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PD-1 expression profile and phenotypes of peripheral blood T lymphocytes in breast cancer

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A Michael, sin el cual este doctorado no hubiese sido posible.

Content

1. INTRODUCTION

1.1 Tumor immune escape

Tumor immunity means the success of the immune system to reject malignant tumor cells. If either innate or adaptive immunity becomes impaired or suppressed, tumor development can occur (Bui and Schreiber, 2007; Dunn et al., 2006; Smyth et al., 2006).

The central regulator of the anticancer adaptive immune response is the T lymphocyte. It is still an unsolved question why existing tumor-specific T cells in cancer patients fail to effectively prevent tumor progression. The presence of tumor-specific $CD8^+$ cytotoxic T cells (CTLs), which are the principal effector cells of the adaptive immunity, does not necessarily lead to complete or even partial tumor rejection in both mice and humans (Overwijk et al., 2003; Prevost-Blondel et al., 1998; Robbins et al., 2004; Valmori et al., 2002; Yee et al., 2002).

The tumor immune escape is a process whereby tumor cells are concealed from tumor-specific T cells leading to tumor survival. It has been clearly shown that in a very active way the tumor can mimic some of the signaling pathways of the immune system to escape tumor specific immune recognition and that the microenvironment uses tumor-mediated processes to evade tumor-specific immunity. This results in reprogramming of immune cells, which become dysfunctional and is finally responsible for the lack of immunity specific to so-called tumor-associated antigens (TAAs) (Gilboa, 2004; Pardoll, 2003; Romero et al., 1998; Schreiber et al., 2002; Zou, 2005).

Two major strategies are found in the phenomenon of tumor escape to evade T cell response, tumor-induced peripheral $CD8^+$ T cell tolerance and tumor-immune evasive strategies. Some of the mechanisms used by tumors are: secretion of immunosuppressive and/or apoptotic factors, inefficient T cell stimulation due to dysfunctional antigen presenting cells (APCs) at the tumor site, recruitment of high numbers of suppressive regulatory T cells in tumors and tumor-draining lymph nodes (Curiel et al., 2004; Zou, 2006). One additional factor contributing to the described phenomenon is due to the presence of high levels of co-inhibitory molecules by APCs, stromal cells and tumor cells, leading to activation of the negative regulatory pathways (Chen, 2004; Zou, 2005).

In concert, these mechanisms cooperate in advanced stages of cancer to limit the anti-tumor immunity and the effectiveness of immunotherapy strategies to successfully eradicate malignant cells (Pardoll and Allison, 2004), having disastrous effects on our patients.

1.2 PD-1:PD-L1 interactions

Focusing on co-inhibitory molecules as a mechanism of tumor immune escape, there is one membrane-associated factor expressed by tumor cells that has been identified and has the capability of inhibiting T cell function. It is called programmed death-ligand-1 (PD-L1; also known as B7-H1 or CD274) receptor. Studies from several groups have shown that engagement of the CD28 family member programmed cell death (PD-1, also known as CD279) receptor with the B7-family member PD-L1 negatively regulates T cell activation and T cell functions like cytokine production, proliferation and cell survival (figure 1) (Carter et al., 2002; Chemnitz et al., 2004; Freeman et al., 2000).

The wide expression of PD-1 contrasts with restricted expression of other CD28 family members on T cells, which suggest that PD-1 regulates a wider spectrum of immune response compared with other members of the same family. PD-1 is expressed on thymocytes (Blank et al., 2003; Nishimura et al., 1996), mature B and T cells following activation (Agata et al., 1996; Blank et al., 2004), on myeloid cells (Nishimura and Honjo, 2001) and in the cytoplasm of a regulatory T cell population (Pentcheva-Hoang et al., 2007; Raimondi et al., 2006).

The strong and widespread expression of PD-L1 on tumor cells led to the hypothesis of PD-L1:PD-1 interaction as a tumor escape mechanism. In fact, the assumption that tumors evade through this mechanism the immune destruction protecting themselves from activated T lymphocytes actually occurs, as it has been demonstrated in several experiments using animal tumor models (Blank et al., 2004; Blank et al., 2005; Dong et al., 2002; Iwai et al., 2002).

PD-L1-expressing cells use at least six distinct mechanisms to evade T cell immunity which are inducing apoptosis, anergy or exhaustion of T cells, forming a molecular shield to protect tumor cells from lysis, inducing production of the immunosuppressive cytokine IL-10 and promoting T_{REG} cell-mediated suppression (Zou and Chen, 2008).

According to this, the presence of PD-L1 protein has been reported in solid tumors, the majority by immunohistochemistry either of frozen or paraffin-embedded specimens, or reverse transcription in a wide variety of cancer types, including breast cancer (Dong et al., 2002). Most interestingly, recent data exists about the correlation between PD-L1 expression in cancer patients and cancer progression with the subsequent unfavorable prognosis in breast cancer (Ghebeh et al., 2008; Ghebeh et al., 2006) and other tumors like renal cell carcinoma (Thompson et al., 2004; Thompson et al., 2006), esophageal cancer (Ohigashi et al., 2005), gastric carcinoma (Wu et al., 2006), ovarian cancer (Hamanishi et al., 2007), bladder cancer (Inman et al., 2007b), pancreatic cancer (Nomi et al., 2007) and human

hepatocellular carcinoma (Gao et al., 2009). Furthermore, PD-1 expression in urothelial cancer correlates with disease progression (Boorjian et al., 2008).

It is known that PD-1 expression is up-regulated on some tumor infiltrating lymphocytes (TILs), and this may also contribute to tumor immunosuppression (Blank et al., 2003). In breast cancer patients, PD-1 expression on TILs was found to correlate with a bad prognosis (Ghebeh et al., 2008) and the expression of the ligand PD-L1 on TILs was also associated with poor prognosis (Ghebeh et al., 2006; Ghebeh et al., 2007).

Immunomodulatory approaches have emerged as promising alternative treatment strategies like the concept of blockade of inhibitory receptors. These could revive T cell function and overcome immune resistance and have generated hope that manipulating second signals may lead to enhanced antitumor immune responses (Carreno and Collins, 2002; Subudhi et al., 2005). Therefore, the manipulation of the inhibitory PD-1:PD-L1 interaction can be applicable in a therapeutic way to treat human cancer, given that many tumor-associated APCs and tumor cells express the molecule PD-L1 mediating T cell suppression.

Despite considerable success in preclinical studies, the outcome of immunotherapy is often disappointing when translated to clinical trials. The tumor microenvironment may restrict the effectiveness of antitumor responses by displaying a variety of immunosuppressive strategies. That is why combination therapy approaches are now being investigated. One successful example was the triple treatment therapy in mice with anti-PD-L1 blockade, depletion of $CD4^+$ T cells to avoid inhibitory regulation of T_{REG}, and irradiated tumor cell vaccination, which induced complete elimination of large established renal cancer cell (RENCA) tumors with long-lasting tumor-specific immunity (Webster et al., 2007).

A better understanding of PD-1:PD-L1 signal will help us to reveal new ways to overcome T cell exhaustion and peripheral tolerance and to enable an effective clearance of tumors.

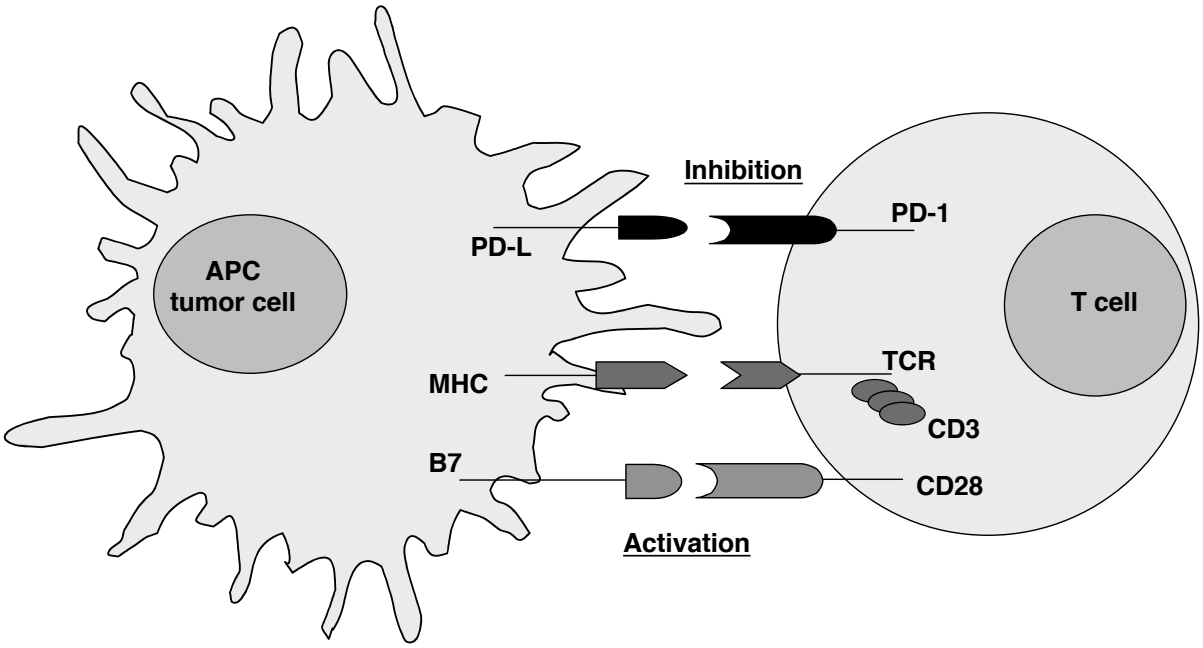

Figure 1: Interaction between antigen presenting cell or tumor cell and T lymphocytes

Interaction of protein presented by MHC on the side of the antigen presenting cell or a tumor together with costimulatory interactions such as B7-CD28 leads to activation of T lymphocytes. Recognition of PD-L expressed on the tumor cell by the T lymphocyte via PD-1 leads to inhibition of T lymphocytes, resulting in weakened antitumor response.

1.3 CD8+ T cell phenotypes

Previous studies showed that CD8+ T cells change the expression of costimulatory surface molecules (CD27, CD28 and CD45RA) according to their differentiation and maturation (Hamann et al., 1997; Hamann et al., 1999).

A phenotypic complexity among CD8+ T cells in both human and mouse has been identified thanks to the two cell surface markers CD45RA and CC chemokine receptor 7+ receptor (CCR7). This brings the concept of four T cell subsets: naive T cells (T_{naive} CD45RA+ CCR7+), effector T cells (T_{EFF}; CD45RA+ CCR7-), central memory T cells (T_{CM}; CD45RA- CCR7+) and effector memory T cells (T_{EM}; CD45RA- CCR7-) (Masopust et al., 2001; Sallusto et al., 2004; Sallusto et al., 1999).

Recently, it was shown that an even more complex classification of functional CD8+ T cells using the co-stimulatory receptors CD27 and CD28 could be achieved. These cell surface markers divide CD8+ T cell in several subpopulations with different functional characteristics in healthy donors (Romero et al., 2007; Rufer et al., 2003).

This functional division agrees with the T cell differentiation model according to which there is a differentiation pathway with progressive loss of CCR7, CD28, and CD27 cell surface expression concomitant with up-regulation of cytolytic capacity and reduced ability to proliferate (Appay et al., 2002). Tumor growth is comparable to chronic viral infections because antigens are persistently presented to CD8+ T cells and T cells have must respond to continuous antigenic stimulus.

The use of the different CTLs in antitumor therapy with their different functional abilities has provided some promising successes, but until now there is not one single approach with superior antitumor response. However, there are two significant T cell subsets used in cancer therapy: T_{EFF} cells and memory T cells. Which subset provides the best protective immunity against cancer it is not known so far. However, the experimental evidence suggests that both types can each confer a protective advantage in different tumor models (Kaech et al., 2002; Sallusto et al., 2004).

1.4 Breast cancer

Cancer is one of the three leading causes of death in industrialized nations. Breast cancer is the leading malignancy among women worldwide and is the second most common cause of mortality after lung cancer (Jemal et al., 2008).

In Germany and other industrialized countries, breast cancer is responsible for close to 28% of all new cancer cases among women. It is estimated that there are 57.000 new breast cancer cases and that 17.500 women die because of its consequences each year (Robert Koch-Institut, Germany).

1.4.1 Breast cancer and HER2 overexpression

Currently, all breast cancer should be tested for expression of the hormonal receptors and the human epidermal growth factor receptor 2 (HER2) protein by immunohistochemistry. This is made by using the HER2 DAKO-Score, an immunohistochemical protein-based test that is used to provide an assessment of the amount of HER2 protein receptors expressed on the surface of the cancer cells. The results, the so-called DAKO-score, assign a value between 0 and 3 to every tissue. A HER2-negative breast cancer is considered when the result is score 0 and 1, while HER2-positive breast cancers are assigned with a score 3. A tumor with a score 2 is considered a borderline or equivocal result and it should be followed by doing a fluorescence in situ hybridization (FISH) test to determine the definitive HER2 status (Gown, 2008).

The HER2 receptor is a transmembrane glycoprotein with intrinsic tyrosine kinase activity, whose structure is homologous to the epidermal growth factor receptor (Bargmann et al., 1986; Schechter et al., 1984). HER2 overexpression is found in 25-30% of breast cancer patients and is related to increased tumor aggressiveness, increased rates of recurrence, and increased mortality (Slamon et al., 1987). The prognostic value in node-positive patients has been widely demonstrated, its value in node-negative cases remains controversial (Menard et al., 2008).

The HER2 oncogenic protein is also a tumor antigen (Disis and Cheever, 1997). The first reference about the HER2 immunogenicity as an antigen was made on account of the detection of pre-existing antibodies and $CD4^+$ HER2-reactive T cells in breast cancer patients with HER2 overexpression (Disis et al., 1994). The HER2 antigen is known to be expressed in many healthy tissues, although the expression level is lower than in tumor cells (Press et al., 1990).

With time, several CTL epitopes derived from numerous TAAs have been described (Lindauer et al., 1998; Rosenberg, 1999). In the classification made by Ribas (Ribas et al., 2003), HER2 is considered

an overexpressed epitope derived from genes transcribed at abnormal levels in tumor compared to normal cells.

The immunogenicity of HER2 was shown owing to the existence of the HER2-reactive antibodies and T cells from breast cancer patients with a HER2 overexpression (Disis et al., 1994; Kiessling et al., 2002).

To date, the HER2 oncogenic protein has been used very often as a target for new breast cancer therapies like kinase inhibitors and immunotherapeutical approaches (Hynes and Lane, 2005). Indeed, our group has shown that the transfer of HER2-reactive CTLs can lead to the elimination of disseminated HER2-overexpressing tumor cells *in vivo* (Bernhard et al., 2008).

For this study, we used the nonameric $HER2_{369-377}$ (KIFGSLAFL) peptide. This HER2-derived peptide is naturally processed and serves as an epitope for HLA-A2-restricted T cells, which is known to be recognized by several CTL lines and was described in 1995 (Fisk et al., 1995). The immunodominant HLA-A*0201 restricted peptide is being used often in tumor therapies based on dendritic cell immunization, preferentially used for patients with HER2 overexpression. Our group used T cell clones reactive for $HER2_{369-377}$ for an adoptive T cell transfer of a patient with HER2 overexpressing breast cancer, showing that the transfer of HER2-reactive CTLs can lead to the elimination of disseminated HER2-overexpressing tumor cells in vivo (Bernhard et al., 2008).

The HER2-derived peptide 369-377 (KIFGSLAFL) is also a common epitope expressed by various tumor types including ovarian (Kono et al., 1997), renal cell (Brossart et al., 1998), melanoma (Rongcun et al., 1999) and prostate cancer (Sotiropoulou et al., 2003a).

To visualize HER2-reactive T cells we used HLA-A*0201/$HER2_{369-377}$-multimer. A multimer is a soluble tetrahedral complex artificially generated by using a fluorochrome-coupled avidin to join four biotinylated MHC class I molecules with a peptide of interest and $ß_2$-microglobulin. MHC class I multimer allow the direct *ex vivo* identification of antigen-specific $CD8^+$ T cell population permitting the analysis of the endogenous T cell response to infection or immunization.

Lack of sensitivity when evaluating rare events because of background staining limits its use.

It is important to say that there are several HER2 multimer epitopes, which are loaded with different peptides. In the case of HER2, the epitope p654-662 (IISAVVGIL) could also have been used.

Finally, it is important to emphasize that there is a clear evidence for immune defects in breast cancer patients, including a lower absolute number of peripheral blood lymphocytes (Caras et al., 2004) and an elevated number of functionally immunosuppressive $CD4^+$ $CD25^+$ T_{REG} lymphocytes in both peripheral blood and tumor microenvironment (Liyanage et al., 2002). DCs obtained from the peripheral blood and

lymph nodes of patients with operable breast cancer had a substantial decrease in MHC II and CD86 expression, and IL-12 production, which are essential elements for an effective immune response (Pockaj et al., 2004).

1.5 Objectives

Currently, all breast cancer patients should be tested for expression of the hormonal receptors and the HER2 protein by immunohistochemistry. HER2 overexpression is found in 25-30% of breast cancer patients and is related to increased tumor aggressiveness, increased rates of recurrence, and increased mortality (Slamon et al., 1987). The confirmed existence of HER2-reactive T cells in human blood calls for an analysis of the HER2-reactive T lymphocytes in breast cancer patients and their different phenotypes. Our group has previously used T cell clones reactive for $HER2_{369-377}$ for an adoptive T cell transfer of a patient with HER2-overexpressing breast cancer, showing that the transfer of HER2-reactive CTLs can lead to the elimination of disseminated HER2-overexpressing tumor cells in vivo (Bernhard et al., 2008).

