

The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer

S. Muenst · S. D. Soysal · F. Gao · E. C. Obermann ·
D. Oertli · W. E. Gillanders

Received: 21 May 2013 / Accepted: 25 May 2013 / Published online: 12 June 2013
© Springer Science+Business Media New York 2013

Abstract Programmed death 1 (PD-1) is a co-inhibitory receptor in the CD28/CTLA-4 family, and functions as a negative regulator of the immune system. Tumor-infiltrating lymphocytes (TIL) in many epithelial cancers express PD-1, suggesting that antitumor immunity may be modulated by the PD-1/PD-L1 signaling pathway, and promising results from two recent clinical trials with monoclonal antibodies targeting PD-1 or PD-L1 confirm the clinical relevance of this pathway in human cancer. To explore the role of PD-1⁺ TIL in human breast cancer, we performed immunohistochemistry studies on a tissue microarray encompassing 660 breast cancer cases with detailed clinical annotation and outcomes data. PD-1⁺ TIL were present in 104 (15.8 %) of the 660 breast cancer cases. Their presence was associated with tumor size, grade, and lymph node status, and was differentially associated with the intrinsic subtypes of breast cancer. In univariate survival analyses, the presence of PD-1⁺ TIL was associated with a significantly worse overall survival (HR = 2.736,

$p < 0.001$). In subset analyses, the presence of PD-1⁺ TIL was associated with significantly worse overall survival in the luminal B HER2⁻ subtype (HR = 2.678, $p < 0.001$), the luminal B HER2⁺ subtype (HR = 3.689, $p < 0.001$), and the basal-like subtype (HR = 3.140, $p < 0.001$). This is the first study to demonstrate that the presence of PD-1⁺ TIL is associated with poor prognosis in human breast cancer, with important implications for the potential application of antibody therapies targeting the PD-1/PD-L1 signaling pathway in this disease.

Keywords PD-1 · Tumor infiltrating lymphocytes · Breast cancer · Prognostic factor

Introduction

Upon antigen recognition, T cells integrate signals from the T cell receptor, and costimulatory receptors of the CD28/CTLA-4 family [1]. Signaling from costimulatory receptors can be either activating or inhibitory, and the balance between costimulatory and co-inhibitory signals regulates T cell activation and tolerance [1]. Programmed death-1 (PD-1) is a member of the CD28/CTLA-4 family of costimulatory receptors, and, together with Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) [2] and B and T lymphocyte attenuator (BTLA) [3], conveys an inhibitory signal to the T cell. PD-1 is constitutively expressed on a subset of thymic T-lymphocytes, and is upregulated on activated T-cells, B-cells, and myeloid cells [4, 5]. PD-1 is particularly important in peripheral tolerance to self-antigens [6]. PD-1 signaling leads to cell cycle arrest in G0/G1 but does not increase cell death [4]. Persistent high-level PD-1 expression on antigen-experienced CD8⁺ T cells is associated with a CD8⁺ T cell phenotype defined by impaired effector

S. Muenst and S. D. Soysal have contributed equally to this study.

S. Muenst (✉) · E. C. Obermann
Institute of Pathology, University Hospital Basel,
Schönbeinstrasse 40, Basel 4031, Switzerland
e-mail: muensts@uhbs.ch

S. Muenst · S. D. Soysal · W. E. Gillanders
Department of Surgery, Washington University School
of Medicine, St. Louis, USA

S. D. Soysal · D. Oertli
Department of Surgery, University Hospital Basel,
Basel, Switzerland

F. Gao
Division of Biostatistics, Washington University School
of Medicine, St. Louis, USA

function and the persistent expression of inhibitory receptors [7], termed “T cell exhaustion”.

Recent studies have underscored the significance of PD-1 in human disease. PD-1 is significantly upregulated on HIV-specific T cells in patients with chronic infection. Its expression is associated with impaired T cell function, and with predictors of disease progression including plasma viral load [8] and CD4⁺ T cell count [9–12]. In vitro blockade of PD-1 significantly enhances HIV-specific T cell function, clearly defining a reversible immunoregulatory pathway. In addition, there is increasing evidence that it is equally important in human cancer. PD-1 is significantly upregulated on cancer-specific T cells [13–17], and the PD-1 ligand, PD-L1, is expressed by a variety of epithelial cancers [18–20], suggesting that these malignancies may use the PD-1/PD-L1 signaling pathway to attenuate or escape antitumor immunity by maintaining an immunosuppressive tumor microenvironment. Supporting this hypothesis is the fact that despite the induction of cancer-specific T cells in many trials of adoptive cell therapy, with concomitant infiltration of tumor sites, tumor growth is seldom controlled [21].

Based on these findings, targeting the PD-1 pathway to enhance antitumor immunity is under investigation in multiple human cancers [22–24]. Two recently reported phase I clinical trials investigated the effects of fully human anti-PD-1 and anti-PD-L1 antibodies in patients with various types of advanced solid cancers [25, 26]. The antibodies were administered intravenously in patients with melanoma, renal cell carcinoma, and non-small-cell lung cancer, and both studies showed objective responses (response rates 6–28 %). Of note, success was documented in cancers that have long been considered to be resistant to immunotherapy, such as non-small-cell lung cancer. In addition, some of these responses were durable, suggesting that targeting the PD-1/PD-L1 signaling pathway is likely to develop into an important treatment modality for patients with advanced malignancies. However, neither of these two trials included a significant number of breast cancer patients. In the anti-PD-1 antibody study by Topalian et al., there were no breast cancer patients, and in the anti-PD-L1 antibody study by Brahmer et al. [26], there were only four breast cancer patients. Therefore, defining the importance of the PD-1/PD-L1 signaling pathway in breast cancer is of significant clinical relevance, with the potential to provide significant insights into whether antibody therapies targeting this pathway will be appropriate in breast cancer patients.

