

Down-regulation of CD28, TCR-zeta (ζ) and Up-regulation of FAS in Peripheral Cytotoxic T-cells of Primary Breast Cancer Patients

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Abstract. *Background:* Several studies have supported the hypothesis that the concept of immuno-surveillance would not be effective in cancer patients. One reason for suppression of anti-tumor immunity may be attributed to immune impairment of T-lymphocytes, which extends beyond the tumor-microenvironment and might effect the peripheral blood. Therefore the aim of this study was to investigate the expression of immunoregulatory antigens in peripheral blood lymphocytes of primary breast cancer patients in comparison with healthy donors. *Materials and Methods:* The peripheral blood immune status of 61 patients with primary breast cancer was analysed by FACS-analysis. The different lymphocytic subpopulations were identified by intracellular/extracellular monoclonal antibodies in three-color flow cytometry. The distribution was compared to age-matched healthy female donors (n=29). *Results:* The expression of TCR zeta-chain, an important signal complex for T-cell activation and functional integrity of specific immune response, was significantly reduced in the cytotoxic specific T-cell population. Cytotoxic T-cells (CD3⁺/CD8⁺) also showed a down-regulation of CD28, the important ligand to the co-stimulatory molecule CD80 (B7.1) on antigen-presenting cells. Moreover, breast cancer patients had significantly more CD95 (FAS) expressing cytotoxic T-cells than their healthy counterparts ($p<0.05$). *Conclusion:* The significant up-regulation of CD95 and down-regulation of TCR zeta and CD28 in peripheral cytotoxic T-cells of breast cancer patients leads to the hypothesis of systemic immunosuppression, which could open the door for tumor cell dissemination via the blood stream and which is the subject of ongoing studies.

Cancer arises from uncontrolled proliferation and spread of clones of transformed cells. The growth of malignant tumors

is determined in large part by the proliferative capacity of the tumor cells and their ability to invade host tissue and metastasize to distant sites. In addition, it is believed that malignant tumors are able to evade or overcome the mechanisms of host defense. The possibility that cancer can be eradicated by specific immune response has been proposed in the concept of immuno-surveillance (1).

However, immuno-surveillance is not always effective in preventing and/or controlling tumor growth. One reason for suppression of antitumor immunity can be attributed to functional impairment of T-lymphocytes, which extends beyond the tumor microenvironment and is seen in tumor-infiltrating lymphocytes (TILs) as well as peripheral blood lymphocytes (PBLs) of patients with melanoma and head and neck cancer (2, 3).

Currently, breast cancer is seen as a systemic disease with micrometastases and general immune dysfunction (4). It has been shown that detection of disseminated tumor cells (DTC) is an independent prognostic factor in early breast cancer (5) and presence of circulating peripheral blood tumor cells as well as bone marrow tumor cells were significantly associated with poor prognosis (6). In this context, we analysed the expression of important immunomodulatory antigens of circulating peripheral T-cells in breast cancer patients as the concept of immuno-surveillance against micrometastasis has to be questioned.

The ability of naïve T-cells for clonal expansion and tumor antigen-specific effector function depends on signals received by the T-cell receptor (TCR) and on effective T-cell priming.

The latter requires co-stimulatory receptors on naïve T-cells, the most prominent of which is CD28 (7, 8). For optimal T-cell priming, the CD80 (B7.1) molecule on APCs binds to the T-cell ligand CD28. CD28-mediated co-stimulation complements the TCR signal (9). Recent studies described the depressed functional potential of T-lymphocytes as a link between ineffective TCR signalling and spontaneous apoptosis of circulating lymphocytes and those infiltrating the tumor (7, 10-14). Moreover immune response is also regulated by interaction of CD95 (Apo-1/FAS) and CD 95L (ligand) (7-9).

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Human tumors that express FAS ligand could directly induce death of activated FAS + immune cells via the FAS/FAS ligand pathway.

