PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes

H. R. Ali\textsuperscript{1,2}, S-E. Glont\textsuperscript{1}, F. M. Blows\textsuperscript{3}, E. Provenzano\textsuperscript{4,5}, S-J. Dawson\textsuperscript{1,3,5*}, B. Liu\textsuperscript{1}, L. Hiller\textsuperscript{6}, J. Dunn\textsuperscript{6}, C. J. Poole\textsuperscript{6}, S. Bowden\textsuperscript{7}, H. M. Earl\textsuperscript{3,5}, P. D. P. Pharoah\textsuperscript{3,8}, C. Caldas\textsuperscript{1,3,5}

Affiliations
1. Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge, UK
2. Department of Pathology, University of Cambridge, Cambridge, UK
3. Department of Oncology, University of Cambridge, Addenbrooke’s Hospital, Cambridge, UK
4. Department of Histopathology, Addenbrooke’s Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
5. Cambridge Experimental Cancer Medicine Centre and NIHR Cambridge Biomedical Research Centre, Cambridge, UK
6. Warwick Clinical Trials Unit, University of Warwick, Coventry, UK
7. Cancer Research UK Clinical Trials Unit, Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham, UK
8. Strangeways Research Laboratory, University of Cambridge, Cambridge, UK

*Current affiliation:
Division of Cancer Medicine, Division of Research, Peter MacCallum Cancer Centre, Melbourne, Australia.

Corresponding author:
Dr H R Ali
CRUK Cambridge Institute
University of Cambridge
Robinson Way
Cambridge
CB2 0RE
raza.ali@cruk.cam.ac.uk
Tel: +44 1223 769650
Key Message

PD-L1 protein expression has been shown to predict response to PD-L1 inhibition in solid tumours. In this analysis of breast tumours from three studies, we detected PD-L1 protein in tumour and immune cells to reliably estimate what proportion of patients might benefit from PD-L1 inhibition. Expression of the protein was rare overall but it was expressed in around one fifth of basal-like tumours.
Abstract

Background
Expression of PD-L1 in solid tumours has been shown to predict whether patients are likely to respond to anti-PD-L1 therapies. To estimate the therapeutic potential of PD-L1 inhibition in breast cancer we evaluated the prevalence and significance of PD-L1 protein expression in a large collection of breast tumours.

Patients and methods
Correlations between CD274 (PD-L1) copy-number, transcript and protein levels were evaluated in tumours from 418 patients recruited to the METABRIC genomic study. Immunohistochemistry was used to detect PD-L1 protein in breast tumours in tissue microarrays from 5763 patients recruited to the SEARCH population-based study (N=4079) and the NEAT randomised controlled trial (N=1684).

Results
PD-L1 protein data was available for 3916 of the possible 5763 tumours from the SEARCH and NEAT studies. PD-L1 expression by immune cells was observed in 6% (235/3916) of tumours and expression by tumour cells was observed in just 1.7% (66/3916). PD-L1 was most frequently expressed in basal-like tumours. This was observed both where tumours were subtyped by combined copy-number and expression profiling (39% (17/44) of IntClust 10 i.e. basal-like tumours were PD-L1 immune cell positive; P<0.001) and where a surrogate IHC-based classifier was used (19% (56/302) of basal-like tumours were PD-L1 immune cell positive; P <0.001). Moreover, CD274 (PD-L1) amplification was observed in five tumours of which four were IntClust 10. Expression of PD-L1 by either tumour cells or infiltrating immune cells was positively correlated with infiltration by both cytotoxic and regulatory T-cells (P<0.001). There was a nominally significant association between PD-L1 and improved disease-specific survival (hazard ratio 0.53, 95% CI 0.26–1.07; P=0.08) in ER-negative disease.

Conclusions
Expression of PD-L1 is rare in breast cancer, markedly enriched in basal-like tumours and is correlated with infiltrating lymphocytes. PD-L1 inhibition may benefit the 19% of patients with basal-like tumours in which the protein is expressed.