Although breast cancer is commonly thought to be less immunogenic than melanoma or renal cell carcinoma, there is increasing evidence of a dynamic crosstalk between the immune system and breast cancer. Evidence of this

crosstalk includes the presence and clinical significance of immune infiltrates in breast cancer [27, 28], the increased prevalence of regulatory T cells [14, 29], as well as reported upregulation of inhibitory molecules of the CD28 receptor family on breast cancer-specific T cells [13–15] and of PD-L1 on breast cancer cells [15]. Ghebeh et al. [14] analyzed 62 breast cancer specimens and found that PD-1 was expressed in up to 70 % of tumor-infiltrating lymphocytes (TIL) compared to 30 % in normal breast tissue, and the presence of PD-1⁺ TIL was associated with histologic grade, estrogen receptor (ER), and progesterone receptor (PR) status. In a similar study, the same authors also found expression of PD-L1 on breast cancers cells as well as on TIL in 50 % of cases ($n = 44$). Expression of PD-L1 on either cancer cells or TIL was associated with tumor size, histologic grade, ER status, PR status, and human epidermal growth factor receptor 2 (HER2) status [15]. This finding was supported by Brown et al. [18] who showed that 9 out of 12 breast carcinomas expressed PD-L1, while very low expression was found on adjacent normal breast tissue. In addition, PD-L1 is also expressed by a number of human breast cancer cell lines [4]. Taken together, these results suggest that activation of the PD-1/PD-L1 signaling pathway in the breast cancer microenvironment may modulate antitumor immunity, permitting cancer progression.

BTLA, a recently identified co-inhibitory receptor of the CD28 receptor family, also inhibits proliferation of T cells and cytokine secretion [30]. Investigating the role of BTLA in cancer, Wang et al. [31] showed BTLA to be upregulated in pleural fluid T cells of patients with lung cancer. It has also been shown that tumor antigen-specific effector CD8⁺ T cells in melanoma express high levels of BTLA [32], and that simultaneous blockade of both PD-1 and BTLA enhances the expansion, proliferation, and function of these cells [33]. These data suggest that similar to PD-1, BTLA also could play a role in limiting cancer immunosurveillance.

So far, studies investigating the roles of PD-1 and PD-L1 in human breast cancer have involved relatively small series, and the role of BTLA in breast cancer has not been analyzed. To further explore the prevalence and roles of PD-1⁺ and BTLA⁺ TIL in human breast cancer, we conducted immunohistochemistry studies using a breast cancer tissue microarray (TMA) encompassing a total of 1460 formalin fixed breast cancer cases with detailed clinical annotation and outcomes data. The aim of the present study was to investigate the association between PD-1⁺ TIL, and/or BTLA⁺ TIL, and clinicopathological parameters in breast cancer, with a particular focus on any potential association with prognosis. The data are reported according to the reporting recommendations for tumor marker prognostic studies (REMARK) [34].

Materials and methods

Tissue microarray

We used a TMA encompassing 1460 breast cancer tissue punches from formalin-fixed and paraffin-embedded tumor samples collected from patients diagnosed with primary breast cancer between 1985 and 2007 at the Institute for Pathology, University of Basel and the Viollier Institute in Basel, Switzerland. Of these 1460 tissue punches, a total of 660 were evaluable for our study. The tissue samples were brought into a TMA format as previously described [35]. Briefly, 0.6 mm tissue cylinders were punched out of donor tumor tissue blocks and transferred into a recipient paraffin block using a semi-automated tissue arrayer. Histopathologic data was obtained from the pathology reports, and raw patient survival data was obtained from the Cancer Registry of Basel or from the patient's attending physician. Retrieval of tissue and clinical data was performed according to the regulations of the local institutional review boards and data safety laws with specific regard to ethical standards and patient confidentiality. The mean follow up time was 65 months (range 1 to 174 months), and the mean age of the patients at diagnosis was 64 years (range 27 to 101 years). Demographic information of the patients can be found in Table 1.

Immunohistochemistry

For immunohistochemical staining, 4 μ m sections of the TMA blocks were incubated overnight with a prediluted mouse antihuman PD-1 monoclonal antibody (Cell Marque, Clone MRQ-22, Rocklin, CA, USA) or the mouse antihuman BTLA monoclonal antibody (dilution 1:50, Clone FLO67B, a kind gift from G. Roncador, Centro Nacional de Investigaciones Oncologicas, Madrid, Spain) after heat induced antigen retrieval with Citrate buffer at pH 6 and TEA buffer at pH 8, respectively. Standard DAB-technique (Dako EnVision⁺ System-labeled polymer anti-mouse followed by Liquid DAB⁺ Substrate Chromogen System) was employed for immunostaining. Counterstaining was performed with hematoxylin solution. The number of PD-1⁺ and BTLA⁺ TIL were counted in each breast cancer tissue punch. Normal human lymph node tissue was used as a positive control. The staining intensity of ER, PR, and HER2 was scored as described previously [36].

Flow cytometry of human breast cancer specimens

Fresh human breast cancer specimens were cut into small pieces (5 \times 5 mm), and digested with collagenase B (Roche Diagnostics, Mannheim, Germany) at 37 °C for 15 min. The mixture was then put on the Gentlemacs

Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 s and filtered through a 70 μ m filter. The remaining cell suspension was washed and resuspended in FACS staining buffer, at a concentration of 1×10^6 cells/50 μ l in 5 mL round-bottom polystyrene tubes. Subsequently antihuman CD16/CD32 Fc-block (BD Biosciences, San Jose, CA, USA) was added to the staining cocktail and incubated for 10 min at room temperature. For PD-1 staining, APC-conjugated mouse antihuman CD3 (BD Pharmingen, Franklin Lakes, NJ, USA), PE-conjugated mouse antihuman CD4 (BD Pharmingen), Alexa-Fluor 488-conjugated mouse antihuman CD8 (BioLegend, San

Table 1 Basic demographic data for 660 evaluable breast cancer cases

Mean tumor size (mm) \pm standard deviation (SD)	33.6 \pm 16.8	
Mean age at diagnosis (years) \pm standard deviation (SD)	64.8 \pm 14.3	
	Number (n)	Percent (%)
Tumor stage		
pT1	184	27.9
pT2	357	54.1
pT3	35	5.3
pT4	84	12.7
Lymph node involvement		
pN0	360	54.6
pN1	231	35.1
pN2	68	10.3
Tumor grade		
1	147	22.3
2	261	39.5
3	252	38.2
Histologic subtype		
Invasive ductal	489	76.3
Invasive lobular	75	11.7
Mucinous	23	3.6
Apocrine	3	0.5
Cribriform	14	2.2
Papillary	8	1.2
Medullary	29	4.5
Intrinsic subtype		
Luminal A (ER ⁺ and/or PR ⁺ , HER2 ⁻ , Ki-67 < 14 %)	85	12.9
Luminal B (HER2-negative) (ER ⁺ and/or PR ⁺ , HER2 ⁻ , Ki-67 \geq 14 %)	314	47.7
Luminal B (HER2-positive) (ER ⁺ and/or PR ⁺ , HER2 ⁺)	75	11.4
HER2 type (ER ⁻ or PR ⁻ , HER2 ⁺)	56	8.5
Basal-like (ER ⁻ , PR ⁻ , HER2 ⁻)	128	19.5

