Histopathology

Histopathology 2016, **69**, 25–34. DOI: 10.1111/his.12904

Programmed death ligand 1 expression in triple-negative breast cancer is associated with tumour-infiltrating lymphocytes and improved outcome

Rhiannon K Beckers,[1] Christina I Selinger,[1] Ricardo Vilain,[1,2,3] Jason Madore,[3] James S Wilmott,[2,3] Kate Harvey,[1,4] Anne Holliday,[4] Caroline L Cooper,[1,2] Elizabeth Robbins,[1] David Gillett,[2,5] Catherine W Kennedy,[2,5] Laurence Gluch,[2,5,6] Hugh Carmalt,[2,5,7] Cindy Mak,[7] Sanjay Warrier,[2,7,8] Harriet E Gee,[9,10] Charles Chan,[2,11] Anna McLean,[2] Emily Walker,[2] Catriona M McNeil,[2,12] Jane M Beith,[2,12] Alexander Swarbrick,[4,13] Richard A Scolyer[1,2,3] & Sandra A O'Toole[1,2,4,13]

[1]*Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia,* [2]*Sydney Medical School, University of Sydney, Sydney, NSW, Australia,* [3]*Melanoma Institute Australia, Sydney, NSW, Australia,* [4]*The Kinghorn Cancer Centre and Cancer Research Program, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia,* [5]*The Strathfield Breast Centre, Strathfield, NSW, Australia,* [6]*Department of Breast and Endocrine Surgery, Concord Repatriation General Hospital, Sydney, NSW, Australia,* [7]*Department of Breast Surgery, Royal Prince Alfred Hospital, Camperdown, NSW, Australia,* [8]*Department of Surgery, Prince of Wales Hospital, Randwick, NSW, Australia,* [9]*Department of Radiation Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Camperdown, NSW, Australia,* [10]*Department of Oncology, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK,* [11]*Anatomical Pathology Department, Concord Repatriation General Hospital, Concord, NSW, Australia,* [12]*Department of Medical Oncology, Chris O'Brien Lifehouse, Camperdown, NSW, Australia, and* [13]*St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Darlinghurst, NSW, Australia*

Date of submission 18 June 2015
Accepted for publication 14 November 2015
Published online *Article Accepted* 20 November 2015

Beckers R K, Selinger C I, Vilain R, Madore J, Wilmott J S, Harvey K, Holliday A, Cooper C L, Robbins E, Gillett D, Kennedy C W, Gluch L, Carmalt H, Mak C, Warrier S, Gee H E, Chan C, McLean A, Walker E, McNeil C M, Beith J M, Swarbrick A, Scolyer R A & O'Toole S A
(2016) *Histopathology* **69**, 25–34. DOI: 10.1111/his.12904

Programmed death ligand 1 expression in triple-negative breast cancer is associated with tumour-infiltrating lymphocytes and improved outcome

Aims: Triple-negative breast cancer (TNBC) patients generally have a poor outcome; there is a pressing need to identify more effective therapeutic strategies. Clinical trials targeting programmed death 1/programmed death ligand 1 (PD1/PDL1) in melanoma and non-small-cell lung cancer have reported high response rates, and tumoral PDL1 expression has been suggested as a potential biomarker to enrich for patient response to these treatments. There are only very limited data to date reporting the expression of PDL1 in TNBC.

Methods and results: PDL1 immunohistochemistry was performed on 161 primary TNBCs and assessed in the tumour as well as immune cells in the stromal compartment. PDL1 expression was very common in TNBC, expressed in the tumour cell membrane (64%), cytoplasm (80%) and stromal (93%) cellular compartments. Cytoplasmic tumoral expression of PDL1 was

Address for correspondence: S A O'Toole, Department of Tissue Pathology and Diagnostic Oncology, Building 94, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW, 2050, Australia. e-mail: sandra.otoole@sswahs.nsw.gov.au

associated with a lower risk of breast cancer-specific death [hazard ratio (HR) 0.45, $P = 0.035$] while stromal PDL1 expression was associated with a lower rate of deaths from all causes (HR 0.305, $P = 0.0042$). Membranous expression of PDL1 was not associated with outcome. While both PDL1 expression and tumour-infiltrating lymphocytes were associated with a better outcome, only lymphovascular invasion and high tumour-infiltrating lymphocytes were independently prognostic for breast cancer-specific death.

Conclusion: While PDL1 expression is frequent in TNBC, it was not independently prognostic. There were differences in outcome depending on the cellular compartment of PDL1 expression. These data provide further impetus for investigating the utility of immune checkpoint therapies in TNBC, given the clinical significance of tumour-infiltrating lymphocytes (TILs) and PDL1 expression in this cohort.

Keywords: PDL1, triple-negative breast cancer, tumour-infiltrating lymphocytes

Introduction

The use of mammographic screening, and particularly the use of targeted therapies against hormone and human epidermal growth factor receptor 2 (HER2) receptors in breast cancer, have contributed to marked improvements in outcome during the past two decades, with the 5-year survival rates in several countries now approaching 90%.[1] However, for the group of patients lacking expression of hormone and HER2 receptors, the 'triple-negative breast cancer' (TNBC) group, the prognosis is often poor, with limited effective treatment options. With a 5-year survival for TNBC of only 60%,[2] there is a pressing need to identify potential new therapeutic strategies for this generally poor-prognosis cancer.

