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PRECLINICAL STUDY

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

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Abstract Programmed death 1 (PD-1) is a co-inhibitory receptor in the CD28/CTL-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

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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 μm 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 × 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 μm filter. The remaining cell suspension was washed and resuspended in FACS staining buffer, at a concentration of 1×10^6 cells/50 μl 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) ± standard deviation (SD)	33.6 ± 16.8	
Mean age at diagnosis (years) ± standard deviation (SD)	64.8 ± 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 ≥ 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

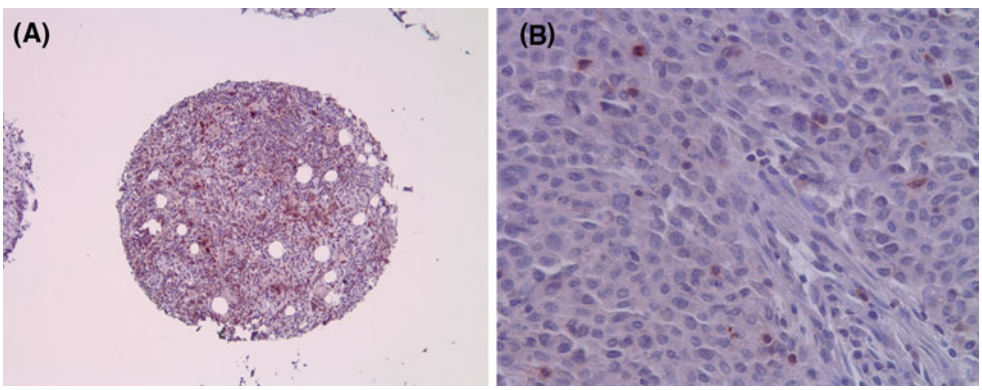

Fig. 1 Representative photographs of PD-1^+ TIL in a breast cancer tissue punch. **a** Tissue punch with PD-1^+ TIL. Magnification 20× **b** PD-1^+ TIL infiltrating a case of invasive ductal carcinoma. Magnification 400×

Table 2 Association between PD-1 expression and clinicopathological parameters

Clinicopathologic parameter	PD-1$^+$		PD-1$^-$		*p* value
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	(*n*)				<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$^-$		*p* value
	(*n*)	(%)	(*n*)	(%)	<0.0001
Luminal A (ER$^+$ and/or PR$^+$, HER2$^-$, Ki-67 < 14 %)	4	4.7	81	95.3	
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)	*p* 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

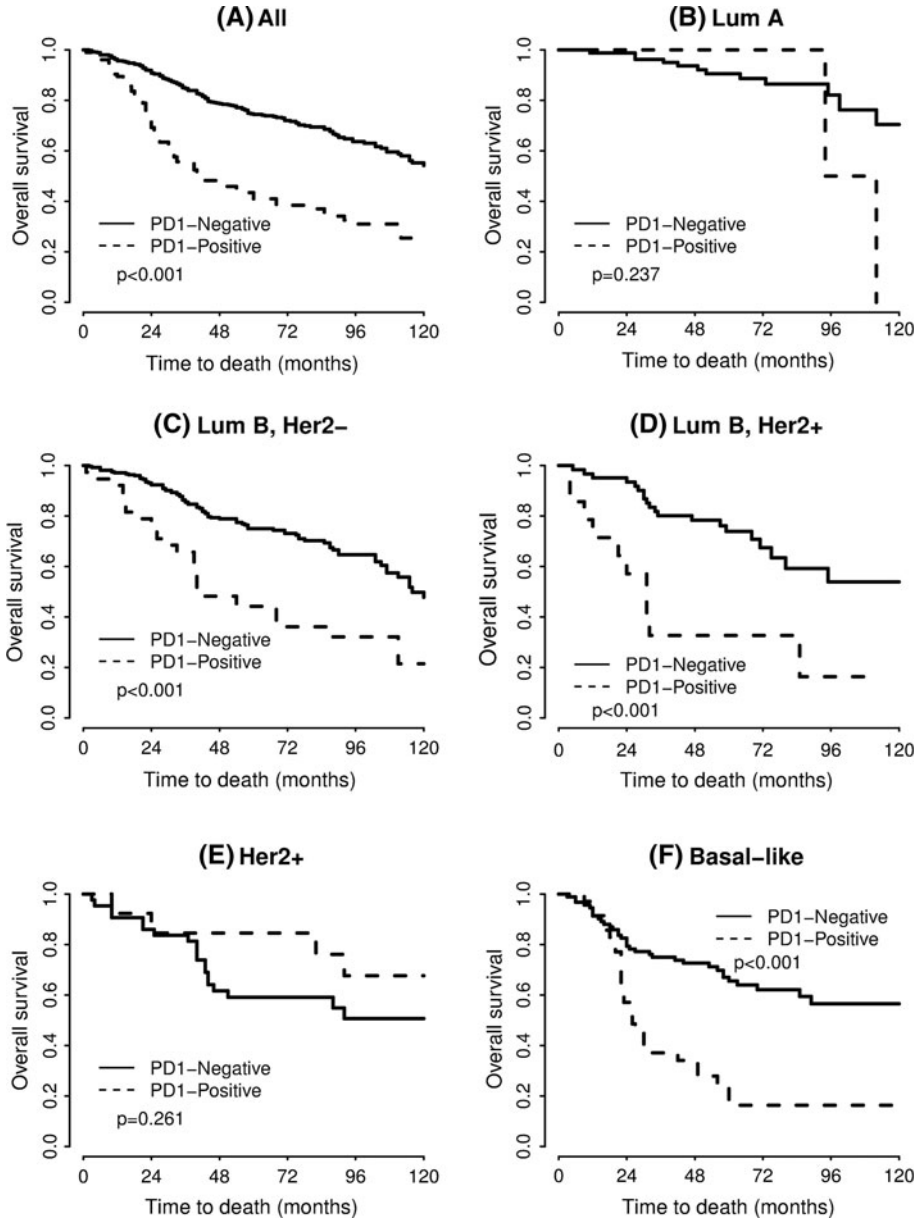

Fig. 2 **a** Kaplan–Meier survival curve for overall survival depending on the presence of PD-1$^+$ TIL (univariate analysis); **b–f** Kaplan–Meier survival curves for overall survival depending on the presence of PD-1$^+$ TIL for the indicated breast cancer intrinsic subtypes

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

Clinicopathologic parameter	Hazard ratio (95 % CI)	*p* 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		
$PD\text{-}1^+$	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 $PD\text{-}1^+$ TIL in a large cohort of clinically annotated primary breast cancer specimens. We observed that $PD\text{-}1^+$ TIL are present in 15.8 % of primary breast cancers, and the presence of $PD\text{-}1^+$ 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 $PD\text{-}1^+$ 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 $PD\text{-}1^+$ 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 $PD\text{-}1^+$ TIL are present in 60 % of primary breast cancers, a significantly higher 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 $PD\text{-}1^+$ 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 $PD\text{-}1^+$ 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.

Table 6 Flow cytometry results for 3 breast cancer specimens

	$PD\text{-}1^+$ cells	$PD\text{-}1^+$ $CD3^+$ cells	$PD\text{-}1^+$ $CD4^+$ cells	$PD\text{-}1^+$ $CD8^+$ 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 %

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 $PD\text{-}1^+$ cells in the breast cancer tissue specimens, confirms our assumption that the $PD\text{-}1^+$ 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 $PD\text{-}1^+$ cells are $CD4^+$ T cells, a finding that is surprising considering that most of the studies so far have reported that $PD\text{-}1^+$ TIL are predominantly $CD8^+$, and it is believed that suppression of $CD8^+/PD\text{-}1^+$ 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

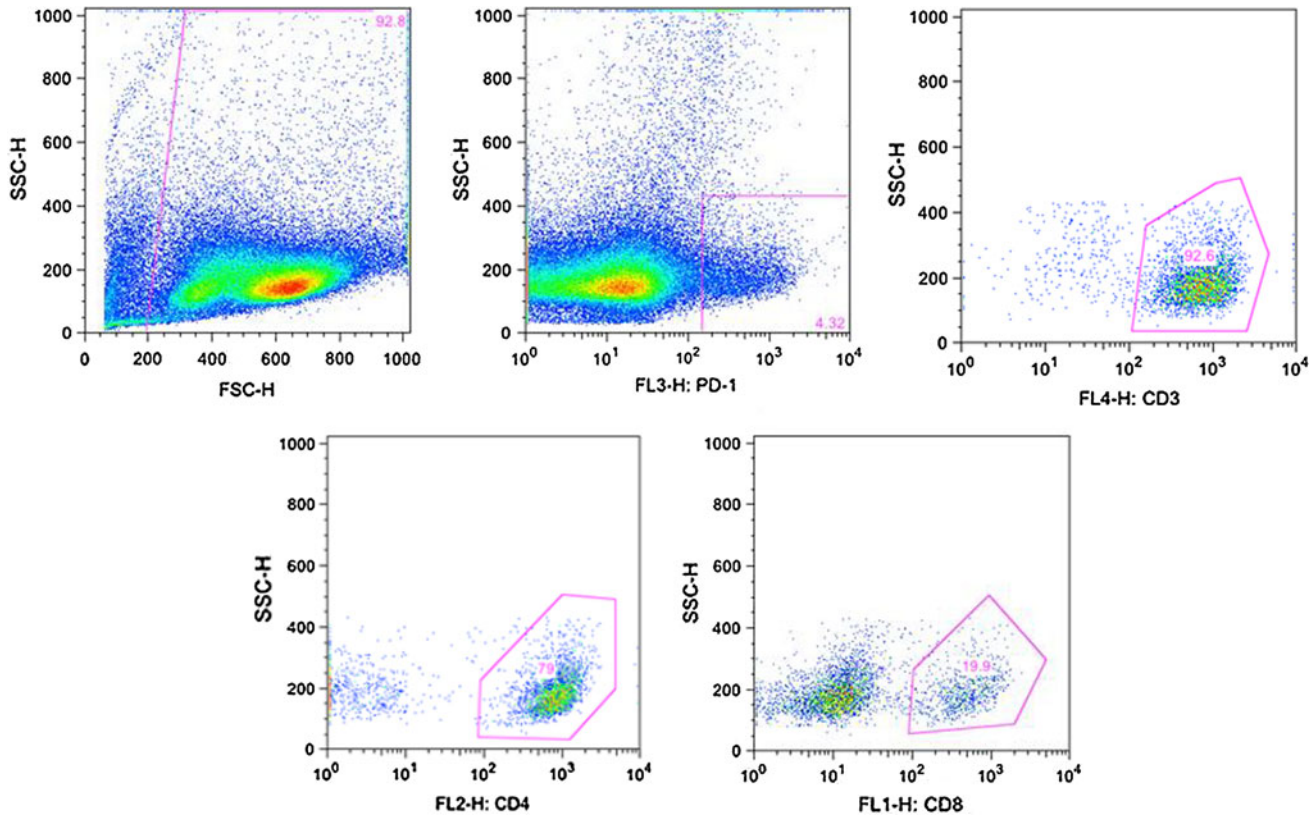

Fig. 3 Representative flow cytometry data for PD-1 expression in human breast cancer. Consecutive gating on live cells/PD-1$^+$ cells/CD3$^+$ cells and subsequent gating on CD4$^+$ and CD8$^+$ cells in this subpopulation

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.

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

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