Breast Cancer Res Treat (2014) 147:459–462
DOI 10.1007/s10549-014-3064-x

REBUTTAL LETTER

Author's response to "Letter to the editor: unvalidated antibodies and misleading results"

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Published online: 1 August 2014

To the Editor,

Rimm et al. challenge our study demonstrating an association between PD-L1 expression and poor prognosis in human breast cancer [1] raising a concern about the antibody we used for immunohistochemistry.

Rimm et al. refer to ab58810 (Abcam) as an unvalidated antibody. We share Rimm et al.'s concern about the use of unvalidated antibodies in immunohistochemistry studies, and we performed validation studies of the ab58810 antibody prior to using it in our study. Specifically, we screened several anti-PD-L1 antibodies for tissue specificity using human term placenta and lymphoid tissue, as mentioned in our paper [1]. Human term placenta is commonly used for the validation of anti-PD-L1 antibodies and was used by Rimm et al. for validation of anti-PD-L1 antibodies in their manuscript on PD-L1 expression in non-small cell lung cancer (NSCLC) [2]. We tested several antigen retrieval and staining protocols until we were satisfied with the specificity of tissue staining. With an optimized protocol using ab58810, we observed strong staining of the trophoblast layer in human term placenta but the absence of staining in the stromal cells and vessels of the chorionic villi, as can be appreciated in Figs. 1–3. In tonsils, strong granular cytoplasmic staining of PD-L1 was observed in follicular dendritic cells (Fig. 4). It is important to recognize the fact that Rimm et al. report a lack of tissue specificity with ab58810 in their study [2] does not preclude the possibility that a positive result can be obtained with a different staining protocol. The ab58810 antibody also recognizes the PD-L1 antigen in Western blot analysis, as demonstrated on the Abcam website (http://www.abcam.com/cd274-antibody-ab58810.html).

Rimm et al. also comment on nuclear staining observed in some breast cancer cells in Fig. 1 of our manuscript [1]. Of note, the majority of breast cancer cells in Fig. 1 clearly show cytoplasmic staining. We have attached additional images from our breast cancer tissue micro array (TMA), confirming that the staining was predominantly cytoplasmic (Figs. 5–7). We acknowledge that staining is observed in some apoptotic nuclei, but this nuclear staining was not considered for the evaluation of PD-L1 expression. As stated in the Material & Methods section of our paper [1], only cytoplasmic and membranous staining was considered specific.

Of note, we have previously evaluated our TMA for the presence of PD-1^+ tumor-infiltrating lymphocytes (TIL) [3]. We observed a statistically significant association between PD-L1 expression by tumor cells and the presence of PD-1^+ TIL in the same tumors ($p < 0.001$). This observation provides additional evidence to support the integrity of immunohistochemical staining in our manuscript.

In preparation of this response, we stained our breast cancer TMA with an additional, newly developed anti-PD-L1 antibody (clone E1L3 N, Cell Signaling), which has been validated by Rimm et al. according to their recent ASCO

This rebuttal letter refers to the article availbale at doi:10.1007/s10549-014-3061-0.

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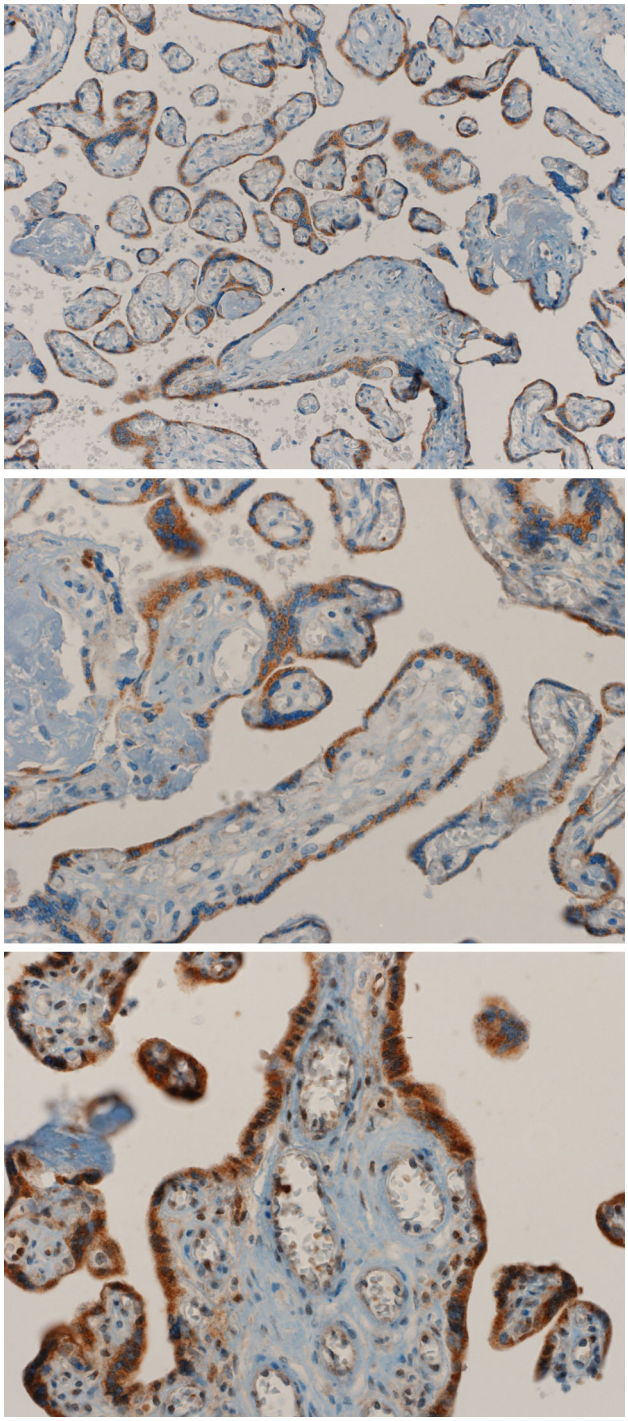


Figs. 1–3 Human placental tissue stained with the anti-PD-L1 antibody clone ab58810, demonstrating intense staining of the trophoblast layer and the absence of staining in the stromal and vascular regions

poster (*Domain-specific PD-L1 protein measurement in non-small cell lung cancer;* http://meetinglibrary.asco.org/content/132010-144). With this clone, we observed a strong association between PD-L1 expression and poor tumor differentiation (G3), estrogen receptor (ER) negativity and

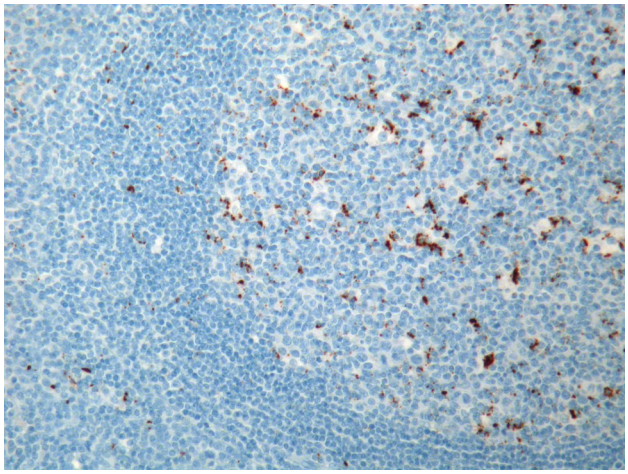
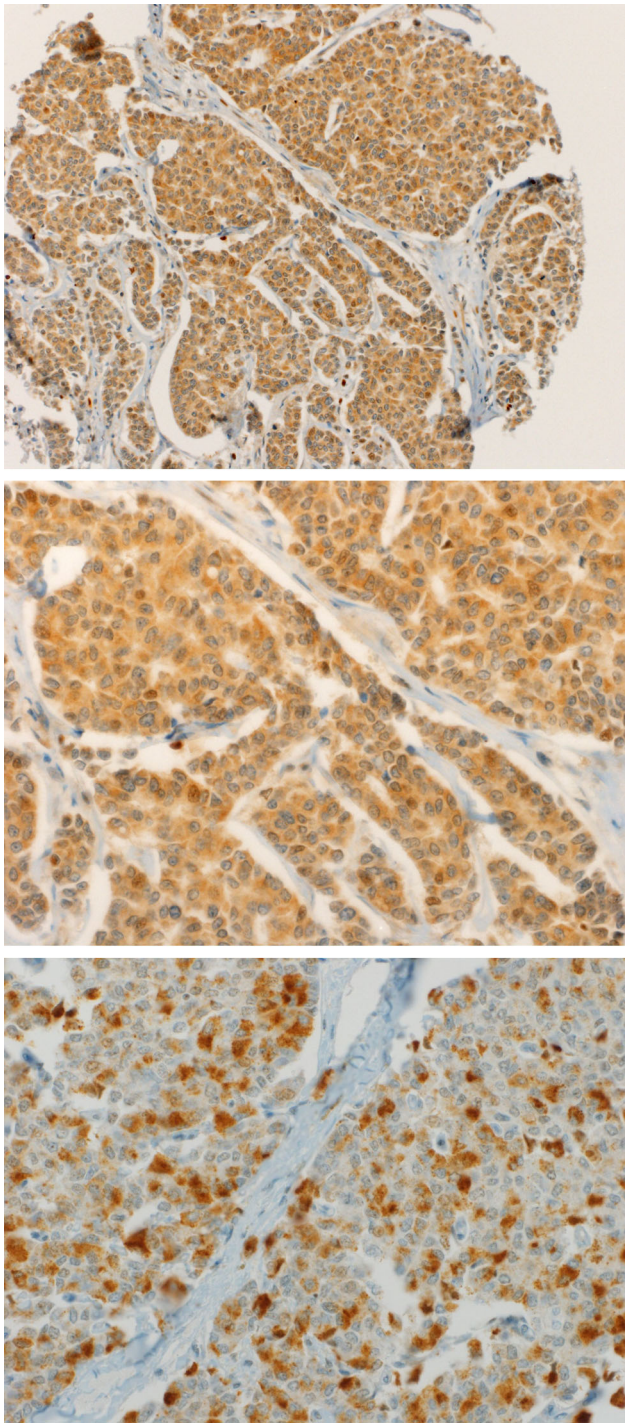


Fig. 4 Human tonsil stained with the anti-PD-L1 antibody clone ab58810, demonstrating strong granular cytoplasmic staining of the follicular dendritic cells and the absence of staining in the surrounding lymphocytes

worse survival. These findings further support the notion that PD-L1 protein expression is associated with poor prognosis in breast cancer.

We acknowledge that our results are not consistent with Schalper's et al. findings that PD-L1 mRNA expression is associated with better prognosis in breast cancer [4]. However, our study is consistent with the results of Ghebeh et al. who reported an association in breast cancer between PD-L1 expression and higher tumor grade, HER2 expression, absence of ER expression [5], and high Ki-67 expression [6], which are all known negative prognostic factors. Furthermore, there are numerous reports in the literature that expression of PD-L1 is associated with poor prognosis in melanoma [7], NSCLC [8], renal cancer [9], ovarian cancer [10], esophageal cancer [11], pancreatic cancer [12], and gastric cancer [13]. Importantly, the study about renal cancer was performed utilizing the PD-L1 clone M5H1, which Rimm et al. claim to be the only validated antibody [2], and the study on esophageal cancer was performed analyzing *PD-L1* gene expression [11].

There are important differences between our study and that of Schalper et al. [4] that may explain the disparate results. First, our study evaluated PD-L1 protein expression by immunohistochemistry, while Schalper et al. evaluated PD-L1 mRNA expression. Although PD-L1 mRNA expression appears to be associated with PD-L1 protein expression, additional studies will need to be performed to confirm this observation. Second, there is a significant difference in the percentage of patients who are positive for PD-L1 protein expression (23.1 % in our study) vs. PD-L1 mRNA expression (55.7, and 59.5 % in Schalper et al.). The significantly different thresholds for considering tumors positive for PD-L1 expression could substantially



Figs. 5–7 Breast cancer TMA punches stained with the anti-PD-L1 antibody clone ab58810, demonstrating cytoplasmic staining and accumulation of antibody in apoptotic cell nuclei, the latter not being considered in our study [1]

affect the results observed. Third, there is a significant difference in the cohorts studied. Our cohort appeared to include patients with more aggressive disease as 72 % had primary tumors that were pT2 and above (compared to 18 % in Schalper et al.), and 44 % had lymph node metastases (compared to 19.6 % in Schalper et al.). Although the lack of proliferation rate and other biomarkers in the cohort of Schalper et al. precludes an approximation of intrinsic subtype, it appears that this cohort has a greater representation of smaller, ER-positive and HER2-negative tumors (most probably Luminal type). Of note, we observed that there was no association between PD-L1 expression and survival in Luminal A breast cancers ($p = 0.132$), while it was highly significant in the HER2- ($p = 0.013$) and basal-like subtypes ($p < 0.001$).

We agree that the absence of a validated standardized staining and analysis protocol for PD-L1 has contributed to concerns about the reliability of immunohistochemistry staining for PD-L1. We were aware of this limitation when we initiated our study and openly discussed this issue in our paper [1]. We also acknowledge that we failed to sufficiently explain the antibody validation in the Materials & Methods section of our paper.

The role of PD-L1 expression in various human cancers is still not completely understood, and conflicting results have been reported. The impact of PD-L1 expression on cancer biology may depend on the level of PD-L1 expression, tissue localization, and clinical context. It is therefore clear that additional studies need to be performed in order to validate preliminary results and define the clinical significance. This is particularly important given the promising results to date with novel therapeutics targeting the PD-1/PD-L1 pathway.

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