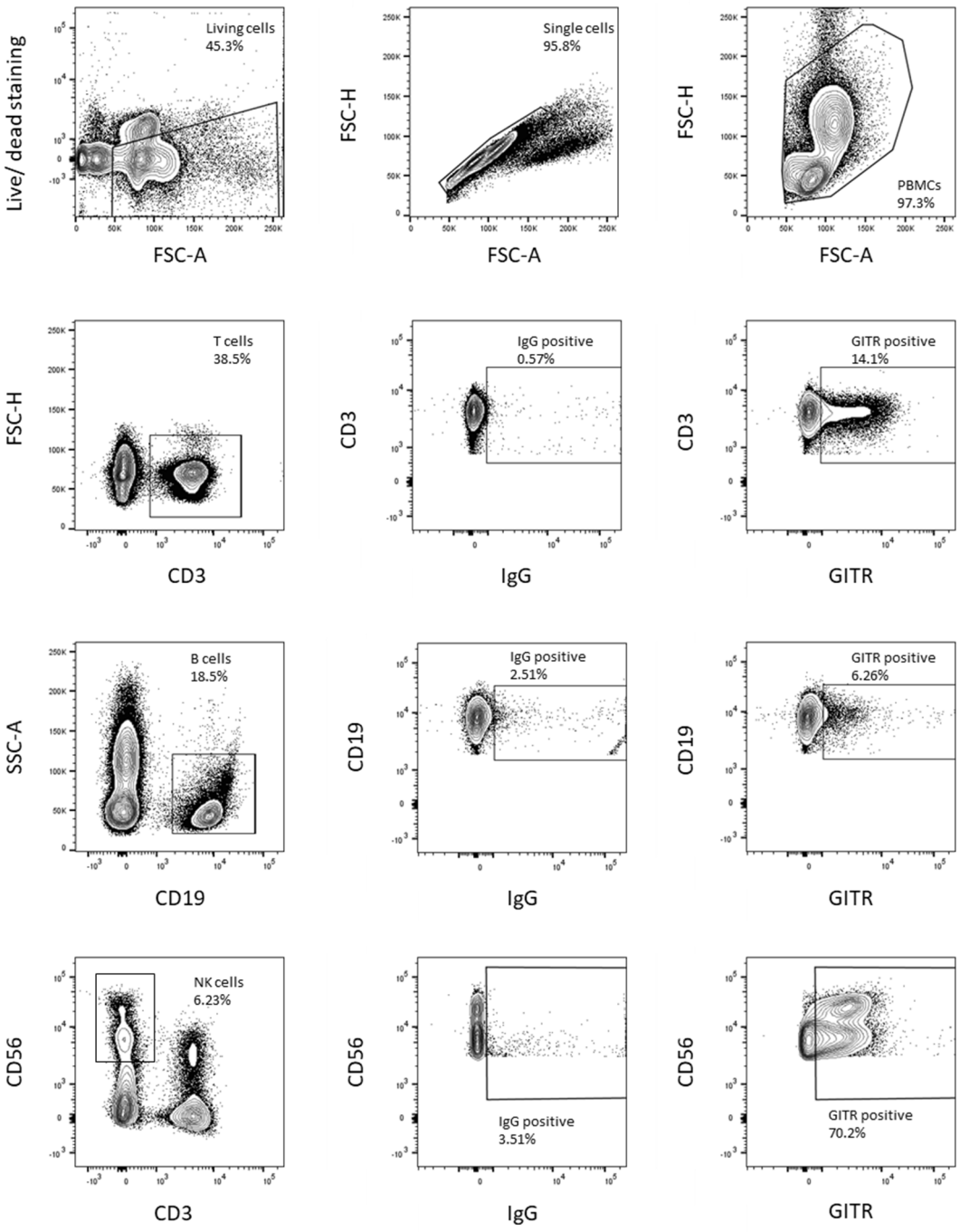
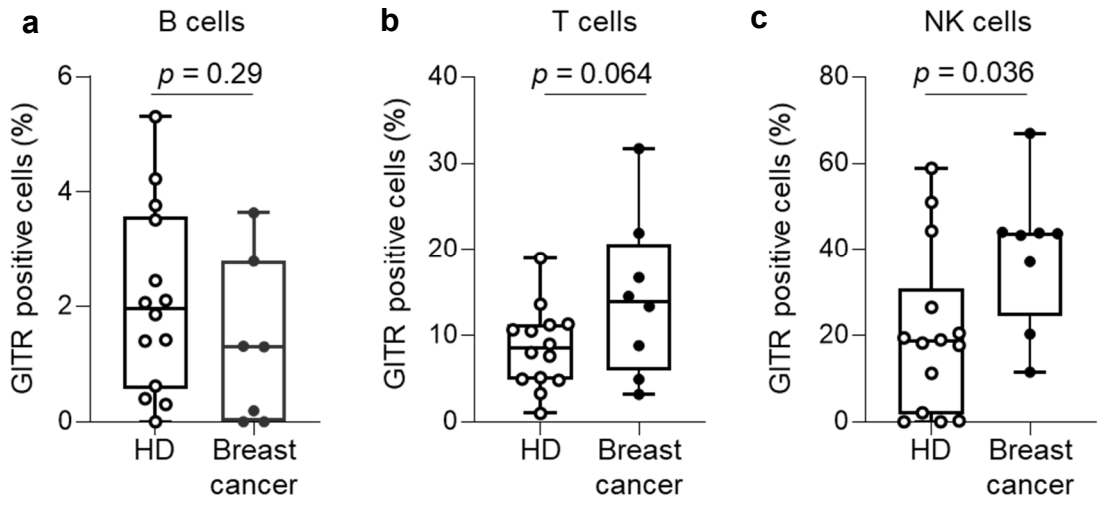


Figure 3: Gating strategy used to analyze GITR expression on PBMCs. Aqua fluorescent reactive dye was used to distinguish living and dead cells. Expression of GITR on the surface of PBMCs from healthy donors and breast cancer patients was determined by flow cytometry using either specific GITR mAb or isotype controls followed by goat-anti-mouse-PE. Cells were co-stained by APC/fire-conjugated-CD3 mAb, Pecy7-conjugated-CD56 mAb and FITC-conjugated-CD19 mAb or isotype control. B cells were characterized by CD19 positive, T cells by CD3 positive, and NK cells by CD56 positive CD3 negative.



F**igure 4: Expression of GITR on B, T, and NK cells.** Freshly isolated PBMCs from HD and breast cancer patients were analyzed for surface expression of GITR by flow cytometry. GITR levels in B cells (a), T cells (b), and NK cells (c) obtained in a total of 14 HD (open dots) and 7 breast cancer patients (shaded dots) are depicted. Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITR levels. Whiskers indicate minimum and maximum of pGITRL levels. Unpaired t test was used for statistical analysis. Statistically significant differences were defined by $p < 0.05$.

3.1.3 Correlation of pGITRL levels and GITR expression on lymphocytes

To unravel the involvement of pGITRL in the immune surveillance in solid tumors, we studied the association of pGITRL and its cognate receptor GITR in breast cancer patients. pGITRL expression was negatively associated with GITR levels on T cells in our cohort (p = 0.04, linear regression) **(Figure 5a),** while there was no correlation between pGITRL and GITR on NK cells (p = 0.97, linear regression) **(Figure 5b)**. This may indicate that GITR on T cells - upon interaction with pGITRL - is reduced, which may serve as negative feedback mechanism to modulate T cell activation via the GITR/GITRL-axis.