Inhibition of the immune checkpoint regulators PD1/PDL1 is a new anticancer therapy, with results from recent clinical trials in other poor-prognosis malignancies such as melanoma and non-small-cell lung cancer (NSCLC) showing promising results thus far. PD1 is often referred to as a 'checkpoint molecule' in immune regulation, and assists in maintaining tolerance to self. PD1 is expressed on many tumour-infiltrating $CD8^+$ T cells, as well as $CD4^+$ T cells, natural killer (NK) T cells, B cells, activated monocytes and dendritic cells.[3] PD1 is involved in T cell regulation, and functions as a negative regulator of the immune system by forming a complex with its ligands (either PDL1 or PDL2), transmitting inhibitory signals, resulting in reduced proliferation of activated $CD8^+$ T cells.

PDL1 is expressed on haematopoietic, endothelial and epithelial cells in response to inflammation[4] and is expressed on a number of tumours, including ovarian, lung and renal cell carcinomas, where it is believed to play a major role in immune suppression within the tumour microenvironment. Specifically, PDL1 expression in tumour cells has been shown to suppress T cell activation and induce T cell apoptosis.[5] Data in clinical trials of PD1/PDL1 inhibitors in melanoma and NSCLC have suggested that the expression of PDL1 is associated with a higher response rate,[6] and recent clinical trials utilizing PD1/PDL1 antibodies in melanoma, NSCLC and renal cell carcinoma have shown safety and durable tumour regression.[7]

Early data from clinical trials on anti-PD1/PDL1 monoclonal antibodies in TNBC have shown promising results; preliminary results from clinical trials of pembrolizumab (an anti-PD1 monoclonal antibody) in a heavily pretreated TNBC cohort ($n = 27$) reported an overall response rate of 18.5%, including one complete response (3.7%).[8] The clinical trial on MPDL3280A (an anti-PDL1 monoclonal antibody) in PDL1 expressing metastatic TNBC ($n = 9$ evaluable) has reported a 33.3% response rate to therapy, with one patient showing a complete response.[9]

Only very limited data have been reported to date on the expression of PDL1 in breast cancer. Recent studies have identified PDL1 expression as both a poor prognostic[10] and a protective factor[11] in breast cancer. There has been only one study to date investigating PDL1 expression in a pure cohort of TNBC, although other studies have included TNBC patients in their cohorts.[10,12] Mittendorf *et al.*[12] reported membranous PDL1 expression in 19% of their TNBC patient cohort ($n = 105$), with no outcome data reported. The study also found that PDL1 expression was significantly higher in TNBC versus non-TNBC and that a greater number of intratumoral $CD8^+$ T cells were associated with PDL1-positive disease than PDL1-negative disease. Validation of these data in a larger, independent cohort with outcome data is essential.

Given the promising results of clinical trials of PD1/PDL1 antibodies in other cancer types and the recent preliminary results in TNBC, we investigated

the expression of PDL1 and quantified tumour-infiltrating lymphocytes (TILs) in a cohort of TNBC and determined their association with clinicopathological features and patient outcome.

Materials and methods

PATIENTS AND SPECIMENS

The study was approved by the Royal Prince Alfred Hospital Human Ethics Review Committee (X14-0241). This cohort was derived from a large database of more than 7000 patients maintained by the Strathfield Breast Centre, from which 190 TNBC patient samples for which blocks were available within our hospital network were selected initially for this study. Twenty-eight patients were excluded due to their tumour showing hormone and/or HER2 positivity upon central repeat testing, with a single case of metaplastic carcinoma (adenosquamous type) excluded from the cohort as an outlier. The size of the tumours was based on assessment by the reporting pathologist, as was grade.[13]

TISSUE MICROARRAYS AND IMMUNOHISTOCHEMISTRY

Tissue microarrays were utilized in the study (see Data S1) containing at least three cores taken from different areas of the tumour. TNBC status was confirmed by testing the samples for oestrogen receptor (ER), progesterone receptor (PR) (both <1% weak expression) and HER2 via immunohistochemistry [IHC = 0 or 1, 2+ cases were confirmed as negative by *HER2* fluorescence *in-situ* hybridization (FISH)]. Basal-like breast cancers (BLBCs) were defined further as triple-negative for ER, PR and HER2 as well as showing any expression (≥1%) of the surrogate IHC markers cytokeratin (CK) 5/6 (D5/16 B4 mouse monoclonal antibody; Dako, Glostrup, Denmark) and epidermal growth factor receptor (EGFR) (H11 mouse monoclonal antibody; Dako), as described by Cheang *et al.*[14] IHC was performed for PDL1 using the E1L3N, XP rabbit monoclonal antibody #13684 (Cell Signaling Technology, Danvers, MA, USA).

