PD-L1 and CD8\(^+\)PD1\(^+\) lymphocytes exist as targets in the pediatric tumor microenvironment for immunomodulatory therapy

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**Abbreviations:** AR, alveolar rhabdomyosarcoma; ER, embryonal rhabdomyosarcoma; ES, Ewing’s sarcoma; HR, high risk neuroblastoma monoclonal antibody; NHR, non-high risk neuroblastoma; OS, osteosarcoma; mAb, monoclonal antibody; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; TIL, tumor-infiltrating lymphocyte.

Recent monoclonal antibody trials targeting the PD1/PD-L1 immune-checkpoint pathway have shown remarkable success in treating adult malignancies, with PD-L1-expressing tumors showing the most objective response. However, little is known as to whether pediatric cancers have also adopted this immune evasion mechanism. We evaluated 115 pediatric tumors (taken at diagnosis) for PD-L1 expression and the presence of CD8\(^+\)TILs. Tumors with >5% PD-L1 membrane staining were scored positive. The presence of CD8\(^+\) TILs expressing PD-1 was assessed using dual-labeling immunohistochemistry. Data were evaluated against clinical demographics. The proportion of PD-L1\(^+\) tumors was 86% for alveolar rhabdomyosarcoma (12/14), 72% for high-risk neuroblastoma (31/43), 57% for Ewing’s sarcoma (8/14), 50% for embryonal rhabdomyosarcoma (8/16) and 47% for osteosarcoma (7/15).

Increased proportions of CD8\(^+\) TILs significantly correlated with PD-1 expression. When grouped by cancer type, those with the highest proportion of PD-L1 positivity showed poorest survival. PD-L1\(^+\) patients with a particularly high frequency of CD8\(^+\) TILs (but not those with low numbers CD8\(^+\) TILs) had significantly better survival compared to PD-L1 negative patients. This study reveals the presence of an active PD-L1 pathway in a high proportion of pediatric cancers, as demonstrated by strong PD-L1 positivity and the presence of PD-1 expressing CD8\(^+\) TILs. In addition, patients with high proportions of CD8\(^+\) TILs showed better survival, suggesting that bolstering CD8\(^+\) T-cell responses through PD-1/PD-L1 blockade would be a viable treatment strategy, providing support for expediting these targeted immunotherapies in children.

**Introduction**

Immunomodulatory therapies based on monoclonal antibodies (mAb) have recently shown considerable success in harnessing an effective immune response against adult malignancies.\(^1,3\) However, there is as yet little clinical experience administering these agents against pediatric malignancies. Although survival rates for many childhood cancers have improved substantially over the last decades, the treatment burden and long-term deleterious effects from typically intensive multi-modal therapies is high. Additionally, a number of tumor types such as high-risk neuroblastoma remain challenging to treat and still carry a poor prognosis.\(^4\) In the case of high-risk neuroblastoma, treatment related mortality from current treatment regimens is substantial and there is little room to further intensify therapy.\(^5,6\) Immunotherapy offers an attractive therapeutic option for these children, as this approach potentially provides a much more specific, and therefore less toxic, alternative therapy. Immunomodulatory monoclonal antibodies offer a powerful means of generating therapeutic tumor immunity and are sufficiently practical to deliver in a pediatric setting.