Diego, CA, USA), and PerCP/Cy5.5-conjugated mouse antihuman PD-1 (BioLegend) were added and incubated for 15 min at room temperature. For BTLA staining, APC-conjugated mouse antihuman CD19 (BD Pharmingen), PE-Cy7-conjugated mouse antihuman CD8 (eBioscience, San Diego, CA, USA), PE-conjugated mouse antihuman CD4 (BD Pharmingen), and Alexa Fluor 488-conjugated mouse antihuman CD272/BTLA (AbD Serotec, Raleigh, NC, USA) were added and incubated for 15 min at room temperature. Samples were then washed twice with FACS buffer, resuspended in FACS buffer and analyzed by flow cytometry on a FACScalibur flow cytometer (BD Biosciences) or a LSR II flow cytometer (BD Biosciences). The acquired data was analyzed with FlowJo software.

Statistical analysis

The distributions of patient and clinical characteristics between tumors with PD-1⁺ TIL and tumors without PD-1⁺ TIL were compared using Chi square test, Wilcoxon rank sum test, or two-sample *t* test, deemed appropriate. Overall survival (OS) was defined as the time from the first operation to death due to any cause. Survivors were censored at the date of last contact. Survival curves by occurrence of any PD-1⁺ TIL were estimated using the Kaplan–Meier product-limit method and compared by log-rank test. Univariate Cox proportional hazard models were fit to identify factors significantly related to OS. To assess whether the occurrence of any PD-1⁺ TIL was an independent predictor of survival, a multivariate Cox model was constructed to adjust other patient/clinical characteristics that were significant in the univariate analyses. Two-way interaction terms between PD-1⁺ TIL and other factors in the multivariate Cox model were also assessed. All analyses were two-sided and significance was set at a *p* value of 0.05. Statistical analyses were performed using SAS (SAS Institutes, Cary, NC) Fig. 1.

Results

PD-1⁺ TIL were present in a total of 104 (15.8 %) of the 660 evaluable primary breast cancers. The mean number of PD-1⁺ TIL present in the 104 breast cancer cases was 6 (range 1 to 50 TIL). The presence of PD-1⁺ TIL was significantly associated with tumor size, AJCC primary tumor staging system (TNM), tumor grade, and lymph node status (Table 2). The presence of PD-1⁺ TIL was positively associated with Ki-67 expression (*p* = 0.0051) and negatively associated with ER expression (*p* < 0.0001) and PR expression (*p* = 0.0004) (Table 2, and data not shown). There was no significant association between the presence of PD-1⁺ TIL and HER2 expression (*p* = 0.0921, Table 2). Of note, the prevalence of PD-1⁺ TIL differed significantly among the different intrinsic subtypes of breast cancer, as defined by the St Gallen consensus conference [37]. The breast cancer intrinsic subtypes were originally defined by gene expression profiling [38, 39] but can be approximated using immunohistochemistry for ER, PR, Ki-67, and HER2 [37, 40]. These subtypes are known to have differing epidemiological risk factors, prognosis, and response to therapy [37]. The prevalence of PD-1⁺ TIL was the highest in the basal-like subtype (27.4 %) and the lowest in the luminal A subtype (4.7 %, *p* < 0.0001) (Table 3).

In univariate survival analyses, breast cancer cases with any PD-1⁺ TIL present had a significantly worse OS (HR = 2.736, *p* < 0.0001, Table 4; Fig. 2). In subset analyses by intrinsic subtype, the presence of PD-1⁺ TIL was associated with decreased OS in the luminal B HER2⁻ subtype (HR = 2.678, *p* < 0.0001), the luminal B HER2⁺ subtype (HR = 3.689, *p* = 0.0009), and the basal-like subtype (HR = 3.140, *p* < 0.0001) (Table 4; Fig. 2). In multivariate analysis, after adjusting for age, grade, tumor size, lymph node status, and intrinsic subtype, the presence of PD-1⁺ TIL proved to be an independent negative prognostic

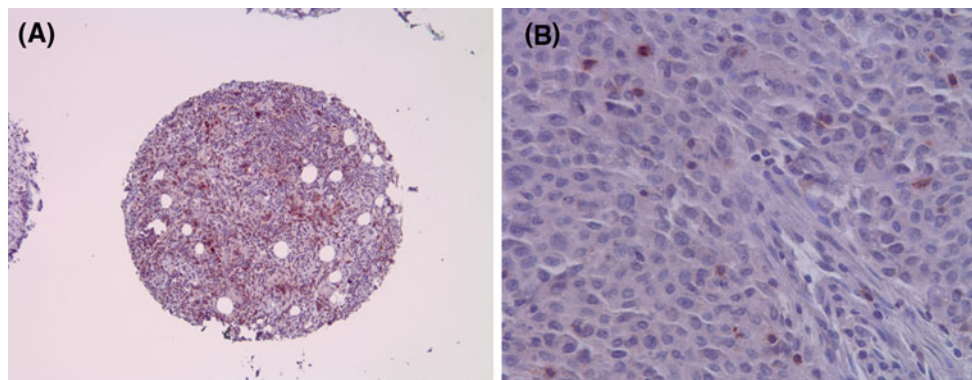


Fig. 1 Representative photographs of PD-1⁺ TIL in a breast cancer tissue punch. **a** Tissue punch with PD-1⁺ TIL. Magnification 20× **b** PD-1⁺ TIL infiltrating a case of invasive ductal carcinoma. Magnification 400×