PATHOLOGICAL ASSESSMENT OF TUMOUR-INFILTRATING LYMPHOCYTES AND PDL1 EXPRESSION

TILs were assessed in the stromal compartment of the TMA sections using a semi-quantitative grading scheme as described by Schalper *et al.*[11] The quantity of TILs in a sample was determined by the percentage of TILs covering the area of stromal tissue, and was defined as 0, virtual absence; 1, mild infiltrate <30%; 2, moderate (30–60%); and 3, marked (>60%). PDL1 immunohistochemistry was evaluated in consensus viewing by two observers (R.B., S.O.'T.), one a specialist breast pathologist (S.O.'T.). The samples were scored as the percentage of tumour cells in samples expressing PDL1 and the intensity of the IHC signal intensity (0–3). Scoring was assessed in both the tumour and stromal compartments: tumoral membranous or cytoplasmic expression and stromal immune cell compartments. Similar to many other studies of other cancers,[15–17] tumours/patients were classified as PDL1-positive if there was ≥ 1% tumoral membranous or cytoplasmic PDL1 expression or ≥1% immune cells in the stromal compartment were positive (Figure 1). Because the most appropriate cut-off value for PDL1 expression in TNBC remains uncertain, analyses were also performed utilizing a ≥5% cut-point for tumour PDL1 positivity in each compartment, as this cut-off was utilized in the only other study of PDL1 in a cohort of TNBC (Mittendorf *et al.*[12]), as well as using the criteria of Muenst *et al.*[10] of a tumoral membranous or cytoplasmic H score of >100.

Results

PATIENT CHARACTERISTICS

A total of 161 surgically resected FFPE primary TNBCs sampled in tissue microarrays could be assessed for the markers, 81.4% of which were defined as basal-like using CK5/6 and EGFR immunohistochemistry.[14] There was a median follow-up of 55 months (range 0–213 months) and a median age at diagnosis of 57 years (range 28–89 years). The vast majority of tumours (90.6%) were grade III, while 40% of patients had lymph node metastasis and 31% of tumours had lymphovascular invasion (LVI). Fifty-two of the 161 (32.3%) patients had experienced an event: 15 local recurrences (9.3%), 24 distant metastases (14.9%), 29 breast cancer-specific deaths (18.0%) and 42 deaths in total (overall deaths) (26.0%), as shown in Table 1.

TILS IN TNBC

TILs were seen commonly in TNBC cases (Figure 1A–C), with mild TILs (score 1) seen in 45%, moderate TILs (score 2) in 21.3% and marked TILs (score 3) in 3.7%. A TIL score of ≥ 2 was associated with