Targeting the PD-1/PD-L1 immunee checkpoint pathway using immunomodulatory antibodies has perhaps shown the most remarkable results in cancer clinical trials, with significant objective responses in patients with notoriously treatment-refractory advanced solid malignancies such as non-small cell lung cancer and melanoma.\(^7,8\) Programmed cell death 1 (PDCD1, better known as PD-1), a member of the CD28 co-stimulatory receptor family, is expressed naturally on chronically activated CD8\(^+\) cytotoxic T-lymphocytes (CTL) that have tracked to sites of inflammation and infection within the peripheral tissue. Interaction with its ligand PD-1 ligand (PD-L1; CD274) allows negative regulation of the T-cell effector response by inducing T-cell exhaustion thereby preventing autoimmunity and damage to healthy tissue.\(^9,10\) However, the PD-1/PD-L1 pathway can be adopted by tumor cells as an immune evasion mechanism to...
suppress the activity of tumor-infiltrating CTLs through the expression of PD-L1. In support, studies in pancreatic cancer, renal cell carcinoma and breast cancer have reported PD-L1 tumor expression to be associated with poor prognosis. Furthermore, results from early clinical trials using anti-PD-1 antibody suggest patients with PD-L1 positive tumors may be better responders. These studies have demonstrated that by blocking PD-1+ CTL from interacting with PD-L1 CTLs can maintain a durable effector function. These results have vast implications as targeting the PD-1/PD-L1 pathway would not be limited to a specific tumor type but could be relevant to the immune microenvironment of a wide range of cancers. Thus, PD-1/PD-L1 is an opportune candidate target for the treatment of pediatric cancers that, in comparison to adult malignancies, are relatively rare, making tumor-specific therapies challenging to establish and against which more encompassing therapies are likely to have the biggest impact. In addition, unlike stimulating antibodies, which may indiscriminately activate immune cells, antibodies targeting the PD-1 checkpoint pathway would have a more focused effect in maintaining an already primed CTL response within the tumor site and indeed these trials have shown manageable toxicities.

Immunotherapies remain poorly characterized in the pediatric field, and the development of the approach has been necessarily cautious in terms of identifying which of the emerging monoclonal antibody therapies is the most appropriate for children already subject to intense therapies to avoid exposing them to additional toxic therapies. Anti-PD-1 antibody is of particular interest because unlike other immunotherapies, a specific predictive biomarker, namely PD-L1, has already been linked to clinical outcome and provides a means to determine whether targeting the PD-1/PD-L1 pathway is a worthwhile target for the treatment of malignant disease in children.

This is the first study to report on the frequency of PD-L1 expression in pediatric malignancies and to assess the frequency of tumor-infiltrating CD8+ cytotoxic T-lymphocytes and their level of PD-1 expression. We show that the tumor microenvironment of several pediatric tumors present with a favorable effector cell profile potentially susceptible to antibody therapy targeting the PD-1/PD-L1 pathway.

Results

Patient demographics are summarized in Table 1. Selection was based on the availability of primary tumor samples taken at diagnosis, with no other selection criteria applied.

All the samples that were scored as PD-L1 positive showed unequivocal membrane staining, with some samples having both membrane and cytoplasmic staining. Representative images for each tumor type are shown in Figure 1A–E. The level of CD8+ T cells infiltrating the tumor was assessed using dual labeling immunohistochemistry to identify PD-1 expressing CD8+ T cells. Representative images of single stained CD8+ T-cells and dual labeled PD1+CD8+ T-cells are shown in Figure 1F.

The results show that the percentage of PD-L1 positive samples for each tumor type varied, with alveolar rhabdomyosarcoma (AR) and high risk neuroblastoma (HR) having the highest frequency of positive samples (86% and 72% respectively), followed by Ewings sarcoma (ES;57%), embryonal rhabdomyosarcoma (ER;50%) and osteosarcoma (OS;47%) (Fig. 2A). PD-L1 expression did not significantly correlate with either the gender or age of the patients (Fig. 2B, C). The overall survival for both PD-L1 positive and negative patients taken altogether was low, and although PD-L1 positive patients had a higher overall survival than did PD-L1 negative patients (57% vs 39%), the difference was not statistically significant (Fig. 2D). However, when assessing the survival curves of PD-L1 positive samples fractionated according to tumor type it becomes apparent that PD-L1 expression uniquely impacts survival in certain tumor types, with alveolar rhabdomyosarcoma and high-risk neuroblastoma patients having particularly low overall survival at 40% and 52% respectively (Fig. 2E).

CD8+ tumor-infiltrating lymphocytes (TILs) were present in many samples from all tumor types including PD-L1 positive and negative tumors (Fig. 3A). The number of CD8+ TILs did not relate to PD-L1 tumor expression. When survival was assessed based on CD8+ counts regardless of PD-L1 status, patients harboring tumors with a relatively higher occurrence of CD8+ TILs (>20 cells per tumor area) showed better survival as compared to those with low or no CD8+ TILs (65% vs 48%) (Fig. 3B). Furthermore, when we assess PD-L1 positivity in relation to CD8+ TIL counts, PD-L1+ patients with higher proportions of CD8+ TILs but not those with infrequent CD8+ TILs exhibited significantly better overall survival as compared to patients with PD-L1 negative samples (Fig. 3C). The number of PD-1+CD8+ TILs was assessed using dual labeling immunohistochemistry (IHC). Both PD-1 positive and PD-1 negative CD8+ populations were identified in many of the tumors (Fig. 3D) but no association could be made between these CD8+ TIL subsets and tumor PD-L1 expression (Fig. 3E). However, there was a high positive correlation (R² = 0.76) between the total number of CD8+ TILs and the number PD-1 expressing CD8+ TILs (Fig. 3F). Interestingly, when the PD-L1+ tumors with high CD8+ TILs were further grouped according to the percentage of PD-1+CD8+ TILs, those with a greater percentage of PD1+CD8+ TILs had better overall survival as compared to those with a lower percentage of PD-1+CD8+ TILs (92% vs 47%), giving a further significant improvement in survival relative to PD-L1 negative tumors (Fig. 3G).

We further investigated whether PD-L1 expression in neuroblastoma and the level of CD8+ TIL differed between primary HR, primary non-high risk (NHR) and metastatic neuroblastoma samples. Interestingly, all the NHR samples (12/12) were PD-L1 positive whereas only 6/13 metastatic samples (46%) and 25/30 HR samples (83%) were PD-L1 positive (Fig. 4A). The PD-L1 staining for the NHR primary tumors and metastatic samples demonstrated a similar intensity of membrane staining to that of the HR samples. Although the NHR samples had the greatest proportion of PD-L1 positivity, they had the lowest number of CD8+ TIL contrasting with HR patient samples.
having not only the greatest number of CD8$^+$ TILs, but also the greatest percentage of PD-1 expressing CD8$^+$ TILs (Fig. 4A–D), demonstrating a more prevalent presence of the PD-L1/PD-1 pathway in HR vs NHR patient tumors.

**Discussion**

PD-L1 expression in pediatric cancers has not been previously reported. Here, we have shown that the staining pattern in several common pediatric cancers is similar to that previously described for adult malignancies such as renal cell carcinoma, B-cell lymphomas and soft tissue sarcomas, with a distinct strong membranous and some cytoplasmic staining. The percentage of PD-L1 positive tumors in AR and neuroblastoma samples was particularly high compared to the other pediatric malignancies. The presence of CD8$^+$ tumor-infiltrating cells in many of the pediatric tumors also suggests that there may be an existing underlying immunogenicity toward many of the tumors. Moreover, PD-1 is essentially an activation marker upregulated on

**Table 1.** Patient demographics according to tumor type. The neuroblastoma samples (55) consisted of high risk (43), including primary high-risk (30) and metastatic high-risk (13), and primary non high-risk samples (12)

| Tumor Type                           | Number of cases | Age at diagnosis: Median (Range) | Male:Female ratio |
|--------------------------------------|-----------------|----------------------------------|-------------------|
| Ewing’s sarcoma (ES)                 | 14              | 11.5 (1.4–16.5)                  | 9:5               |
| Alveolar rhabdomyosarcoma (AR)       | 15              | 6.5 (1.3–16.2)                   | 6:9               |
| Embryonal rhabdomyosarcoma (ER)      | 16              | 6.0 (1.2–12.8)                   | 14:2              |
| Osteosarcoma (OS)                    | 15              | 13.8 (0.8–16.6)                  | 8:7               |
| Neuroblastoma (NB):                  | 55              | 2.6 (0.1–16.9)                   | 25:30             |
| Non-high-risk (NHR)                  | 12              | 1.6 (0.3–16.9)                   | 3:9               |
| High-risk (HR):                      | 43              | 2.7 (0.1–15.9)                   | 22:21             |
| Primary (HR-primary)                 | 30              | 2.2 (0.1–15.9)                   | 17:13             |
| Metastatic (HR-Mets)                 | 13              | 3.7 (1.3–7.3)                    | 5:8               |

**Figure 1.** Immunohistochemistry staining of various types of pediatric tumors for PD1/PD-L1. (A–E) Representative images immunohistochemistry staining for programmed cell death ligand 1 (PD-L1; brown) and counterstained nuclei (blue) of formalin-fixed paraffin embedded (FFPE) primary tumors. Images demonstrate both PD-L1 positive tumors (A–E) and PD-L1 negative tumors (Aii–Eii) at x100 and x400 magnification (inset) for each tumor type: neuroblastoma (A); Ewing sarcoma (B); osteosarcoma (C); embryonal rhabdomyosarcoma (D) and alveolar rhabdomyosarcoma (E). (F) A representative image is shown for CD8 (brown) and PD-1 (red) dual labeling with counterstained nuclei (blue) of FFPE tumor at x400 magnification; arrows indicate a cluster of PD-1$^+$CD8$^+$ double positive T cells (a) and PD-1 negative CD8$^+$ cells (b).
interferon γ (IFNγ) producing cells, with the increased IFNγ production being responsible for inducing PD-L1 expression.19 Greater frequency of CD8+ TILs gives better survival, and those tumors displaying PD-L1 positivity with high proportions of CD8+ TILs may be indicative of an effective immune response that could be enhanced and sustained by blockade of tumor PD-L1 interaction and signaling through PD-1 on the surface of CD8+ TILs. Furthermore, those with PD-L1 positivity concomitant with low CD8+ TILs may indicate that the tumor has been more effective in executing immune evasion as the PD-L1/PD-1 interaction is also known to induce CD8+ apoptosis, and therefore a corresponding reduction in CD8+ cells.20 The analysis of the PD-1 status of CD8+ TILs provides a rather unexpected result, with the best overall survival in the PD-L1 positive tumors containing high numbers of CD8+ TILs, of which a high proportion are also PD-1 positive. As functionally active CD8+ T cells naturally upregulate PD-1, the number of PD-1+CD8+ TILs may actually be an indicator of an effective T-cell response. However, those with a high frequency of PD-1 low (or negative) CD8+ TIL may be suggestive of an effective PD-L1/PD-1 tumor
evasion mechanism whereby PD-1$^+$CD8$^+$ TIL deletion has occurred through interaction with tumor cells expressing PD-L1. In other words, the presence of PD-1$^+$CD8$^+$ TILs could be regarded as a good indicator of effector response if they can be maintained without apoptotic induction caused by PD-L1 interaction; this maintenance could be provided by therapeutic administration of anti-PD-L1/PD-1 antibodies.

We can infer from our own results that patients with tumors that are PD-L1$^+$ with a large presence of CD8$^+$ TILs are most likely to benefit from PD-1/PD-L1 monoclonal antibody therapy blocking this pathway in order to prevent the induction of CD8$^+$ exhaustion and apoptosis. In particular, high-risk neuroblastoma would appear to be an ideal target for this type of immunotherapy, with a high proportion of tumors expressing PD-L1 that display poor survival. Blocking the PD-1/PD-L1 interaction could help sustain an effective CD8$^+$ response.

Of further interest is the changing landscape of the tumor microenvironment following conventional therapy or even during immunotherapy. Assessing the tumor microenvironment of solid malignancies during therapy is not a practical option; even post-therapy pediatric tumor samples are not readily available. However, clinical studies have shown that pre-treatment PD-L1 negative patients can also respond to PD-1 antibody therapy. In addition, several studies have shown that the release of
IFNγ from activated lymphocytes can induce expression of PD-L1 on tumor cells.\textsuperscript{20,22} This suggests that tumors could adopt this immune escape mechanism at a later stage post-therapy, once an effective immune response ensues and this may explain the presence of PD-1 expressing CD8\textsuperscript{+} TILs preceding upregulation of PD-L1. Assessing PD-L1 induction, and PD-1 expression on CD8\textsuperscript{+} TILs during treatment, would significantly further our understanding of the induction of this pathway to potentially stratify and design responsive modes of patient interventions.

In conclusion, what is evident from our study is that PD-L1 expressing tumors and PD-1 expressing CD8\textsuperscript{+} TILs are clearly present in many pediatric cancers even before any intervention. This is indicative of a favorable tumor microenvironment that will lend itself to antibody therapy targeting the PD-1/PD-L1 pathway with the potential to benefit many pediatric patients through harnessing an effective CD8\textsuperscript{+} T cell immune response.

\section*{Methods}

\subsection*{Cases}

Formalin-fixed paraffin-embedded (FFPE) primary tumor samples taken at diagnosis were received from and ethically approved for use by the Children’s Cancer and Leukemia Group (CCLG), UK Tissue Bank and consisted of high risk neuroblastoma (HR, 18 cases), Ewing’s sarcoma (ES, 14 cases), osteosarcoma (OS, 15 cases), embryonal rhabdomyosarcoma (ER, 18 cases) and alveolar rhabdomyosarcoma (AR, 15 cases). A further 32 neuroblastoma samples were sourced from the Cellular Pathology Department, University Hospitals Southampton, and consisted of primary non-high risk neuroblastoma (NHR, 12 cases), primary HR (12 cases) and metastatic HR tumor (Mets, 13 cases). Wherever possible, clinical characteristics were obtained (Table 1). All samples were taken at diagnosis (pre-therapy) after obtaining informed consent and were processed by NHS Trust accredited pathology laboratories according to a standard protocol, before being deposited to the Human Tissue Act (HTA) licensed CCLG UK Tissue Bank for research use.

\subsection*{Immunohistochemistry}

FFPE samples were cut into 4 \(\mu\)m-thick sections and mounted on slides for immunohistochemistry. A standard deparaffinization and epitope retrieval protocol was used with the Leica Bond-max\textsuperscript{TM} automated system (Leica Biosystems). In brief, this involved heat-induced epitope retrieval at 90°C using Leica Bond\textsuperscript{TM} Epitope Retrieval Solution 1 (ER1) (pH = 6) for 30 min. Antibodies were titrated on control tissue to determine their optimal concentration before use, and to assess for non-specific binding. PD-L1 expression was determined using 5 \(\mu\)g/ml

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\caption{Comparison of PD-1/PD-L1 expression and CD8\textsuperscript{+} TILs in high-risk, non-high risk and metastatic neuroblastoma pediatric patient samples. (A) The percentage of programmed cell death ligand 1 expressing (PD-L1\textsuperscript{+}) patient samples, the number of CD8\textsuperscript{+} tumor-infiltrating lymphocytes (TILs) and the percentage PD-1\textsuperscript{+}CD8\textsuperscript{+} TILs for each indicated neuroblastoma subtype: primary high-risk (HR), non-high risk (NHR) and metastatic high-risk (mets). Error bars show the SEM. (B-D) The total number of CD8\textsuperscript{+} TIL per neuroblastoma subtype, with the number of PD-1\textsuperscript{+} shaded in black.}
\end{figure}
rabbit anti-human PD-L1 antibody (Abcam, anti-CD274 ab58810), detected using the Leica Bond™ Polymer Refine (diaminobenzidine) detection system and nuclei were counterstained with haematoxylin. For dual staining of CD8 and PD-1, 1:200 anti-CD8 (Abnova, MAB11248) and 1:50 rabbit anti-PD-1 (Abnova, anti-PDCD1 PAB19608) antibodies were used and detected with Bond™ Polymer Refine DAB and Red Detection system respectively (Leica). Nuclei were counterstained with haematoxylin.

Sample evaluation

Haematoxylin and eosin stained slides were evaluated by a Consultant Cellular Pathologist (M.A-K) to ensure the quality and presence of tumor for each sample. Samples were scored blind and independently by S.M and M.A-K. Samples were considered PD-L1 positive when >5% tumor cells showed PD-L1 membrane staining which is in accordance with the scoring used in clinical trials.8 Samples with only cytoplasmic staining were classed as negative. The number of CD8+ tumor-infiltrating T cells was calculated as the total count from 10 high power fields at 400x magnification per sample with more than 20 CD8+ cells being scored as high. PD-1 expressing CD8+ T cells were calculated as the number of dual stained CD8+ cells as a percentage of the total number of CD8+ cells. The total area of tumor assessed was 2.9 mm².

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Statistics

Statistical significance was assessed using non-parametric t-test. Correlation between datasets was assessed using the Pearson’s correlation test. The log-rank (Mantel-Cox) test was used to assess differences between Kaplan-Meier survival curves. Error bars show standard error of the mean. All statistical analyses were performed using Microsoft Excel and GraphPad Prism software.

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