NEAT ClinicalTrials.gov: NCT00003577

Key words: breast cancer, pd-l1, cd274, immune checkpoint, lymphocytes, basal-like
Introduction

The potential of enlisting the immune system as an endogenous tumouricidal therapy has been realised in recent clinical trials of inhibitors of immune checkpoint proteins such as cytotoxic T-lymphocyte associated protein 4 (CTLA4)[1], programmed cell death 1 (PD-1)[2] and programmed death ligand 1 (PD-L1)[3, 4]. PD-L1 binds PD-1[5, 6] and CD80[7] to reduce the cellular immune response by inducing T-cell tolerance which, amongst other roles, helps prevent autoimmunity in certain organs and is thought to act as a mechanism of immune evasion by tumours[8]. Therefore inhibition of PD-L1 will, in a sense, ‘release the brakes’ on the immune response enabling an unfettered T-cell mediated attack. In solid tumours PD-L1 has been found to be expressed by both tumour cells and infiltrating immune cells[4, 9] and its inhibition has been found to result in an enduring clinical response in a variety of solid tumours including melanoma[1], renal cell carcinoma[3], lung carcinoma[3] and, most recently, bladder carcinoma[4]. Breast cancer has not generally been thought of as an immunogenic malignancy and, unlike bladder cancer, no therapy designed to enhance the anti-tumour immune response is currently used. However, breast cancer is an exceptionally heterogeneous disease and it has become apparent that a subset of tumours is subject to an effective immune response. In a large scale analysis of four studies we found that the presence of infiltrating cytotoxic T-cells was associated with improved outcome but that this effect was limited to ER-negative and HER2-positive disease[10].

Since clinical trials have found that expression of PD-L1 on both tumour and immune cells predicts whether a patient is likely to respond to its inhibition[9], we used large clinical studies to investigate the prevalence of PD-L1 expression in breast cancer in order to estimate the proportion of the population that may benefit from these therapies.
Methods

Patient population
Primary tumour samples from three clinical studies were used for this analysis: the SEARCH observational study[11], the NEAT randomised controlled trial[12] and the METABRIC genomic study[13, 14]. Patient characteristics are summarised in Supplementary Table 1. Details of clinical studies are provided in Supplementary Methods.

Molecular subtyping
Tumour molecular subtype was assigned using both combined copy-number and expression profiling for tumours from the METABRIC study[13, 15] and a surrogate immunohistochemical classifier for the SEARCH and NEAT studies[16] which is summarised in Supplementary Table 2. Genomic instability and TP53 status were determined as previously described[13, 17, 18]. Further details are provided in the Supplementary Methods.

PD-L1 assay validation and scoring
A rabbit monoclonal antibody raised against residues near the carboxy terminus of PD-L1 (Cell Signalling Technology, catalogue #13684) was investigated for use in immunohistochemistry (IHC). Two breast cancer cell lines (MDA-MB-231 and MCF7) were used to assess the specificity of the antibody by western blot analysis. To modulate PD-L1 expression, cell lines were treated with interferon gamma and knockdown experiments were conducted using siRNA directed against human CD274 (PD-L1). Specificity of the immunohistochemical assay was tested using formalin fixed paraffin embedded pellets of the cell lines with concurrent quantitative western blot analysis of fresh cell lysates for comparison. Detailed experimental procedures are described in Supplementary Methods.

Tumour samples were represented by a single 0.6 mm core in tissue microarrays. Stained slides were scanned using an Aperio Scanscope AT2 digital slide scanner. PD-L1 was scored as reported in recent trials[9] was used where tumour and immune cells were attributed separate scores on a four-point scale as follows: 0 = <1% positive cells, 1 = 1-5% positive cells, 2 = 5-10% positive cells and 3 = >10% positive cells.

Statistical analyses
Correlations between continuous and ordinal variables were assessed using Spearman’s rank correlation coefficient. Associations between categorical variables were assessed using Pearson’s chi-squared test. The Kruskal-Wallis rank test was used to investigate the relationship between CD274 (PD-L1) copy number status and gene expression. The relationship between expression of PD-L1 and
disease specific survival was investigated using a Cox proportional-hazards regression model stratified by study. Since expression of ER violates the proportional-hazards assumption[16], analyses were conducted separately by ER-status. To investigate whether expression of PD-L1 modified the association between infiltrating lymphocytes and outcome previously reported, an interaction term was included in survival models. Similarly, whether expression of PD-L1 could predict differential benefit from the addition of epirubicin to CMF was assessed using an interaction term in survival models. Estimates of absolute five and ten year survival were calculated using the Kaplan-Meier method. All analyses were conducted in Intercooled Stata version 11.2 (Stata Corp, College Station, TX, USA). Further details are provided in Supplementary Methods.
Results

PD-L1 assay validation

Western blot analysis using a rabbit monoclonal antibody detected a protein of 50 kDa, approximately the size of glycosylated PD-L1[19], in lysates of the MDA-MB-231 breast cancer cell line while no protein was detected in lysates of MCF7 (Supplementary Figure 1). Treatment with interferon gamma increased the amount of protein detected in lysates of MDA-MB-231 and a band of similar atomic mass became detectable in lysates of MCF7 (Supplementary Figure 1). Immunohistochemistry was applied to formalin fixed paraffin embedded (FFPE) cell pellets of MDA-MB-231 cells revealing strong circumferential membranous staining. Antibody specificity was confirmed by knockdown using siRNA against PD-L1, resulting in a more than 70% reduction in the amount of protein detected by quantitative western blot analysis and a proportionate reduction in the signal observed by IHC (Supplementary Figure 2A-D). Immunohistochemistry of human tonsil revealed weak membranous staining of macrophages and staining of human placenta showed strong expression by syncytiotrophoblasts (Supplementary Figure 2E) consistent with previous observations[19]. Collectively these analyses confirmed the specificity of the immunohistochemical assay for PD-L1.

CD274 (PD-L1) amplification occurs in a subset of almost exclusively basal-like tumours

We used the METABRIC dataset comprising 1,980 tumours to investigate the relationship between CD274 (PD-L1) copy-number status, gene and protein expression and molecular subtype (Figure 1). CD274 (PD-L1) gene expression was found to be positively associated with copy number status ($P=0.0001$). The distribution of the ten integrative clusters of breast cancer (IntClust)[13, 14] also significantly differed according to CD274 (PD-L1) copy number status ($P<0.001$). Of the ten clusters, IntClust 10, which approximately equates to basal-like tumours, was significantly enriched amongst tumours with either a copy number gain or amplification of CD274 (PD-L1). Only five tumours showed amplification and, of these, four belonged to IntClust 10 while of 65 tumours with copy number gain of CD274 (PD-L1), 37 (57%) were IntClust 10. Immunohistochemistry for PD-L1 protein was conducted on full face sections of three IntClust 10 tumours with CD274 (PD-L1) amplification, and strong expression was observed in tumour cells. However, the distribution of expression differed between these cases with only one showing diffuse expression in a large proportion of the tumour, whereas the others showed focal PD-L1 expression largely limited to the tumour-stroma interface (Figure 1). Immunohistochemistry for PD-L1 was also conducted on a subset of 418 cases represented in TMAs from the METABRIC study. PD-L1 protein expression was modestly correlated with CD274 (PD-L1) mRNA expression. The Spearman’s rank correlation between tumour PD-L1 expression and gene expression was 0.17 ($P=0.0005$) and 0.15 ($P=0.002$) between PD-L1 immune cell expression and gene expression in bulk tumour samples (Supplementary Figure 3). Supplementary Figure 4 depicts box-plots illustrating that both tumour and immune cell
PD-L1 expression was significantly associated with a greater number of genomic breakpoints (PD-L1+ immune cells $P=0.003$; PD-L1+ tumour cells $P=0.004$) but not with a genome instability index. Supplementary Figure 5 depicts cross-tabulations of PD-L1 expression versus TP53 mutation status, separately for ER-positive and ER-negative disease. PD-L1 expression by either tumour or immune cells was not significantly associated with mutation of TP53.

**PD-L1 protein is rarely expressed in breast cancer**

Data on expression of PD-L1 was successfully generated in 3916 tumours from the SEARCH ($N=2453$) and NEAT ($N=1463$) studies (Supplementary Figure 6); data was not generated on the remaining 1847 owing to technical reasons such as core dropout or lack of adequate representation of tumour cells. Expression in >1% of immune cells was observed in 235 (6%) tumours while just 66 (1.7%) tumours expressed PD-L1 in >1% of tumour cells (Supplementary Figure 6). Expression of PD-L1 in tumour and/or immune cells was observed in 245 tumours.

**PD-L1 expression is associated with infiltrating lymphocytes and basal-like tumours**

Expression of PD-L1 showed a significant positive correlation with levels of infiltrating intratumoural CD8+ and FOXP3+ lymphocytes (Spearman’s rank correlation = 0.3; $P<0.0001$ for both comparisons). Supplementary Figure 7 depicts the distribution of infiltrating intratumoral or stromal lymphocytes by level of PD-L1 expression. Of the 418 cases from the METABRIC study for which both PD-L1 protein data and genomic subtyping data were available, 50 showed expression in >1% of infiltrating immune cells and 17 of these belonged to IntClust 10, while only 15 showed expression in >1% of tumour cells and 9 of these belonged to IntClust 10. Thirty nine percent (17/44) of IntClust 10 cases showed PD-L1 expression in >1% of immune cells (Supplementary Figure 8). The distribution of IHC-defined molecular subtypes also differed significantly according to the level of PD-L1 expression ($P<0.00001$; Figure 2). The proportion of tumours which belonged to the basal-like (CBP, i.e. triple negative tumours with expression of CK56 and/or EGFR) subtype steadily increased with the proportion of PD-L1 positive immune cells where the proportion of basal-like tumours at each level of <1%, 1-5%, 5-10% and >10% PD-L1 positive immune cells was 8% (246/2899), 20% (9/44), 33% (19/58) and 47% (28/59); collectively amounting to 19% (56/302) of basal-like tumours with PD-L1 protein expression in >1% of immune cells. In addition when limited to basal-like tumours, expression of PD-L1 was not significantly associated with grade, the number of positive lymph nodes, tumour size or patient age.

**Association with disease specific survival**

Expression of PD-L1, by either tumour cells or immune cells, was not significantly associated with outcome in either ER-positive or ER-negative breast cancer (Supplementary Tables 3 and 4) irrespective of whether the variable was modelled as categorical, continuous or dichotomous. However, in ER-negative disease, a subgroup in which immune infiltration has previously been found
to be associated with outcome [10], PD-L1 expression in >10% of immune cells was associated with reduced disease specific mortality although this was nominally statistically significant (HR 0.53, 95% CI 0.26–1.07; P=0.08). There was no significant interaction between the prognostic effect of cytotoxic or regulatory T-cells and PD-L1 expression nor was any significant interaction between randomisation arm in the NEAT trial and PD-L1 (data not shown). Supplementary Table 5 details estimates of absolute survival at five and ten years for patient subgroups defined by PD-L1 expression, the presence of intra-tumoural CD8+ (iT-CD8) lymphocytes and ER-status. These estimates require cautious interpretation owing to the rarity of PD-L1 expression.
Discussion

We pooled large clinical studies to investigate the prevalence and significance of PD-L1 protein expression in breast cancer using a validated assay. Expression of PD-L1 by immune cells was observed in 6% of tumours, while expression by tumour cells occurred in just 1.7%. These frequencies significantly differed by tumour subtype. Basal-like tumours (CBP, i.e. triple negative tumours with expression of CK56 and/or EGFR) were characteristically enriched in PD-L1 expression with 19% containing PD-L1 positive immune cells. Although expression of PD-L1 was significantly correlated with infiltrating lymphocytes, a large proportion of tumours which contained lymphocytic infiltration did not contain PD-L1 positive cells. There was, however, no interaction between the prognostic effect of infiltrating lymphocytes and expression of PD-L1. There was a nominal association between PD-L1 expression and longer disease specific survival in ER-negative disease.

This study is the first large scale analysis of PD-L1 protein expression in breast cancer. Previous studies have included many fewer patients[20] and their analytic validity has been questioned[21, 22]. We used a validated assay and two large clinical studies including a population-based cohort in order to derive reliable estimates of the probable prevalence of PD-L1 expression in the breast cancer population. We report lower frequencies of PD-L1 expression than previous studies. Two prior studies evaluated PD-L1 at the level of gene expression using RNA fluorescence in situ (FISH) in one[23], and pooled microarray data in the other[24]. Based on RNA FISH, Schalper et al. report that up to 60% of breast tumours show CD274 (PD-L1) expression[23] whereas Sabatier et al. report that CD274 (PD-L1) is upregulated in 20% of tumours based on microarray data[24]. These estimates themselves differ enormously and both are far greater than the frequencies observed here. This disparity may be due to the poor correlation between PD-L1 protein and RNA, as well as differences between study populations.

Although we find that PD-L1 expression is rare overall and though expressed in around one-fifth of basal-like tumours, clinical benefit from inhibition of PD-L1 may extend beyond this population. Recent studies have shown, based both in mouse models[25] and human tumour samples[26, 27], that mutational patterns can influence the efficacy of immune checkpoint inhibitors. Most notable amongst these characteristics are mutations which result in genetic alterations predicted to be immunogenic to the individual’s immune system by encoding neo-antigens. Identifying the best predictor of immune checkpoint inhibition whether it’s genomic, tissue based or a combination, will necessitate a head-to-head comparison in representative clinical cohorts of sufficient size.

We did not find a convincing association between PD-L1 expression by immune cells and clinical outcome. There was, however, a nominally significant association between high levels (>10%) of
immune cell PD-L1 expression and improved survival (P=0.08). Given that both Schalper et al. and Sabatier et al. report a significant association between high $CD274$ (PD-L1) gene expression and improved survival[23, 24], it is plausible that we did not robustly detect this effect owing, for example, to a different threshold for ‘high’ PD-L1 expression or due to insufficient power. This difference may be addressed by pooling data from additional studies and using a quantitative system for scoring PD-L1 expression. However, our findings are supportive of the association between improved survival and PD-L1 expression previously reported.

The main limitation of this study was the use of TMAs for representation of tumours. In some tumours immune infiltration may be heterogeneous and this heterogeneity will not be captured by TMAs. However, the reduced power associated with this sampling error was here attenuated by a large sample size. Indeed, TMAs enable the conduct of large scale pathology studies and in this way ultimately lead to more reliable conclusions. A second limitation is that we have not assessed PD-L1 protein expression in the metastatic setting in which trials of PD-L1 inhibitors have so far been conducted.

We find that PD-L1 protein expression is rare in primary breast cancer overall but that around one fifth of basal-like tumours contain infiltrating immune cells that express it. Basal-like breast cancer is again implicated as subject to the effects of the PD-L1 axis by the observation that amplification of $CD274$ (PD-L1), though rare, occurs almost exclusively in basal-like tumours. This implies that within the microenvironment of these tumours expression of PD-L1 confers a survival advantage. Moreover, amplification of $CD274$ (PD-L1) has been observed in the setting of EBV-positive gastric cancer[28] and in samples of Hodgkin’s lymphoma from patients who have had a clinical response to PD-1 inhibition[29]. Taken together, these observations have important implications for the potential of inhibitors of PD-L1 in breast cancer, particularly for the design and analysis of clinical trials. Our observations suggest that PD-L1 inhibitors may benefit a small subset of women with breast cancer with tumours that express PD-L1, most of which will prove to be basal-like. Previous observations, both in cell-lines and in tumour samples are concordant with this conclusion[30].

**Conclusions**

To our knowledge we have conducted the first large scale analysis of PD-L1 protein expression in breast cancer. We find that PD-L1 expression, both by immune and tumour cells is rare, associated with infiltrating lymphocytes and significantly enriched in basal-like tumours. Our findings together with those previously published[23, 24] suggest that high PD-L1 expression is associated with improved survival. These findings imply that clinical trials of PD-L1 inhibitors in breast cancer ought
to be enriched for patients with basal-like breast cancer and, given the rarity of expression, may need to recruit across a large number of centres.
Acknowledgements
We thank the participants of the SEARCH, NEAT and METABRIC studies who have permitted use of their tissue for research and the many individuals who have made this work possible. We thank Oscar M. Rueda for advice regarding bioinformatic analyses.

Funding
This work was supported by Cancer Research UK (C490/A10119 and C490/A10124), the Cambridge Experimental Cancer Medicine Centre and the NIHR Cambridge Biomedical Research Centre. HRA is an NIHR Academic Clinical Lecturer and supported by a Career Development Fellowship from the Pathological Society of Great Britain and Northern Ireland and a Starter Grant for Clinical Lecturers from the Academy of Medical Sciences, UK.

Disclosure
The authors have declared no conflicts of interest.
References

Figure Legends

Figure 1
Distribution of PD-L1 (CD274) gene expression and molecular subtypes of breast cancer by PD-L1 (CD274) copy number status based on aCGH in the METABRIC study. Values within coloured boxes are the absolute number of cases. Right panel depicts immunohistochemistry applied to full-face sections from three IntClust 10 tumours shown to harbour PD-L1 amplification by aCGH.

Figure 2
Distribution of molecular subtypes of breast cancer by PD-L1 protein expression level in pooled cases from the SEARCH and NEAT studies. Values within coloured boxes are the absolute number of cases. Abbreviations: HER2 – human epidermal growth factor receptor 2; CBP – core-basal phenotype; 5NP – five marker negative phenotype; TNBC – triple negative breast cancer.
Supplementary Material

Supplementary Methods
Study populations, statistical analyses and experimental procedures for cell culture, CD274 knockdown and immunohistochemistry.

Supplementary Tables

Supplementary Table 1
Summary of clinical characteristics of SEARCH and NEAT studies.

Supplementary Table 2
Scheme for molecular subtyping of tumours within the SEARCH and NEAT studies.

Supplementary Table 3
Univariate Cox-regression of PD-L1+ immune cells in ER-positive breast cancer for disease-specific survival, stratified by study.

Supplementary Table 4
Univariate Cox-regression of PD-L1+ immune cells in ER-negative breast cancer for disease-specific survival, stratified by study.

Supplementary Table 5
Survival estimates at five and ten years for subgroups defined by PD-L1 and iT-CD8 status, separately for ER-positive and ER-negative disease.

Supplementary Figures

Supplementary Figure 1
Top panel: Formalin-fixed paraffin-embedded cell pellets of breast cancer cell lines (MDA-MB-231 and MCF7) variably treated with interferon gamma as indicated with PD-L1 expression detected by immunohistochemistry.

Supplementary Figure 2
A. Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 untreated and treated with interferon gamma.
B. Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 untreated and treated with interferon gamma.
C. Western blot analysis for PD-L1 protein using lysates from MDA-MB-231 following treated with control (scramble) and PD-L1 targeting siRNA.
D. Immunohistochemistry for PD-L1 on formalin-fixed paraffin-embedded cell pellets of MDA-MB-231 treated with control (scramble) and PD-L1 targeting siRNA.
E. Immunohistochemistry for PD-L1 applied to human tonsil (left) revealing weak membranous expression by macrophages and on human placenta (right) revealing strong expression by syncytiotrophoblasts.

Bottom panel: Concurrent western blot analysis for PD-L1 protein.

**Supplementary Figure 3**
Box-plots depicting the correlation between PD-L1 protein expression and PD-L1 mRNA expression in tumour cells and immune cells.

**Supplementary Figure 4**
Box-plots depicting the distribution of the number of genomic breakpoints (top) and of a genomic instability index (bottom) by the proportion of immune or tumour cells expressing PD-L1 protein by immunohistochemistry.

**Supplementary Figure 5**
Cross-tabulations of TP53 mutation status versus the proportion of immune or tumour cells expressing PD-L1, separately for ER-positive and ER-negative tumours. Boxes are shaded with an intensity in proportion to the depicted value.

**Supplementary Figure 6**
Distribution of scores for PD-L1 expression by immunohistochemistry on immune and tumour cells in the SEARCH and NEAT studies.

**Supplementary Figure 7**
Box-plots depicting the distribution of CD8+ and FOXP3+ lymphocytes by PD-L1 protein expression in tumour and immune cells in the cases pooled from the SEARCH and NEAT studies. Abbreviations: iT – intratumoural; S – stromal.

**Supplementary Figure 8**
Distribution of Integrative Clusters of breast cancer by PD-L1 protein expression in immune and tumour cells. Values within coloured boxes are the absolute number of cases.