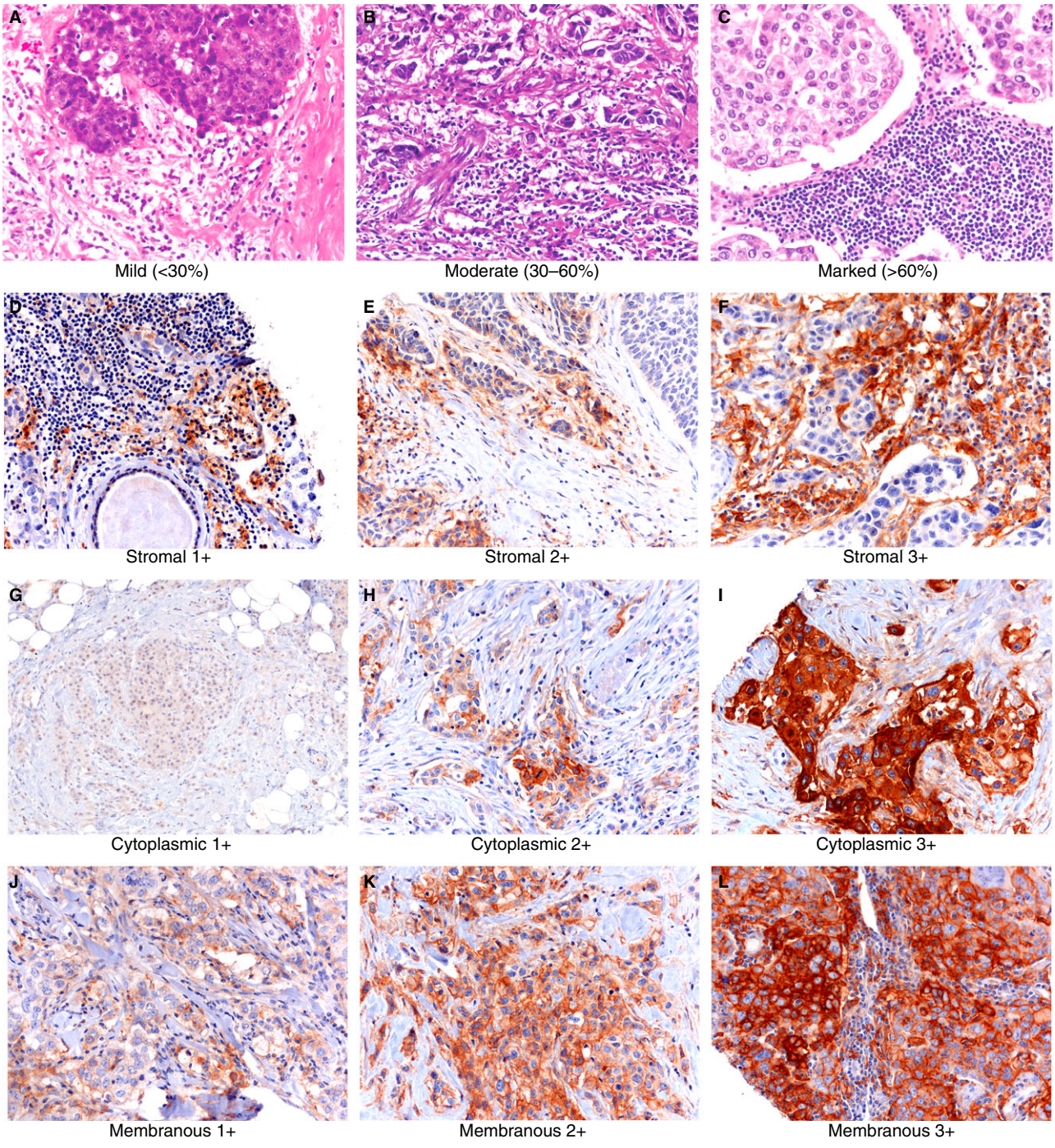

Figure 1. Representative microphotographs of sections from triple-negative breast cancer samples showing the different tumour infiltrating lymphocyte (TIL) categories and cellular PDL1 expression compartments. Haematoxylin and eosin-prepared samples showing mild (**A**), moderate (**B**) and marked (**C**) TILs. Programmed death ligand 1 (PDL1) protein 1+ (**D**), 2+ (**E**) and 3+ (**F**) expression in immune cells in the stromal cellular compartment. Tumoral cytoplasmic PDL1 protein 1+ (**G**), 2+ (**H**) and 3+ (**I**) expression. Tumoral membranous PDL1 protein 1+ (**J**), 2+ (**K**) and 3+ (**L**) expression.

improved patient survival (HR = 0.220, $P = 0.039$, Table 7) for breast cancer-specific death, and based on this was selected as the cut-point for further analysis. There were no significant differences in TILs between the cohort as a whole and the BLBC subgroup.

Table 1. Clinicopathological characteristics of the triple-negative breast cancer cohort

Patient characteristics	*n*	%
TNBC 'subtype'		
Basal-like	131	81.4%
Non-basal-like	30	18.6%
Tumour size (mm)		
0–20	65	40%
21–50	85	53%
>50	11	7%
Tumour grade		
II	16	10%
III	145	90%
Lymph node metastasis		
0	88	55%
1–3	47	29%
4–10	12	7%
>10	6	4%
Unknown	8	5%
Lymphovascular invasion (LVI)		
Positive	50	31%
Negative	100	62%
Unknown	11	7%
Patient outcome		
Local recurrences	15	9%
Distant metastasis	24	15%
Total deaths	42	26%
Breast cancer-specific deaths	29	18%

PDL1, Programmed death ligand 1; TNBC, triple-negative breast cancer.

PDL1 EXPRESSION

PDL1 expression (defined as ≥1% for the following results) was seen in the tumoral membrane in 64% of cases (Table 2; Figure 1J–L), as tumoral cytoplasmic staining in 80% (Table 2; Figure 1G–I) and as stromal immune cell expression in 93% (Table 2; Figure 1D–F). The levels of expression at ≥5% in each compartment are shown in Table 2. Low-level expression (1+) intensity was seen most commonly, followed by an intermediate (2+) and then strong (3+) intensity (Table 2). The intensity of expression of PDL1 in each compartment was non-significant in the assessment of association with survival. A TILs score of 2 or greater was associated with PDL1 expression in all compartments (Table 3).

Table 2. PDL1 expression in the membranous tumoral, cytoplasmic tumoral and stromal immune compartments of the cell

PDL1 expression	Membranous tumoral	Cytoplasmic tumoral	Stromal immune
Absent (0)	36%	22%	7%
Low (1 +)	41%	62%	50%
Intermediate (2 +)	14%	14%	40%
Strong (3 +)	9%	2%	3%
PDL1 expression ≥1%	64%	80%	93%
PDL1 expression ≥5%	60%	77%	93%

PDL1: Programmed death ligand 1.

Table 3. Relationship of PDL1 expression in each compartment by (non-parametric) Spearman's rank correlation

PDL1 expression	Cytoplasmic tumoral	Membranous tumoral	Stromal immune
Membranous tumoral	Rho 0.673	–	–
	$P < 0.0001$		
Stromal immune	Rho 0.476	Rho 0.618	–
	$P < 0.0001$	$P < 0.0001$	
TILs	Rho 0.416	Rho 0.598	Rho 0.579
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

PDL1, Programmed death ligand 1; TILs, tumour-infiltrating lymphocytes.

PDL1 expression, assessed as a continuous variable in each compartment (Table 4), showed that only stromal PDL1 was associated with histological grade. There were no other associations with important clinicopathological variables in each compartment.