Materials and Methods

Patients and normal controls. Patients with primary breast cancer were recruited routinely from the Department of Obstetrics and Gynecology of the University Hospital of Tübingen between 2005-2006. 10 ml heparinized venous blood was drawn from each primary breast cancer patient before surgery and also from healthy female volunteers. Informed consent was obtained routinely from all participants. The criteria for exclusion from this retrospective study were previous simultaneous secondary malignant disease, breast cancer recurrence, neo-adjuvant chemotherapy or hormone therapy, serious functional disorders of liver and kidney and metabolic disease. The approval of the local ethical committee (University of Tübingen) was obtained for the retrospective analyses (59/2007V). Clinicopathological features of the patients are shown in Table I.

Cell isolation and staining for flow cytometry. After collection, blood samples were obtained on ice and stained/analysed within the next 5 hours. A volume of 0.5 ml of FACS-Lyse-Reagent (Becton Dickinson, Heidelberg, Germany) was added to 100 µl EDTA blood in order to recover peripheral blood mononuclear cells (PBMCs) by erythrocytic and thrombocytic lysis. After incubation (10 min), cells were washed in Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies, Inc., Grand Island, NY, USA) by centrifugation.

Aliquots of 5×10^5 PBMCs were stained with 10 µl directly conjugated mouse anti-human monoclonal antibodies, such as CD3-PerCP, CD4-APC, CD8-APC, CD16-FITC, CD19-FITC, CD20-APC, CD95-PE, CD95-FITC (Becton Dickinson, Heidelberg, Germany), CD28-RPE and CD56-RPE (Dako, Hamburg, Germany). Intracellular staining with 10 µl TCR- ζ -FITC (Biozol, Munich, Germany) was enabled by addition of 10 µl/ml digitonin working solution (Sigma, Deisendorf, Germany). All cells were incubated with the antibodies up to 60 min on ice, then washed twice in PBS and fixed with 0.5% paraformaldehyde in PBS prior to flow cytometric analysis.

Propidium iodide-negative viable cells were detected by three-color flow cytometry using a FACSCalibur™ with CELLQuest software (Becton Dickinson). Typically, 20,000 events were collected and the data were expressed as dot plots.

Statistical analysis. Cell populations of 61 breast cancer patients (34-to 84-years-old) were compared with healthy donors (20- to 83-years-old) by their absolute number of cells. The statistical significance of differences between groups was determined by a nonparametric Mann-Whitney *U*-test for the unpaired analysis. Statistical analysis was performed using SPSS for Windows (Version 11.5, Chicago, IL, USA). *P*-values <0.05 were considered to be significant.

Results

Quantitative distribution of T-, B- and NK-cells in primary breast cancer patients. Breast cancer patients showed a significant reduction of peripheral T-cells ($p < 0.046$, Table II), which particularly affected the cytotoxic T-cell

Table I. Clinicopathological features of primary breast cancer patients in this study.

	N	%
Total	61	100
Tumor stage		
pT1	43	70
pT2-4	18	30
Nodal status		
N0	44	72
N+	17	28
Estrogen receptor		
Positive	51	84
Negative	10	16
Menopausal status		
Pre-	12	20
Post-	49	80
Bone marrow tumor cell dissemination		
Positive	11	18
Negative	50	82

subpopulation (CTL). A mean of 384 ± 192 (CD3 $^+$ /CD8 $^+$) cells/ μ l were seen in cancer patients vs. 489 ± 225 (CD3 $^+$ /CD8 $^+$) cells/ μ l in healthy donors ($p = 0.018$). Compared to healthy controls, T-helper cells were not significantly reduced ($p = 0.3$). Moreover the number of CD19 $^+$ /CD20 $^+$ B-cells and CD3 $^-$ /CD16 $^+$ /CD56 $^+$ NK-cells showed no significant change in breast cancer patients.

Down-regulation of TCR ζ -chains and CD28 in primary breast cancer patients. The quantitative expression of TCR ζ -chain and CD28 on cytotoxic T-cells was significantly reduced in breast cancer patients (Table II). Concerning CD28 expression we found 229 ± 113 CD8 $^+$ cells/ μ l in breast cancer patients compared to 303 ± 125 CD8 $^+$ cells/ μ l in normal controls ($p = 0.031$). The CD8 $^+$ /TCR ζ $^+$ population amounted to 364 ± 184 cells/ μ l vs. 469 ± 217 cells/ μ l in healthy controls ($p = 0.013$). Analyses of the T-helper subpopulation showed also fewer CD28 $^+$ and TCR ζ $^+$ cells in breast cancer patients, however the total number of viable cells / μ l was not significantly reduced.