In this study, PBMCs from patients with breast cancer are analyzed to investigate different aspects of the $CD3^+$ $CD8^+$ T cells reactive for the nonameric $HER2_{369-377}$-peptide at the time point of primary diagnosis by combining the simultaneous analysis of surface markers by multiparameter flow cytometry.

CTLs, which are known to be responsible for the clearance of tumors, are divided in four major subsets according to the cell surface markers CD45RA and CCR7: naive T cells (T_{naive} $CD45RA^+$ $CCR7^+$), effector T cells (T_{EFF}; $CD45RA^+$ $CCR7^-$), central memory T cells (T_{CM}; $CD45RA^-$ $CCR7^+$) and effector memory T cells (T_{EM}; $CD45RA^-$ $CCR7^-$). These $CD8^+$ T cell populations with different phenotype and functional antitumor capacities can even be further divided in more subpopulations according to the costimulatory markers CD28 and CD27. The description of the different CTLs subsets in peripheral blood as well as their relation to different breast cancer HER2 status is one of the major objectives of this thesis.

Another objective is the analysis of the expression of the PD-1 co-inhibitory molecule as a tumor immune escape mechanism. Interaction between the PD-1 receptor expressed on antitumor specific T cells with its ligand PD-L1 expressed on tumor cells negatively regulates T cell activation and T cell functions and it is considered an outstanding immune evasion mechanism. In this study, the molecule PD-1 is also analyzed in peripheral blood from breast cancer patients by flow cytometry.

It is an objective to ascertain whether there is a correlation between the frequency of peripheral HER2 reactive T cells expressing PD-1, the different HER2 breast tumor scores and the several $CD8^+$ T cell populations and subpopulations. Differences between PD-1 frequencies among the A2/HER2-multimer positive T cells could be an indication that the tumor has reacted to the specific antitumor immune response as the PD-1:PD-L1 interactions are, resulting in tumor escape and tumor progression. Investigation of the mechanisms underlying this tumor-induced immunosuppression may provide clues about how to treat breast cancer more efficiently.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Patients and controls

The study was performed at the Technical University of Munich, Germany. The enrollment of the patients was made in the gynecology department. A total of 107 HLA-A2$^+$ patients, of whom 85 had breast cancer and 22 were diagnosed with a benign breast disease, gave written informed consent to participate in the study. All the patients were female. The average age was 67 years. Peripheral blood and tumor tissue were obtained from patients undergoing resection for breast cancer. Blood and tissue samples were also obtained from patients undergoing surgery, which were afterwards diagnosed with a benign breast disease. None of the patients during the study had been treated with chemo-, radio- or hormonal therapy.

PBMCs were isolated from the patients´ peripheral blood samples and stored at -160°C in liquid nitrogen tanks.

The tissue sections of HER2 breast cancer were performed at the pathology routine laboratory according to the DAKO immunohistochemical score system (see Methods).

For this project, a total of 41 patients were finally included in the study of whom 34 had breast cancer. The TNM Classification was determined in every case by a gynecologist from our department. From the 41 patients, “T” ranged from T1 to T4, and “N” from N0 to N1. None of them had metastasis at the time point of diagnosis (M0). The most prevalent histological type was invasive ductal carcinoma (82%). The remaining 7 patients had benign breast disease and the majority of them were classified as fibrocystic mastopathy. From the breast cancer group, 6 patients were classified as a HER2 score 0 and 9 patients were HER2 score 1. These 15 patients were gathered as a negative score group. There were 13 patients with a 3 or positive HER2 score gathered as a positive group. Finally, the samples of the 6 patients classified as a HER2 score 2 were analyzed with the FISH test at the pathology department of our hospital to confirm the HER2 status. Subsequently 5 patients were included in the negative group whereas 1 patient was included in the positive group (table 1).

As a control for the multicolor flow cytometric analysis, PBMCs were used from HLA-A2$^+$ healthy donors, who voluntarily underwent leukapheresis.

HER2 tumor status	Grouped as	Number of patients
Score 0	Negative score	11
Score 1	Negative score	9
Score 3	Positive score	14
Benign breast disease	Benign breast disease	7

Table 1: Patients´ classification according to the breast cancer HER2 DAKO-score and the FISH analysis used in this study

2.1.2 Reagents

Aqua irrigation solution	Aqua DeltaSelect, Dreieich
Cryopreservation medium	DMSO (Dymethyl sulfoxide), glucose, HSA (Human serum albumin)
Dulbeccos phosphat buffered saline (PBS) 10-fold	PAA Laboratories GmbH, Pasching, Austria
DMSO	SERVA, Heidelberg
Ethylenediaminetetraacetic acid (EDTA)	Fluka, Deisenhofen
Ethidium monoazide bromide (EMA)	Invitrogen, USA
FACS staining buffer	1% BSA in PBS (pH 7.45)
Ficoll	Biochrom GmbH, Berlin
Fixation buffer	2% PFA in PBS
Fetal Bovine Serum Gold	PAA Laboratories GmbH, Pasching, Austria
Glucose-40%	B.Braun, Melsungen
HSA 20%	CSL Behring, Margburg
Heparin-Natrium-25000	Ratiopharm, Ulm
Human serum type AB	Milan Analytica, La Roche, Switzerland
Paraformaldehyde (PFA)	Rhot, Karlsruhe
PBS 10x, liquid	Invitrogen GmbH, Karlsruhe
Propidium iodide	Sigma-AldrichChemie, Deisenhofen
Trypan Blue	Invitrogen GmbH, Karlsruhe
Roswell Park Memorial Institute (RPMI) 1640, cell culture medium	Invitrogen GmbH, Karlsruhe

2.1.3 Antibodies

Antibody	Clone	Company
Anti-human CCR7-FITC	150503	R&D Systems, Wiesbaden-Nordenstadt
Anti-human CD8-PE	3B5	Invitrogen GmbH, Karlsruhe
Anti-human CD19-PE-Alexa Fluor 610	SJ25-C1	Invitrogen GmbH, Karlsruhe
Anti-human CD45RA-Pacific Blue	MEM-56	Invitrogen GmbH, Karlsruhe
Anti-human CD3-AmCyan	SK7	BD Pharmingen, Franklin Lakes, USA
Anti-human CD8-APC	DK25	Dako GmbH, Eching
Anti-human CD28-PE-Cy7	CD28.2	Natutec, Frankfurt am Main
Anti-human CD27-Alexa Fluor 700	0323	ebioscience, San Diego, USA
Anti-human HLA-A2-FITC	BB7.2	BD Pharmingen, Franklin Lakes, USA
Anti-human PD-1-APC	MIH4	ebioscience, San Diego, USA
Mouse IgG2b isotype control-FITC	27-35	BD Pharmingen, Franklin Lakes, USA

2.1.4 Peptides and Multimers

$HER2_{369-377}$ KIFGSLAFL	Biosyntan, Berlin
HER2 p369-377-Multimer	Dr. Busch, Insitute of Microbiology, *Rechts der Isar* hospital, Munich
$HIVpol_{476-484}$ ILKEPVHGV	Biosyntan, Berlin

2.1.5 Equipment

Centrifuge Multifuge 3s	Heraeus
Centrifuge 5810 R	Eppendorf, Hamburg
Centrifuge Rotina 420 R	Hettich, Tuttlingen
Centrifuge Rotanta 460 R	Hettich, Adelsried
Flow Cytometer (Coulter®Epics®)	Beckman-Coulter, Krefeld
Flow Cytometer BD™ LSR II	BD; Houston, USA
Light microscop Axiovert 25	Zeiss, Jena
Light microscop Axiovert 40 C	Zeiss, Jena
Multichannel pipette	Eppendorf, Hamburg
Neubauer Chamber	Reichert, New York, USA
Vortex REAX-Top	Heidolph, Nürnberg

2.1.6 Standard dilutions and buffers

Cryopreservation medium	20 ml DMSO 25ml 6% Glucose 55ml 20% HSA
Ethidium monoazide bromide (EMA)	Stock solution: 2mg/ml in DMSO by -20°C Working solution: 1µl EMA stock solution x to 1ml of FACS buffer stored at - 4°C
Erythrocyte lysis buffer (EL)	8.3 g Ammonium chloride 1.0 g Potassium carbonate 37 mg EDTA
FACS staining buffer	1% BSA in PBS (pH 7.45)
Fixation buffer	2% PFA in PBS
PFA	4% in warm PBS (pH 7.45) at -4°C
PBS	10-fold in Aqua irrigation solution

2.2 Methods

2.2.1 Isolation of PBMCs

Mononucleated cells of peripheral blood were separated with a two-phases-density gradient centrifugation with Ficoll from the high-density granulocytes, erythrocytes and dead cells. 200 ml heparinized patient or donor blood was diluted in several 50 ml tubes with 1x PBS and layered on 15 ml Ficoll. After centrifugation at 887 x g (18 min at room temperature) with switched off brakes, the mononucleated cells were harvested from the interphase between the sedimented cells and a mix from plasma and PBS above the Ficoll. The PBMCs were washed with 1x PBS (centrifugation 340 x g, 10 min) two times. After the first washing step the cells were pooled. Contaminating red blood cells (RBCs) were lysed by incubating in 5 ml erythrocytes lysing buffer and washed twice in cold PBS (by 340 x g centrifugation).

2.2.1.1 Cell count

Cells were counted in a Neubauer-counting chamber. 50 µl from the cell suspension were placed in a microscope in a ratio of 1:2 with a 0.5% trypan blue solution. Trypan blue stains only dead cells and detritus cells, making it possible to differentiate them from intact viable cells. Cells were counted in the four predetermined quadrants and calculated by the concentration of the cells per millimeter. The average of cells per quadrant was multiplied by the dilution factor and also by the coefficient 10^4.

2.2.1.2 Cryopreservation of the cells

After the cell count, the patients´ PBMCs were cryopreserved in human serum albumin 20% with cryopreservation medium. Vials containing an amount of cells between $8x10^6$ cells and $1x10^7$ were frozen 24 hours to -80°C in a freezing container before stored at -160°C in liquid nitrogen tanks.

2.2.2 Assignment of the HER2 breast cancer status

The most common HER2 testing approaches are at the protein level by immunohistochemistry (ICH) and at the DNA level by fluorescence in situ hybridization (FISH), which have been reported to have a high degree of fidelity. All patients included in the study were evaluated by routine hospital IHC test, a protein-based test that is used to provide an assessment of the amount of HER2 protein receptors on the surface of the cancer cells. The HER2 protein is visualized by attaching a color-emitting molecule.
After the breast surgery, breast tissues on formalin-fixed, paraffin-embedded samples were analyzed using the IHC-based HER2-testing kit, HercepTest (DAKO), which employs a HER2 antibody to measure the magnitude of HER2 overexpression in tumor cells. The results, the so-called DAKO-score, assign a value between 0 and 3 to every tissue sample. A HER2-negative breast cancer is considered when the result is score 0 and 1, while HER2-positive breast cancers are assigned with a score 3. A tumor with a score 2 is considered a borderline or equivocal result and therefore should be subjected to fluorescence in situ hybridization (FISH) to determine the definitive HER2 status (Gown, 2008).

For the purpose of this study, patients whose DAKO score was 0 or 1 were considered to be HER2 negative and those classified as score 3 were categorized as HER2 positive. An expert pathologist from the pathology department of our hospital analyzed the patients, whose DAKO score was 2, with FISH. According to the result they were included in the HER2 negative or positive group.

2.2.3 Flow cytometry measure (FACS analysis)

2.2.3.1 Basics of FACS

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. Several detectors are used for that purpose. One is directed in line with the light beam, the forward scatter, which measures the size of the cell. Several detectors are perpendicular to the light beam. The side scatter measures the intracellular granularity. Separate fluorescence channels detect fluorescent light associated with the particle. The surface antigens are marked with the correspondent labeled antibody to a fluorescence dye (direct immunofluorescence) or a first unlabeled antibody would be coupled with a second fluorescence-labeled antibody (indirect immunofluorescence).

The multimer technology has contributed to our ability to detect and estimate the frequency of T cells specific for tumor epitopes. T cells are able to recognize MHC class I- or class II-restricted peptides which can now be measured in the circulation or tissues of patients with cancer using multimers and flow cytometry (Altman et al., 1996; Meidenbauer et al., 2003).

In this project, direct FACS-measurement was performed for the surface antigen analysis, for the identification of the patients´ HLA status as well as for the visualization of CTLs reactive for HLA-A2:HER2-multimer.

Fluorochrome	**Peak excitation wavelength (nm)**	**Peak emission wavelength (nm)**
FITC	494	518
PE	565	578
PE-Alexa 610	565	627
EMA	462	625
PerCP	490	675
PE-Cy7	480	767
APC	650	660
PB	403	455
AmCyan	458	491
PE-Alexa 700	696	719

Table 2: Overview on excitation and emission wavelengths maxima of the fluorochromes used in this study

2.2.3.2 Staining HLA-A2 surface marker

Between the years 1999 and 2008, patients´ PBMCs with and without breast cancer isolated from peripheral blood were used for evaluating the HLA status. As a control for HER2, an irrelevant HLA-A2-multimer labeled with the HLA-A2-binding peptide HIVpol$_{476-484}$ was used. Propidium iodide-negative T cells were gated and the frequencies of HLA-A2^{+} cells were analyzed by flow cytometry (Coulter®Epics; Beckman-Coulter).

2.2.3.3 Staining cell surface markers

Recently it has become possible to identify particular antigen-specific T cells because of their receptor specificity. The affinity between T cell receptor (TCR) and MHC:peptid-complex is very low for tracking T cells with their specific MHC:peptid-complexes. This problem was resolved thanks to the introduction of multimers of MHC:peptid-complexes, which bind with a higher avidity to specific TCR on the surface of T cells (Romero et al., 1998).

In this study, which deals with the evaluation of the circulating $CD3^+$ $CD8^+$ $HER2_{369-377}$-reactive T cells, patients´ PBMCs were prepared and visualized with the multimeric complex containing HLA-A2:HER2 derived peptide (amino acids 369 to 377) as previously described (Knabel et al., 2002). When interpreting these data, one must bear in mind that, depending on past interaction with certain antigens, crossreaction between HER2 and other antigens may occur. Antigens that have been described in this context are certain viral peptides. Therefore, in our study it cannot be excluded entirely that all positive HLA-A2:HER2 T cell signals truly account for T cell specificity. Even though this bias caused by cross reactivity is though to be very low for the multimer technique, didactically in this study the term HER2-reactive T cells is used instead of HER2-specific T cells, even though the two can be seen as synonyms under the above stated restriction. The event count for each flow cytometric analysis was between 0.6 and 2 million cells.

PBMCs were used for seven colors cell surface staining using antibodies against cell surface molecules. All antibodies fluorochrome conjugates were first titrated in order to know the optimal staining intensities (table 3).

Antibody	**Titration**	**Antibody**	**Titration**
CCR7-FITC	1:10	CD45RA-PB	1:20
CD8-PE	1:200	CD3-AmCyan	1:20
CD19-PE-Alexa610	1:50	CD8-APC	1:200
CD8-PerCP	1:10	CD27-Alexa700	1:20
CD28-PE-Cy7	1:20	PD-1-APC	1:5

Multimer	**Titration**
$HER2_{369-377}$Multimer-PE	1:25

Table 3: Titration of the antibodies and multimer used in this study

2.2.3.4 Controls

a) Single colors

As the cells intercept the light source, they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Each fluorochrome has its own excitation and emission spectra. The inherent overlap of emission spectra from antibody fluorescent labels demands compensation. For determining and adjust accurately the fluorescence compensation, the so-called *single colors* were used, which are individually stained samples with the antibody-fluorochrome components of the multi-color samples. Unstained cells were used to determinate the optimal voltages of the flow cytometer detectors (table 4).

Unstained	CCR7-FITC	CD8-PE	CD8-PE-A610	CD45RA-PB	CD3-AmCyan	CD8-APC	CD8-PerCP	CD28-PE-Cy7	CD27-A700

Table 4: Schema of the single colors used in this study

b) Fluorescence Minus One (FMO)

In multicolor experiments, it is not proper to set gates based on an entirely unstained or fully isotype control stained cells. The best control for multicolor experiments is to stain cells with all reagents except the one of interest, the so-called FMO control. This FMO control allows to define between positivity and negativity for every used antibody.

In this study, every test composed by “all the antibodies” means antibodies: CCR7-FITC plus CD19-PE-Alexa610 plus CD8-PerCP plus CD28-PE-Cy7 plus CD45RA-PB plus CD3-AmCyan plus CD27-Alexa700 plus PD-1-APC, unless it is specifically indicated that one antibody was not added (table 5).

A FMO without multimer was also performed to help to determine the cut-point between positive and negative $HER2_{369-377}$ events.

All the antibodies	All the antibodies minus CCR7-FITC	All the antibodies minus $HER2_{369-377}$-Multimer	All the antibodies minus CD19-PE-A610	All the antibodies minus CD28-PE-Cy7	All the antibodies minus CD45RA-PB	All the antibodies minus PD1-APC	All the antibodies minus CD27-A700

Table 5: Schema of the FMO used in this study

c) Multimer control

A positive $HER2_{369-377}$-multimer-PE control was also performed by using a corresponding epitope-specific cell population, which is 90% composed of HLA-A2 restricted T cells. The control was generated as following: first the NvB 2/12 clone was expanded and cultured for 2 weeks. A $HER2_{369-377}$-multimer-PE staining was performed in order to determine the percentage of HER2-reactive cells and 78% of the cells were HER2 positive (data not show). The clone was titrated with healthy donor PBMCs to yield a cell suspension with estimated 5% proportion of HER2-reactive cells. Each vial containing $1.4x10^7$ clone cells and PBMCs were cryopreserved in cryopreservation medium and HSA 20%. Vials were stored 24 hours at -80°C in a freezing container and then stored at -160°C in a liquid nitrogen tank.

With this control it is possible to detect false negative results due to a patients cells´ HER2 reactivity. It is also important for the control of the proper $HER2_{369-377}$-multimer-PE functionality.

2.2.3.5 MHC:multimer and multicolor staining protocol

As already explained, PBMCs from HLA-A*0201 patients were carefully thawed, harvested, washed and stained with different surface markers antibodies and the HLA-A*0201/$HER2_{369-377}$ (A2/HER2) multimer. A2/HER2 multimer-positive cells´ frequencies were measured and compared between patients with histologically confirmed breast cancer and benign breast disease and/or within the different HER2 breast tumor scores.

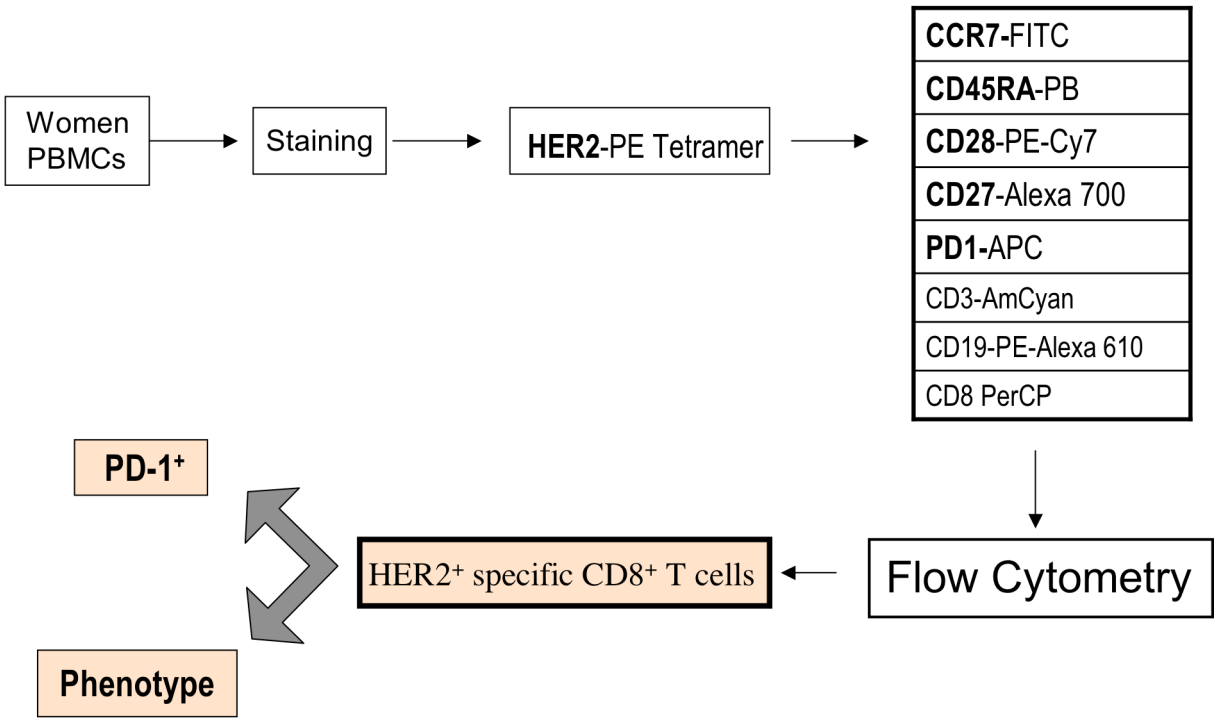

Figure 2: Set up from the nine colors flow cytometry used in this study

The multimer HLA-A2:HER2 and multicolor staining protocol for PBMCs was performed as follows:

1) Cell count

PBMCs were thawed for a few minutes by 37°C, harvested and transferred to a 50 ml tube with 40 ml RPMI cell culture medium supplemented with 10% serum. They were washed twice with RPMI medium (Centrifugation: 5 min at 423 x g at 4°C). Cells were counted as already described. 3-4x10^6 patient cells were distributed in wells of an U-bottom 96well plate. At the same time, 1-2x10^6 donor cells were also distributed per well for the controls as well as the positive control for the multimer function. Cells were washed with FACS buffer (centrifugation: 5 min by 423 x g by 4°C).

2) Ethidium monoazide bromide staining

All cells were resuspended in 50 µl per well of the fluorescent photoaffinity label ethidium monoazide bromide (EMA). Due to the fact that EMA binds covalently upon light irradiation to nucleic acids in cells that have compromised membranes, viable cells can be discriminated from such cells, which have taken up EMA.

After 20 minutes of incubation on ice in the dark followed by 10 minutes on ice to strong light, the cells were washed twice with FACS buffer (centrifugation: 5 min by 423 x g by 4°C).

3) Multimer staining

Thereupon, cells were resuspended in 50µl FACS buffer per well. The staining of PBMCs with the MHC:$HER2_{369-377}$-multimer-PE-labeled was realized by adding multimer to the corresponding wells. After incubation of 20 minutes on ice in the dark, a second surface staining antibody mix solution was added followed by an incubation of 30 minutes on ice in the dark. Cells were washed twice with PBS (centrifugation: 5 min at 423 x g at 4°C).

d) Fixation of the cells

To proceed with the fixation of the cells, 100µl PFA 2% was added per well followed by an incubation for 30 minutes at 4°C in the dark.

Finally, cells were washed two times with 200µl FACS buffer per well and they were stored at 4°C during the next few hours until the FACS analysis. Before the measurement with FACS BD™ LSR II, the cells were one last time resuspended by pipetting.

2.2.4 Patients´ analysis strategy

A total of 41 patients were analyzed by multicolor flow cytometry. Figure 3 and figure 4 show the general gating strategy with one of the patients PBMCs as an example.

First, a lymphocytes gate was created using the forward scatter and side scatter. EMA-negative T cells were identified for nonviable cell exclusion and afterwards, a gate made in both $CD3^+$ and $CD8^+$ T cells allowed to identify the CTL cells from the rest of the lymphocytes. A final gate was made to measure the frequencies of reactive HLA-A*0201/$HER2_{369-377}$-multimer T cells. Both $HER2_{369-377}$-reactive and non-reactive populations of cells were analyzed in all patients for the expression of the different surface antibodies. The combination of CCR7 and CD45RA cell surface markers was used to characterize

T_{naive}, T_{CM}, T_{EFF}, T_{EM} cell subsets and the two markers CD27 and CD28 to determine the different phenotypic subpopulations. Finally the expression of PD-1 was examined in each subset.

To permit a more accurate analysis, the $CD3^+$ $CD8^+$ non-reactive $HER2_{369-377}$ population of T cells was compared with the $CD3^+$ $CD8^+$ $HER2_{369-377}$-reactive T cells.

To make objective the selection of patients, three criteria were applied for each patient after the flow cytometry analysis: the percentage of cells within the lymphocyte gate had to be more or equal than 10% of the total acquired events, the number of $CD3^+$ $CD8^+$ T cells had to be more than 17.000 and the spreading should be acceptable.

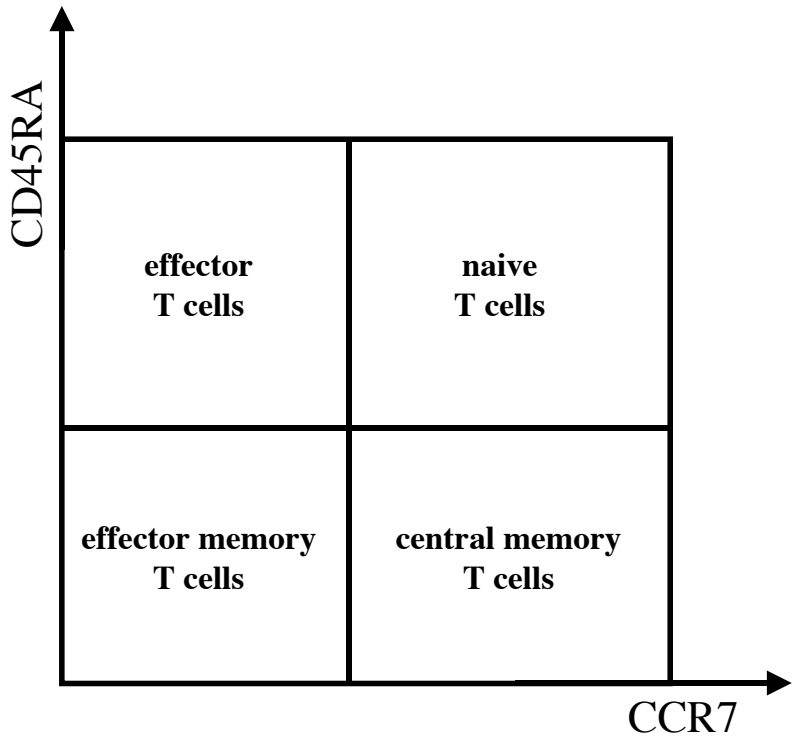

Figure 3: Distribution of T cell in FACS analysis

Patients were analyzed according to the cell surface markers CCR7 and CD45RA. Distribution of the naive T cells (T_{naive}; $CD45RA^+$ $CCR7^+$), effector T cells (T_{EFF}; $CD45RA^+$ $CCR7^-$), central memory T cells (T_{CM}; $CD45RA^-$ $CCR7^+$) and effector memory T cells (T_{EM}; $CD45RA^-$ $CCR7^-$) are shown schematically as they appear in FACS analysis.

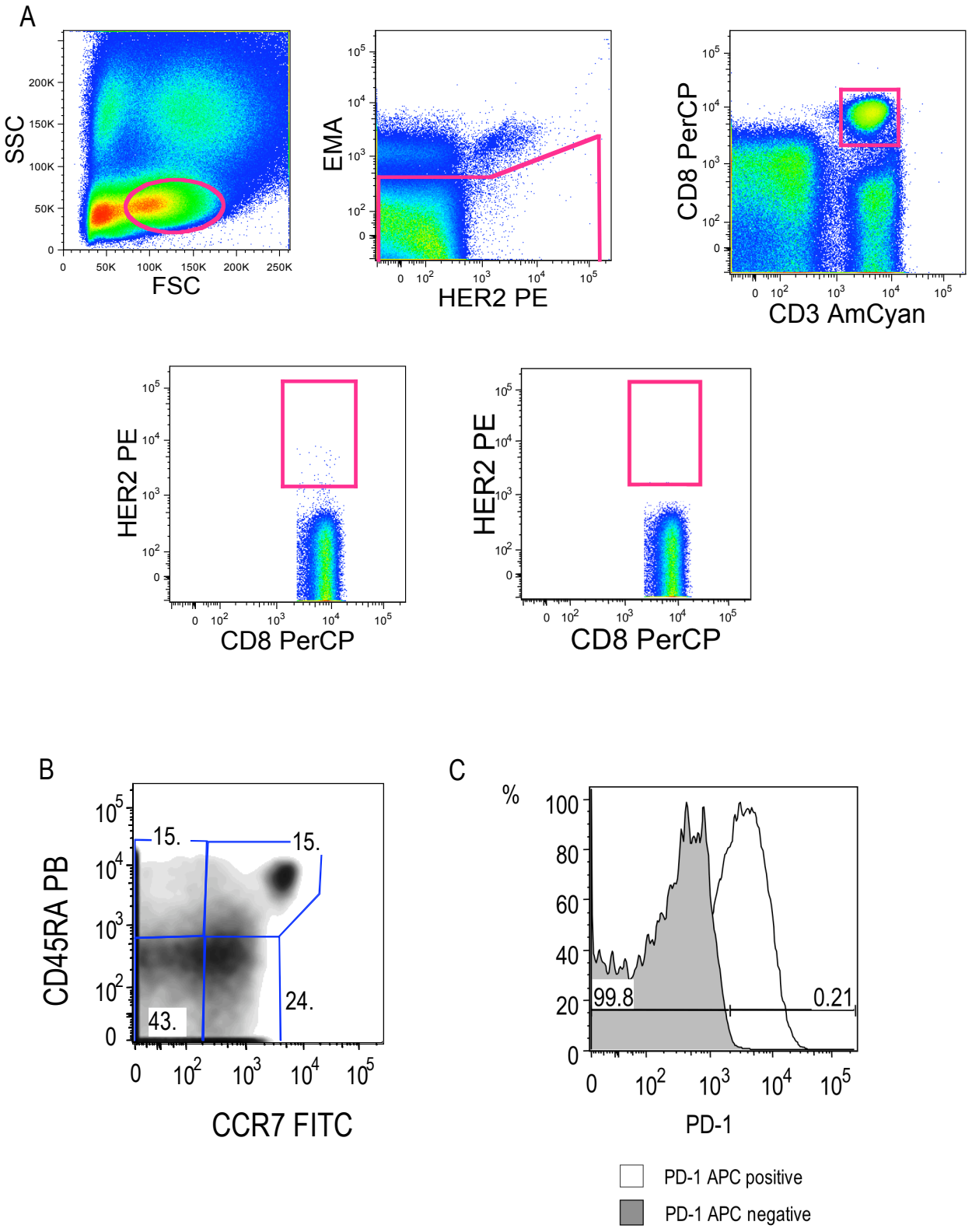

Figure 4: Flow cytometric analysis of CD8⁺ T cells using the A2/HER2-multimer

Dot plot analysis of the gating strategy from a representative experiment of one of the patients used in this study is shown in figure 4A. Figure 4B shows staining of CD45RA PB and CCR7 FITC cells in peripheral blood from the same patient. The expression of PD-1 is shown in figure 4C.

2.2.5 Statistics and analysis

The software used in the Flow Cytometer BD™ LSR II was the BD FACSDiva™ 6.0 software. For the analysis of the flow cytometric data, the FlowJo software (version 8.8.3) was used. Results from FlowJo were examined in the Pestle program (version 1.6.1). Pestle is designed to formats and recodes data generated in FlowJo for use in Spice. For the graphics and statistical analysis the Spice program (version 4.2.2) was used, which analyzes data from multiparametric flow cytometry experiments as well as Microsoft Excel and Microsoft PowerPoint programs (Microsoft Cooperation, CA, USA). The significant statistical differences were made either with a Student's T-test or the Wilcox Rank test. The p-value was set up either $p<0.05$ for significant statistic differences or $p<0.001$ for highly significant differences. All data show the standard deviation (SD).

3. RESULTS

3.1 Frequency of $CD3^+$ $CD8^+$ T cells reactive to the A2/HER2 multimer

3.1.1 Frequency of $CD3^+$ $CD8^+$ T cells reactive to the A2/HER2 multimer does neither differ between patients with a benign breast disease and with a breast cancer nor does it differ within the different tumor scores

Tumor antigens that can be immunogenic in patients have been identified in some human cancers. However, the prevalence of endogenous T cell response in breast cancer patients remains currently unclear. In this study, it was questioned whether HER2-overexpressing and HER2 non-overexpressing breast cancer patients had similar pre-existing A2/HER2-reactive cellular immune responses at the time point of primary diagnosis.

Frequencies of $CD3^+$ $CD8^+$ T cells reactive for HER2 did not differ neither between patients with benign breast disease and with breast cancer nor within the different HER2 status with a p-value of <0.05 (figure 5). The average value in patients with a benign breast disease was 0.05%, very similar to the average in breast cancer patients with a negative HER2 score 0.045% or a positive HER2 score 0.05%.

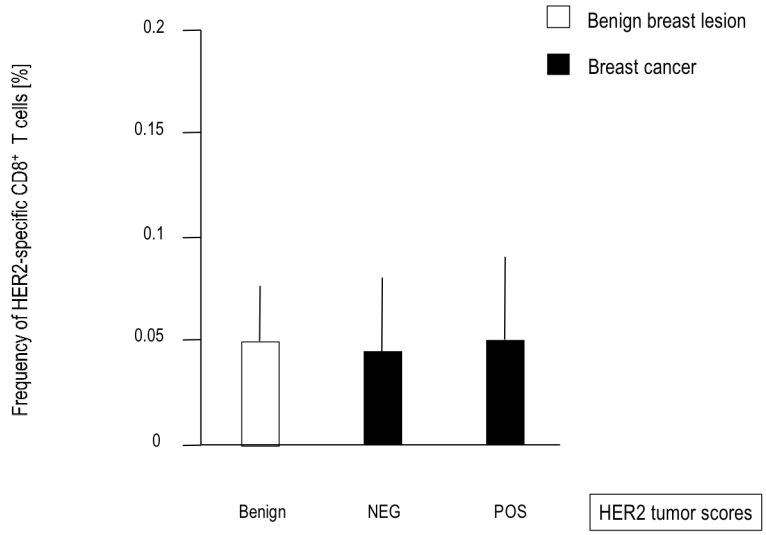

Figure 5: Analysis by flow cytometry, of $CD3^+$ $CD8^+$ A2/HER2-multimer reactive T cells

PBMCs frequencies of circulating A2/HER2-reactive T cells were measured in patients with benign breast lesions (n=7) and breast cancer (n=34). The HER2 breast cancer division was made in compliance with the DAKO immunohistochemical score system. The average value in peripheral blood was very low and did not differ neither between benign breast disease and breast cancer patients nor within the different HER2 tumor scores.

3.1.2 PD-1 expression is significantly higher in reactive A2/HER2 $CD3^+$ $CD8^+$ T cells compared to other $CD3^+$ $CD8^+$ T cells

The molecule PD-1 is expressed on activated T lymphocytes and it is known to negatively regulate T cell activation and T cell functions through engagement with its ligand PD-L1 expressed in tumors (Carter et al., 2002; Chemnitz et al., 2004; Freeman et al., 2000). Our hypothesis was that this immunoresistance might play a role also in breast cancer since the tumor may express PD-L1, which interacts with the receptor PD-1 in tumor-specific CTLs and in this case, especially with CTLs reactive for the HER2 molecule overexpressed in breast tumors. Quantification of A2/HER2-reactive CTLs in the peripheral blood of breast cancer patients with a high PD-1 expression would be indicative of the tumor escape mechanism playing a role.

PD-1 expression was analyzed specially with regard to its relation between reactive A2/HER2 and A2/HER2-multimer negative $CD3^+$ $CD8^+$ T cells among the 34 breast cancer patients. The receptor PD-1 was significantly higher expressed in A2/HER2-multimer positive T cells compared to A2/HER2-multimer negative T cells with a p-value of <0.001. Figure 6A shows the total number of cells in which the average value for A2/HER2-multimer positive and negative cells was 74.4 and 27.4, respectively, of 62.331 $CD3^+$ $CD8^+$ T cells. Figure 6B shows the frequency of cells as a percentage, in which the average value of frequency for A2/HER2-multimer positive cells was 37.22% and 13.73% for A2/HER2-multimer negative cells.

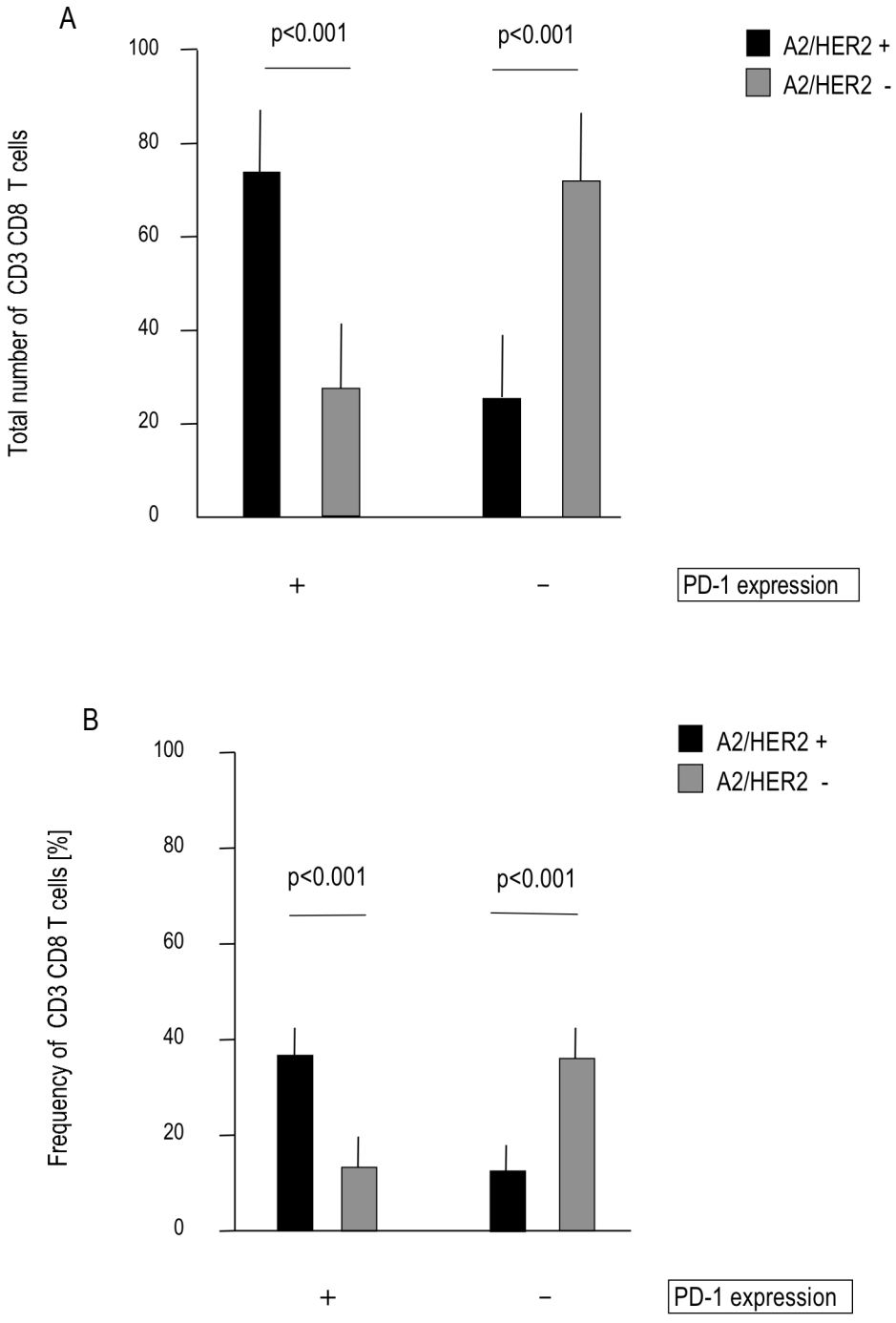

Figure 6: PD-1 expression was significantly higher in A2/HER2-multimer positive compared to A2/HER2-multimer negative T cells

PD-1 expression on PBMCs was measured and compared between positive and negative A2/HER2 T cells among breast cancer patients. (A) shows the total number of cells while (B) shows the average value as a percentage. In A2/HER2-reactive T cells the PD-1 expression is significantly higher with a p-value of <0.001 compared to A2/HER2-multimer negative T cells.

3.1.3 Frequency of A2/HER2 $CD3^+$ $CD8^+$ T cells between benign breast disease and breast cancer patients does not differ in their PD-1 expression pattern

The PD-1 expression pattern in PBMCs from benign breast disease and breast cancer patients is currently not known. In this context, A2/HER2 multimer-positive $CD3^+$ $CD8^+$ T cells from these two groups were compared regarding their PD-1 expression.

No significant differences were found between these two groups (figure 7). The average value as a percentage was 54.71% and 50.93%, respectively (figure 7A). The total number of cells expressing the molecule PD-1 in patients with a benign breast disease was 109.4 of the 64.331 $CD3^+$ $CD8^+$ T cells, while it was 101.8 in breast cancer patients of the 61.423 $CD3^+$ $CD8^+$ T cells (figure 7B).

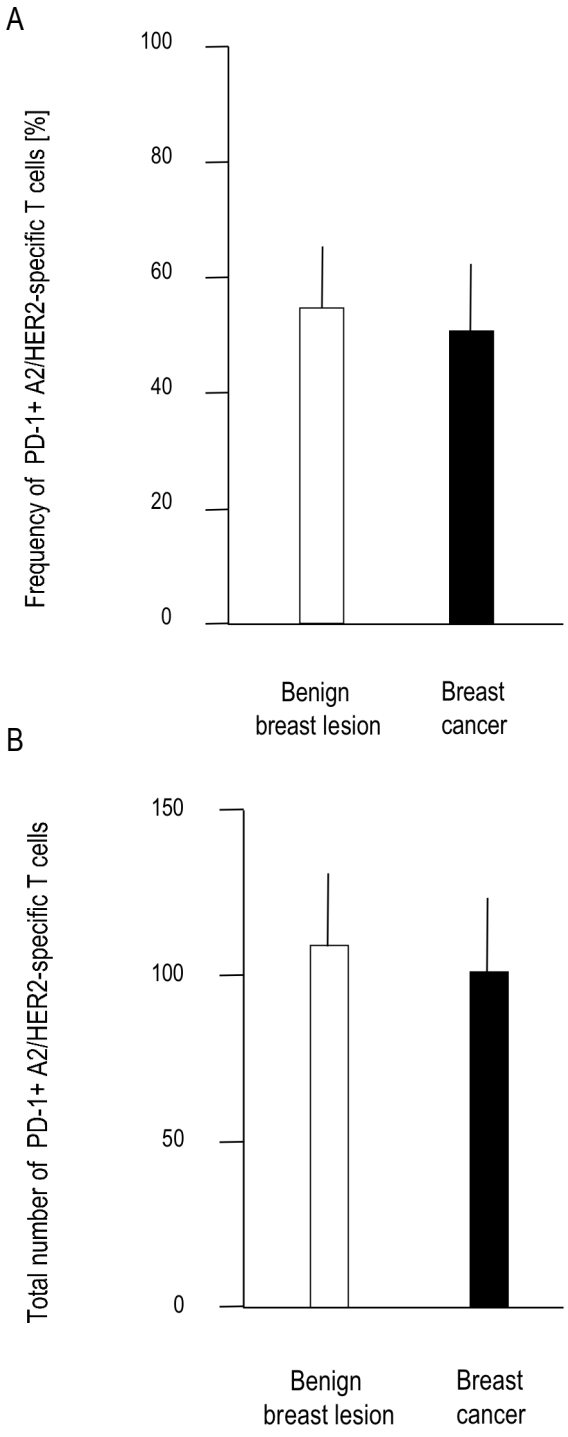

Figure 7: A2/HER2-reactive CD3$^+$ CD8$^+$ T cells did not differ in their PD-1 expression between patients with a benign breast disease and breast cancer

The expression of PD-1 in PBMCs from patients with benign breast disease and breast cancer was analyzed as a percentage (A) as well as a total number of cells (B). No significant differences were found between these two groups regarding their PD-1 expression.

3.1.4 PD1 expression on A2/HER2-reactive CD3+ CD8+ T cells is overrepresented independently of the HER2 status compared to all CD8+ T cells

The incidence, magnitude and clinical significance of PD-1 expression within the different tumor stages of breast cancer were previously unknown. Once it was clear that A2/HER2-reactive T cells have a higher PD-1 expression, a comparison was made between A2/HER2-reactive cells and the remaining CD3+ CD8+ T cells related to the histological HER2-tumor score.

Reactive A2/HER2 CD3+ CD8+ T cells expressing PD-1 molecule were overrepresented in both negative and positive scores. The total number of PD-1-positive cells for the negative and positive score in the A2/HER2+ population was 73.67% and 75.55% respectively compared to all CD3+ CD8+ T cells in which the average value among the negative score was 27.88% and 26.76% for the positive score with a p-value of <0.001 (figure 8).

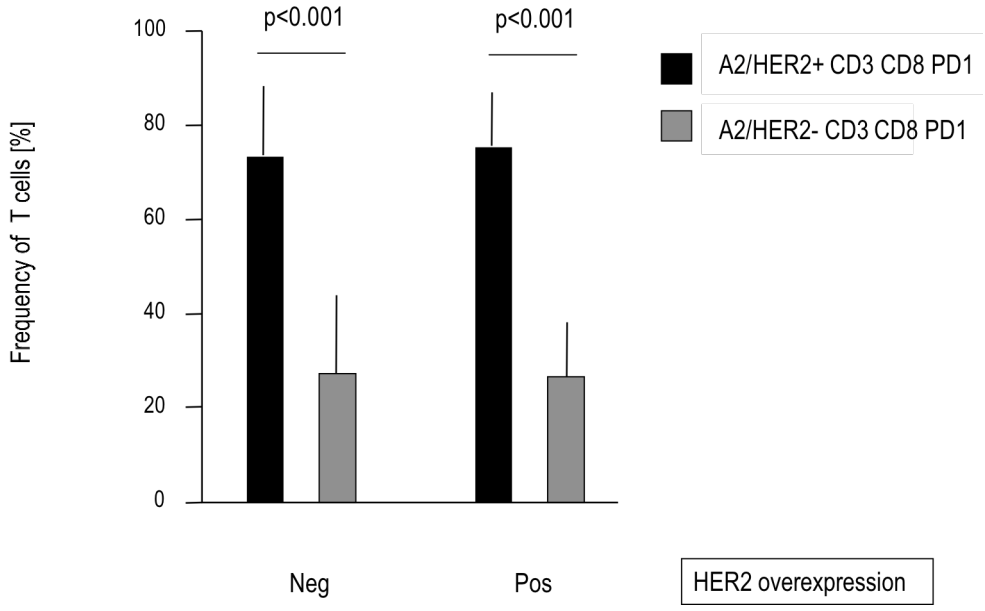

Figure 8: A2/HER2-positive T cells expressing PD-1 molecule were overrepresented independent of the HER2 status compared to all CD3+ CD8+ cells

The PD-1 expression on PBMCs from breast cancer patients was measured and compared between the two HER2 tumor scores and the A2/HER2 restriction. A2/HER2-reactive T cells were significantly higher in both scores compared to all CD3+ CD8+ T cells.

3.2 $CD8^+$ T cell phenotypes in A2/HER2-multimer positive cells

3.2.1 Distribution of the four defined $CD3^+$ $CD8^+$ T cell phenotypes in peripheral blood of breast cancer patients

Initially, three $CD8^+$ T cell subpopulations (naive, memory and effector) distinguished by their cell surface phenotype were described (Hamann et al., 1997). More recently, a new phenotypic complexity among $CD8^+$ T cells in both human and mouse has been shown thanks to two cell surface markers: CD45RA and CC chemokine receptor 7^+ receptor (CCR7), bringing the concept of four subsets: naive T cells (T_{naive}; $CD45RA^+$ $CCR7^+$), effector T cells (T_{EFF}; $CD45RA^+$ $CCR7^-$), central memory T cells (T_{CM}; $CD45RA^-$ $CCR7^+$) and effector memory T cells (T_{EM}; $CD45RA^-$ $CCR7^-$) (Masopust et al., 2001; Sallusto et al., 2004; Sallusto et al., 1999).

According to this classification and by using the cell surface markers CCR7 and CD45RA, an overview of the distribution of the four $CD8^+$ T cell phenotypes was obtained in order to analyze PBMCs from the 34 breast cancer patients within the A2/HER2-multimer positive and negative $CD3^+$ $CD8^+$ T cell populations as well as according to their HER2 tumor score (figure 9A).

T_{CM} were the population of cells with the lowest frequency (6.43%) and no significant differences were found between A2/HER2-multimer positive and negative T cells.

Frequency of naive T cells was significantly increased in the A2/HER2-reactive population (38.71%) compared to the A2/HER2-multimer negative population (7.16%) with a p-value of <0.001, while the opposite results were seen in the T_{EM} population, where the frequency in the A2/HER2-multimer negative population and positive population was 52.5% and 17.51%, respectively.

When the different HER2-status was analyzed among the A2/HER2-reactive T cell population no significant differences were found among the subsets of naive, effector, central or effector memory T cells (figure 9B). The highest frequencies were observed for T_{naive} and T_{EFF} subpopulations of cells with a percentage of 37.54% and 39.58%, respectively, while the lowest frequency was found for the T_{CM} subpopulation (6.32%).

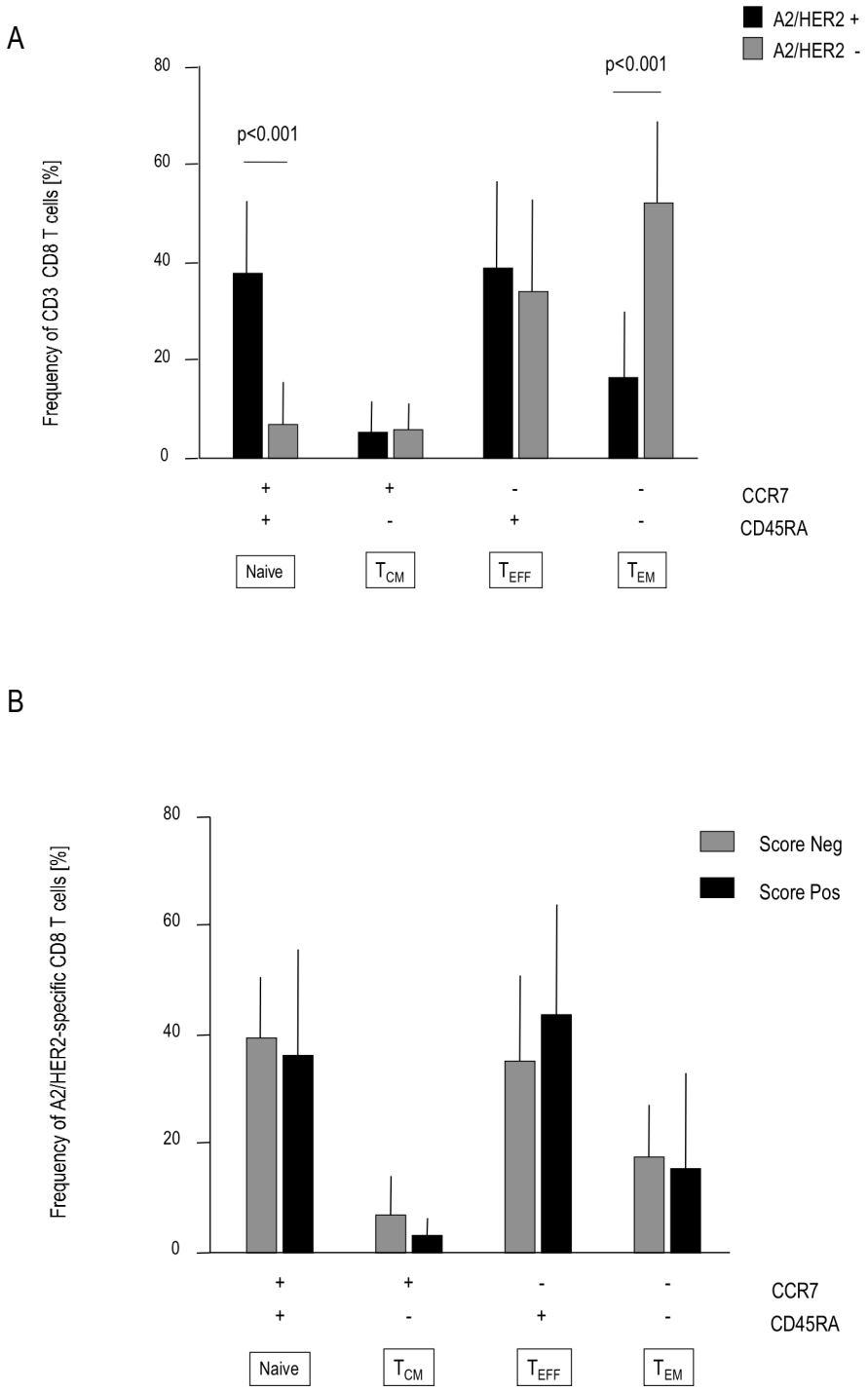

Figure 9: Distribution of the naive T cells (T_{naive}; CD45RA$^+$ CCR7$^+$), effector T cells (T_{EFF}; CD45RA$^+$ CCR7$^-$), central memory T cells (T_{CM}; CD45RA$^-$ CCR7$^+$) and effector memory T cells (T_{EM}; CD45RA$^-$ CCR7$^-$) in peripheral blood from breast cancer patients

Figure A: Among A2/HER2-multimer positive and negative T cells, frequencies of PBMCs from breast cancer patients were analyzed according to the cell surface markers CCR7 and CD45RA.

Figure B: Distribution of the naive T cells (T_{naive}; CD45RA$^+$ CCR7$^+$), effector T cells (T_{EFF}; CD45RA$^+$ CCR7$^-$), central memory T cells (T_{CM}; CD45RA$^-$ CCR7$^+$) and effector memory T cells (T_{EM}; CD45RA$^-$ CCR7$^-$) A2/HER2-multimer specific CD8 T cells phenotypes in peripheral blood from breast cancer patients according to their HER2 tumor score.

3.2.2 Naive CD3+ CD8+ T cells reactive to A2/HER2 were overrepresented compared to A2/HER2-multimer negative T cells in the two tumor scores

The phenotype of naive CD8+ T cells is CD45RA+ CCR7+. Naive CD8+ T cells are mature T cells that circulate within the blood predominantly between secondary lymphoid organs, constantly in search for their specific antigen. Upon activation with peptide recognition specificity and restricted by MHC class I or MHC class II, they reenter the cell cycle and divide rapidly to proliferate and afterwards differentiate into effector T cells.

In all tumor scores A2/HER2-reactive naive T cells were overrepresented compared to A2/HER2-multimer negative T cells ($p<0.001$). The percentage of A2/HER2-reactive T cells was 38.56% compared to 5.97% in A2/HER2-multimer negative T cells. However, there were no significant differences within the two different tumor scores (figure 10).

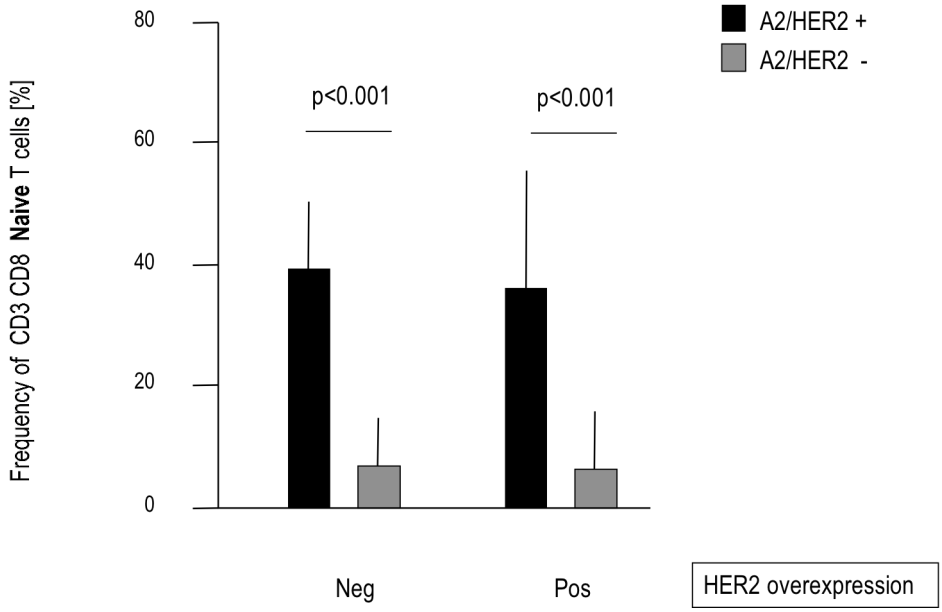

Figure 10: Naive (CCR7+ CD45RA+) CD3+ CD8+ T cells specific to A2/HER2 were overrepresented compared to A2/HER2-multimer negative T cells independent of the tumor score

The naive T cell subset was analyzed in peripheral blood of breast cancer patients according to the cell markers CCR7 and CD45RA. A higher total number of A2/HER2-multimer positive cells were found in both HER2 tumor scores compared to A2/HER2-multimer negative cells.

3.2.3 Frequency of T_{CM} cells in peripheral blood from breast cancer patients

The T_{CM} subset phenotype is CCR7$^+$ CD45RA$^-$. They express CCR7 and would therefore be expected to migrate more easily to the T cell zones of peripheral lymphoid tissues, as do naive CCR7$^+$ T cells, and eventually encounter antigen presented by mature dendritic cells (DCs). They take longer than T_{EM} to differentiate into effector T cells but have a higher capacity to proliferate upon antigen stimulation compared with T_{EM} (Bachmann et al., 2005; Wherry et al., 2003).

The frequency of A2/HER2-positive T_{CM} cells did not differ between negative and positive HER2 tumor score. The A2/HER2-negative T_{CM} cells in patients with a negative HER2 tumor score were significantly higher compared to that in patients with a positive HER2 tumor score with a p-value of <0.05 (figure 11).

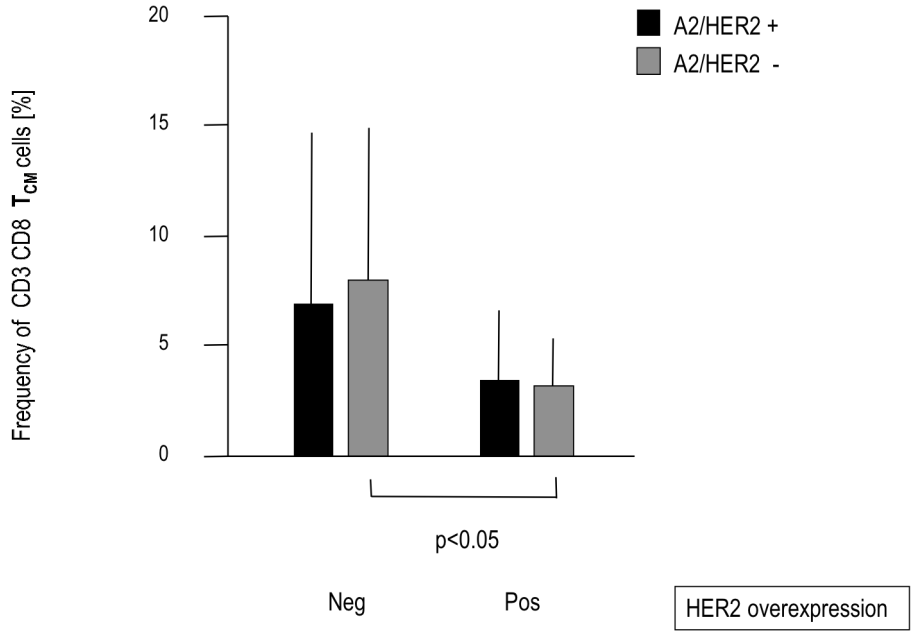

Figure 11: Frequency of A2/HER2-multimer negative T_{CM} cells (CCR7+ CD45RA-) in breast cancer patients was decreased in the positive HER2 tumor score

Among A2/HER2-multimer positive and negative T cells, frequencies of T_{CM} were analyzed in PBMCs from breast cancer patients according to the cell surface markers CCR7 and CD45RA.

3.2.4 A2/HER2 positive T_{EM} cells are overrepresented in all scores compared to A2/HER2-multimer negative T_{EM} cells

The T_{EM} subset phenotype is CCR7$^-$ CD45RA$^-$. They can rapidly mature into effector T cells and secrete large amounts of interferon gamma (IFNγ), interleukin 4 (IL-4), and interleukin-5 (IL-5) early after restimulation. They lack the chemokine receptor CCR7 and thus circulate in the peripheral blood having the property to populate non-lymphoid peripheral tissues and mucosal sites. They express high levels of β1 and β2-integrins, as well as receptors for inflammatory chemokines, suggesting that these T_{EM} are specialized for rapidly entering inflamed tissues and in general to respond rapidly to peripheral challenges (van Panhuys et al., 2005; Weninger et al., 2001).

In contrast to the naive T cell population, the T_{EM} subset was found to be overrepresented in A2/HER2-multimer negative T cells with 52.03% of cells compared to 16.67% of reactive A2/HER2 T cells (figure 12). There were no significant differences within the HER2 tumor scores.

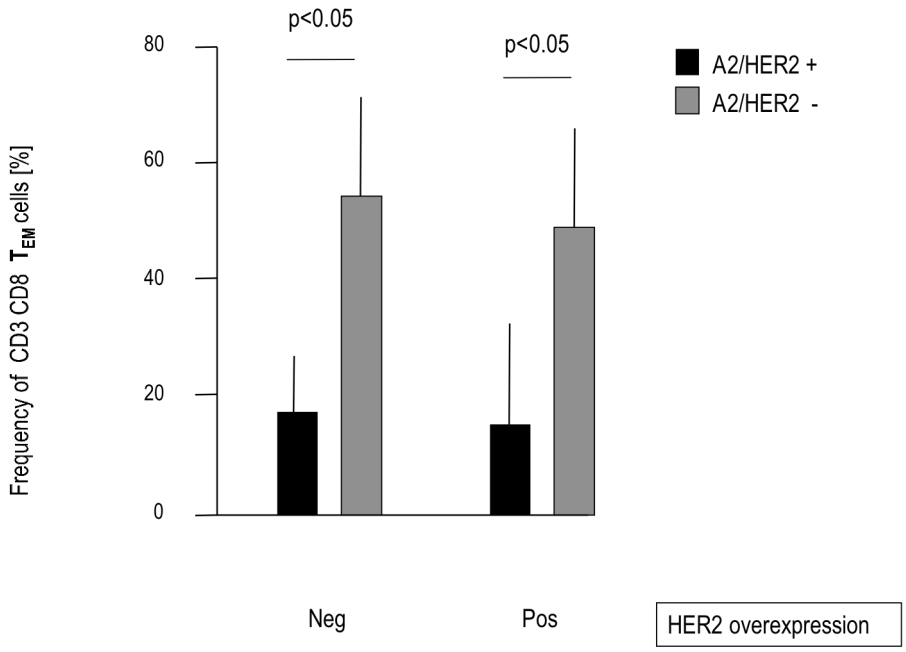

Figure 12: A2/HER2-multimer negative T_{EM} cells (CCR7- CD45RA-) are overrepresented independent of the HER2 status compared to A2/HER2 positive T_{EM} cells

Frecuencies of T_{EM} cells were analyzed in PBMCs from patients with breast cancer. In the two HER2 tumor scores, T_{EM} cells in peripheral blood from breast cancer patients were significantly more numerous in the negative A2/HER2 T cell population than in the positive A2/HER2 T cell population.

3.2.5 Frequency of reactive A2/HER2 $CD3^+$ $CD8^+$ T_{EFF} cells do not differ significantly within the HER2 tumor scores

Once primed, naive T cells differentiate into effector T cells (T_{EFF}) that perform antigen-specific functions without the need for co-stimulation. Their function, which among others is the clearance of intracellular pathogens and tumor, depends on the cell-mediated immune response. These cells have a strong cytotoxic potential compared with memory T cells. Their phenotype is $CD45RA^+$ $CCR7^-$.

Frequency of cells in the A2/HER2-reactive T cell population was 40.31%, very similar to the A2/HER2-multimer negative T cells, which was 35%. Among the frequency of T_{EFF} cells there were neither significant differences between the HER2 tumor scores nor between A2/HER2-multimer positive and negative T cells (figure 13).

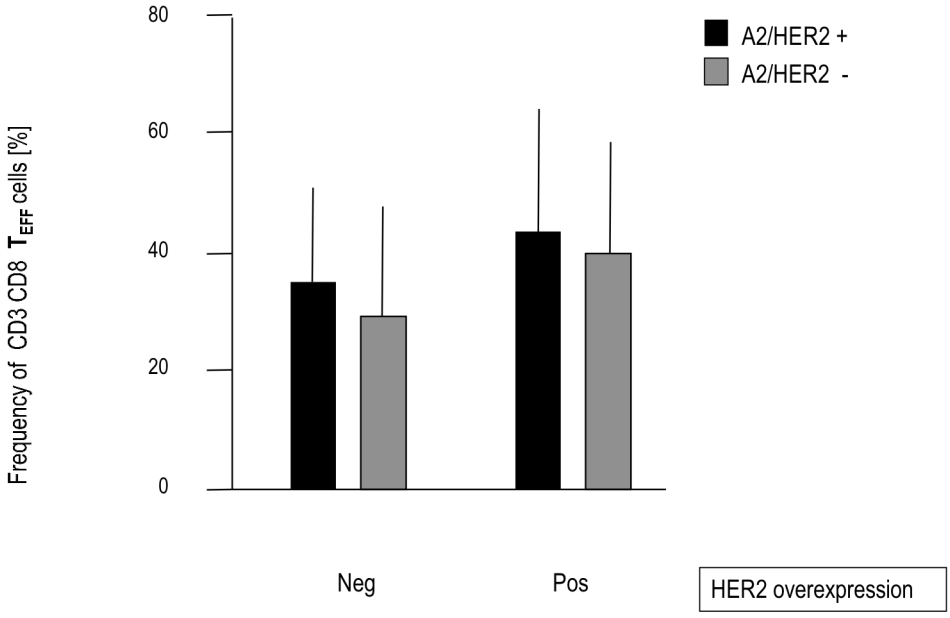

Figure 13: Specific HER2 T_{EFF} cells (CCR7- CD45RA+) have no significant differences within the different HER2 tumor score

T_{EFF} cells were analyzed on PBMCs from breast cancer patients thanks to the cell markers CCR7 and CD45RA by flow cytometry. Positive and negative A2/HER2 T cells are compared within the different HER2 tumor scores.

3.3 CD8+ T cell phenotypes in A2/HER2 multimer-positive cells expressing PD-1

Given that each phenotypical subset of CD3+ CD8+ T cells has different functional capacities and plays a different role in antitumor immunity, the four different populations were analyzed according to their PD-1 expression in order to find a possible correlation with the described tumor immune escape mechanism.

3.3.1 Frequency of naive PD-1 reactive CD3+ CD8+ T cells is higher in the A2/HER2-positive T cell population compared to the A2/HER2-multimer negative T cell population

As well as in naive T cells, frequencies in PD-1-expressing naive T cells among the reactive HER2 population were overrepresented compared to the non-reactive HER2 population. Frequency of cells among PD-1-expressing A2/HER2-reactive naive T cells was 31.62%, while among the non-reactive A2/HER2 T cells the frequency was 0.87% making a significant difference with a p-value of <0.001 (figure 14).

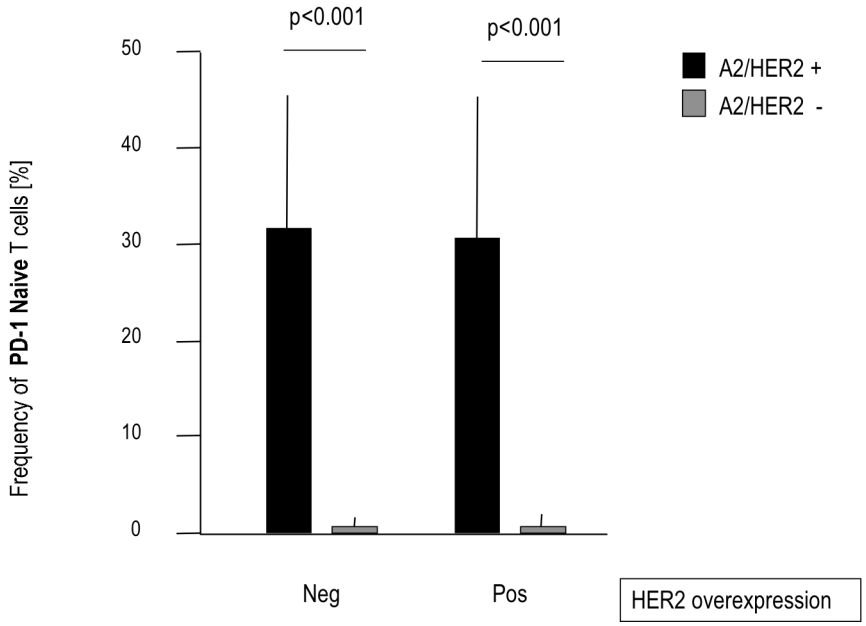

Figure 14: Frequency of naive (CCR7+ CD45RA-) PD-1+ T cells is higher in the A2/HER2+ T cell population

Naive T cells were analyzed in peripheral blood from breast cancer patients by flow cytometry according to the cell markers CCR7+ CD45RA-. Frecuency of PD-1+ naive T cells are shown in both A2/HER2 positive and negative populations.

3.3.2 The positive HER2 tumor score is characterized by high frequency of PD-1-positive A2/HER2 CD3+ CD8+ T_{CM} cells

In the positive HER2 tumor score the frequency of A2/HER2-reactive CD3+ CD8+ T_{CM} cells expressing PD-1 was found to be significantly increased compared to that of A2/HER2-multimer negative CD3+ CD8+ T_{CM} cells (figure 15).

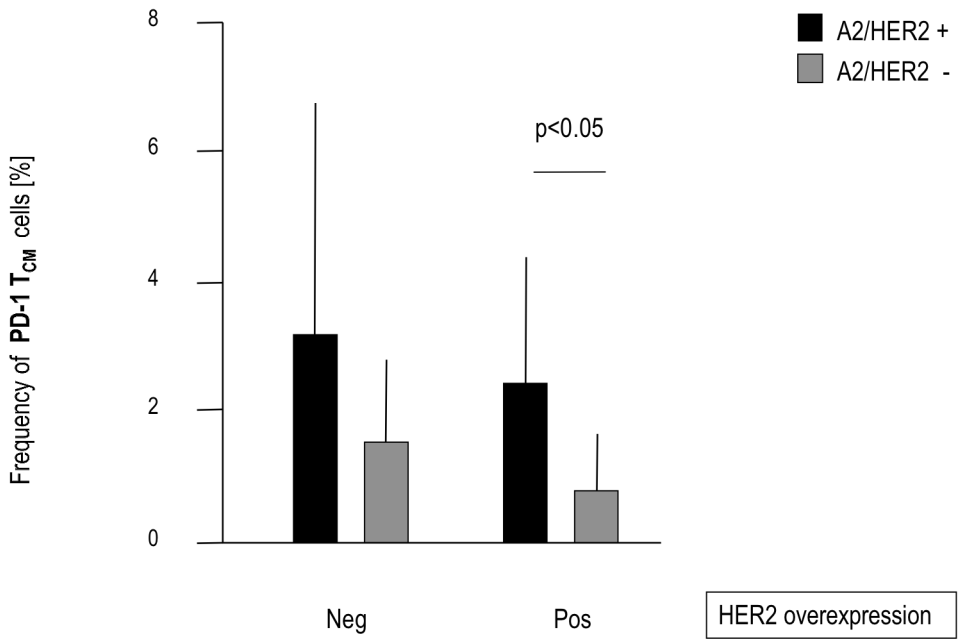

Figure 15: The positive tumor score is characterized by high numbers of PD-1 reactive A2/HER2 T_{CM} cells (CCR7+ CD45RA-) compared to A2/HER2-multimer negative T_{CM} cells

The frequency of A2/HER2-reactive CD3+ CD8+ T_{CM} cells expressing PD-1 in peripheral blood from breast cancer patients was analyzed between A2/HER2-reactive and non-reactive T cells.

3.3.3 The negative tumor score is characterized by low frequency of cells in the PD-1-positive A2/HER2-reactive CD3+ CD8+ T_{EM} subset

A2/HER2-reactive CD3+ CD8+ T_{EM} cells expressing PD-1 in the HER2 negative score were found to be significantly decreased compared to A2/HER2-multimer negative CD3+ CD8+ T_{EM} cells while no significant differences where found among the HER2 positive score (figure 16).

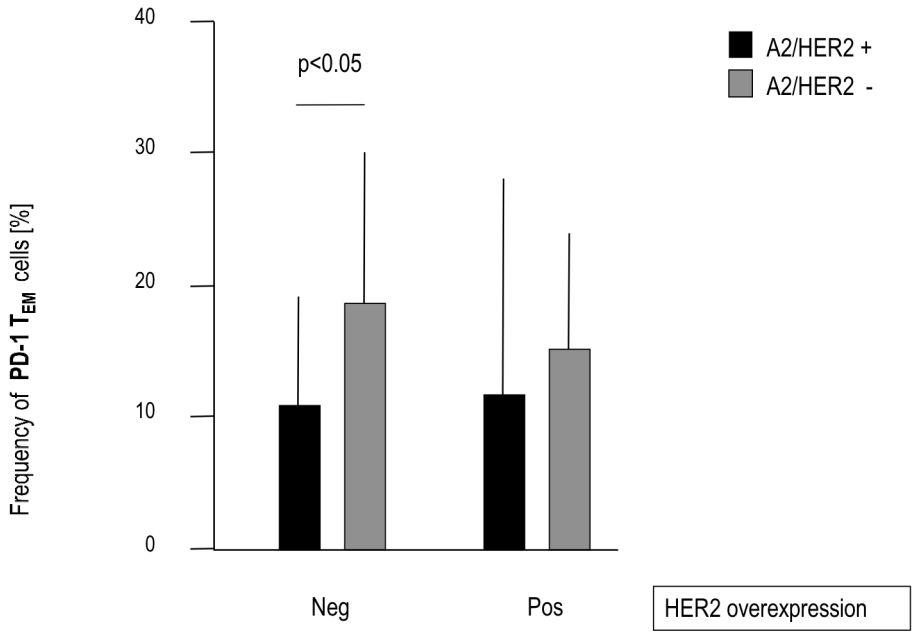

Figure 16: The negative tumor score is characterized by low frequency of PD-1-positive A2/HER2-reactive CD3+ CD8+ T_{EM} cells (CCR7- CD45RA-)

The frequency of A2/HER2-reactive T_{EM} cells expressing PD-1 in peripheral blood from breast cancer patients was measured and compared among A2/HER2-multimer positive and negative T cells.

3.3.4 Frequency of PD-1 is higher in A2/HER2-multimer positive T_{EFF} cells independent of the HER2 status compared to A2/HER2-multimer negative T_{EFF} cells

In spite of the PD-1-negative T_{EFF} cells, which had no significant differences between the different A2/HER2 positive and negative populations, frequencies of PD-1-positive A2/HER2-reactive $CD3^+$ $CD8^+$ T_{EFF} cells were overrepresented in all scores compared to A2/HER2-multimer negative T cells. No correlation within the different tumor scores was found (figure 17).

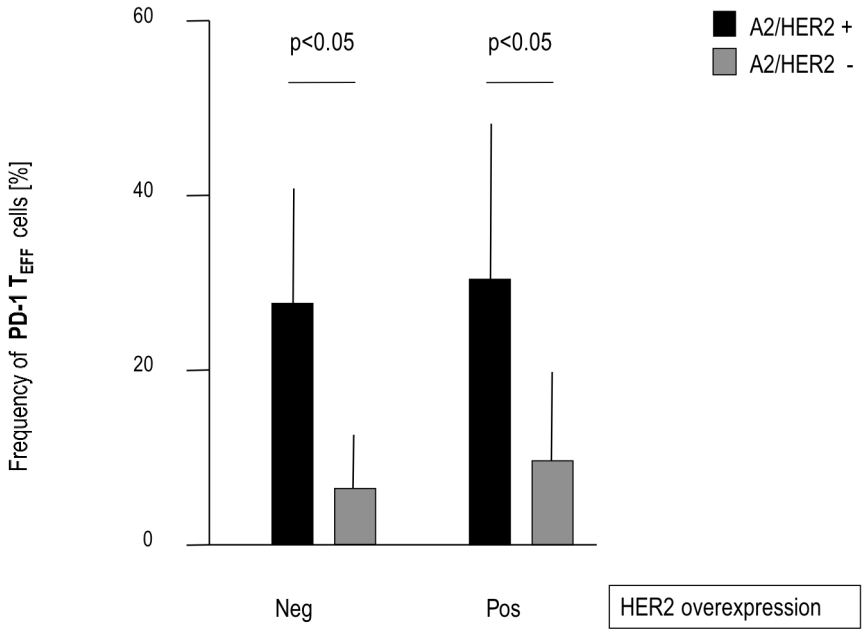

Figure 17: Frequency of PD-1 cells is higher in A2/HER2$^+$ $CD3^+$ $CD8^+$ T_{EFF} cells ($CCR7^-$ $CD45RA^+$), independently of the HER2 status compared to A2/HER2-negative T_{EFF} cells

Peripheral blood cells from breast cancer patients expressing PD-1 and among the A2/HER2-reactive T_{EFF} cell subset were measured and compared among A2/HER2-multimer positive and negative T cells.

3.4 $CD8^+$ T cell phenotypes in A2/HER2-multimer-positive cells expressing CD27 and CD28

In addition to the phenotypical $CD8^+$ T cell classification, a highly heterogeneous functional $CD8^+$ T cell population has been shown with the help of the co-stimulatory receptors CD27 and CD28. These cell surface markers divide $CD8^+$ T cell in several subpopulations with different functional characteristics in healthy donors (Romero et al., 2007; Rufer et al., 2003).

The four phenotypical subsets defined by the differential expression of CCR7 and CD45RA of A2/HER2-reactive $CD3^+$ $CD8^+$ T cells were additionally analyzed in respect to the CD27 and CD28 surface expression. To obtain an overview of the distribution of the nine $CD8^+$ T cell subsets defined by the expression of the CCR7, CD45RA, CD27 and CD28 markers, figure 18 shows the distribution of the cell subsets among the 34 breast cancer patients as a mean percentage. Although the proportion of each T cell subset varied between donors, all nine subpopulations were found in every single individual, with the preferential dominance for the populations T_{EFF} with the $CD27^-$ $CD28^-$ phenotype (39.5%) and the population T_{EM} with the $CD27^-$ $CD28^-$ (28.95%) and $CD27^-$ $CD28^+$ (17.78%) phenotypes.

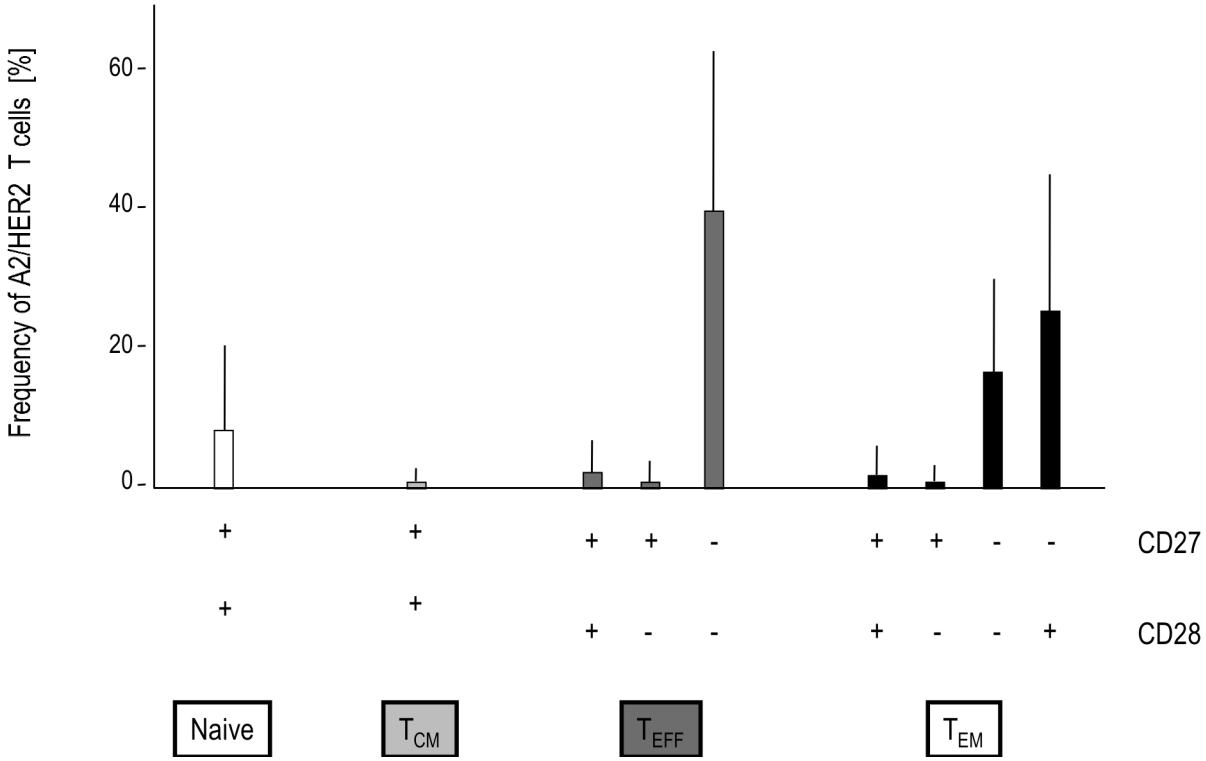

Figure 18: Distribution of the nine defined $CD8^+$ T cell subsets among the 34 breast cancer patients

Differential expression of CD45RA, CCR7, CD27 and CD28 cell surface markers on total $CD3^+$ $CD8^+$ T cells from breast cancer patients shown as a mean percentage.

3.4.1 Naive T cells and T_{CM} with the $CD27^+$ $CD28^+$ phenotype and PD-1 expression

Naive T cells are known to uniformly co-express CD27 and CD28 (Romero et al., 2007). When the naive $CD27^+$ $CD28^+$ T cell population was analyzed within the HER2 scores, there was a highly significant difference between A2/HER2-multimer positive T cells compared to A2/HER2-multimer negative T cells (data not shown). The frequency of cells among the A2/HER2-positive subpopulation was 11.35%, while it was 0.79% among the A2/HER2-multimer negative subpopulation. A2/HER2-multimer reactive T cells expressing PD-1 in the negative and positive HER2 tumor scores have a higher significant expression, 9.57% and 9.59%, respectively, compared to A2/HER2-multimer negative T cells, 2.75% and 6.73%, respectively (data not shown). T_{CM} cells uniformly co-expressed CD27 and CD28 (Romero et al., 2007). Frequencies of A2/HER2 reactive T_{CM} cells with the $CD27^+$ $CD28^+$ phenotype have no significant differences compared to A2/HER2-multimer negative T cells (data not show).

3.4.2 Frequency of T_{EM} according to the CD27 CD28 cell surface markers

The T_{EM} subset can be further divided in four different phenotypical and functional subpopulations according of the expression of CD27 and CD28: EM_1 ($CD27^+$ $CD28^+$; memory-like cells), EM_2 ($CD27^+$ $CD28^-$; partial effector functions and power of replication), EM_3 ($CD27^-$ $CD28^-$; effector-like cells) and EM_4 ($CD27^-$ $CD28^+$; very similar to EM_1) (Romero et al., 2007).

Among these four subsets, cells with the phenotype $CD27^-$ were clearly represented at a statistically greater number in both positive and negative A2/HER2 T cells compared to the population of cells with the $CD27^+$ phenotype (figure 19). The third ($CD27^-$ $CD28^+$) and fourth subpopulations ($CD27^-$ $CD28^-$) had significant higher total numbers of A2/HER2-multimer negative T cells (24.96) compared to A2/HER2-reactive T cells with a p-value of <0.05.

In all four subpopulations of cells, the A2/HER2-multimer negative T cells were more numerous than A2/HER2- reactive T cells with a p-value of <0.05.

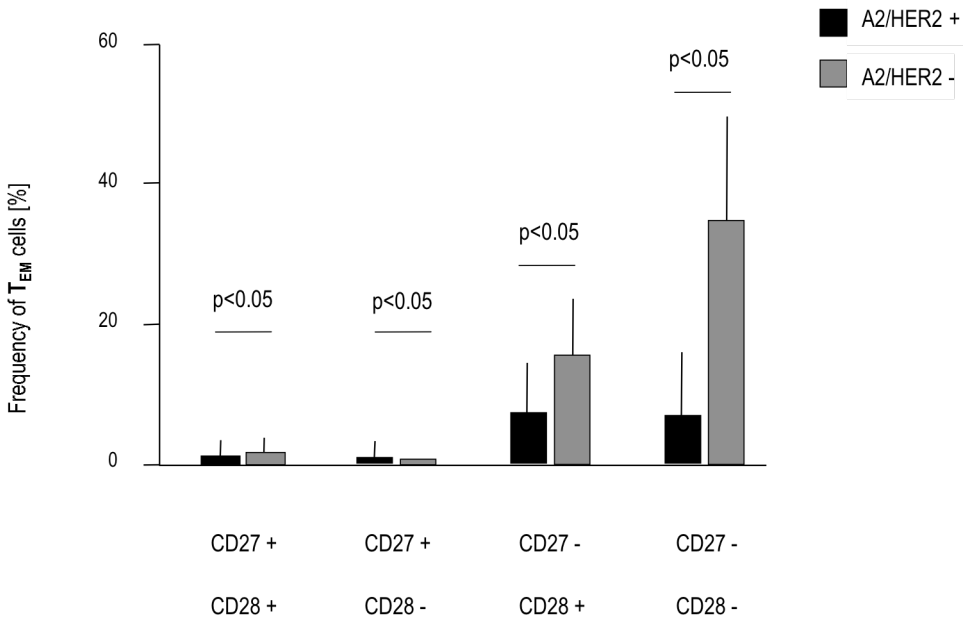

Figure 19: Frecuency among the T_{EM} (CCR7- CD45RA-) subsets

T_{EM} cells were characterized according to the two markers CCR7 and CD45RA on PBMCs from breast cancer patients. The expression of CD27 and CD28 was analyzed and compared between A2/HER2-multimer positive and negative T_{EM} cells.

3.4.3 Frequencies of T_{EFF} according to the CD27 CD28 cell surface markers

T_{EFF} cells can be divided in three functionally distinct subsets: pE_1 ($CD27^+$ $CD28^+$), PE_2 ($CD27^+$ $CD28^-$), pE_3 ($CD27^-$ $CD28^-$) (Romero et al., 2007). As well as the T_{EM} subset, T_{EFF} cells with a $CD27^-$ phenotype represented the majority of the total cells compared to the cells with a $CD27^+$ phenotype (figure 20). The A2/HER2-positive T cells in the first ($CD27^+$ $CD28^+$) and second subpopulations ($CD27^+$ $CD28^-$), were overrepresented compared to the A2/HER2-multimer negative T cells (p-value <0.05).

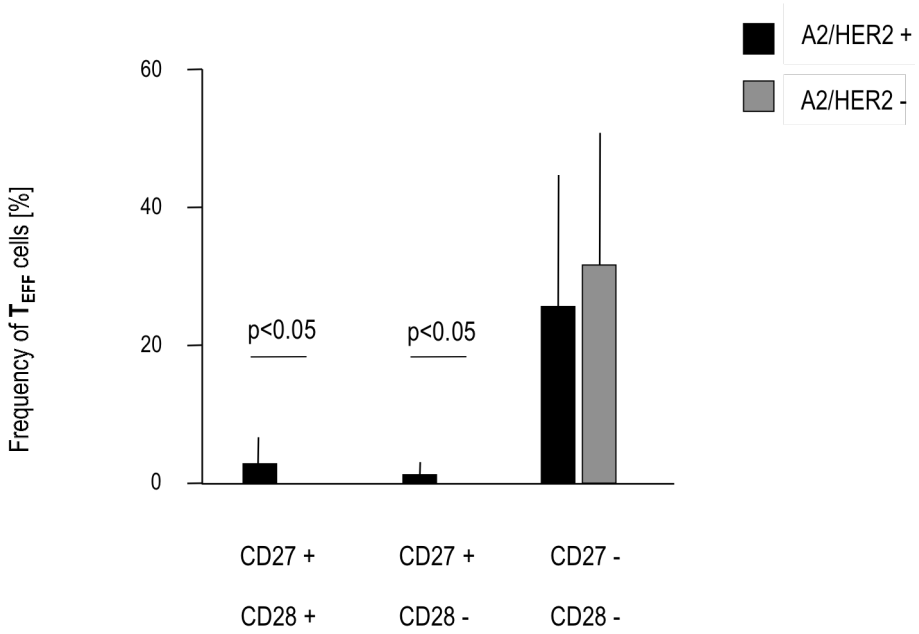

Figure 20: Frequency of cells among the four T_{EFF} ($CCR7^+$ $CD45RA^-$) subsets

The two markers CD27 and CD28 and its expression on peripheral blood from breast cancer patients were analyzed and compared between T_{EFF} positive and negative A2/HER2 T cells.

4. DISCUSSION

4.1 Frequencies of A2/HER2-multimer reactive CD3+ CD8+ T cells in the peripheral blood

PBMCs from benign breast lesions´ and breast cancer patients were analyzed to compare HER2-multimer reactive CD3+ CD8+ T cell frequencies in peripheral blood at the time point of primary diagnosis, at a stage where patients had not received any therapy yet. The median cell frequency was low (0.05%) and did not differ between breast and non-breast cancer patients.

CTLs can recognize and destroy tumor cells expressing epitopes derived from tumor-associated antigens (TAAs) (Berke, 1994; Ikeda et al., 1997; van der Bruggen et al., 1991; Wolfel et al., 1995). New immunomodulating strategies against cancer are based on the assumption that the T cell repertoire of individuals contains TAA-specific CTLs or tumor-primed memory T cells. Pre-existing T cell responses to TAAs have been reported in patients with solid tumors, including melanomas, colorectal, lung, breast, ovarian, prostate, and pancreatic carcinomas (Crosti et al., 2006; Lurquin et al., 2005; McNeel et al., 2001; Sotiropoulou et al., 2003b). However, the prevalence of pre-existing T cell responses is very limited and it is present at low T cell frequencies that are usually not detected with the exception to melanoma patients (Bioley et al., 2006; Mortarini et al., 2003).

Pre-existing immunity to HER2 MHC class I-restricted peptides recognized by CTLs has been described in the literature, however it has been mainly evaluated in terms of cytokine detection after stimulation and by intracellular staining methods. Although the different staining methods used to stain intracellular and cell surface markers are not comparable, a more general understanding of this important matter is necessary. In contrast to our experiments, one study used antigen-dependent cytokine release assays for both protein and mRNA detection and multimer staining in PBMCs to assess the specificity and magnitude of T cell responses. This study analyzed HLA-A*0201 breast cancer patients and showed that 19% of the patients had pre-existent CD8+ T cell reactions against HER2 epitopes at the time point of primary diagnosis in contrast to healthy donors (Rentzsch et al., 2003). In addition, it was shown that breast cancer patients had significantly higher frequencies of TAA melanoma-associated protein 3 (MAGE-A3), HER2 and carcinoembryonic antigen (CEA)-specific CD8+ T cells measured as T cell cytokine responses (Inokuma et al., 2007). However, another group reported a case of HLA-A*02 breast and ovarian cancer patients, where no CD8+ T cells responses against the HER2 HLA-A*02 epitope (p369-377) could be detected despite presensibilisation prior to the analysis (Disis et al., 2000). Very low precursor frequencies of CD8+ T cell responses were reported to the $HER2_{369-377}$ peptide in the course of vaccination protocols assessing the efficacy of the HER2 peptide vaccine study (Disis et al.,

2002), as well as to the HER2-reactive peptide (9_{689}) among non-immunized HER2-overexpressing breast and ovarian cancer patients (Knutson et al., 2001).

The data presented in this work showed similar HER2-reactive T cell frequencies between breast cancer patients and patients with benign breast lesions. It is important to understand that this analysis was from peripheral blood and the cell populations in every patient were very low, and that the results are therefore challenging to interpret.

The assumption that selected patients with benign breast lesions are actually a representative healthy cohort and that they may not have an endogenous HER2 T cell response should be considered carefully. First of all, we could not confirm that these patients with a benign lesion were a real representative healthy population. All of them were diagnosed with benign breast disease namely fibroadenoma, fibrocystic mastopathy or intraductal papilloma. It is known that immunohistochemical staining for HER2 protein varies in different reports focusing on benign breast disease, such as fibrocystic mastopathy and fibroadenoma. In general, they have been absent to weak in intensity. When Southern blot analysis was performed on both benign female breast tissue and male breast tissue showing gynecomastia, no significant HER2 amplification was identified (Hanna et al., 1990; Odagiri et al., 1994). Second, the patients with benign breast lesions were diagnosed with a benign breast disease after surgery, which cannot rule out a missed malignant disease.

It is important to notice why we could not use other PBMCs from healthy patients as a control group. PBMCs from breast cancer patients had an autofluorescence emission due to the storage of many years in the nitrogen tanks. For an adequate comparison it would have been important to use the same quality PBMCs stored at the same conditions as the patients´ PBMCs.

Frequency of $CD3^+$ $CD8^+$ cells reactive for A2/HER2 had no significant difference within the HER2 breast cancer scores (figure 7). It could have been expected to find more A2/HER2-multimer positive cells in positive HER2 status compared to HER2-negative status due to a major endogenous HER2 response. However, despite the fact that our findings are somewhat challenging to interpret because very high numbers of patients are needed in order to identify small differences**,** these findings support the theory that despite the role of HER2 in cancer cell proliferation, under physiologic conditions, this receptor is not a common allogen recognized by the immune system when combating breast cancer. However, it has been shown that both HER2-overexpressing and non-overexpressing breast, colorectal, lung and prostate cancer are immunogenic and are recognized by CTLs directed against several HER2-derived epitopes (Sotiropoulou et al., 2003b).

In addition to these results, our group recently reported that the frequency of Melan-A-reactive T cells increased significantly with the tumor stage in melanoma patients (data not shown). We should not

compare the melanoma tumor stage based on the TNM staging system with the breast HER2 histological status in breast cancer, but these results should be considered to better understand the tumor biology and malignancy of each cancer. It is important to emphasize that most of our patients had a low clinical stage and none of them had metastasis at the time point of diagnosis and acquisition of the material. In contrast, the melanoma patients had the four different tumor stages, including more advanced tumor scores and stages. This might be one reason why the T cell frequency in our analysis of breast cancer patients was so low compared to the data found in patients with melanoma.

4.1.1 PD-1 expression in A2/HER2-multimer reactive $CD3^+$ $CD8^+$ T cells

Different tumor escape mechanisms play a role in immune evasion of tumors from host immunity. It has been shown that inadequate, inappropriate, or inhibitory T cell costimulatory pathway signaling plays a key role in the host's inability to generate an immune response against cancer. One of these mechanisms is mediated by PD-1. Among other cells, the molecule PD-1 is expressed on activated T lymphocytes and negatively regulates T cell activation (Freeman et al., 2000). It has been demonstrated that the interaction between PD-L1:PD-1 is a tumor escape mechanism by which tumors evade the immune response protecting themselves from activated tumor-specific T lymphocytes (Blank et al., 2004; Blank et al., 2005; Dong et al., 2002; Iwai et al., 2002).

Different frequencies of PD-1 among the A2/HER2-multimer positive T cells could be an indication that tumor cells have reacted to the specific antitumor immune response resulting in tumor escape and by this in tumor progression. We found a significantly higher PD-1 expression in reactive A2/HER2-multimer positive T cells compared to A2/HER2-multimer negative T cells. This may be a hint that tumor-reactive cells against the $HER2_{369-377}$ epitope have been inhibited by breast cancer immunosuppressive mechanisms. Moreover, the subpopulation of A2/HER2-multimer positive T cells that expressed PD-1 significantly higher were actually $CD3^+$ $CD8^+$ T cells, confirming that these cells were CTLs.

However, $CD3^+$ $CD8^+$ HER2-multimer positive cells from patients with a benign breast disease and breast cancer patients were compared regarding their PD-1 expression and no significant differences were found between these two groups. As already mentioned above, the selected patients with benign breast lesions may not be the correct representative healthy cohort for comparison with the breast cancer patients used in this study. It is important to notice that the patients enrolled in this study had no metastases at the time point of primary diagnosis, leading to the hypothesis that the inhibitory molecule PD-1 may not play a major role in patients without metastases, and that is why no difference could be found between breast cancer patients and patients with benign breast disease. However, as stated

above, definite interpretation of these findings is challenging due to the low frequencies of cells found throughout this study.

4.2 Phenotypes of $CD8^+$ T cells

The adoptive T cell transfer (ACT) is based on the attempt to circumvent tolerance by taking out the potentially tumor-reactive T cells from the tumor-bearing host and stimulating the T cells *ex vivo*. The use of different CTLs in this cell-based antitumor therapy has provided some promising successes. These $CD8^+$ T cell populations are heterogeneous and characterized with unique functions, phenotypes, and anatomic localization. So far they have been characterized by the coexpression of CD45RA and CCR7, as already mentioned above.

The two different cell types that are currently used in cancer therapy are T_{EFF} cells or memory T cells. Highly activated T_{EFF} cells have the advantages of rapid cytotoxic function, cytokine production and the ability to traffic to the tumor site.

However, increasing amount of evidence is beginning to point in favor of the memory T cells. Some of the reasons that lead to these cells being the superior cell type for the use in adoptive T cell transfer are long-term survival, a rapid responsiveness to low doses of antigen upon secondary antigen challenge, higher potential to proliferate, homing to lymphoid tissues and the characteristic of responding to tumor antigens presented by APCs (Kaech et al., 2002; Sallusto et al., 2004).

We found higher percentage of naive (37.54%) and T_{EFF} cell subsets (39.58%) among the A2/HER2-multimer positive cells in peripheral blood from breast cancer patients, according to data already published (Romero et al., 2007), while T_{CM} was the population of cells with the lowest frequencies (6.32%). As we will explain, these could be to the fact that T_{CM} have been identified in the bone marrow of cancer patients, while in peripheral blood samples the frequency was much lower.

4.2.1 Memory T cells

When compared with naive T cells, memory T cells have the capacity to mount a more rapid response to secondary antigen challenge, that means they require less co-stimulation to be activated, release a broader spectrum of cytokines, and are multifunctional after reactivation (Veiga-Fernandes et al., 2000). In the absence of antigenic stimulation, they maintain their homeostatic proliferation ability (Kaech et al., 2002). Experimental evidence in cell-based studies suggests that both types of memory T cells, T_{CM} and

T_{EM} can each confer a protection in different tumor models, and opinions remain divided memory T cell subset provides the best tumor protection.

It may be a direct association between lower memory T cell population and the positivity in the HER2 breast tumor score, which is a poor prognostic factor in breast cancer patients due to its higher aggressiveness. This finding could be partially explained by a direct relation between aggressive tumors and lower frequencies of superior tumor-reactive cells resulting in lower anti-cancer functions. It is known that dysfunctional immunity characterize both a tumorous and chronic inflammatory process where T cells are driven to a state of senescence and exhaustion without the formation of T_{CM}.

Supporting this theory there are some studies using a virus model which showed how T_{CM} cells conferred superior protective and therapeutic immunity (Wherry et al., 2003; Zaph et al., 2004). Another publication reported that short-term adoptively transferred CTLs, that were expanded by IL-15, persisted in mice, uniformly expressed properties of T_{CM} and rejected a tumor challenge (Bathe et al., 2001). Moreover, it was shown that the phenotype T_{CM} was superior to T_{EM} in suppressing tumor growth and correlated with enhanced survival *in vivo*, when administered in combination with vaccination and exogenous IL-2 (Klebanoff et al., 2005). More evidence that favored T_{CM} was shown in a very recent paper. *In vitro* generated T_{CM}-like cells form a persistent population when transferred *in vivo* and were able to reject established tumors in mice (Rolle et al., 2008).

In our study no significantly differences were found among the memory T cell population when comparing positive and negative HER2 breast tumors (figure 11). However, HER2-reactive T_{CM} cells expressing PD-1 in the positive HER2 tumor scores are significantly increased compared to HER2-multimer negative T_{CM}-cells. We interpret this to be a possible association between lower T_{CM} population and the positivity in HER2 tumor scores, which potentially could be a prognostic factor in breast cancer patients (figure 15). Due to preferential homing to lymphoid organs, it remains possible that tumor-reactive memory T cells were not detectable in the peripheral blood. It is noteworthy that several studies already reported that few functional tumor-specific memory $CD8^+$ T cells were found in the peripheral blood of cancer patients (Anichini et al., 1999; D'Souza et al., 1998). In addition, high numbers of functionally active and tumor-specific memory T cells have been identified in the bone marrow of patients with breast cancer (Feuerer et al., 2001), myeloma (Choi et al., 2005), pancreatic cancer (Schmitz-Winnenthal et al., 2005) and melanoma (Letsch et al., 2003), while in peripheral blood the cell numbers were much lower.

The T_{EM} subset was found to be overrepresented in A2/HER2-multimer negative T cells compared to A2/HER2-reactive T cells (figure 12). It is well known that T_{EM} cells possess an anti-tumor effect. Some evidence to this is that in colon cancer tumor infiltration by T_{EM} was associated with a better prognosis

(Pages et al., 2005). Other studies have reported that T_{EM} were the best population for adoptively transferred cells able to infiltrate and destroy tumors (Kemp et al., 2005). T_{EM} sorted and activated *ex vivo*, transferred without concomitant vaccination or cytokines, induced tumor rejection more effectively than unsorted T cells (Seeley et al., 2001; Wang et al., 2002). In addition, in tumor infiltration of T_{EM} correlated with an objective response against different tumors (Labarriere et al., 1998; Mortarini et al., 2003). Moreover, experiments in tumor bearing mice, following adoptive cell transfer with tumor-reactive T cells and T_{REG} depletion using anti-CD4 antibodies, showed that the T_{EM} subset has a successful anti-tumor response even to secondary tumor challenges (Zhang et al., 2007).

4.2.2 Effector T cells

Highly activated T_{EFF} cells have the advantage of rapid cytotoxic function, cytokine production and ability to traffic to the tumor site. Among the frequency of T_{EFF} cells there were neither significant differences between the HER2 breast tumor scores nor between A2/HER2-multimer positive and negative T cells. However, when the PD-1 expression was analyzed, it was found that the A2/HER2- reactive T cell population expressing PD-1 was overrepresented compared to the A2/HER2 PD-1-negative population. This may be an indication of a specific T_{EFF}-antitumor immune response, which is inhibited by a PD-1:PD-L1 interaction resulting functional exhaustion of CTLs.

However, the role of anti-tumor T_{EFF} cells in tumor immunity is though to be less and less important and evidence is pointing toward memory T cells as the best antitumor cells. It was recently described that T_{EFF} cells can be generated *in vitro* and, with the appropriate conditions, are able to establish a population of memory T cells, including both T_{EM} and T_{CM}. This new population with characteristics of a memory subset is capable to mediate both immediate and long-term antitumor activity *in vivo* (Perret and Ronchese, 2008).

4.2.3 Naive T cells

Naive $CD3^+$ $CD8^+$ T cells are mature T cells that have not yet encountered their cognate antigens. They circulate between the blood and secondary lymphoid organs and are characterized by the phenotype $CD45RA^+$ $CCR7^+$. After activation with their cognate peptides (via TCR), they reenter the cell cycle and proliferate rapidly, afterwards they differentiate into effector T cells.

In peripheral blood from breast cancer patients, we found 37.54% of naive T cells. These results are in agreement with already published data by Romero and colleagues (Romero et al., 2007). It was

interesting to find that this population of T cells was significantly increased in the A2/HER2-multimer reactive population (38.71%) compared to the A2/HER2-multimer negative cells (7.16%), while no differences where seen between the two HER2 breast tumor scores. Why this was so we can only speculate. One reason that could explain these unexpected findings is that despite the high affinity for the A2/HER2-multimer for its binding site certain crossreaction to other peptides does occur (see material and methods for details). Another reason could be that many of the cells classified as naive T cells by the criteria mentioned in this study in and in accordance with the current literature as CD45RA+ CCR7+ have really already had contact with their cognate antigen and are in the process of maturation, and their surface characteristics are soon to change. This would be in accordance with the fact that most of our patients had low tumor scores and no distant metastasis. In order to evaluate this interesting finding more profoundly, a larger patient population including patients in higher tumor scores and with distant metastasis should be analyzed in a similar way.

Naive T cells exhibited the highest levels of CD27 with a percentage of 15.6% compared with the other T cell populations (data not shown). The interaction of CD27 with its ligand CD70 augments TCR-stimulated proliferation of $CD8^+$ T cells (Hintzen et al., 1995), it is involved in the generation of antigen-primed cells and it is required in part for the generation and maintenance of T cell memory (Hendriks et al., 2000). This data are in the same line as those already shown by Romero and colleagues and support the condition of undifferentiated cells in naive T cells. In a next step we will analyze the expression of the HER2 by immunohistochemistry in breast cancer tissue to confirm the presence of PD-L1 on tumor cells. By this we hope to get more detailed understanding of the association of PD-1 expression in breast cancer.

4.3 Frequencies of A2/HER2-multimer reactive CD27 and CD28

An even more complex classification of functional $CD8^+$ T cells was shown using the co-stimulatory receptors CD27 and CD28. These cell surface markers divide $CD8^+$ T cell in several subpopulations with different functional characteristics in healthy donors (Romero et al., 2007; Rufer et al., 2003).

The distribution of phenotypical different A2/HER2-multimer reactive $CD8^+$ T cell subsets from breast cancer patients in peripheral blood showed high numbers of T_{EFF} cells with the $CD27^-$ $CD28^-$ phenotype (28.68%) and T_{EM} cells with the $CD27^-$ $CD28^-$ (34.12%) and $CD27^-$ $CD28^+$ (15.79%) phenotypes (figure 18). The costimulatory molecule CD27 has been implicated in T cell memory generation and it serves as a marker for terminal differentiation (Hamann et al., 1997; Hendriks et al., 2000). In our study, the great majority of T_{EFF} (34.4% of total number of cells) as well as the T_{EM} subpopulations (33.62%) had a $CD27^-$ phenotype. These well-differentiated $CD3^+$ $CD8^+$ $CCR7^-$ $CD27^-$ cells were associated with progressive impairment of proliferative capacity and impaired *in vivo* antitumor efficacy (Gattinoni et al., 2005). The significantly increased $CD27^-$ population may be an evidence that there are many cells without the optimal proliferative and antitumor capacity in peripheral cells repertoire from breast cancer patients.

T_{EM} cells can be divided in four different phenotypical and functional subsets. The first T_{EM} subpopulation ($CD27^+$ $CD28^+$) was reported to be closely related to the T_{CM} subpopulation and to be dominant in peripheral blood from healthy donors (Romero et al., 2007). Moreover, after ACT in melanoma patients, stable numbers of T_{EM} cells co-expressing the $CD27^+$ $CD28^+$ markers were related to objective tumor responses (Powell et al., 2005). In our data, these T_{EM} $CD27^+$ $CD28^+$ cells have a very low frequency (3.38%) compared to the other subsets of T cells. Additionally A2/HER2-multimer positive T_{EM} cells were significantly decreased (p-value <0.05) compared to the A2/HER2-multimer negative T cells within the four different subsets of T_{EM}. Similar results are seen when the PD-1 expression was analyzed.

The second T_{EM} subpopulation, with a $CD27^-$ $CD28^+$ phenotype was reported to be closely related to T_{EM} $CD27^+$ $CD28^+$ despite the loss of CD27. We found that T_{EM} $CD27^-$ $CD28^+$ cells had a higher frequency compared to T_{EM} $CD27^+$ $CD28^+$ in contrast to the frequencies found in healthy donors by Romero and colleagues.

The third ($CD27^-$ $CD28^-$) and fourth populations ($CD27^-$ $CD28^+$) had significantly higher frequencies in A2/HER2-multimer negative cells compared to A2/HER2-multimer positive cells within the two scores (p-value <0.05). The ($CD27^-$ $CD28^-$) subpopulation have been described to have a high *ex vivo* lytic activity (Romero et al., 2007) and its low frequency in A2/HER2-reactive T cells may be a sign of a decreased lytic activity against breast tumor in these patients.

If we focus on T_{EFF} cells, they can be divided in three functionally distinct subsets: pE_1 ($CD27^+$ $CD28^+$), pE_2 ($CD27^+$ $CD28^-$), pE_3 ($CD27^-$ $CD28^-$). The T_{EFF} cells have a high *ex vivo* lytic activity. According to our data the preferred dominance was for T_{EFF} $CD27^-$ $CD28^-$ cells according to the publication by Romero et al. (Romero et al., 2007). Cells with the $CD27^-$ phenotype were described as cells with intermediate effector-like functions (Rufer et al., 2003). In our data, T_{EFF} cells in the first ($CD27^+$ $CD28^+$) and second subpopulations ($CD27^+$ $CD28^-$) had the lowest frequencies, and among them, the A2/HER2-multimer positive cells were overrepresented compared to the A2/HER2-multimer negative T cells (p value <0.05).

The $CD8^+$ T cell cytokine response to the HER2 antigen in breast cancer patients was found to be very similar in phenotype consisting almost exclusively of $CD28^+$ $CD45RA^-$ cells (Inokuma et al., 2007). In this data presented here, the cells with the $CD28^+$ $CD45RA^-$ phenotype represented 13.24% of the PBMCs in breast cancer patients. The most predominant phenotype was $CD28^-$ $CD45RA^+$ with a frequency of 44.13%. These differences could be explained by the treatment of the patients. In our study all the patients were analyzed prior to chemotherapy treatment, however in the study by Inokuma et al., patients were analyzed after receiving surgery and/or chemotherapy, making an interesting comparison with our data. A conclusive answer to the issues concerning the function of the different T subsets described in this study and their relationship with the breast cancer will require the in vivo tracking of antigen-reactive T cells in patients before, during and after the tumor therapy.

4.4 Immunomodulatory approaches – outlook –

Malignancy is comparable to chronic viral infections because antigens are persistently presented to $CD8^+$ T cells and T cells respond to continuous antigenic stimulus. It was speculated that persistent tumor-antigen stimulation causes tolerance of tumor-reactive $CD8^+$ T cells during tumor growth (Huang et al., 2005; Redmond and Sherman, 2005). The presence of an existing immune tolerance in patients with cancer limits the clinical outcome of immunotherapeutic strategies, which otherwise may represent a major alternative approach for the management of cancer since they focus on the manipulation of the patients´ immune system to specifically destroy tumor (Dermime et al., 2004).

The induction of a strong and persistent tumor-specific T cell response is critical in the development of an effective cancer immunotherapy, whose activity is the result of a balance between positive and negative signals conferred through interactions between various T-cell co-regulatory receptors and ligands (Inman et al., 2007a). Several lines of evidence indicate that the interaction between the receptor PD-1 and its ligand PD-L1 play a major role in the tumor microenvironment and in immune evasion (Dong and Chen, 2003). The tumor microenvironment may restrict the effectiveness of antitumor responses by displaying a variety of immunosuppressive strategies in concert with others signals and co-inhibitory molecules, and that these could have a synergistic effect (Blackburn et al., 2009). Other candidate molecules that were recently identified to be involved in T cell exhaustion (Wherry et al., 2007) need to be investigated to get a better understanding of the detailed mechanisms.

Due to these struggles, combinations of therapeutic approaches are now been investigated. For example, the triple treatment therapy in mice using anti-PD-L1 blockade, depletion of $CD4^+$ T cells in order to avoid inhibitory regulation of T_{REG}, and irradiated tumor cell vaccination induced complete elimination of large established renal cancer cell (RENCA) tumors with long-lasting tumor-specific immunity (Webster et al., 2007).

Over the past years it has become clear that PD-1:PD-L1 interaction plays a major role in the tumor microenvironment. However, we should not forget that the tumor microenvironment may restrict the effectiveness of antitumor responses by displaying a variety of immunosuppressive strategies in concert with others signals and co-inhibitory molecules besides PD-1:PD-L1, and that these could have a synergistic effects. Other candidate molecules involved in T cell exhaustion need to be investigated in greater detail in order to solve the underlying mechanisms. This could lead us to new and efficient immunotherapeutic anticancer strategies.

5. SUMMARY

It is still a not entirely resolved question why existing tumor-specific T cells in cancer patients fail to effectively prevent tumor progression. Effective antitumor immunity depends on the concordant activation of tumor-specific CTLs, whose activity is the result of a balance between positive and negative signals conferred through interactions between various T-cell regulatory receptors and ligands. CTLs, which are the principal effector cells of the adaptive immunity, can be divided into subsets according to the two cell surface markers CCR7 and CD45RA. This brings the concept of four T cell subsets: naive T cells (T_{naive}; $CD45RA^+$ $CCR7^+$), effector T cells (T_{EFF}; $CD45RA^+$ $CCR7^-$), central memory T cells (T_{CM}; $CD45RA^-$ $CCR7^+$) and effector memory T cells (T_{EM}; $CD45RA^-$ $CCR7^-$) with different functional properties.

The $CD8^+$ T cells with antitumor capacities are thought to be inhibited by tumor immune escape mechanisms, where the tumor cells are protected from the immune detection leading to tumor survival. One of the more investigated tumor escape strategies is the presence of high levels of co-inhibitory molecules on APCs, stromal cells and tumor cells, leading to activation of the negative regulatory pathways of the immune response. Over the past 15 years it has become clear that the primary function of PD-1 receptor is to attenuate the immune response. This co-inhibitory mechanism results from the interaction between the PD-1 receptor expressed on antitumor specific T cells with its ligand PD-L1 expressed on tumor cells. This link negatively regulates T cell activation and T cell functions and it is considered an potent tumor escape mechanism.

Because of the strong association between PD-1:PD-L1 interactions to evade the immune response and the poor clinical prognosis in many cancers, we investigated PBMCs from $HLA\text{-}A2^+$ breast cancer patients prior to chemotherapy treatment and their PD-1 expression within the different T cells subpopulations. We performed an analysis of the HER2-reactive T lymphocytes in breast cancer patients and their different phenotypes by combining simultaneous cell surface markers with multiparameter flow cytometry. As a multimer, HLA-A2:$HER2_{369—377}$ was used.

In the presented work, for the first time, the measurement and characterization of the different CTLs subsets present in peripheral blood from breast cancer patients at the time point of primary diagnosis as well as their PD-1 expression pattern are reported. We were able to identify the following characteristics:

- Frequency of HER2-multimer reactive T cells in peripheral blood was very low in patients with breast cancer as well as patients with benign breast disease.

- Frequency of HER2-multimer reactive T cells did not differ between patients with breast cancer and patients with benign breast lesions.
- Pre-existent $CD8^+$ T cell reactions against $HER2_{369-377}$ at the time point of primary diagnosis do not differ in patients with benign breast disease and breast cancer within different HER2 tumor scores.
- The distribution of the four defined $CD3^+$ $CD8^+$ T cell phenotypes in peripheral blood of breast cancer patients showed the T_{CM} population as the one with the lowest frequency and the T_{naive} and T_{EFF} subsets as those with the highest frequency.
- The distribution of the nine defined $CD3^+$ $CD8^+$ T cell subsets according to the CD27 and CD28 cell surface markers showed that the great majority of HER2-reactive cells had a $CD27^-$ and/or $CD28^-$ phenotype.
- Frequency of potent antitumor T_{CM} cells independently of the HER2 tumor status was decreased in patients with breast cancer. Indeed, HER2-multimer positive T_{CM} cells expressing PD-1 in the positive HER2 tumor score were significantly increased compared to HER2-multimer negative T_{CM}-cells.
- The inhibitory molecule PD-1 may not play a major role in patients without metastases, showing a similar expression pattern between HER2-multimer positive cells from breast cancer patients and patients with a benign breast disease.
- PD-1 expression was significantly higher in A2/HER2-multimer positive T cells compared to A2/HER2-multimer negative T cells.
- A2/HER2-multimer positive T cells expressing PD-1 molecule were overrepresented independent of the HER2 status compared to all $CD3^+$ $CD8^+$ T cells.

Taken together, we hope that these findings help to a better understanding of the immunologic tumor escape mechanisms responsible for tumor progression and poor outcome in breast cancer. Even though recently much knowledge was gained with respect to the PD-1:PD-L1 pathway, there are many open questions that have to be answered in order to manipulate the immune response in a purposeful manner and to create a beneficial anti-tumor effect. Additional studies must show the therapeutic implications and benefits to target PD-1 or PD-L1 with or without other therapies, and clarification is necessary regarding the therapeutic effect of PD-1:PD-L1 blockade in breast cancer models and clinical trials. When used in a accurate manner, these strategies could become an efficient addition to current treatment strategies.

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Abbreviations

ACT	Adoptive cell transfer therapy
AmCyan	ANemonia majano cyan fluorescent protein
APCs	Antigen presenting cells
APC	Allophycocyanine
A700	Alexa-700
BM	Bone marrow
BSA	Bovine Albumin
CCR7	CC chemokine receptor 7^{+} receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CTLs	Cytotoxic T cells
Cy7	Cyanine dye indotricarbocyanine
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EL	Erythrocyte lysis buffer
EMA	Ethidium monoazide bromide
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
HER2	Human epidermal growth factor receptor 2
HLA	Human leucocyte antigen
HSA	Human serum albumin
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
MAbs	Monoclonal antibodies
MAGE-A3	Melanoma-associated protein 3
MHC	Major Histocompatibility Complex
Min	Minute
nm	nanometers

PB	Peripheral blood
PB	Pacific Blue
PBMCs	Peripheral blood mononucleal cells
PBS	Phosphat buffered saline
PD-1	Programmed death-1
PD-L1	Programmed death-ligand-1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein Complex
PFA	Paraformaldehyde
RBCs	Red blood cells
RPMI	*Roswell Park Memorial Institute*
SD	Standard deviation
TAAs	Tumor-associated antigens
T_{CM}	Central memory T cells
TCR	T-cell receptor
$T_{EFF:}$	Effector T cells
T_{EM}	Effector memory T cells
T_H1	T helper type 1 cells
T_H2	T helper type 2 cells
TILs	Tumor infiltrating lymphocytes
T_{naive}	naive T cell
T_{REG}	T regulatory cells

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Publications

Posters, Abstracts and Oral Presentations

1. Presentation: **Salvador MR**
   PD-1 expression and phenotypes on peripheral blood T lymphocytes from breast cancer and melanoma patients.
   Scientific Seminar of the III. Medical Clinic, Hematology and Oncology, Technical University Munich, Germany.

2. Poster: **Salvador MR**, Krönig H, Peschel C, Bernhard H.
   PD-1 expression profile and phenotypes of peripheral blood T lymphocytes in breast cancer.
   DGHO Congress Heidelberg/Mannheim, Germany 2009.

Publications

1. Krönig H, Falchner KJ, Odendahl M, **Salvador MR,** Conrad H, Brackertz B, Hein R, Muck D, Blank C, Peschel C, Haller B, Schulz S, Bernhard H
   Significantly elevated MelanA-specific and PD-1+ CD8+ T cells correspond to advanced stage of malignant melanoma.
   2010, manuscript submitted

2. Conrad H, **Salvador MR**, Schmitt M, Krönig H, Bernhard H, Peschel C
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