STROMAL AND CYTOPLASMIC PDL1 EXPRESSION IS ASSOCIATED WITH A GOOD PROGNOSIS

Cytoplasmic and stromal PDL1 expression were both associated with a good outcome in this cohort. Cytoplasmic expression of PDL1 ≥5% was associated with improved patient survival for breast cancer-specific

deaths (Table 7; Figure 2A); however, no significant association between tumoral membranous PDL1 expression was seen for deaths from all causes or for breast cancer-specific death (Tables 5 and 7). PDL1 stromal expression fell short of significance for breast cancer-specific death at this cut-point (Table 7), but was associated significantly with death from all causes at ≥1% (Figure 2B, Table 5) and at ≥5% (Table 5). Tumoral cytoplasmic PDL1 expression ≥1% (Figure 2C, Table 5) and ≥5% (Table 5) was also associated with improved overall survival. There was no significant association between tumoral membranous PDL1 expression and outcome.

There were no significant differences in PDL1 expression between the cohort as a whole and the BLBC subgroup. Intensity of PDL1 expression did not associate with outcome in any compartment.

Upon univariate and multivariate analysis, only LVI was associated with death from all causes (Table 5) and on multivariate analysis only LVI was associated with overall deaths (Table 6). Univariate analysis showed that tumour size, LVI and a TIL score ≥2 were associated with breast cancer-specific death (Table 7). Upon multivariate analysis, a TIL score ≥2 and LVI were independent prognostic factors for breast cancer-specific survival in TNBC (Table 8).

There were no significant differences in these variables between the whole cohort and the BLBC subgroup.

Table 4. Relationship of PDL1 expression in each compartment as a continuous variable with dichotomized clinicopathological features assessed by Mann–Whitney (non-parametric) statistics

PDL1 expression	Cytoplasmic tumoral	Membranous tumoral	Stromal immune
Histological grade	$P = 0.60$	$P = 0.1093$	$P = 0.02$
Tumour size	$P = 0.7460$	$P = 0.2917$	$P = 0.8741$
LVI-positive	$P = 0.2487$	$P = 0.3408$	$P = 0.8521$
Lymph node-positive	$P = 0.7234$	$P = 0.3991$	$P = 0.5051$
BLBC subtype	$P = 0.4182$	$P = 0.643$	$P = 0.7484$

PDL1, Programmed death ligand 1; LVI, lymphovascular invasion; BLBC, basal-like breast cancers.

Discussion

Although several studies have investigated TILs and PDL1 expression in breast cancer, this is the largest to date in the poor-prognosis TNBC subgroup and highlights several important findings that may have implications for immune therapy for such patients. PDL1 expression, a putative biomarker of response to PD1 therapy, is expressed very commonly in TNBC, but to our knowledge this is the first large study ($n = 161$) focusing solely on TNBC to assess stromal immune cell expression of PDL1 and to report outcome in this subtype, highlighting the association of both cytoplasmic and stromal PDL1 with good outcome in this cohort. Upon multivariate analysis, only LVI was associated with deaths from all causes, while LVI and high TILs were independently prognostic for breast cancer-specific death.

The results of this study provide further evidence of a biologically significant relationship between TNBC and TILs. Recent findings from a breast cancer clinical trial have highlighted the predictive and prognostic value of TILs in breast cancer; Adams *et al.*[18] evaluated the density of intra-epithelial and stromal

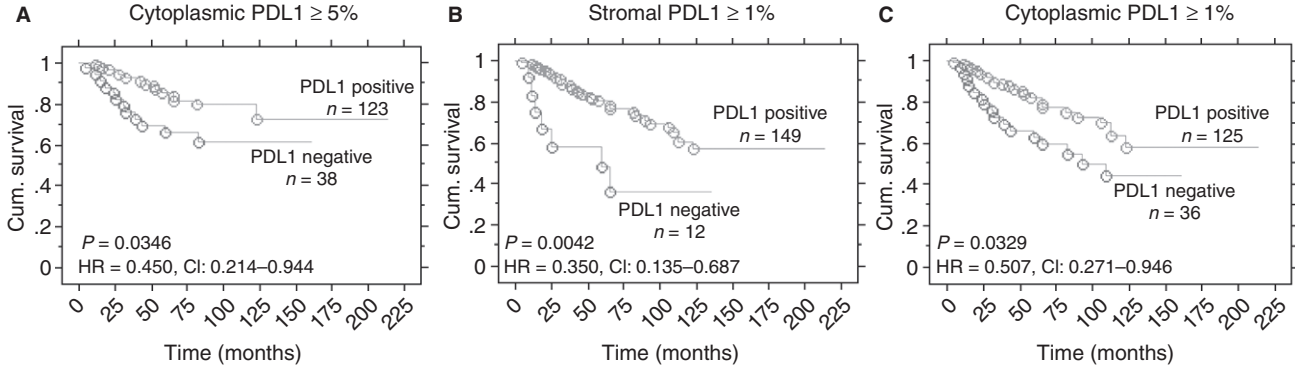

Figure 2. Programmed death ligand 1 (PDL1) protein expression location is associated with patient outcome in triple-negative breast cancer (TNBC). Kaplan–Meier analysis of PDL1 expression and survival: **A,** breast cancer-specific survival in TNBC patients expressing ≥ 5% cytoplasmic PDL1; **B,** overall survival in TNBC patients expressing ≥1% stromal PDL1; **C,** overall survival in TNBC patients expressing ≥ 1% cytoplasmic PDL1.