Table 2 Association between PD-1 expression and clinicopathological parameters

Clinicopathologic parameter	PD-1 ⁺		PD-1 ⁻		<i>p</i> value
	(<i>n</i>)	(%)	(<i>n</i>)	(%)	
Mean tumor size (mm) ± SD	39.5 ± 23.1		27.8 ± 14.6		<0.0001
Mean age at diagnosis (years) ± SD	66.2 ± 14.1		63.4 ± 14.2		0.0619
Tumor stage	(<i>n</i>)				<0.0001
pT1	14	7.6	170	92.4	
pT2	48	13.5	309	86.5	
pT3	12	34.3	23	65.7	
pT4	30	35.7	54	64.3	
Lymph node involvement					<0.0001
pN0	30	8.3	330	91.7	
pN1	29	12.5	202	87.5	
pN2	45	66.2	23	33.8	
Tumor grade					<0.0001
1	7	4.8	140	95.2	
2	34	13.0	227	87.0	
3	63	25.0	189	75.0	
Estrogen receptor					<0.0001
ER ⁺	56	12.0	409	88.0	
ER ⁻	48	24.9	145	75.1	
HER2					0.0921
HER2 ⁺	27	20.6	104	79.4	
HER2 ⁻	77	14.6	450	85.4	
Ki67					0.0051
Ki67 ⁺	95	17.9	437	82.1	
Ki67 ⁻	9	7.5	111	92.5	

Table 3 Association between PD-1 expression and breast cancer intrinsic subtype

Intrinsic subtype	PD-1 ⁺		PD-1 ⁻		<i>p</i> value
	(<i>n</i>)	(%)	(<i>n</i>)	(%)	
Luminal A (ER ⁺ and/or PR ⁺ , HER2 ⁻ , Ki-67 < 14 %)	4	4.7	81	95.3	<0.0001
Luminal B (HER2-negative) ER ⁺ and/or PR ⁺ , HER2 ⁻ , Ki-67 ≥ 14 %)	38	12.1	276	87.9	
Luminal B (HER2-positive) (ER ⁺ and/or PR ⁺ , HER2 ⁺)	14	18.7	61	81.3	
HER2 type (ER ⁻ , PR ⁻ , HER2 ⁺)	13	23.2	43	76.8	
Basal-like (ER ⁻ , PR ⁻ , HER2 ⁻)	35	27.3	93	72.7	

factor for OS (HR = 1.532, *p* = 0.0198) (Table 5). In this multivariate analysis, we excluded the HER2 subtype for two reasons: 1) the univariate analysis indicated that the effect of PD-1⁺ TIL may be different in this subtype (Table 4). 2) the HER2 subtype consists of only 56 cases and may preclude a reliable testing of interaction between PD1 and intrinsic subtypes. When looking at all breast cancer cases, The

number of PD-1⁺ TIL was associated with worse OS (HR = 1.031, *p* = 0.0175, data not shown). However, owing to the relatively low number of cases with PD-1⁺ TIL (*n* = 104), this association may not be representative.

BTLA⁺ TIL were present in 15 of the 660 breast cancer cases (2.3 %, range of BTLA⁺ TIL 1–452). Owing to the small number of breast cancer cases with BTLA⁺ TIL, we

Table 4 Univariate analyses for all cases, and by intrinsic subtype, for the effect of PD-1 expression on overall survival

PD-1 expression, all cases	Hazard ratio (95 % CI)	<i>p</i> value
PD-1 ⁺	2.736 (2.066–3.625)	<0.0001
PD-1 expression, by intrinsic subtype		
Luminal A	2.474 (0.551–11.120)	0.2374
Luminal B (HER2 ⁻)	2.678 (1.703–4.212)	<0.0001
Luminal B (HER2 ⁺)	3.689 (1.712–7.949)	0.0009
HER2 type	0.536 (0.181–1.588)	0.2607
Basal-like	3.140 (1.886–5.230)	<0.0001

did not perform statistical analyses to determine if there is an association between BTLA⁺ TIL and clinicopathological parameters or prognosis. Of note, all cases that showed BTLA⁺ TIL also contained PD-1⁺ TIL.

To investigate the phenotype of PD-1⁺ TIL in more detail, we performed flow cytometry of cells freshly isolated from three human breast cancers of the invasive ductal subtype. A mean of 3.9 % of all cells in the tumors expressed PD-1. 89.1 % of the PD-1⁺ cells were CD3⁺ lymphocytes, and 80.9 % of the PD-1⁺/CD3⁺ cells were CD4⁺, and 17.9 % were CD8⁺, suggesting that PD-1 is primarily expressed on CD4⁺ T cells in human breast cancer. 6.3 % of all CD3⁺ lymphocytes expressed PD-1

Fig. 2 a Kaplan–Meier survival curve for overall survival depending on the presence of PD-1⁺ TIL (univariate analysis); **b–f** Kaplan–Meier survival curves for overall survival depending on the presence of PD-1⁺ TIL for the indicated breast cancer intrinsic subtypes

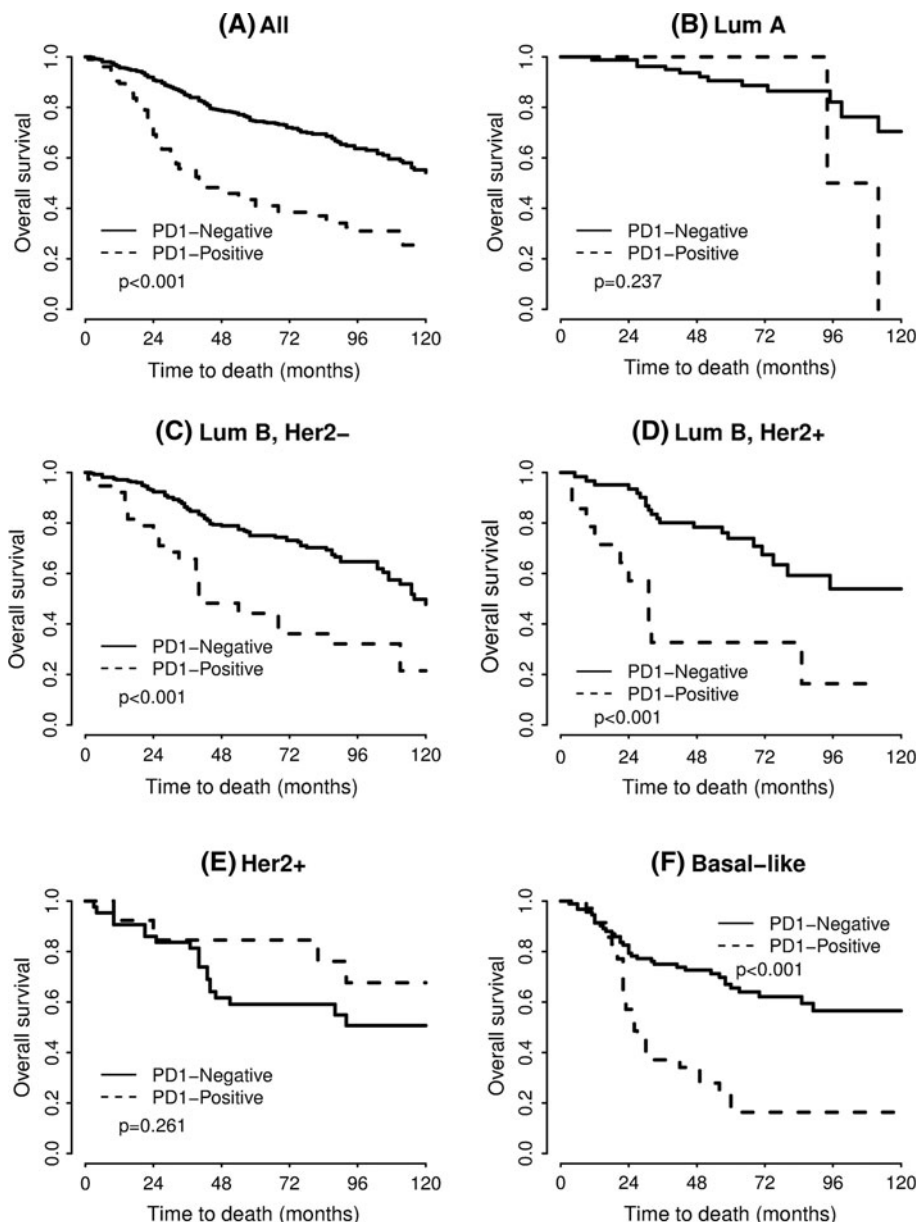


Table 5 Multivariate analysis for the effect of clinicopathologic parameters and PD1 expression on overall survival

Clinicopathologic parameter	Hazard ratio (95 % CI)	<i>p</i> value
Age (per 1-year)	1.038 (1.027–1050)	<0.0001
Tumor stage		
pT1 (reference)	1	
pT2	1.559 (1.036–2.347)	0.0334
pT3	2.157 (1.120–4.154)	0.0216
pT4	2.588 (1.572–4.261)	0.0002
Lymph node involvement		
pN1 (reference)	1	
pN1	1.310 (0.955–1.798)	0.0940
pN2	2.315 (1.499–3.576)	0.0002
Tumor grade		
BRE grade 1 (reference)	1	
2	1.751 (1.119–2.740)	0.0142
3	2.435 (1.535–3.863)	0.0002
Intrinsic subtypes		
Luminal A	1	
Luminal B (HER2 ⁻)	1.558 (0.877–2.770)	0.1306
Luminal B (HER2 ⁺)	1.838 (0.951–3.551)	0.0702
Basal-like	2.761 (1.482–5.143)	0.0014
PD-1 expression, all cases		
PD-1 ⁺	1.532 (1.070–2.194)	0.0198

(Table 6; Fig. 3). We performed similar flow cytometric analyses to investigate the phenotype of BTLA⁺ cells in human breast cancer. Less than 1 % of all cells in the tumor expressed BTLA, and BTLA expression could not be detected on CD4⁺ or CD8⁺ T cells (data not shown).

Discussion

In this study, we investigated the significance of PD-1⁺ TIL in a large cohort of clinically annotated primary breast cancer specimens. We observed that PD-1⁺ TIL are present in 15.8 % of primary breast cancers, and the presence of PD-1⁺ TIL is associated with tumor size, AJCC primary tumor staging system (TNM), tumor grade, lymph node status, and biomarker profile (ER, PR, and HER2 status). In addition, the presence of PD-1⁺ TIL is differentially associated with the intrinsic subtypes of breast cancer. Of particular note, our study is the first to show that the presence of PD-1⁺ TIL in breast cancer is associated with a significantly worse OS.

Our findings confirm and extend the results of Ghebeh et al., but there are important differences between ours and theirs. Ghebeh et al. [14] found that PD-1⁺ TIL are present in 60 % of primary breast cancers, a significantly higher

Table 6 Flow cytometry results for 3 breast cancer specimens

	PD-1 ⁺ cells	PD-1 ⁺ CD3 ⁺ cells	PD-1 ⁺ CD4 ⁺ cells	PD-1 ⁺ CD8 ⁺ cells
Patient 1	2.52 %	97.2 %	88.8 %	10.9 %
Patient 2	4.32 %	92.6 %	79 %	19.9 %
Patient 3	4.79 %	77.6 %	75.1 %	23 %

prevalence than what we observed. However, their study was relatively small ($n = 62$), and the authors evaluated whole tumor sections, which may increase the likelihood of finding PD-1⁺ TIL. They also used a different PD-1 monoclonal antibody, which may have a distinct staining pattern. Despite these differences, Ghebeh et al. also found a correlation between the presence of PD-1⁺ TIL and higher tumor grade in their series [14], although the small size of their series and lack of clinical outcome data precluded a survival analysis.

Flow cytometry analyses confirm that PD-1 is expressed mainly by CD3⁺ lymphocytes in human breast cancer. This result, as well as the morphologic appearance of the PD-1⁺ cells in the breast cancer tissue specimens, confirms our assumption that the PD-1⁺ cells identified by immunohistochemistry are indeed TIL, obviating the need for double staining with PD-1 and a T cell marker. Of note, the flow cytometry analyses also demonstrate that the majority of PD-1⁺ cells are CD4⁺ T cells, a finding that is surprising considering that most of the studies so far have reported that PD-1⁺ TIL are predominantly CD8⁺, and it is believed that suppression of CD8⁺/PD-1⁺ tumor-specific T cells may be a primary mechanism by which cancers evade immune responses [7, 14, 16, 17]. However, a recent study found that PD-1 is expressed on 73.4 % of CD4⁺ TIL in gastric cancer tissue and that these cells had impaired function [41]. Similarly, PD-1 expression was found on up to 76.4 % of CD4⁺ T cells in Hodgkin's lymphoma specimens [42] and their presence is associated with reduced overall survival [43]. In HPV-positive head and neck cancer, PD-1 expression is also higher in CD4⁺ T cells than in CD8⁺ T cells [44], and studies conducted in melanoma patients show that PD-1 is upregulated on both CD4⁺ as well as CD8⁺ TIL [17, 45].

Two recent phase I clinical trials have targeted the PD-1/PD-L1 signaling pathway using fully human monoclonal antibodies. These studies were associated with objective clinical responses in cancers that have previously been refractory to immunotherapy. Of note, Topalian et al. [25] were able to assess PD-L1 expression in a subset of cancers, and preliminary results suggest that PD-L1 expression by the cancer is associated with improved outcome following anti-PD-1 antibody therapy, suggesting that PD-L1 is a candidate biomarker for anti-PD-1 immunotherapy. The data from this study, particularly the association

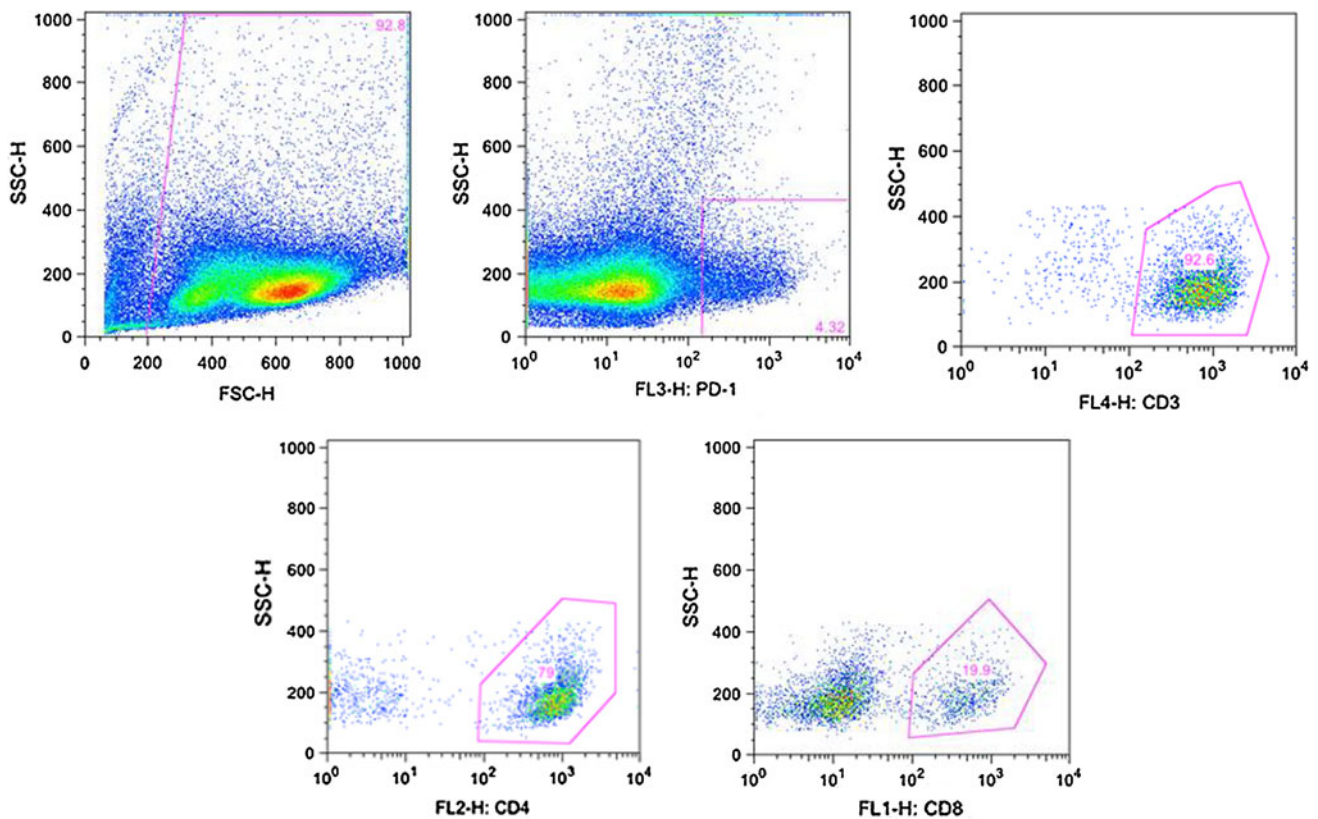


Fig. 3 Representative flow cytometry data for PD-1 expression in human breast cancer. Consecutive gating on live cells/PD-1⁺ cells/CD3⁺ cells and subsequent gating on CD4⁺ and CD8⁺ cells in this subpopulation

between the presence of PD-1⁺ TIL and higher stage, grade and worse survival, suggest that PD-1 may also be a candidate biomarker for predicting response to therapy. PD-1 has two physiologic ligands, PD-L1 and PD-L2; assessing PD-L1 expression may underestimate the number of tumors that modulate the PD-1/PD-L1 signaling pathway. However, if PD-1⁺ TIL are present in the cancer, it suggests that tumor-specific T cells have been primed but were subsequently functionally inactivated. Reactivation of these cells through PD-1/PD-L1 checkpoint blockade may lead to enhanced antitumor immunity and improved clinical outcome [25, 26, 46, 47].

It is important to note that the presence of PD-1⁺ TIL is differentially associated with the intrinsic subtypes of breast cancer. The prevalence of PD-1⁺ TIL is the highest in the basal-like subtype and the lowest in the luminal A subtype. In subset analyses, the presence of PD-1⁺ TIL proved to be a negative prognostic factor for OS in the luminal B HER2⁻ type, the luminal B HER2⁺ type, and the basal-like subtype. The increased prevalence of PD-1⁺ TIL, and strong association with survival in the basal-like subtype are particularly relevant, as treatment options are limited in this subtype, and PD-1-targeted therapies may represent an attractive alternative or additive therapy in this subset of breast cancer patients.

We also evaluated BTLA expression, a second co-inhibitory receptor of the CD28/CTLA-4 family. However, we found very few breast cancers with BTLA⁺ TIL in our series, suggesting that this co-inhibitory receptor does not play a biologically relevant role in breast cancer immunosurveillance. This finding was supported by flow cytometry analyses of human breast cancers, where less than 1 % of all cells expressed BTLA, and no BTLA⁺ T cells were detectable.

In summary, our findings suggest that PD-1 plays a functional inhibitory role in human breast cancer immunosurveillance, a fact that should encourage immunotherapeutic approaches targeting the PD-1/PD-L1 signaling pathway in breast cancer. Further studies investigating the roles of PD-1 and PD-L1 in breast cancer are recommended.

Acknowledgments This study was supported by a research grant of the Swiss National Foundation (SNF) (PBBSP3-138709). We thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, Mo., for the use of the Biostatistics Core. The Siteman Cancer Center is supported in part by NCI Cancer Center Support Grant #P30 CA091842. We thank Professor D. Craig Allred for his contribution to the development of the BTLA immunohistochemical assay. We thank Professor Alexander Tzankov for his valuable insights and critical review of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Porichis F, Kaufmann DE (2012) Role of PD-1 in HIV pathogenesis and as target for therapy. *Curr HIV/AIDS Rep* 9(1):81–90. doi:10.1007/s11904-011-0106-4
2. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA (1994) CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1(5):405–413. doi:10.1074-7613(94)90071-X
3. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, Hurchla MA, Zimmerman N, Sim J, Zang X, Murphy TL, Russell JH, Allison JP, Murphy KM (2003) BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 4(7):670–679. doi:10.1038/nri944
4. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2(3):261–268. doi:10.1038/85330
5. Kitazawa Y, Fujino M, Wang Q, Kimura H, Azuma M, Kubo M, Abe R, Li XK (2007) Involvement of the programmed death-1/programmed death-1 ligand pathway in CD4⁺ CD25⁺ regulatory T-cell activity to suppress alloimmune responses. *Transplantation* 83(6):774–782. doi:10.1097/01.tp.0000256293.90270.e8
6. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M (2005) Resting dendritic cells induce peripheral CD8⁺ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 6(3):280–286. doi:10.1038/nri1165
7. Flies DB, Sandler BJ, Sznol M, Chen L (2011) Blockade of the B7–H1/PD-1 pathway for cancer immunotherapy. *Yale J Biol Med* 84(4):409–421
8. Aaltomaa S, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E, Syrjanen K (1992) Tumor size, nuclear morphometry, mitotic indices as prognostic factors in axillary-lymph-node-positive breast cancer. *Eur Surg Res* 24(3):160–168
9. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443(7109):350–354
10. Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, Miura T, Palmer S, Brockman M, Rathod A, Piechocka-Trocha A, Baker B, Zhu B, Le Gall S, Waring MT, Ahern R, Moss K, Kelleher AD, Coffin JM, Freeman GJ, Rosenberg ES, Walker BD (2007) Upregulation of CTLA-4 by HIV-specific CD4⁺ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol* 8(11):1246–1254
11. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, Koup RA (2006) PD-1 is a regulator of virus-specific CD8⁺ T cell survival in HIV infection. *J Exp Med* 203(10):2281–2292
12. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP (2006) Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat Med* 12(10):1198–1202
13. Czerniecki BJ, Koski GK, Koldovsky U, Xu S, Cohen PA, Mick R, Nisenbaum H, Pasha T, Xu M, Fox KR, Weinstein S, Orel SG, Vonderheide R, Coukos G, DeMichele A, Araujo L, Spitz FR, Rosen M, Levine BL, June C, Zhang PJ (2007) Targeting HER-2/neu in early breast cancer development using dendritic cells with staged interleukin-12 burst secretion. *Cancer Res* 67(4):1842–1852. doi:10.1158/0008-5472.CAN-06-4038
14. Ghebeh H, Barhoush E, Tulbah A, Elcum N, Al-Tweigeri T, Dermime S (2008) FOXP3⁺ Tregs and B7–H1⁺/PD-1⁺ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: implication for immunotherapy. *BMC Cancer* 8:57. doi:10.1186/1471-2407-8-57
15. Ghebeh H, Mohammed S, Al-Omar A, Qattan A, Lehe C, Al-Qudaihi G, Elcum N, Alshabanah M, Bin Amer S, Tulbah A, Ajarim D, Al-Tweigeri T, Dermime S (2006) The B7–H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia* 8(3):190–198. doi:10.1593/neo.05733
16. Sfanos KS, Bruno TC, Meeker AK, De Marzo AM, Isaacs WB, Drake CG (2009) Human prostate-infiltrating CD8⁺ T lymphocytes are oligoclonal and PD-1⁺. *Prostate* 69(15):1694–1703. doi:10.1002/pros.21020
17. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA (2009) Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 114(8):1537–1544. doi:10.1182/blood-2008-12-195792
18. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, Greenfield EA, Freeman GJ (2003) Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol* 170(3):1257–1266
19. Zou W, Chen L (2008) Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol* 8(6):467–477. doi:10.1038/nri2326
20. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L (2002) Tumor-associated B7–H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8(8):793–800. doi:10.1038/nm730nm730
21. Blank C, Gajewski TF, Mackensen A (2005) Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother* 54(4):307–314. doi:10.1007/s00262-004-0593-x
22. Strome SE, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, Salomao D, Chevillat J, Hirano F, Lin W, Kasperbauer JL, Ballman KV, Chen L (2003) B7–H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res* 63(19):6501–6505
23. Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, Krzysiek R, Knutson KL, Daniel B, Zimmermann MC, David O, Burow M, Gordon A, Dhurandhar N, Myers L, Berggren R, Hemminki A, Alvarez RD, Emilie D, Curiel DT, Chen L, Zou W (2003) Blockade of B7–H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 9(5):562–567. doi:10.1038/nm863
24. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, Rietz C, Flies DB, Lau JS, Zhu G, Tamada K, Chen L (2005) Blockade of B7–H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 65(3):1089–1096. doi:10.1158/0008-5472.CCR-04-1089
25. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM,

- McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 366(26):2443–2454. doi: [10.1056/NEJMoa1200690](https://doi.org/10.1056/NEJMoa1200690)
26. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I, Topalian SL (2010) Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 28(19):3167–3175. doi: [10.1200/JCO.2009.26.7609](https://doi.org/10.1200/JCO.2009.26.7609)
 27. Aaltomaa S, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E, Syrjanen K (1992) Lymphocyte infiltrates as a prognostic variable in female breast cancer. *Eur J Cancer* 28A(4–5):859–864
 28. Marrogi AJ, Munshi A, Merogi AJ, Ohadike Y, El-Habashi A, Marrogi OL, Freeman SM (1997) Study of tumor infiltrating lymphocytes and transforming growth factor-beta as prognostic factors in breast carcinoma. *Int J Cancer* 74(5):492–501. doi: [10.1002/\(SICI\)1097-0215\(19971021\)74:5<492:AID-IJC3>3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1097-0215(19971021)74:5<492:AID-IJC3>3.0.CO;2-Z)
 29. Droezer R, Zlobec I, Kilic E, Guth U, Heberer M, Spagnoli G, Oertli D, Tapia C (2012) Differential pattern and prognostic significance of CD4⁺, FOXP3⁺ and IL-17⁺ tumor infiltrating lymphocytes in ductal and lobular breast cancers. *BMC Cancer* 12:134. doi: [10.1186/1471-2407-12-134](https://doi.org/10.1186/1471-2407-12-134)
 30. Krieg C, Boyman O, Fu YX, Kaye J (2007) B and T lymphocyte attenuator regulates CD8⁺ T cell-intrinsic homeostasis and memory cell generation. *Nat Immunol* 8(2):162–171. doi: [10.1038/ni1418](https://doi.org/10.1038/ni1418)
 31. Wang XF, Chen YJ, Wang Q, Ge Y, Dai Q, Yang KF, Fang X, Zhou YH, Hu YM, Mao YX, Zhang XG (2007) Distinct expression and inhibitory function of B and T lymphocyte attenuator on human T cells. *Tissue Antigens* 69(2):145–153. doi: [10.1111/j.1399-0039.2006.00710.x](https://doi.org/10.1111/j.1399-0039.2006.00710.x)
 32. Derre L, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, Michielin O, Olive D, Speiser DE (2010) BTLA mediates inhibition of human tumor-specific CD8⁺ T cells that can be partially reversed by vaccination. *J Clin Invest* 120(1):157–167. doi: [10.1172/JCI40070](https://doi.org/10.1172/JCI40070)
 33. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Olive D, Kuchroo V, Zarour HM (2012) CD8⁺ T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* 72(4):887–896. doi: [10.1158/0008-5472.CAN-11-2637](https://doi.org/10.1158/0008-5472.CAN-11-2637)
 34. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM (2006) Reporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res Treat* 100(2):229–235. doi: [10.1007/s10549-006-9242-8](https://doi.org/10.1007/s10549-006-9242-8)
 35. Bubendorf L, Nocito A, Moch H, Sauter G (2001) Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. *J Pathol* 195(1):72–79. doi: [10.1002/path.893](https://doi.org/10.1002/path.893)
 36. Tapia C, Schraml P, Simon R, Al-Kuraya KS, Maurer R, Mirlacher M, Novotny H, Spichtin H, Mihatsch MJ, Sauter G (2004) HER2 analysis in breast cancer: reduced immunoreactivity in FISH non-informative cancer biopsies. *Int J Oncol* 25(6):1551–1557
 37. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ (2011) Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* 22(8):1736–1747. doi: [10.1093/annonc/mdr304](https://doi.org/10.1093/annonc/mdr304)
 38. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* 406(6797):747–752. doi: [10.1038/35021093](https://doi.org/10.1038/35021093)
 39. Prat A, Perou CM (2011) Deconstructing the molecular portraits of breast cancer. *Mol Oncol* 5(1):5–23. doi: [10.1016/j.molonc.2010.11.003](https://doi.org/10.1016/j.molonc.2010.11.003)
 40. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkilä P, Heikkinen T, Nevanlinna H, Akslen LA, Begin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, Garcia-Closas M, Caldas C, Pharoah PD, Huntsman D (2010) Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLoS Med* 7(5):e1000279. doi: [10.1371/journal.pmed.1000279](https://doi.org/10.1371/journal.pmed.1000279)
 41. Saito H, Kuroda H, Matsunaga T, Osaki T, Ikeguchi M (2012) Increased PD-1 expression on CD4⁺ and CD8⁺ T cells is involved in immune evasion in gastric cancer. *J Surg Oncol*. doi: [10.1002/jso.23281](https://doi.org/10.1002/jso.23281)
 42. Yamamoto R, Nishikori M, Kitawaki T, Sakai T, Hishizawa M, Tashima M, Kondo T, Ohmori K, Kurata M, Hayashi T, Uchiyama T (2008) PD-1-PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. *Blood* 111(6):3220–3224. doi: [10.1182/blood-2007-05-085159](https://doi.org/10.1182/blood-2007-05-085159)
 43. Muenst S, Hoeller S, Dirnhofer S, Tzankov A (2009) Increased programmed death-1⁺ tumor-infiltrating lymphocytes in classical Hodgkin lymphoma substantiate reduced overall survival. *Hum Pathol* 40(12):1715–1722. doi: [10.1016/j.humpath.2009.03.025](https://doi.org/10.1016/j.humpath.2009.03.025)
 44. Badoual C, Hans S, Merillon N, Van Ryswick C, Ravel P, Benhamouda N, Levionnois E, Nizard M, Si-Mohamed A, Besnier N, Gey A, Rotem-Yehudar R, Pere H, Tran T, Guerin CL, Chauvat A, Dransart E, Alanio C, Albert S, Barry B, Sandoval F, Quintin-Colonna F, Bruneval P, Fridman WH, Lemoine FM, Oudard S, Johannes L, Olive D, Brasnu D, Tartour E (2012) PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV associated head and neck cancer. *Cancer Res*. doi: [10.1158/0008-5472.CAN-12-2606](https://doi.org/10.1158/0008-5472.CAN-12-2606)
 45. Chapon M, Randriamampita C, Maubec E, Badoual C, Fouquet S, Wang SF, Marinho E, Farhi D, Garcette M, Jacobelli S, Rouquette A, Carlotti A, Girod A, Prevost-Blondel A, Trautmann A, Avril MF, Bercovici N (2011) Progressive upregulation of PD-1 in primary and metastatic melanomas associated with blunted TCR signaling in infiltrating T lymphocytes. *J Invest Dermatol* 131(6):1300–1307. doi: [10.1038/jid.2011.30](https://doi.org/10.1038/jid.2011.30)
 46. Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, Koren-Michowitz M, Shimoni A, Nagler A (2008) Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clin Cancer Res* 14(10):3044–3051. doi: [10.1158/1078-0432.CCR-07-4079](https://doi.org/10.1158/1078-0432.CCR-07-4079)
 47. Topalian SL, Drake CG, Pardoll DM (2012) Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* 24(2):207–212. doi: [10.1016/j.coi.2011.12.009](https://doi.org/10.1016/j.coi.2011.12.009)