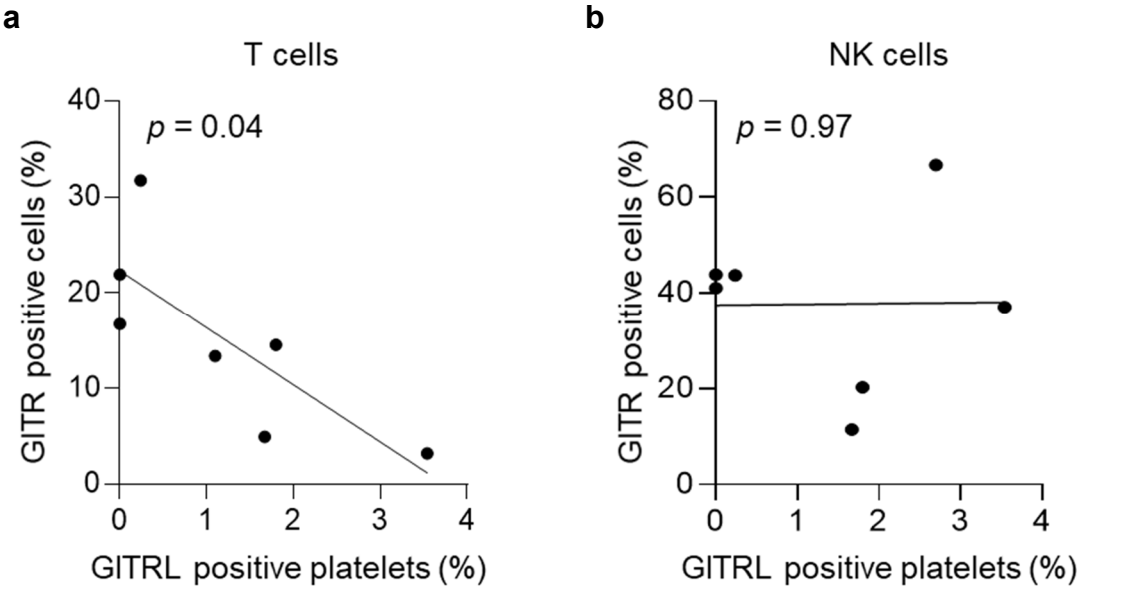


Figure 5: Correlation of pGITRL levels and GITR expression on lymphocytes. T cells (a) and NK cells (b) from breast cancer patients were analyzed for surface expression of GITR and platelets from the same patients were analyzed for surface expression of GITRL by flow cytometry. Surface expression of a total of 7 breast cancer patients is depicted. The solid line is the regression line by simple linear regression.

3.2 pGITRL expression upon platelet activation in breast cancer

3.2.1 Induction of pGITRL upon platelet activation

pGITRL expression can be enhanced upon treatment with platelet agonists including ADP, thrombin, and collagen in vitro[52]. However, the regulation of pGITRL following platelet activation remains to be elucidated in the context of solid tumors, where platelets have been demonstrated to play a crucial role in tumor promotion. We here studied the association of pGITRL levels and platelet activation in 79 breast cancer patients and 31 HD. The gating strategy used to analyze pGITRL upon platelet activation is shown in **Figure 6**.

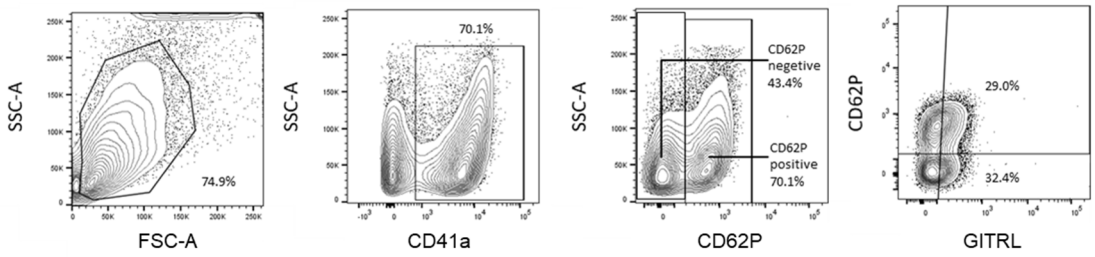


Figure 6: Gating strategy used to analyze pGITRL expression upon platelet activation. Expression of GITRL on the surface of platelets from HD and breast cancer patients was determined by flow cytometry using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. Platelets were costained by PE-Cy-5-conjugated CD41a mAb and FITC-conjugated CD62P mAb. Resting platelets were characterized as CD41a positive/CD62P negative, activated platelets were characterized as CD41a positive/CD62P positive.

First, the expression levels of the activation marker CD62P were compared on both, platelets from breast cancer patients and HD. Similar platelet activation was observed in HD (19.4% CD62P positive platelets, 95% confidence interval (CI): 10.4-38.6) compared to breast cancer patients (18.6%, 95% CI: 6.3-49.8) (p = 0.57, unpaired t test) (**Figure 7**).

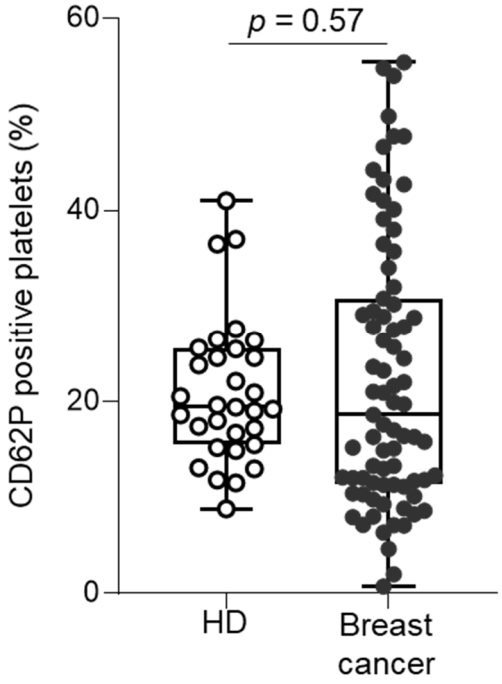


Figure 7: Preexisting activation of platelets *ex vivo*. Expression of the platelet activation marker CD62P on the surface of platelets from HD and breast cancer patients was determined by flow cytometry using FITC-conjugated CD62P mAb. CD62P levels in platelets obtained in a total of 31 HD (open dots) and 79 breast cancer patients (shaded dots) are depicted. Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of CD62P levels. Whiskers indicate minimum and maximum of CD62P levels. Unpaired t test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$.

Second, pGITRL expression upon platelet activation was analyzed. pGITRL levels in 31 HD did not change in dependence of the platelet activation status ($p = 0.78$, unpaired t test). On the contrary, pGITRL expression was significantly increased in activated platelets (CD62P positive) as compared to resting platelets (CD62P negative) of breast cancer patients ($p = 0.002$, unpaired t test) **(Figure 8)**.

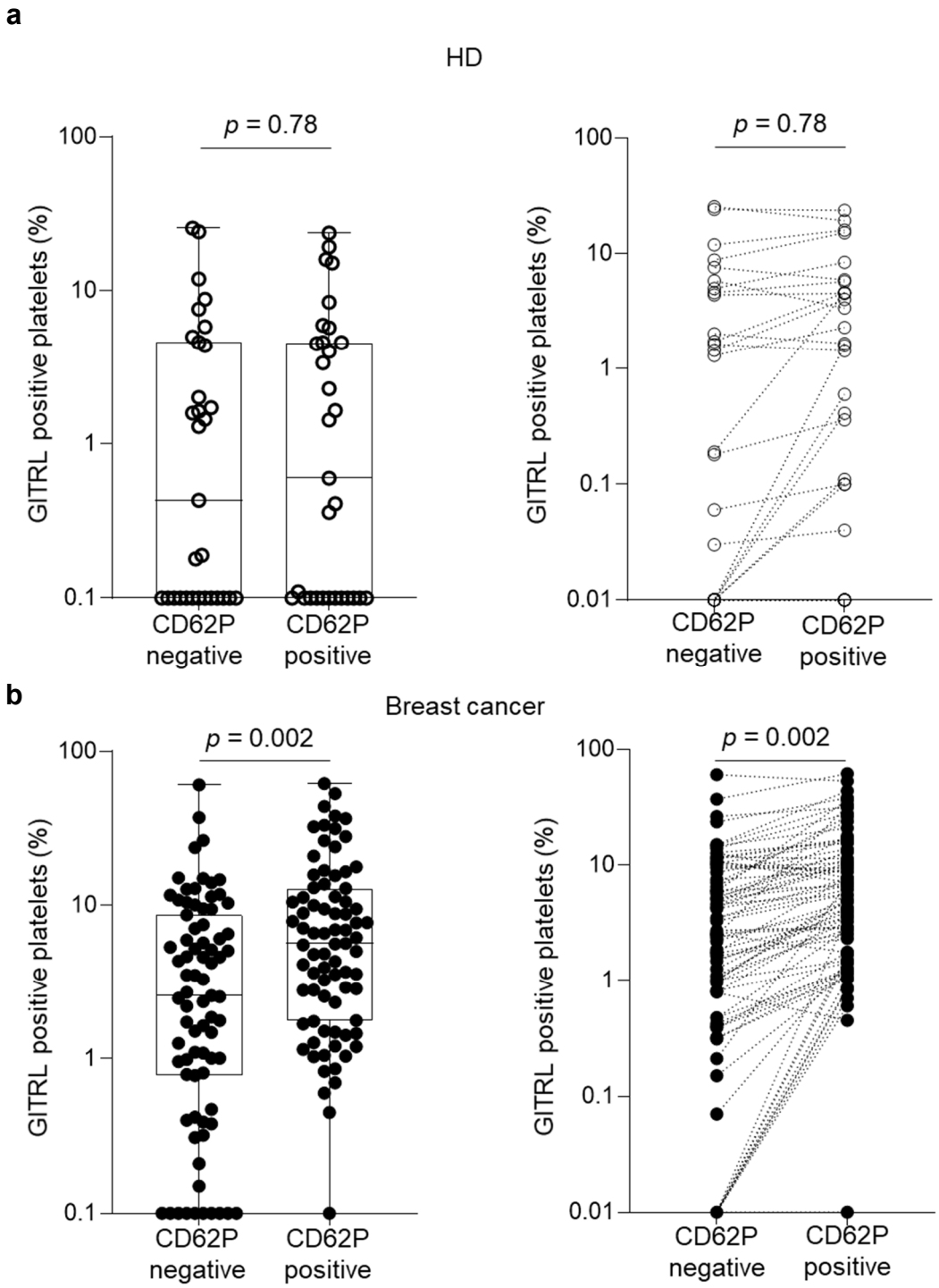


Figure 8: pGITRL levels upon platelet activation. (a) The pGITRL levels upon platelet activation (CD62P) status in 31 HD are depicted in the left panel. (b) pGITRL levels upon platelet activation in 79 breast cancer patients are depicted in the left panel. (a-b) pGITRL levels in resting platelets (CD62P negative) and activated platelets (CD62P positive) in a given donor are connected by dotted lines in the right panel. (a-b) Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITRl levels. Whiskers indicate minimum and maximum of GITRL levels. Unpaired t test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$.

3.2.2 Capacity of pGITRL upregulation upon platelet activation in breast cancer

Next, we assessed the extent of potential pGITRL upregulation upon platelet activation (Δ GITRL) in both HD and breast cancer patients. Interestingly, there was a positive correlation between basal pGITRL levels in resting platelets and Δ GITRL in HD and breast cancer cohorts ($p < 0.001$ and $p = 0.002$, linear regression) **(Figures 9a and 9b).** pGITRL expression levels on resting and activated platelets in breast cancer patients were significantly higher compared to HD ($p = 0.004$ and $p < 0.001$, respectively, unpaired t test) **(Figure 9c)**. In the breast cancer cohort, pGITRL was positively correlated with the percentage of CD62P negative platelets ($p = 0.03$, linear regression), indicating that the breast cancer patients with larger amounts of resting platelets express higher levels of pGITRL **(Figure 9d)**.

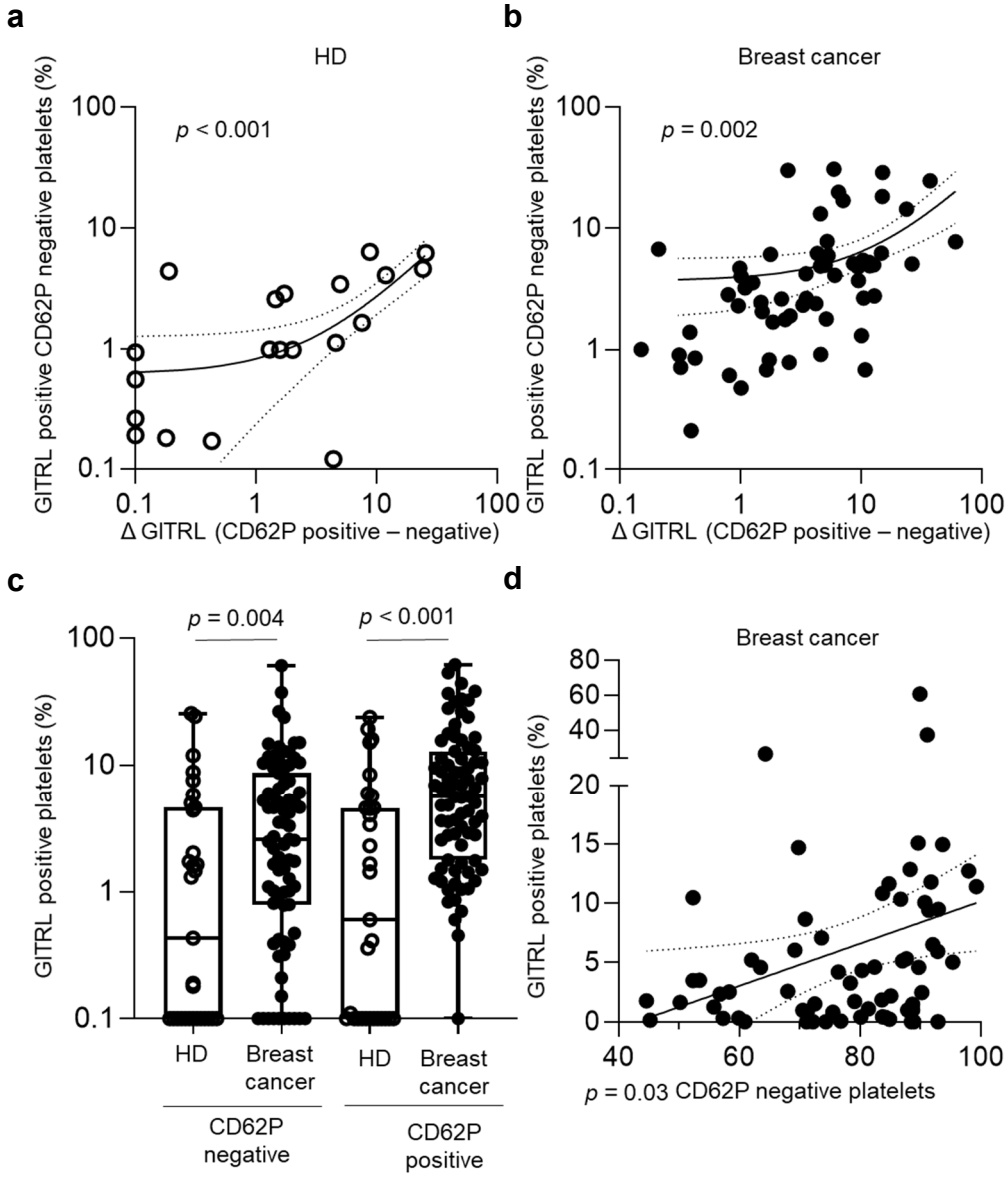


Figure 9: Capacity of pGITRL upregulation upon platelet activation in breast cancer. The percentage of GITRL positive platelets and CD62P levels on platelets were analyzed by flow cytometry using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. Platelets were costained by FITC-conjugated CD62P mAb. (a - b) The capacity of pGITRL upregulation upon platelet activation (Δ GITRL was defined as "the percentage of GITRL positive CD62P positive platelets – the percentage of GITRL positive CD62P negative platelets" in HD and breast cancer patients). The basal pGITRL expression levels were defined as the percentage of pGITRL positive and CD62P negative platelets. The solid lines represent mean and the dashed lines represent the error of the residuals. Simple linear regression was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$. (c) pGITRL expression obtained in a total of 79 breast cancer patients and 31 HD is depicted with regard to CD62P expression. Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITR levels. Whiskers indicate minimum and maximum of pGITRL levels. Unpaired t test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$. (d) pGITRL expression of CD62P negative platelets obtained in a total of 79 breast cancer patients is depicted. Simple linear regression was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$.

3.3 pGITRL and clinical parameters in breast cancer

The enhanced expression of pGITRL and large capacity of pGITRL upregulation upon activation in our patient cohort suggested a critical role of pGITRL in the pathophysiology of breast cancer. We therefore next comparatively studied the association of pGITRL and clinical parameters in breast cancer patients.

3.3.1 Association of pGITRL expression with tumor characteristics in breast cancer

The expression of pGITRL was found to differ among the various primary tumor stages T0 to T4. pGITRL expression was most pronounced in T2 as compared to T0 (p = 0.01). No significant differences were observed between T0 and T1, T0 and T3 or T0 and T4, respectively. However, pGITRL levels were higher in the intermediate (T2) stage than in T1 (p = 0.002) or T4 (p = 0.007) (ordinary one-way ANOVA test) **(Figure 10a)**. With regard to differentiation we next evaluated the association of pGITRL expression and histological grades G1-G3. Highly enhanced pGITRL expression was observed in patients with moderately differentiated tumors (G2). Breast cancer patients with poorly differentiated tumors (G3) displayed lower levels of pGITRL as compared to G2 tumors (p = 0.004, ordinary one-way ANOVA test) **(Figure 10b)**. The cell proliferation index Ki-67 was negatively associated with pGITRL levels in the respective patients (p = 0.001, linear regression) **(Figure 10c)**.

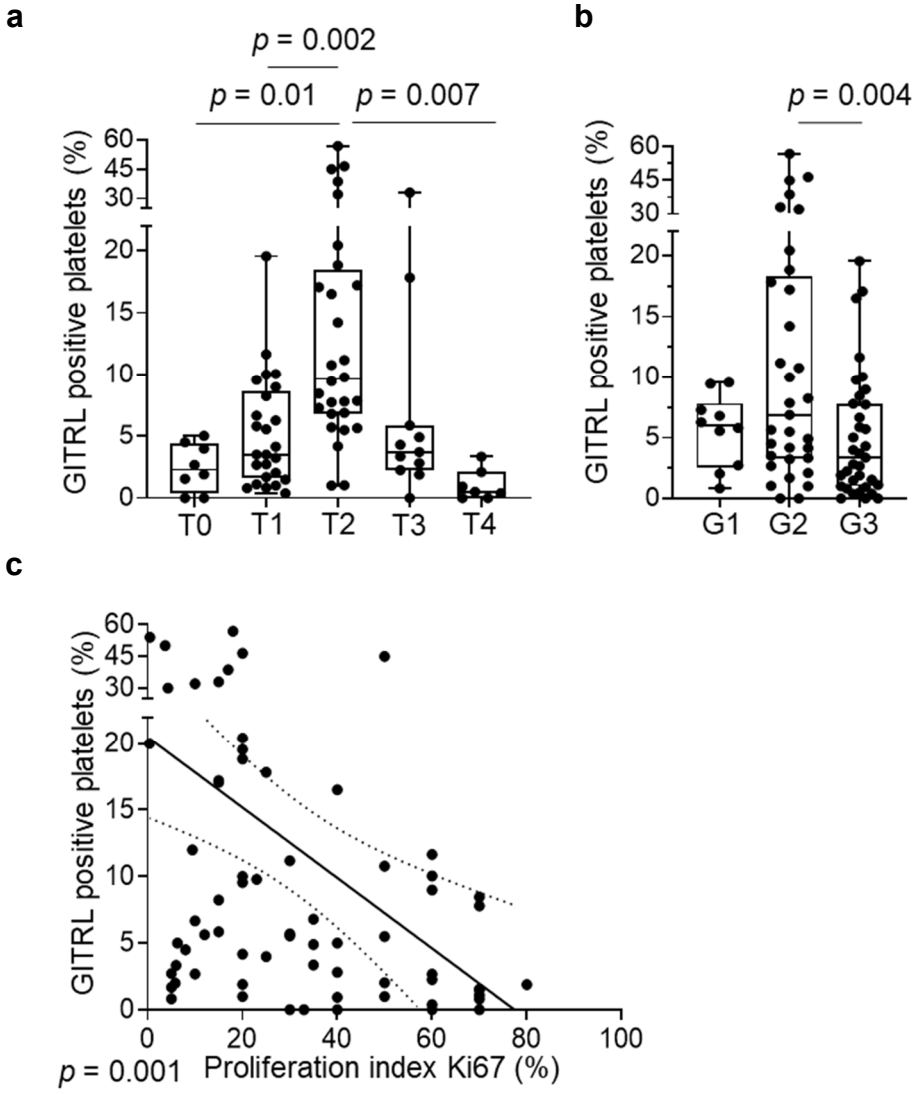


Figure 10: Association of pGITRL expression with clinical parameters including T stage, histological G grading, and cell proliferation index in breast cancer. pGITRL expression obtained in a total of 79 breast cancer patients is depicted with regard to (a) T stage, (b) N stage and (c) proliferation index (Ki-67) of a given patient. (a-b) Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITR levels. Whiskers indicate minimum and maximum of pGITRL levels. Ordinary one-way ANOVA test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$. (c) Simple linear regression was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$.

3.3.2 Association of pGITRL expression with N and M stages in breast cancer

Next we assessed whether pGITRL expression correlates with tumor N and M staging. Notably, no significant correlation among regional lymph node involvement (N0-N3) and pGITRL expression was observed (p = 0.3, Mixed-effects analysis) **(Figure 11a)**. However, pGITRL levels were positively associated with the absence of distant metastatic spread (M0) (p < 0.001, Mann Whitney test) **(Figure 11b),** which is in line with our results showing that pGITRL was upregulated in intermediate T stages (T2, G2) but not in advanced stages of the disease (T4, G3).

As the presence or absence of distant metastatic spread is a prognostic parameter for survival in breast cancer, we further evaluated the predictive value of pGITRL levels using ROC analysis. Notably, with an AUC of 0.75 (95% CI: 0.61-0.87), the specificity of pGITRL expression in predicting metastasis was 97.3% (95% CI: 85.8-98.5%) and the positive predictive value of pGITRL was 90% (95% CI: 54.9-98.5), suggesting that pGITRL may serve as a predictive biomarker for metastasis in breast cancer **(Figure 11c)**. The prognostic value of the combination of pGITRL and G grading (AUC = 0.77, 95% CI: 0.64- 0.88) was better as compared to G grading alone (AUC = 0.51, 95% CI: 0.37- 0.66) (p = 0.0057, DeLong method[106]) **(Figure 11d)**. The prognostic value of the combination of pGITRL and Ki-67 (AUC = 0.85, 95% CI: 0.72 - 0.93) was significantly enhanced as compared to Ki-67 alone (AUC = 0.62, 95% CI: 0.47-0.75) (p = 0.0016, DeLong method) **(Figure 11e)**.

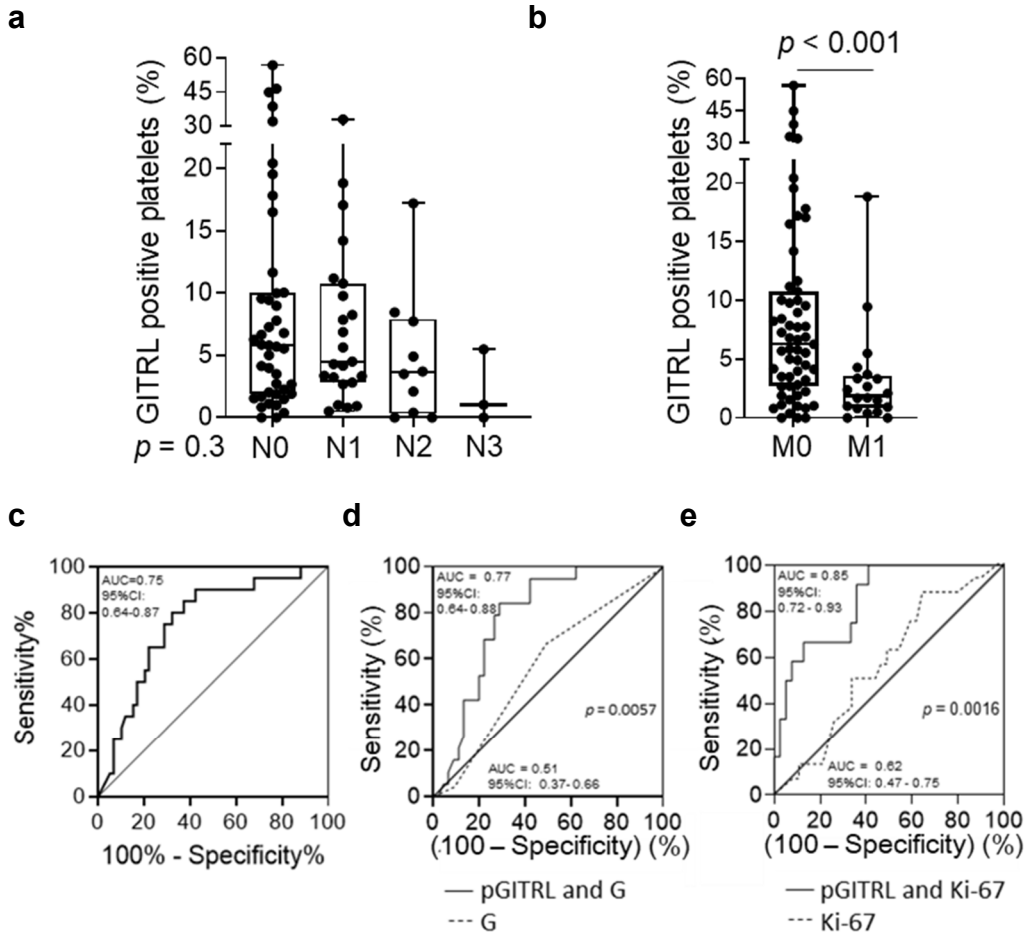


Figure 11: Association of pGITRL expression with N stage and M stage and the predictive value of pGITRL in breast cancer. pGITRL expression obtained in a total of 79 breast cancer patients is depicted concerning (a) the N stage and (b) M stage of a given patient. (Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITR levels. Whiskers indicate minimum and maximum of pGITRL levels. Unpaired t test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$. (c) The predictive value of pGITRL for metastasis is depicted using ROC analysis. (d) The predictive value of G grading alone (dotted curve) and the combination of pGITRL and G grading (solid curve) for metastasis is depicted using ROC analysis. (e) The predictive value of Ki-67 alone (dotted curve) and the combination of pGITRL and Ki-67 (solid curve) for metastasis are depicted using ROC analysis. DeLong method was used to compare the ROC curves. Statistically significant differences were defined as $p < 0.05$.

3.3.3 Expression of pGITRL and clinical parameters in breast cancer

Furthermore, to study the potential relationship between pGITRL expression and clinical parameters in breast cancer, Odds ratio (OR) for multiple endpoints were calculated. The expression level of pGITRL in tumors > 3 cm seemed to be higher (OR 2.8 95% CI 0.66 - 11.83) as compared to tumors ≤ 3 cm. The expression level of pGITRL in patients with regional lymph node involvement (N > 0) (OR

0.39 95% CI 0.09 - 1.71) tended to be lower as compared to patients with lymph node metastasis. The expression level of pGITRL in patients with distant metastasis (M1) (OR 0.25 95% CI 0.03 - 2.14) appeared to be lower as compared to patients without distant metastasis (M0). HER-2 positive breast cancer tended to be related to a higher expression level of pGITRL (OR 2.11 95% CI 0.41-11.4). ER positive breast cancer (OR 0.56 95% CI 0.12-2.5) and PR positive breast cancer (OR 0.67 95% CI 0.17-2.7) tended to be negatively associated with pGITRL expression in breast cancer patients. However, no relevant association between pGITRL expression and treatment, including adjuvant chemotherapy, adjuvant radiation, adjuvant endocrine therapy, and neoadjuvant chemotherapy was observed **(Figure 12).**

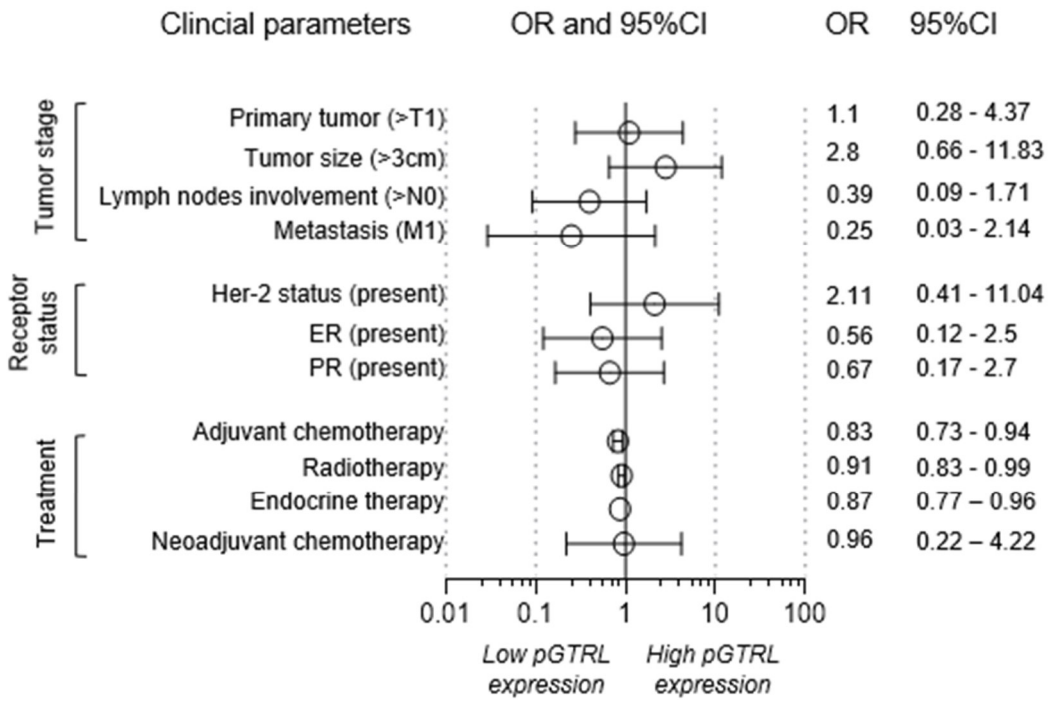


Figure 12: Association of pGITRL expression with clinical parameters in breast cancer by odds ratios (OR). The percentage of GITRL positive platelets was analyzed by flow cytometry. High pGITRL expression was defined as the values above the mean pGITRL (HD) + 2SD pGITRL (HD) while the low pGITRL expression was defined as the values below the mean pGITRL (HD) - 2SD pGITRL (HD). Association of pGITRL expression with clinical parameters in breast cancer was depicted by OR and 95% CI.

3.4 Regulation of expression of GITRL in platelet-precursor

Since GITRL expression was reported to be enhanced during platelet megakaryocyte maturation[51], we explored the regulation of pGITRL using MEG-01 cells as a megakaryopoiesis model. The maturation of MEG-01 cells was induced by VPA.[107] The gating strategy to characterize megakaryocytic MEG-01 cells (generated in the presence of VPA) and their subcellular fragments - considered as MEG-01 platelets - is shown in **Figure 13**.

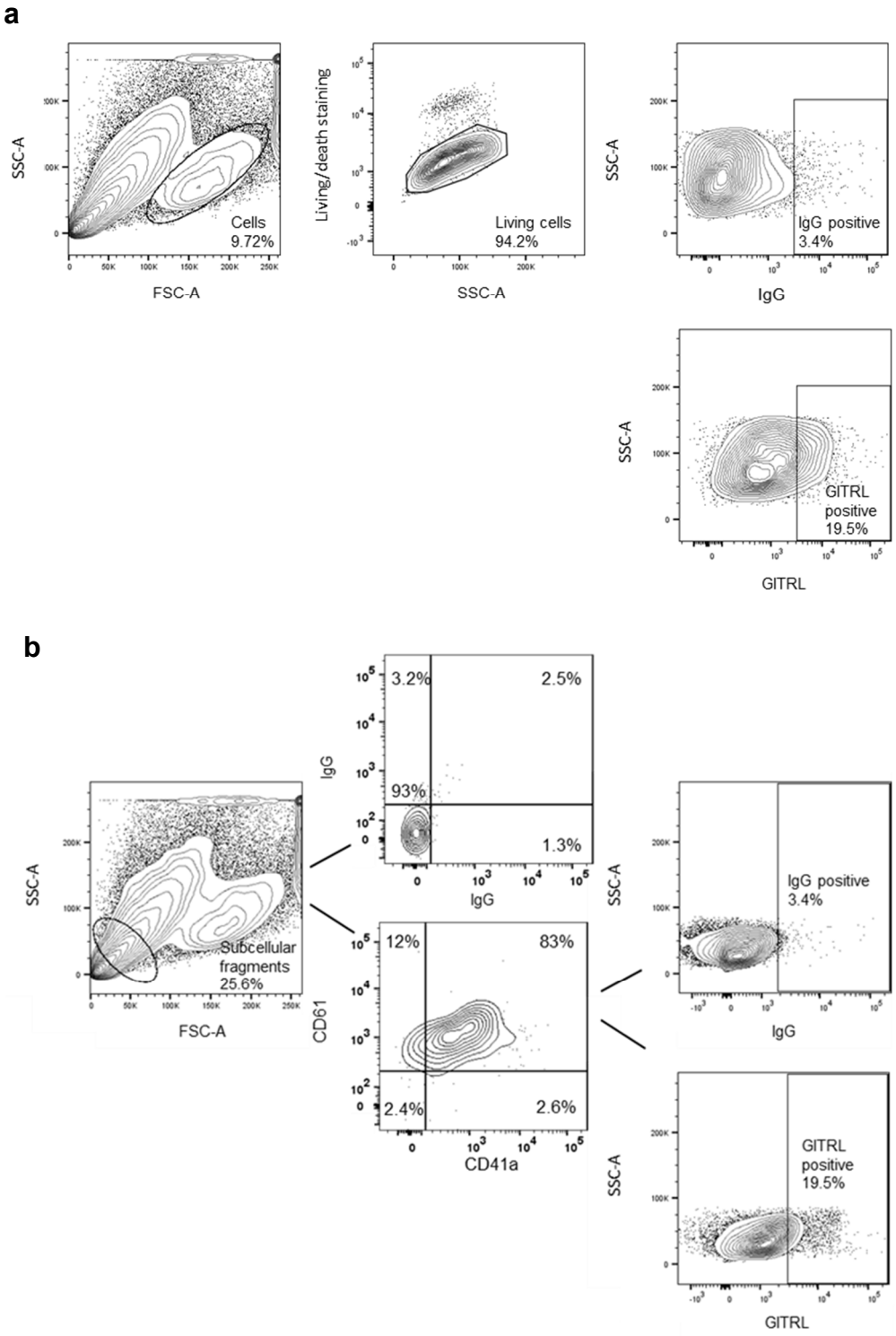


Figure 13: Gating strategy for MEG-01 cells treated with VPA. MEG-01 cells were cultured in the presence of 1 mM VPA for 14 days. Gating strategies to identify megakaryocytic MEG-01 cells (a) or MEG-01 platelets (b) in flow cytometry analysis are depicted. Surface expression of GITRL was analyzed using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. (a) Aqua fluorescent reactive dye was used to distinguish living and dead cells. (b) The subcellular fragments generated by MEG-01 cells were stained by CD61P-FITC and CD41a-PE-Cy-5. The CD61P positive CD41a positive subcellular fragments were considered as platelets generated by MEG-01 cells.

The expression of GITRL on megakaryoblastic (absence of VPA) or megakaryocytic MEG-01 cells and the platelets generated by megakaryocytic MEG-01 cells was analyzed by flow cytometry. GITRL expression on megakaryocytic MEG-01 cells was upregulated during MEG-01 maturation ($p < 0.001$, unpaired t test) **(Figure 14a)**. GITRL expression on platelets generated by megakaryocytic MEG-01 cells is shown in **Figure 14b**.

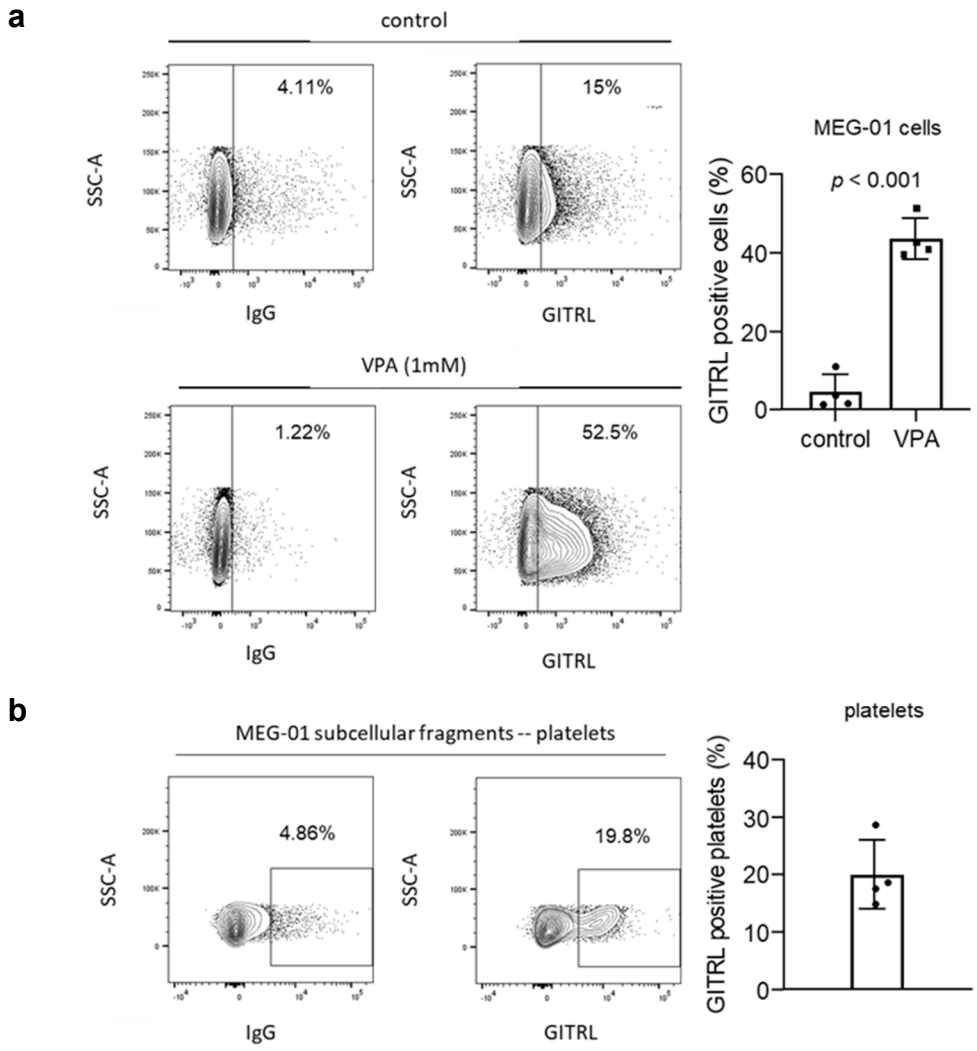


Figure 14: Surface expression of GITRL on MEG-01 cells treated with VPA. MEG-01 cells were cultured in the presence or absence of 1 mM VPA. The percentage of GITRL positive platelets was analyzed by flow cytometry using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. (a) One exemplary result of the expression of GITRL on megakaryoblastic (absence of VPA) and megakaryocytic (presence of VPA) MEG-01 cells is depicted in the left panel. GITRL expression on megakaryoblastic and megakaryocytic MEG-01 cells obtained in a total of 4 experiments are depicted in the right panel. Error bars represent SD. Unpaired t test was used for statistical analysis. Statistically significant differences was defined as $p < 0.05$. (b) One exemplary result out of 4 expriments regarding the expression of GITRL on platelets generated by megakaryocytic MEG-01 cells is depicted in the left panel. GITRL expression on platelets obtained in a total of 3 replicates is depicted in the right panel. Error bars represent SD.

Next, we investigated whether GITRL expression can be modulated by breast cancer cells during megakaryopoiesis. The conditioned medium was derived from 1 million of each breast cancer cell lines, i.e. MDA-MB-231, MDA-MB-468, SK-BR-3, and MCF7, which had been cultured in 10 ml of respective culture medium for 24 hours. 0.3 million of MEG-01 cells were seeded in 2 ml of culture medium for 24 hours. Then 2 ml of conditioned medium from breast cancer cell lines were added into MEG-01 cells and co-cultured for 24 hours, and GITRL levels were subsequently assessed by flow cytometry. GITRL expression on MEG-01 cells did not change in the presence of supernatant of MDA-MB-231 cells (p = 0.28, Mann-Whitney U test), while GITRL was significantly upregulated by soluble factors derived from MDA-MB-468 (p = 0.0006, Mann-Whitney U test), SK-BR-3 (p = 0.01, Mann-Whitney U test) cells and MCF7 (p = 0.01, Mann-Whitney U test) **(Figure 15)**.

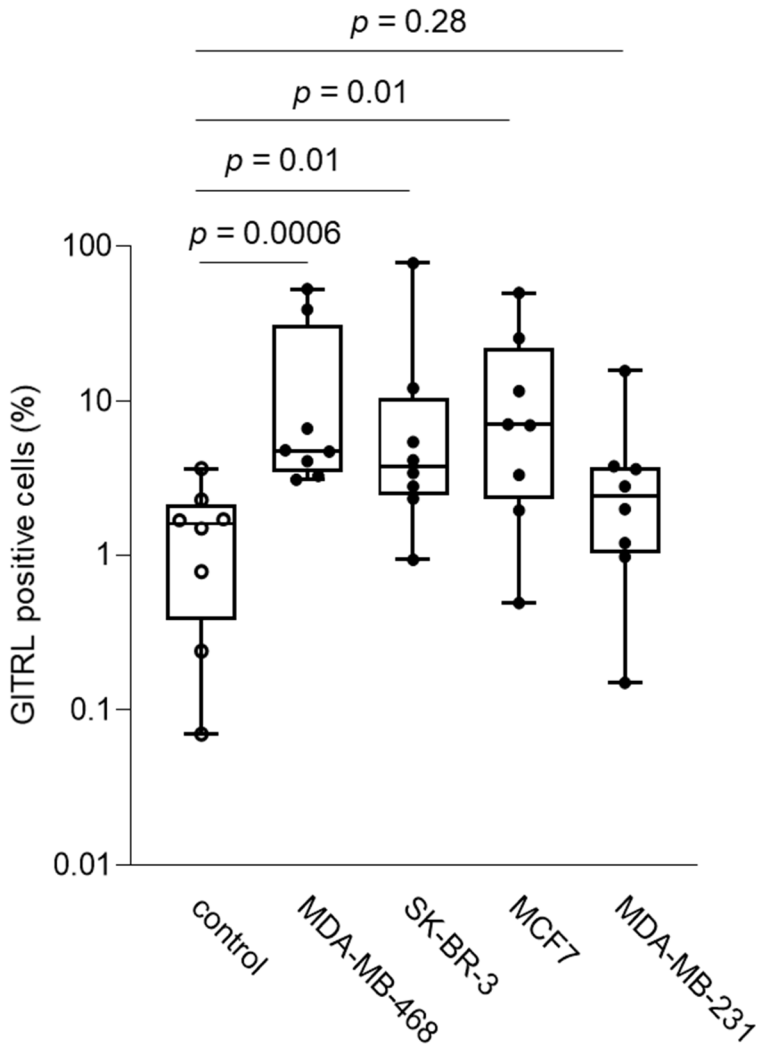


Figure 15: Expression of GITRL on MEG-01 cells treated with conditioned medium from breast cancer cell lines. MEG-01 cells were culture in the presence or absence of conditioned medium from breast cancer cell lines including MDA-MB-468, SK-BR-3, MCF7, and MDA-MB-231 for 24 hours. The percentage of GITRL positive MEG-01 cells was analyzed by flow cytometry using either specific GITRL mAb or isotype controls followed by goat-anti-mouse-PE. GITRL expression on MEG-01 cells obtained in a total of 7 experiments is depicted. Boxes represent the first quartile (25th percentile), median (50th percentile) and third quartile (75th percentile) of GITRL levels. Whiskers indicate minimum and maximum of GITRL levels. Mann-Whitney U test was used for statistical analysis. Statistically significant differences were defined as $p < 0.05$.

4 Discussion

GITRL and its cognate receptor GITR are TNFSF/TNFRSF members. The expression of GITR is well studied on NK and T cells.[96,104,108] The cytoplasmic domain of GITR shows great homology with other co-stimulatory TNFRSF members like 4-1BB, OX40, CD40.[109] Its ligand (GITRL) is expressed on B cells, macrophages, DCs, endothelial cells, and platelets.[51,110] The GITR/GITRL axis plays a key role in regulating both innate and adaptive immune surveillance.[51,96] Tumorigenesis and tumor progression are complex processes that are largely influenced by various molecules derived from the tumor microenvironment including platelets, immune cells, and cancer-cell-intrinsic processes.[111] This is exemplified by a series of studies by the Salih group. Human GITRL was found to be expressed on human tumor cells and to reduce cytokine production and cellular cytotoxicity of NK cells.[104] Furthermore, the Salih lab reported that GITRL provided by platelets inhibits NK cell antitumor activity.[112] GITR inhibits NK cell antitumor activity while it activates T cells.[51,96] Here we provide data indicating that pGITRL may play a role in tumor progression via modulation of NK/T cell-mediated immune surveillance in breast cancer following its regulation during platelet production.

To assess the role of pGITRL in tumor progression, we first studied expression of pGITRL in breast cancer patients and found that pGITRL was significantly upregulated compared to healthy donors. Since we hypothesized that pGITRL expression in patients was modulated to promote immune evasion by solid tumors, we also assessed expression of its cognate receptor GITR on immune effector cells. NK cells displayed a higher GITR expression in breast cancer patients as compared to HD, and T cells also tended to display a higher GITR expression which did not reach statistical significance. B cells of breast cancer patients seemed to have lower GITR expression levels which however, did not reach statistical significance. We next investigated the correlation of pGITRL levels and GITR expression on NK cells and T cells, respectively. GITR expression on NK cells was not correlated with pGITRL levels whereas GITR

expression on T cells was negatively associated with pGITRL, suggesting a negative feedback regulation between pGITRL and GITR expression on T cells.
We further studied the pGITRL expression with respect to the platelet activation state in breast cancer patients. The platelet activation caused by blood collection and sample preparation led to the similar CD62P expression results, which is in line with findings of Best and colleagues, who did not observe any significant platelet activation during blood taking and storage of platelets.[113] And due to the blood collection and sample preparation, it is impossible to determine the exact platelet activation level caused by tumor cells *in vivo*. No significant difference in the level of endogenous platelet activation was observed between healthy donor and breast cancer cohorts *ex vivo*. However, platelets from breast cancer patients demonstrated a higher inter-individual variability with regard to CD62P expression. This indicates a hyperactive state of patient-derived platelets which is a characteristic phenotype in cancer patients.[114] We observed that pGITRL expression was significantly increased in activated platelets as compared to resting platelets of breast cancer patients. pGITRL is upregulated in response to platelet activation, which occurs among others upon encounter of platelets with circulating tumor cells in the bloodstream.[51] Possible reasons for higher pGITRL expression on activated platelets of tumor patients may be: i) protein synthesis in platelets, ii) reprogrammed megakaryopoiesis in cancer patients and iii) preformation/storage of GITRL in platelet granules – which appears to be more plausible than de novo protein synthesis.[115-118] Moreover, we reported that GITRL is found in the cytoplasm of megakaryocytes and that GITRL is rapidly translocated to the platelet surface upon activation.[112] The same phenomenon has also been observed for other platelet-expressed immune checkpoint molecules including CD40L and TWEAK.[119,120] Of note, the findings that the capacity of GITRL upregulation upon platelet activation (Δ GITRL) is positively associated with the basal GITRL expression on resting platelets in both breast cancer and HD cohorts also supports our hypothesis that preformation and storage of GITRL are enhanced in platelet granules in breast cancer patients.
When we further evaluated the association of pGITRL with clinical characteristics, we found that pGITRL expression was only significantly enhanced in patients with

intermediate tumor sizes (T2). Patients with T0, T1, and T4 showed lower pGITRL levels. No correlation of regional lymph node involvement and pGITRL expression was observed. Breast cancer patients with metastasis (M1) displayed lower pGITRL levels. This is in line with our findings that patients with more aggressive tumor phenotypes, such as higher tumor grading (G3) and higher proliferation rates (Ki-67 level), also displayed lower pGITRL levels. Taken together, our data suggest that pGITRL may play a role in certain stages during tumor progression and may serve as a biomarker of metastasis. The negative association of pGITRL and GITR expression on T cells and low pGITRL expression levels in advanced breast cancer stages is in line with the report of an increased fraction of GITR-expressing T-cell subsets in tumor-positive lymph nodes from patients with advanced breast cancer.[121] Of note, GITR inhibits NK cell antitumor activity. Our data may implicate that high pGITRL expression resulting in impaired NK cell activity might provide a favorable condition for tumor growth and spread in early and intermediate tumor stages.[46] GITR can not only inhibit the suppressive properties on regulatory T cells but also act as a co-stimulatory factor for CD4 positive and CD8 positive T cells causing enhanced T cell expansion and cytokine production.[122-124] In the situation of advanced disease, pGITRL might not be beneficial for the tumor onset and cancer cells may engage other T cell co-inhibitory checkpoints such as PD-1 and CTLA4.[125] Previous findings that binding of GITRL on antigen-presenting cells to GITR on T cells abrogates suppression of regulatory T cells and activates effector T cells are in line with our data that pGITRL levels are decreased in advanced tumor stages.[110] This might be due to a downregulation of pGITRL upon interaction with GITR on immune cells and serve as a negative feedback loop to regulate GITRL/GITRL-mediated T cell activation. Notably, different expression levels of GITR have been revealed in various infectious or inflammatory diseases depending on the state of the disease.[105]

The regulation of pGITRL still needs to be fully elucidated in cancer and inflammatory diseases. We hypothesized that the preformation and storage of GITRL might be due to enhanced synthesis of GITRL during megakaryopoiesis in cancer patients. To study this issue, we employed the model of MEG-01 cells

and VPA to induce megakaryopoiesis.[107] Enhanced GITRL expression during megakaryocyte maturation was observed in our study, which is in agreement with our previously reported findings.[51] Next, we investigated the potential effects of breast cancer-derived factors on GITRL expression on MEG-01 cells. Upon culture, with soluble factors released by breast cancer cell lines, GITRL expression on MEG-01 cells was induced. Various cytokines including IL-2, IL-6, IL-8, and IFN-γ are secreted by breast cancer cells, among which IL-2 and IL-8 are predominantly described.[126,127] Further work is needed to identify the responsible factor that mediates GITRL induction.

Particularly high levels of cytokine expression in ER-negative breast cancer cells were observed, which is in line with our data that ER-negative breast cancer patients displayed higher pGITRL.[128] Moreover, increased cytokine levels also were found in patients with HER-2 overexpression *in vitro* and *vivo* [129]. This is also in line with our observation that the Her-2 positive status in our patient cohort was associated with increased pGITRL expression.

Many patients still do not benefit from immune checkpoint blockade. Therefore, there is an urgent need for the identification of immunoregulatory molecules which may be targeted alone or in combination with already existing regimen. The GITR/GTIRL system is a potential candidate for immunotherapy owing to its capacity to promote effector T cell functions, hamper suppression of regulatory T cells and impair NK cell antitumor activity in certain steps during tumor progression.[130] Clinical trials evaluating GITR antibodies as a mean to reinforce antitumor immunity against various malignant entities are ongoing (NCT01239134, NCT02598960). A combination of anti-PD-1 and GITR antibodies may be particularly useful to shift the immunosuppressive tumor milieu to an immunostimulatory environment.[131]

Beyond providing information on the potential use of GITR/GITRL as immunotherapeutic targets we also identified pGITRL as a prognostic and predictive biomarker for metastasis in breast cancer. This is especially useful as platelets are readily available for analysis.

5 Summary

5.1 Summary in English

Breast cancer is the leading cause of cancer-related death in women. Most subtypes of breast cancer are immunologically silent, which makes the immunotherapy of patients with advanced breast cancer particularly challenging and new targets are urgently needed. Platelets play an important role in tumor progression as they promote proliferation, chemotherapy resistance, stemness, invasion, and immune evasion of malignant cells. As a new target of immune checkpoint therapy, GITRL has attracted more and more attention. Little is known about the role of pGITRL in breast cancer. We assumed that pGITRL may serve as a biomarker and a potential immunotherapeutic target in breast cancer. Here, we conducted a comparative analysis of pGITRL in breast cancer patients. pGITRL expression was upregulated in breast cancer patients as compared to healthy donors and especially increased in cases of intermediate tumor stages, low proliferation index, and absence of metastasis. GITR expression on NK cells increased in breast cancer patients as compared to healthy donors. Moreover, GITRL expression was enhanced during platelet activation in breast cancer patients. The capacity of GITRL upregulation upon platelet activation associated with the basal GITRL expression on resting platelets in both breast cancer and healthy donor cohorts. GITRL expression was enhanced during megakaryopoiesis and particularly upregulated in the presence of soluble factors derived from breast cancer cells. All the findings may suggest enhanced pGITRL levels and increased capacity of pGITRL regulation upon platelet activation in breast cancer as part of a tumor-educated platelet phenotype. Based on our data, pGITRL may serve as a readily available biomarker and a potential immunotherapeutic target in breast cancer.

5.2 Summary in German

Brustkrebs ist die häufigste Ursache für krebsbedingte Todesfälle bei Frauen. Die meisten Subtypen von Brustkrebs sind immunologisch stumm, was die Immuntherapie von Patienten mit fortgeschrittenem Brustkrebs besonders schwierig macht und neue Ziele dringend benötigt werden. Thrombozyten spielen eine wichtige Rolle bei der Tumorprogression, da sie die Proliferation, Chemotherapieresistenz, Stammzell-Eigenschaften, Invasion und Immunevasion von malignen Zellen fördern. Als neues Ziel der Immun-Checkpoint-Therapie hat GITRL immer mehr Aufmerksamkeit auf sich gezogen. Über die Rolle von pGITRL bei Brustkrebs ist wenig bekannt. Wir gingen davon aus, dass pGITRL als Biomarker und potenzielles immuntherapeutisches Ziel bei Brustkrebs dienen kann. Hier führten wir eine vergleichende Analyse von pGITRL bei Brustkrebspatientinnen durch. Die pGITRL-Expression war bei Brustkrebspatientinnen im Vergleich zu gesunden Spendern hochreguliert und insbesondere bei mittleren Tumorstadien, niedrigem Proliferationsindex und fehlender Metastasierung erhöht. Die GITR-Expression auf NK-Zellen war bei Brustkrebspatientinnen im Vergleich zu gesunden Spendern erhöht. Darüber hinaus war die GITRL-Expression während der Thrombozytenaktivierung bei Brustkrebspatientinnen erhöht. Die Fähigkeit der GITRL-Hochregulation bei Thrombozytenaktivierung, die mit der basalen GITRL-Expression auf ruhenden Thrombozyten sowohl bei Brustkrebs als auch bei gesunden Spenderkohorten verbunden ist. Die GITRL-Expression wurde während der Megakaryopoese verstärkt und insbesondere in Gegenwart löslicher Faktoren, die von Brustkrebszellen stammen, hochreguliert. Alle Ergebnisse könnten auf erhöhte pGITRL-Spiegel und eine erhöhte Kapazität der pGITRL-Regulation bei Thrombozytenaktivierung bei Brustkrebs als Teil eines Tumor-gebildeten Thrombozyten-Phänotyps hinweisen. Basierend auf unseren Daten kann pGITRL als leicht verfügbarer Biomarker und potenzielles immuntherapeutisches Ziel bei Brustkrebs dienen.

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Appendix

7.1 Declaration of contribution

The dissertation work was carried out in the “Clinical Cooperation Unit Translational Immunology” of the German Consortium for Translational Cancer Research (DKTK) at the German Cancer Research Center (DKFZ) at the partner site Tübingen in the Department of Internal Medicine of the University Hospital Tübingen under the supervision of Prof. Dr. med. Helmut R. Salih.

The study was designed by Prof. Dr. med. Helmut R. Salih.

All experiments were carried out after training by Dr. rer. nat. Stefanie Maurer and Dr. med. Clemens Hinterleitner (see Table 11).

The statistical evaluation was carried out by myself after training by Dr. med. Clemens Hinterleitner.

I declare that I have written the thesis independently and that any additional sources of information have been duly cited.

Table 11: Declaration on contribution

Experiments	**Figures**	**Operator and numbers of experiments**	
Flow cytometry for platelets from breast cancer patients	Figure 1, 2, 6 - 12	Celine Reiss 47	Yanjun Zhou 32
Flow cytometry for platelets from healthy donors	Figure 1, 2, 6 - 12	Celine Reiss 25	Yanjun Zhou 6
Flow cytometry for PBMCs from breast cancer patients	Figure 3 - 5	Kim Larissa Clar 5	Yanjun Zhou 2
Flow cytometry for PBMCs from breast cancer patients	Figure 3 - 5	Kim Larissa Clar 9	Yanjun Zhou 5
Flow cytometry for MEG-01 cells with/ without VPA	Figure 13, 14	Celine Reiss, Clemens Hinterleitner 4	
Flow cytometry for MEG-01 cells with/ without supernatant from breast cancer cell lines	Figure 15	Yanjun Zhou 8	

Tübingen, 28.10.2020

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My special thanks also go to Dr. rer. nat. Stefanie Maurer who brought me closer to the laboratory processes with great dedication from the first day and brought me up to independence. Your quasi-inexhaustible tolerance for frustration and your drive are exemplary. I am proud to be the student you supervised. You were at my side at all times with advice and action on all matters that concerned me. I would especially like to thank you again for the overwhelming support in the course of correcting my doctoral thesis.

I would like to thank Dr. med. Clemens Hinterleitner. You sat tirelessly at my side and support me in my work. Thank you for the interesting discussions we have had and the food for thought that I have learned from you.

I would like to thank all former and current members of the working group, especially Kim Larissa Clar, Claudia Tandler, and Kübra Kaban, for the shared experiences over the past two years. I always had a lot of fun with you. Thank you for the unforgettable time both in the laboratory itself and in the context of various leisure activities. You gave me with a lot of warmth and gave me a feeling of security. Whenever I looked for support, you sent it to me.

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Tübingen, 28.10.2020

7.3 Resume

Personal information

First name: Yanjun Family name: Zhou

Date and place of birth: 14.12.1985 in Kunming

Marital status: Married

Nationality: Chinese

Training

07/2012-10/2018	Resident and junior specialist training	Wuxi Maternity and Child Health Care Hospital China
09/2005-07/2012	Bachelor and master degree in human medicine	Tianjin medical university China

Education

09/2004-07/2005	Tutoring School of Yunnan Normal University
09/2004-07/2001	The high school affiliated to Yunnan normal university
09/2001-07/1998	Kunming number 8 middle school
09/1998-07/1992	Buxin primary school Kunming

Linguistic proficiency

Chinese	Fluent
English	C1

Publications

Yanjun Zhou, Jonas S. Heitmann, Kim L. Clar, Korbinian N. Kropp, Martina Hinterleitner, Tobias Engler, André Koch, Andreas D. Hartkopf, Lars Zender, Helumut R. Salih, Stefanie Maurer, Clemens Hinterleitner. Platelet-expressed immune checkpoint regulator GITRL in breast cancer. Cancer Immunol Immunother. 2021 Feb 4. doi: 10.1007/s00262-021-02866-y. Epub ahead of print. PMID: 33538861.