Table 5. Univariate survival by Cox proportional hazards univariate analysis for death from all causes

Variable	Hazard ratio (CI 95%)	*P*-value
Size >20 mm	1.918 (0.993–3.707)	0.0526
LVI	2.236 (1.210–4.133)	0.0102
Grade 3	0.787 (0.0.308–2.012)	0.6169
TIL ≥2	0.413 (0.162–1.053)	0.0639
Lymph node-positive	1.820 (0.955–3.467)	0.0687
BLBC	0.881 (0.405–1.914)	0.7485
Cytoplasmic PDL1 expression ≥1%	0.507 (0.271–0.946)	0.0329
Cytoplasmic PDL1 expression ≥5%	0.491 (0.264–0.911)	0.0240
Membranous PDL1 expression ≥1%	0.737 (0.401–1.351)	0.3233
Membranous PDL1 expression ≥5%	0.738 (0.404–1.355)	0.3268
Cytoplasmic or membranous expression H score >100	1.194 (0.425–3.352)	0.7366
Stromal PDL1 expression ≥1%	0.305 (0.135–0.687)	0.0042
Stromal PDL1 expression ≥5%	0.305 (0.135–0.687)	0.0042

PDL1, Programmed death ligand 1; TIL, tumour-infiltrating lymphocyte; LVI, lymphovascular invasion; BLBC, basal-like breast cancers; CI, confidence interval.

Table 6. First and final steps of multivariate analysis; revealing only lymphovascular invasion (LVI) was an independent prognostic factor for deaths from all causes

Variable	Hazard ratio (CI 95%)	*P*-value
Step 1 (clinicopathological features)		
Size >20 mm	1.364 (0.628–2.964)	0.1364
TIL score ≥2	0.484 (0.187–1.253)	0.1348
Stromal PDL1 expression ≥5%	0.492 (0.190–1.275)	0.1445
LVI	1.932 (0.949–3.935)	0.0694
Final step: resolved model		
LVI	2.236 (1.210–4.133)	0.01

PDL1, Programmed death ligand 1; TIL, tumour-infiltrating lymphocyte.

compartment TILs in 481 TNBC samples from Eastern Cooperative Oncology Group (ECOG) trials E2197 and E1199, reporting that higher stromal TIL scores were associated with better prognosis and reduction of risk of recurrence or death. Stromal TILs were also found to be an independent prognostic marker of disease-free survival, distant recurrence-free interval and overall survival. Loi *et al.*[19] commented on the consistency of the positive associations between TILs in primary TNBC and prognosis in three clinical trial

Table 7. Univariate analysis of important variables for breast cancer-specific death

Variable	Hazard ratio (CI 95%)	*P*-value
Size >20 mm	2.56 (1.09–6.01)	0.03
BLBC	0.87 (0.35–2.15)	0.77
TIL ≥2	0.220 (0.052–0.928)	0.039
LVI	2.88 (1.38–6.04)	0.005
Lymph node-positive	1.847 (0.848–4.024)	0.1224
Cytoplasmic PDL1 expression ≥1%	0.496 (0.23–1.05)	0.068
Cytoplasmic PDL1 expression ≥5%	0.450 (0.214–0.944)	0.0346
Cytoplasmic or membranous PDL1 H score >100	1.297 (0.391–4.296)	0.67
Stromal PDL1 ≥ 1%	0.394 (1.37–1.13)	0.08
Stromal PDL1 ≥ 5%	0.394 (1.37–1.13)	0.08

PDL1, Programmed death ligand 1; BLBC, basal-like breast cancers; TIL, tumour-infiltrating lymphocyte; LVI, lymphovascular invasion.

Table 8. First and final steps of multivariate analysis; revealing a TIL score ≥2 and lymphovascular invasion as independent prognostic factors for breast cancer-specific death

Variable	Hazard ratio (CI 95%)	*P*-value
Step 1 (clinicopathological features)		
Size >20 mm	1.558 (0.591–4.107)	0.3701
TIL score ≥2	0.250 (0.059–1.062)	0.0603
Stromal PDL1 expression ≥5%	0.444 (0.152–1.300)	0.1385
LVI	2.304 (0.992–5.351)	0.0522
Final step: resolved model		
Breast cancer-specific survival		
TIL score ≥2	0.223 (0.053–0.938)	0.0406
LVI	2.719 (1.298–5.698)	0.0080

TIL, Tumour-infiltrating lymphocyte; LVI, lymphovascular invasion; CI, confidence interval.

cohorts, concluding that immunity is important for the outcome of primary TNBC.

In the single published study on PDL1 in a cohort of pure TNBC, PDL1 was expressed frequently in TNBC (19%).[12] We speculate, however, that this lower percentage of positive cases may be due to differences in the antibody used (5H1) in their study. Our analysis, using the same cut-off for tumoral membranous PDL1 expression (≥5%) as Mittendorf *et al.*,[12] showed no significant association with outcome ($P = 0.3$). Of note, Mittendorf *et al.* did not provide information on outcome and PDL1 expression in their study. Gatalica *et al.*[20] reported in their cohort of different tumour types that strong, consistent membranous staining was uncommon in many cancer types; however, it was a feature of a few specific cancer types, including metaplastic breast cancer. That study also noted that PDL1 tumoral expression was highest in their 53 TNBC cases (59%) compared to other breast cancer subtypes.