CD95 (FAS) expression of T-cells in breast cancer patients. Flow-cytometry distinguishes between CD95 $^+$ and CD95 $^-$ T-cell population (Figure 1). The CD8 $^+$ /CD3 $^+$ subpopulation showed a highly significant increase in the total number of CD95 $^+$ cells ($p < 0.001$; Table II). Compared to healthy counterparts almost twice as many CD8 $^+$ /CD95 $^+$ cells were found (78 ± 64 cells/ μ l in breast cancer patients vs. 43 ± 38 cells/ μ l in healthy donors). Overall 354 ± 168 (CD3 $^+$ /CD95 $^+$) cells / μ l were seen in the breast cancer group and 268 ± 106 (CD3 $^+$ /CD95 $^+$) cells / μ l in the group of healthy donors ($p < 0.014$).

Table II. Cellular composition of peripheral immune cells of primary breast cancer compared to normal controls.

Phenotype	Description	Total number of viable cells/ μ l mean \pm SD		<i>p</i> -value
		Breast cancer (n=61)	Normal control (n=29)	
CD3 ⁺	T-cell	1330 \pm 424	1506 \pm 383	0.046
CD3 ⁺ /CD8 ⁺	T-cytotoxic (T _c) cell	384 \pm 192	489 \pm 225	0.018
CD8 ⁺ /CD28 ⁺	Activated naive T _c -cell	229 \pm 113	303 \pm 125	0.031
CD8 ⁺ /TCR ζ ⁺	ζ -chain expression of T _c -cell	364 \pm 184	469 \pm 217	0.013
CD8 ⁺ /CD95 ⁺	Fas positive T-cytotoxic cell	78 \pm 64	43 \pm 38	0.001
CD3 ⁺ /CD4 ⁺	T-helper (T _h) cell	930 \pm 320	978 \pm 252	*0.3
CD4 ⁺ /CD28 ⁺	Activated naive T _h -cell	848 \pm 298	904 \pm 228	*0.2
CD4 ⁺ /TCR ζ ⁺	ζ -chain expression of T _h -cell	848 \pm 308	938 \pm 262	*0.1
CD4 ⁺ /CD95 ⁺	Fas positive T-helper cell	242 \pm 107	221 \pm 84	*0.3
CD19 ⁺ /CD20 ⁺	B-cell	197 \pm 103	206 \pm 90	*0.5
CD3 ⁻ /CD16 ⁺ /CD56 ⁺	NK-cell	218 \pm 108	239 \pm 120	*0.4

*not significant.

Results are independent of prognostic factors. Concerning the cytotoxic T-cell subpopulations (TCR ζ ⁺, CD28⁺ and CD 95⁺) nonparametric Mann-Whitney *U* test showed no significant difference (*p*>0.05) between the following groups of breast cancer patients: Patients with pT1 vs. patients with pT2-4 ; patients with negative axillary nodal status vs. patients with positive axillary nodal status; patients with negative estrogen receptor vs. patients with positive estrogen receptor; premenopausal patients vs. postmenopausal patients. Consequently the significantly reduction of TCR ζ ⁺-and CD28⁺ in CTLs of breast cancer patients and the up-regulation of CD8⁺/CD95⁺ cells were independent of prognostic factors such as tumor stage, tumor grade, axillary nodal status, hormone receptor status and menopausal status.

Discussion

CD28 – an essential signal for T-cell activation and differentiation. In our study, breast cancer patients presented with a significant reduction of CD28 positive peripheral T-cells, which especially affected the circulating cytotoxic CD8⁺ subpopulation. Analyses of Blake-Mortimer *et al.* showed that CTL (CD3⁺/CD8⁺) counts emerge as a significant predictor of survival in metastatic breast cancer (17). As it is known that CTLs (CD3⁺/CD8⁺) play a critical role in tumor antigen-specific recognition and lysis of tumor cells, a reduced number of activated CD28⁺/CD8⁺ CTLs may lead to impaired immunosurveillance and possible spread of tumor cells. CD28-deficient mice or mice treated with antagonists to CD28-CD80/CD86 interactions produce a reduced response to immune challenges such as infectious pathogens, allograft antigens, graft-versus-host disease (GVHD), contact hypersensitivity and asthma (16, 17).

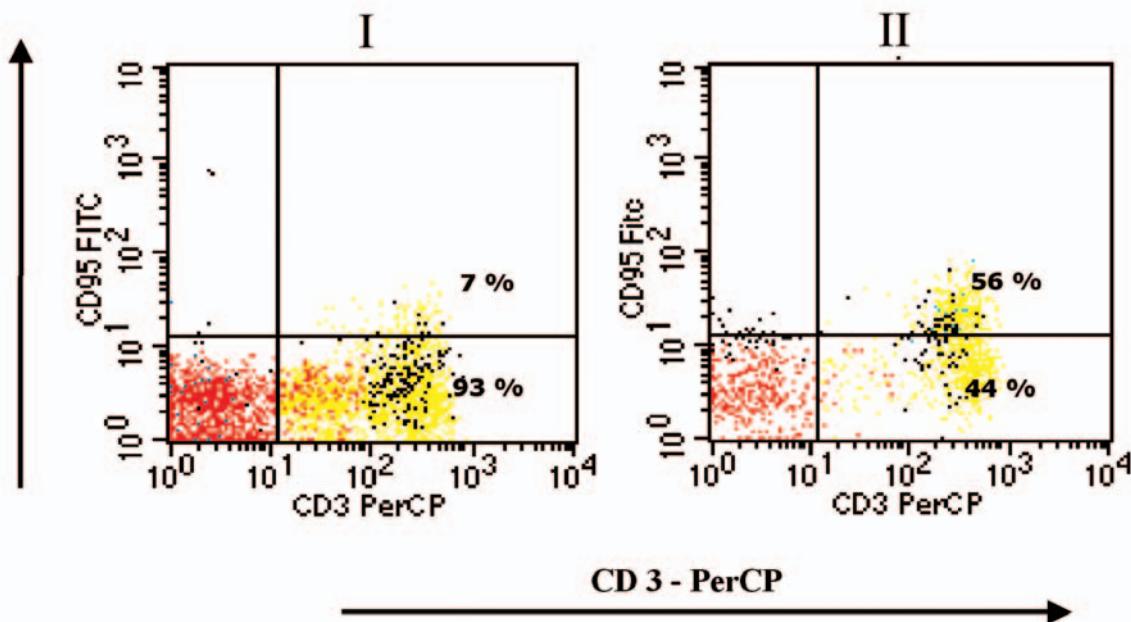
Correspondingly, lack of CD28-mediated co-stimulation results in ineffective T-cell priming and reduced T-cell proliferation *in vitro* and *in vivo* (18, 19). Therefore this ineffective T-cell priming leads to the state of anergy with the manifestation of T-cell apoptosis and less activated tumor-specific effector cells.

CD28-mediated co-stimulation effects TCR signalling. It is known that CD28-mediated co-stimulation complements the TCR signal by activating unique pathways and transcription factors (20). CD28 increases the potential of TCR to signal fully even when interactions with peptide MHC are poor (21). Correspondingly, in our study we saw a reduction of CD28⁺ and TCR- ζ ⁺, cells, concerning the CD8⁺ fraction. Few or no CD28 on T-cells leads to a weak and transient TCR signal that is insufficient to reach a threshold level required for biological T-cell response (22).

Evidence of a reduced number of TCR- ζ ⁺ CTLs in breast cancer patients. The ability of naïve T-cells for clonal expansion and development of an antigen-specific T-cell response depends on the strength of signals produced by the T-cell receptor (TCR) (23). The peptide MHC complex of the antigen-presenting cells (APCs) binds to TCR/CD3 and initiates proximal intracellular signalling events by phosphorylation of TCR ζ -chains (Figure 2), which finally stimulates the expression of various genes involved in an effective T-cell response.

Kurt *et al.*, implicated the detection of dysfunctional T-cells in breast cancer patients by abnormally low levels of signaling T-cell molecules, such as TCR- ζ (24). Our patients showed a significantly lower number of TCR- ζ ⁺ CTLs. As TCR- ζ determines the functional integrity of T-cells,

- a) Peripheral CD3⁺T-cells : Healthy control (I) present with 7% CD95⁺ T-cells; breast cancer patient (II) presents with 56% CD95⁺ T-cells.



- b) Peripheral cytotoxic CD3⁺/CD8⁺ T-cells : Healthy control (I) presents with 1% CD95⁺ cytotoxic T-cells; breast cancer patient (II) presents with 59% CD95⁺ cytotoxic T-cells.

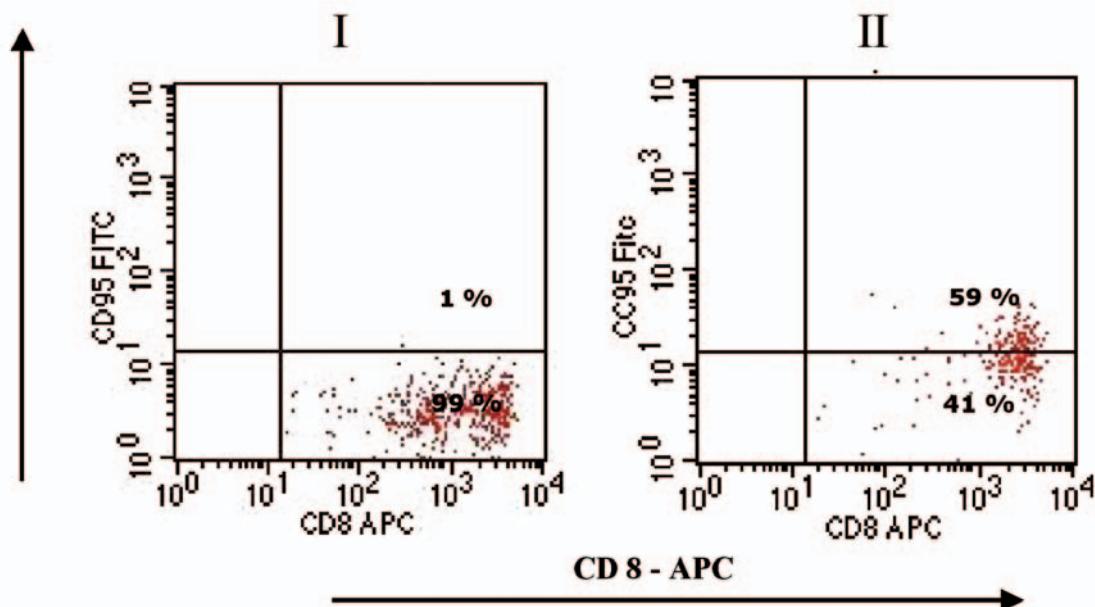


Figure 1. Detection of CD95-positive cells in primary breast cancer patients.

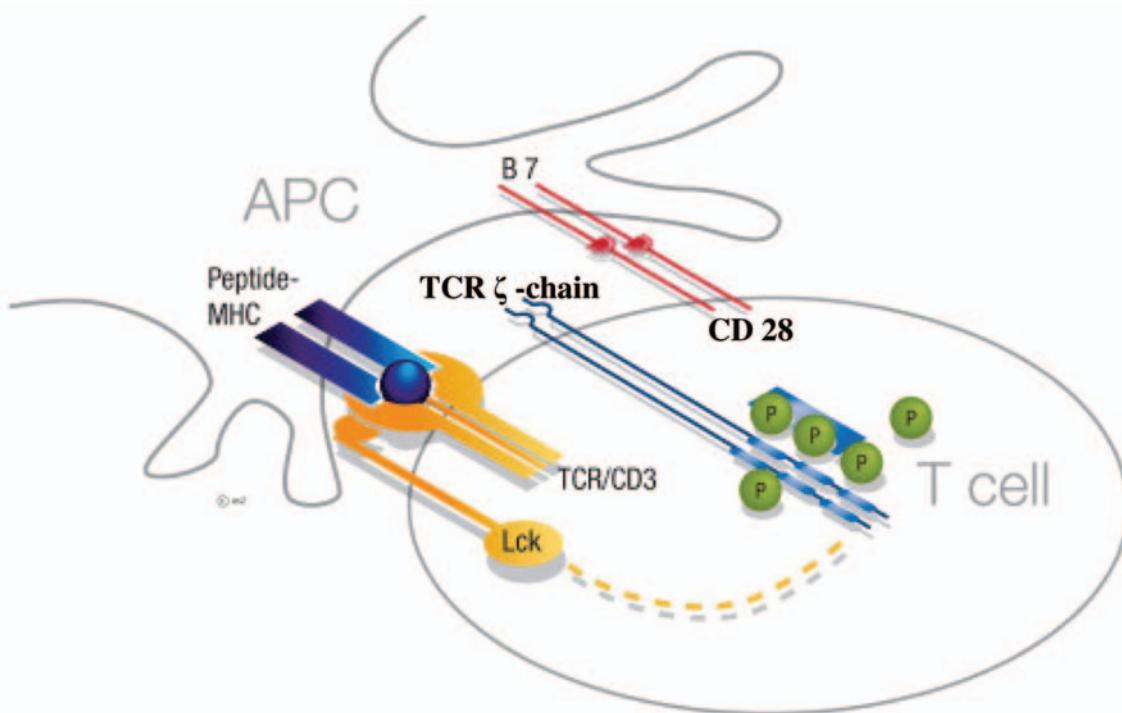


Figure 2. T-cell activation by phosphorylation of TCR-associated ζ -chains and by co-stimulation (B7/CD28 interaction).

spontaneous apoptosis of T-cells was accompanied by down-regulation of ζ (10, 11, 25). In head and neck cancer, low ζ expression in peripheral T-cells was accompanied by apoptosis, and accurately discriminated between patients and healthy controls (11, 25). As Annexin-binding of T-cells was associated with low ζ expression and consistent with membrane changes which lead to apoptosis, it was claimed that TCR- ζ expression might serve as a marker of immune competence (12, 25-27). Signal transduction by TCR links antigen recognition with functional response such as transcriptional activation of particular genes and the entry of cells into cell cycle. TCR associated ζ -chain expression is essential for effective and sustained T-cell activation. A reduced or lack of ζ -chain expression causes inefficient signalling with loss of immune function and induction of T-cell death (7, 8, 11), which could explain the reduced number of cytotoxic T-cells in our breast cancer patients.

Breast cancer patients present with a highly significant number of FAS⁺ CTLs. Müschen et al. (13) proposed that CD95L overexpression in breast cancer cells counterselects tumor-specific (CD95-positive) T-cells and spares resting (CD95-negative) T-cells, which are less sensitive to apoptosis. In our study, compared to healthy donors breast cancer patients presented with a highly significant number of CD95⁺ cytotoxic T-cells and also a significant down-regulation of CD28 and

TCR ζ -expressing CD8-positive T-cells. Lately, other studies showed that survival of activated T-cells is mediated through the Fas/ FasL pathway (12, 26, 28). The loss in expression of T-cell associated ζ - and ϵ -chains was associated with lymphocyte apoptosis induced by FasL-expressing ovarian tumor cells (12). Moreover Walker et al. (28) could demonstrate, that FAS signaling leads to rapid and selective CD28 down-regulation on T-cells and to concomitant apoptosis of these cells.

In accordance with our results this allows the hypothesis that productive signals for T-cell priming and activation might be insufficient in CTLs of primary breast cancer patients and CTLs are primed for apoptosis, independently of clinical prognostic factors. Therefore the body's concept of immunosurveillance would be compromised and T-cell impairment with antitumor dysfunction may result. This might open the "door" to tumor cell escape and tumor cell survival in the peripheral blood of breast cancer patients which is the subject of further ongoing studies.

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