The frequency of PDL1 expression is supported further in mixed breast cancer subtype studies, with 30% of small TNBC subsets expressing PDL1.[10] Higher tumour PDL1 expression is seen generally in receptor-negative breast cancer,[11,21] in keeping with the high levels of PDL1 expression in our TNBC cohort. A recent study also reported that a basal breast cancer cell line shows the highest PDL1 expression of six breast cancer cell lines (including receptor-positive disease).[22]

There have been conflicting results in the literature as to whether PDL1 expression is a favourable or adverse prognostic variable. Our results, showing a positive association with PDL1 expression and good outcome, are in keeping with a recent study (Schalper *et al.*[11]) which assessed mRNA, although other studies of mixed cohorts have shown associations of PDL1 with a poor outcome. It is likely that the differing methods of assessment, cut-offs, antibody clones used (note the published correspondence on this issue between Drs Rimm and Muenst on this issue[23,24]) and composition of cohorts (variable ER-positive/-negative and HER2 status) may account for these results. Intriguingly in our cohort, while PDL1 was associated with a good prognosis, there was a strong association with TILs, and only TILs and LVI were finally independently prognostic for breast cancer-specific death upon MVA. It has been speculated that while PDL1 mediates an immune evasion mechanism[25] and would be expected, therefore, to be associated with a poor prognosis, expression of PDL1 by tumour cells may be ineffective in suppressing the immune response and may merely reflect infiltration by lymphocytes, which are associated with a generally good outcome in a range of malignancies including breast cancer.[26] Others have also found that PDL1 is not associated invariably with a poor prognosis, with recent studies in NSCLC[27], melanoma[28] and colorectal cancer[29] showing that PDL1 expression is associated with a favourable prognosis. Several of these studies described a prominent immune infiltrate whereby TILs were shown to be associated with 98% of PDL1-positive tumours in melanoma,[28] and PDL1 was found to be associated significantly with TILs in NSCLC.[27] It is feasible that PDL1 expression may reflect an association with a TIL-mediated antitumour inflammatory response, rather than always being associated with tumour immune evasion.

Various studies have reported that PDL1 is a good predictive marker for response to PD1 blockade treatment.[6,25] However, a recent study by Tumeh *et al.*[30] reported that in melanoma $CD8^+$ T cell density was found to be the best predictive marker of response to PD1 blocking therapy, and the authors hypothesized that PDL1 may be a marker of adaptive immune resistance in response to TILs rather than a constitutive biomarker. This is supported by Taube *et al.*,[6] who suggested that tumour PDL1 expression is reflective of an active immune environment. PDL1 expression in breast cancer has also been linked to response to neoadjuvant chemotherapy; Wimberly *et al.*[31] report that TILs and PDL1 measured in the epithelium or stroma of presurgery breast cancer biopsies ($n = 94$) predicted a pathological completed response to neoadjuvant chemotherapy in breast cancer, adding to the predictive value of PDL1 in breast cancer.

While membranous expression of PDL1 is often used as the criterion for PDL1 positivity in cancers such as NSCLC,[32] renal cell carcinoma (RCC)[33] and melanoma,[28] we found no association between membranous PDL1 expression and outcome. Mittendorf *et al.*[12] assessed PDL1 expression in 105 TNBC and used the scoring criteria utilized in RCC (>5% cell membrane PDL1 expression) on their TNBC cohort, and reported a low frequency of PDL1-positive samples (19%). However, they did not assess tumoral cytoplasmic and stromal immune cell PDL1 expression and did not provide any outcome data for their cohort. Our findings do not support a biologically significant role for membranous PDL1 expression in TNBC; we do, however, recognize the limitation of utilizing a TMA approach to assess expression of a biomarker that may only be focally present in samples, raising the possibility of false negatives, which could possibly change the significance of PDL1 expression in TNBC.

Stromal immune cell expression of PDL1 has not been well documented in other studies in the literature. A handful of studies mention this finding in malignancies other than breast cancer, but do not describe the patterns, incidence or associations. Taube *et al.*[6] note that in their study on PDL1 in NSCLC, RCC, prostate cancer and melanoma that PDL1 was expressed on tumour cells and was also seen in the stroma. Stromal immune cell expression of PDL1 in breast cancers is a novel finding of our study.

The data represented in this study indicate that PDL1 expression, and TILs in particular, are biologically important in TNBC. Given the recent findings by Tumeh *et al.*[30] that TILs may be the primary predictor of response to immune checkpoint therapy, our data provide support for clinical trials of this therapy in TNBC given the high frequency of TILs and common expression of PDL1 in multiple tumour compartments. It remains to be seen whether TILs or PDL1 expression are effective biomarkers of PD1 blockade therapy response in TNBC, but our data highlight the need to assess PDL1 expression in all tumour compartments and also TILs in any such studies to further understand its significance.

Acknowledgements

Clinical Professor Sandra O'Toole received funding from the CINSW, the Sydney Breast Cancer Foundation and the NHMRC. Professor Richard Scolyer is supported by an Australian National Health and Medical Research Council fellowship. Funding support from the Australian National Health and Medical Research Council, Cancer Institute New South Wales and Melanoma Institute Australia is also gratefully acknowledged.

Conflicts of interest

There are no conflicts of interest to disclose.

References

1. Australian Institute of Health and Welfare (AIHW). *Breast Cancer in Australia: An Overview*. Cancer series no. 71. Cat. no. CAN 67. Canberra: AIHW, 2012.
2. Isakoff SJ. Triple-negative breast cancer: role of specific chemotherapy agents. *Cancer J.* 2010; **16**; 53–61.
3. Keir ME, Liang SC, Guleria I *et al.* Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 2006; **203**; 883–895.
4. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1 (PD-L1) pathway to activate anti-tumor immunity. *Curr. Opin. Immunol.* 2012; **24**; 207–212.
5. Blank C, Gajewski TF, Mackensen A. 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.* 2005; **54**; 307–314.
6. Taube JM, Klein AP, Brahmer JR *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin. Cancer Res.* 2014; **20**; 5064–5074.
7. Brahmer JR, Tykodi SS, Chow LQ *et al.* Safety and activity of anti-PD-l1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 2012; **366**; 2455–2465.
8. Nanda R, Chow LQ, Dees EC. Abstract s1-09: a phase IB study of pembrolizumab (MK-3475) in patients with advanced triple-negative breast cancer. 2014 San Antonio Breast Cancer Symposium (SABCS). San Antonio, Texas, 2014.
9. Emens LA, Braiteh FS, Cassier P. Abstract PD1-6: inhibition of PD-L1 by mpdl3280a leads to clinical activity in patients with metastatic triple-negative breast cancer. 2014 San Antonio Breast Cancer Symposium (SABCS). San Antonio, Texas, 2014.
10. Muenst S, Schaerli AR, Gao F *et al.* Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res. Treat.* 2014; **146**; 15–24.
11. Schalper KA, Velcheti V, Carvajal D *et al. In situ* tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. *Clin. Cancer Res.* 2014; **20**; 2773–2782.
12. Mittendorf EA, Philips AV, Meric-Bernstam F *et al.* PD-L1 expression in triple-negative breast cancer. *Cancer Immunol. Res.* 2014; **2**; 361–370.
13. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991; **19**; 403–410.
14. Cheang MCU, Voduc D, Bajdik C *et al.* Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin. Cancer Res.* 2008; **14**; 1368–1376.
15. Gadiot J, Hooijkaas AI, Kaiser AD, van Tinteren H, van Boven H, Blank C. Overall survival and PD-L1 expression in metastasized malignant melanoma. *Cancer* 2011; **117**; 2192–2201.
16. Hino R, Kabashima K, Kato Y *et al.* Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* 2010; **116**; 1757–1766.
17. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin. Cancer Res.* 2004; **10**; 5094–5100.
18. Adams S, Gray RJ, Demaria S *et al.* Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J. Clin. Oncol.* 2014; **32**; 2959–2967.
19. Loi S. Host antitumor immunity plays a role in the survival of patients with newly diagnosed triple-negative breast cancer. *J. Clin. Oncol.* 2014; **32**; 2935–2937.
20. Gatalica Z, Snyder C, Maney T *et al.* Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. *Cancer Epidemiol. Biomark. Prev.* 2014; **23**; 2965–2970.

21. Ghebeh H, Mohammed S, Al-Omair A *et al.* 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* 2006; **8**; 190–198.
22. Soliman H, Khalil F, Antonia S. PD-L1 expression is increased in a subset of basal type breast cancer cells. *PLoS ONE* 2014; **9**; e88557.
23. Rimm D, Schalper K, Pusztai L. Unvalidated antibodies and misleading results. *Breast Cancer Res. Treat.* 2014; **147**; 457–458.
24. Muenst S, Tzankov A, Gillanders WE, Soysal SD. Author's response to 'Letter to the editor: unvalidated antibodies and misleading results'. *Breast Cancer Res. Treat.* 2014; **147**; 459–462.
25. Topalian SL, Hodi FS, Brahmer JR *et al.* Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. *N. Engl. J. Med.* 2012; **366**; 2443–2454.
26. Loi S, Sirtaine N, Piette F *et al.* Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: Big 02-98. *J. Clin. Oncol.* 2013; **31**; 860–867.
27. Velcheti V, Schalper KA, Carvajal DE *et al.* Programmed death ligand-1 expression in non-small cell lung cancer. *Lab. Invest.* 2014; **94**; 107–116.
28. Taube JM, Anders RA, Young GD *et al.* Colocalization of inflammatory response with B7-H1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci. Transl. Med.* 2012; **4**; 127ra137.
29. Droeser RA, Hirt C, Viehl CT *et al.* Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. *Eur. J. Cancer* 2013; **49**; 2233–2242.
30. Tumeh PC, Harview CL, Yearley JH *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014; **515**; 568–571.
31. Wimberly H, Brown JR, Schalper KA *et al.* PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol. Res.* 2014; **3**; 326–332.
32. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Med. Oncol.* 2011; **28**; 682–688.
33. Thompson RH, Gillett MD, Cheville JC *et al.* Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc. Natl Acad. Sci. USA* 2004; **101**; 17174–17179.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods