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Mini-review

PD-1/PD-L1 blockade in paediatric cancers: What does the future hold?

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ABSTRACT

Checkpoint blockade (CPB) immunotherapy has shown unprecedented success in a wide range of adult malignancies, and is increasingly being employed in the treatment of advanced cancers. However, the experience in the paediatric population remains limited and the small number of single agent studies reported have shown disappointing response rates. Paediatric cancers offer unique challenges that can hinder the translation of CPB into the paediatric clinic, and combinational therapies are likely to be needed to achieve therapeutic success. As the number of paediatric trials using CPB rapidly increases, understanding the challenges that these agents may encounter in this population is of special significance to allow the design of optimal combinatorial strategies for each tumour type. Here, we offer an overview of the unique biological and immunological features of paediatric cancers as compared to adult malignancies, and how these might impact the overall success of CPB in the paediatric population. We review the growing body of pre-clinical and clinical experiences to date, and discuss future strategies involving the combination of CPB with traditionally used therapies (chemotherapy and radiotherapy) or with other newly developed immunotherapies.

1. Introduction

Over the last decade, immunotherapeutic approaches have shown impressive results across different tumour types, leading to a renewed interest in the field. As proposed in the immunoediting theory, the host immune system is capable of surveying against cancerous cells at an early stage of development, recognising and eliminating emerging malignant cells. During this control phase, immune cells exert a selective pressure that favours the outgrowth of less immunogenic clones, which can eventually escape immune recognition and lead to cancer progression [1]. Hence, cancer immunotherapy aims to restore the host's immune function and reinvigorate anti-tumour responses. To date, immunotherapeutic approaches have shown impressive results in adult patients, and are increasingly being explored in childhood cancers.

Generally, paediatric cancers are treated with a combination of surgery, chemotherapy and radiation; advances in these traditional approaches have led to remarkable improvements in outcome, such that overall long term survival rates for paediatric malignancies are in the order of 80% [2]. However, tumours that are refractory to conventional therapies or relapse after initial treatment still represent a major challenge for paediatric oncology. Furthermore, whilst outcome for some paediatric malignancies has improved dramatically, others have proved more challenging, and outcomes remain poor [3]. Immunotherapy has emerged as a promising alternative in the treatment of childhood malignancies thanks to the numerous advantages that it can offer over conventional therapies. Amongst these, the more specific targeting of cancer cells is of great importance as it has the potential to reduce treatment-related side effects, which is vital in paediatric patients, to improve the long-term quality of life of survivors [4]. Ideally, immunotherapy can also offer durable therapeutic effects if immunological memory against antigens on cancer cells can be elicited.

Of all the immunotherapeutic approaches explored in adult cancers, targeting of the programmed cell-death protein (PD)-1 with monoclonal antibodies (mAbs) has perhaps emerged as the most promising strategy, showing therapeutic benefit in a wide range of cancer types [5,6]. Thus, there is a lot of interest in the potential translation of PD-1 blockade into paediatric oncology. Here, we review the rapidly growing body of evidence coming from clinical and preclinical studies focused on establishing the efficacy of PD-1 blockade in paediatric oncology, and highlight the major challenges that the implementation of immunotherapy will need to overcome in childhood cancers.

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2. Immune checkpoint blockade

Immune checkpoints are molecules involved in the maintenance of immune homeostasis and peripheral tolerance to self-antigens, limiting T-cell activation to prevent autoimmune responses [7,8]. However, in the context of cancer, immune checkpoints such as cytotoxic T lymphocyte antigen (CTLA)-4 and PD-1 suppress anti-tumour immunity and can promote tumour growth. The aim of blocking such inhibitory receptors with mAbs is to unleash endogenous anti-tumour T-cell responses, eradicate tumours and establishing immunological memory. However, and despite its unequivocal success, checkpoint blockade (CPB) is not effective in all cancers, nor in all patients within a responsive tumour type [5,9]. Understanding the biology that underlies the differences between responsive and non-responsive tumours is fundamental not only to deliver effective therapies in adult patients, but also to predict the potential translation of CPB immunotherapies into paediatric oncology.

The first checkpoint monoclonal antibodies targeted CTLA-4, an inhibitory co-receptor expressed on activated and regulatory T cells (Tregs). Similar to CD28, CTLA-4 binds CD80 and CD86 expressed on the surface of antigen presenting cells (APCs) [10]. However, CTLA-4 binds with increased affinity, hence out-competing CD28 and decreasing T-cell activation. In addition, ligation of CTLA-4 leads to the direct suppression of T-cell proliferation through the transduction of an inhibitory signal, which also affects effector functions such as IL-2 production [11]. Therapeutic blockade of this receptor achieved durable responses in pre-clinical models and clinical trials, where treatment with the anti-CTLA-4 mAb Ipilimumab resulted in increased overall survival in patients with advanced melanoma [9,10]. However, anti-CTLA-4 antibodies have proved effective in only a relatively small proportion of patients with melanoma, and antibodies targeting the PD-1/PD-L1 axis have proved more effective, and appear to have efficacy in a broader range of tumour types [11,12]. Therefore, subsequent pre-clinical and clinical studies have focussed on PD-1/PD-L1 targeting agents.

2.1. PD-1/PD-L1 axis and therapeutic blockade

PD-1 is a cell-surface receptor largely expressed on activated T and B cells, but absent on naïve and memory lymphocytes [13]. PD-1 acts as a negative regulator of immune responses, inhibiting T-cell proliferation and effector functions upon interaction with its ligands PD-L1 and PD-L2 (see Fig. 1) [7,14]. PD-L1 has a broad expression pattern, being found on several activated immune cell types such as macrophages, T cells or dendritic cells (DCs) and other stromal cells like vascular endothelial cells [15–17]. Although not constitutively expressed in most cancers, tumour cells can up-regulate PD-L1 as a mechanism of adaptive resistance in response to cues in the tumour microenvironment (TME) [18]. As such, cytokines that are normally released during the course of an immune response such as IFN-γ or TNF-α are potent drivers of PD-L1 expression in tumour cells (Fig. 1) [19–22]. In contrast to PD-L1, the role of PD-L2 in immune evasion is less clear, yet most studies suggest that PD-L2 could also dampen tumour immunity in a similar way to PD-L1 by inhibiting T-cell proliferation and cytokine secretion [23,24]. Therefore, PD-1 inhibitory signalling mediated by ligation through PD-L1 or -2 is likely to limit anti-tumour immunity regardless of the tumour type, supporting the broad use of anti-PD-1 mAbs in cancer therapy.

Pre-clinical studies in murine tumour models have demonstrated the capability of PD-1 blockade to promote tumour regression and prolong animal survival in a wide range of histologically distinct tumours, generating a robust immunological memory mediated by memory T cells [25,26]. PD-1 blocking mAbs trigger a polyclonal expansion of tumour-specific cytotoxic T cells that are able to target cancer cells and lead to the regression of primary and metastatic tumours [27,28]. As opposed to direct tumour-targeting strategies (e.g anti-CD20 mAbs), this polyclonal response minimises the rise of antigen-loss tumour variants that can escape the immune attack. In line with these studies, further research using *ex vivo* human cells showed an enhanced T-cell proliferation and cytokine production in response to PD-1 or PD-L1 targeting mAbs [29,30]. Such studies paved the road to assess safety and efficacy of these agents in clinical trials, in which blockade of the PD-1 pathway led to remarkable clinical results in a variety of cancers, most notably metastatic melanoma and non-small cell lung cancer (NSCLC) with an objective response of 28–40% and 18–36%, respectively [5,31,32]. These results culminated in the Food and Drug Administration (FDA) approval of two PD-1 blocking mAbs, Pembrolizumab and Nivolumab, for the treatment of metastatic melanoma and NSCLC, respectively. Three other drugs that inhibit the pathway by blocking PD-L1 (Atezolizumab, Avelumab, Durvalumab) have been more recently approved to be used in NSCLC, bladder cancer and Merkel-cell carcinoma, respectively. More recently, a third anti-PD-1 antibody (Cemiplimab) has been approved as a first line therapy for advanced cutaneous squamous cell carcinoma [33–36]. To date, the number of clinical studies underway is already surpassing the thousand, and the list of cancers with FDA approval currently includes an increasing number of solid carcinomas (renal, head and neck, stomach, bladder, liver) and lymphomas [36].

Due to the systemic immune-modulatory effect of CPB, and given the growing number of patients being treated with this immunotherapy, it is essential to assess any potential toxicities associated with this treatment. To date, compiled experience of PD-1/PD-L1 blockade in adult cancers demonstrates a generally safe profile, with fatigue being the most common adverse event (AE). Although over 70% of the patients experienced immune-related AEs after PD-1/PD-L1 blockade,

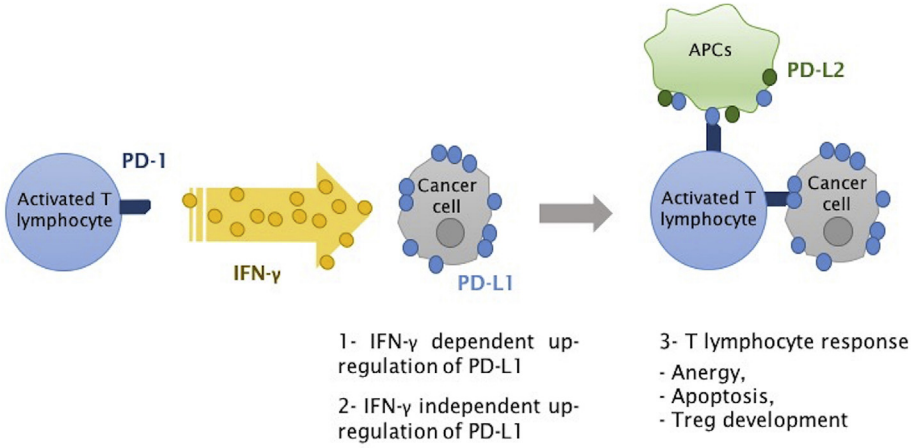

Fig. 1. ***PD-L1 expression as an adaptive mechanism of immune evasion***. During the course of an immune response to cancer, IFN-γ released from activated T lymphocytes can induce the up-regulation of PD-L1 on cancer cells. However, cancer cells can also present constitutive expression of this molecule due to genetic alterations. Engagement of PD-1 by tumour-associated PD-L1 leads to T-cell suppression by inducing T-cell anergy, apoptosis or promoting the development of regulatory T cells. Alternatively, PD-L1/2-expressing antigen presenting cells (APCs) can also trigger PD-1-mediated inhibition on T cells.

only approximately 10% were grade 3–4 AEs. Other common AEs included pruritus and diarrhoea, as well as other site-specific inflammation causing colitis, pneumonitis and hepatitis [5,32]. Despite the remarkable success of single CPB, the number of patients responding is still relatively low, therefore most strategies are now focused on combining CPB with other therapies to increase the number of responders. To date, over a thousand trials are exploring a range of combinatorial approaches with PD-1 blocking drugs; nevertheless, toxicity seems to increase alongside responses when PD-1/PD-L1 blockade is combined with chemotherapy, radiotherapy and other immunotherapies, indicating that tolerability and safety should be evaluated for each combination schedule [37].

2.2. Markers of response to PD-1 blockade in adult cancers

Given that PD-1 blockers are only effective in a minority of patients and tumours, the search for biomarkers to accurately predict response to this immunotherapy has aroused great interest [38]. To date, there is no single biomarker of response in adult cancers; however, the knowledge gathered in recent decades has identified concurring traits of responsive and unresponsive cancers that currently guide clinical practice.

2.2.1. PD-L1 expression

PD-L1 expression by immunohistochemistry (IHC) was one of the earlier biomarkers identified to potentially predict response to PD-1 blockade. Tumour PD-L1 positivity has been reported to correlate with improved objective response rates compared to PD-L1 negative tumours in many adult cancers [39]. One of the first studies where PD-1/PD-L1 interaction was described as an adaptive mechanism for immune evasion established four categories of tumours according to the level of PD-L1 expression and tumour-infiltrating lymphocytes (TILs) [18]. The authors investigated primary and metastatic melanoma lesions and described a first group of tumours with co-localisation of PD-L1 and large TIL infiltration, thereby being classed as immunologically “hot”. In contrast to “hot” lesions, a second group of “cold” tumours that lacked both TILs and PD-L1 expression was observed. Intermediate phenotypes were also identified, with tumours that were infiltrated by TILs but lacked PD-L1, or tumours that expressed diffuse levels of PD-L1 but absence of TILs [18]. This last group of melanoma tumours led the authors to propose that some tumours may constitutively express PD-L1 due to genetic alterations. In accordance to this, subsequent studies have demonstrated that increased MAPK activation or loss of PTEN upregulated PD-L1 expression in melanoma and glioma tumours, respectively [40,41].

A priori, only those groups presenting PD-L1 expression would be the ones predicted to benefit from PD-1 blockade, due to the presence of PD-1 ligand within the tumour. Nevertheless, durable and complete responses to PD-1 blockade have been seen in PD-L1 negative tumours, and thus the absence of PD-L1 expression is not necessarily indicative of unresponsiveness to the therapy [5,42]. Although initial reports focused on PD-L1 expression on tumour cells, novel studies highlight that host-derived PD-L1 on tumour infiltrating immune cells may also play a critical role in immune suppression. While some studies conclude that both malignant and immune cells contribute to immune suppression, others emphasise the dominant role of PD-L1-positive myeloid populations driving T-cell inhibition [43–46], and correlate PD-L1 positivity on myeloid cells with CPB responses [43]. Therefore, determining the role of PD-L1 in different subsets of cells in a cancer-specific basis may help to better predict responsiveness.

2.2.2. Mutational burden

Tumour mutational burden, and more specifically the extent of non-synonymous mutations, which potentially generate novel epitopes to be displayed by MHC molecules, has been shown to correlate with response to PD-1 blockade [47,48]. This highlights the importance of neoantigens in dictating therapeutic anti-tumour immune responses. The strong relationship between mutational load and PD-1 responsiveness was first suggested by the fact that biallelic mismatch repair deficient (biMMRD) tumours are more responsive to PD-1 blockade as compared to non-biMMRD, irrespective of the tumour type. This was thought to be driven by the large number of somatic mutations that biMMRD cancers bear due to biallelic defects of the DNA mismatch repair system, which gives rise to an increased number of potential neoantigens [49]. Besides its role in primary response to PD-1 blockade, the specific mutational landscape of a tumour has been demonstrated to be important in acquired resistance to immunotherapy in adult cancers. In an elegant study by Anagnostou et al. (2017), the analysis of relapsed tumours from patients that had initially responded to PD-1 blockade revealed a loss of 7–18 putative mutation-associated neoantigens compared to pre-treatment counterparts [50]. This evidence suggests that CPB could benefit from combination with cytotoxic therapies that potentially broaden the neoantigen landscape of tumours, both in primary and relapsed cases.

2.2.3. Immune contexture

The dynamic nature of cancer and the interactions between tumour and stromal cells dictate the spatial organisation of immune populations within the TME. As reviewed by Fridman et al., a dense intratumoural lymphocyte infiltrate is a common characteristic of many different tumour types with favourable prognosis (i.e melanoma, head and neck, breast, bladder, ovarian, colorectal, pancreatic), as opposed to an absent or peri-tumoural immune infiltrate [51]. Recent studies are shedding some light on the value of immune infiltrates in determining responsiveness to PD-1 blockade. Two different studies in melanoma patients showed that the best predictive value of response to therapy were pre-treatment biopsies displaying co-localisation of CD8 T cells and PD-L1 expressing macrophages and tumour cells at the invasive margin [28,52]. During the responsive phase, tumour regression was driven by intratumoural proliferation of CD8 T cells that co-localised with a pro-inflammatory response enriched in an IFN-γ signature [28,52]. Interestingly, Zaretsky et al. found that CD8 T cells were restricted to the invasive margin at the time of tumour relapse, supporting the importance of intratumoural immune infiltrates in the therapeutic activity of anti-PD-1 mAbs [28]. Given the apparent importance of the immune contexture in determining response, the International Immunoscore project was initiated as a first step to standardise immune measurements and promote their use in clinical practice. Following from this, findings from a recent large-scale study provide evidence in support of Immunoscore (defined as the CD3/CD8-positive cell densities in the tumour centre and invasive margin) as a prognostic biomarker in colon cancer, being an important step towards the standardisation of immune-measurements in solid tumours [53,54].

3. Immunotherapy in paediatric cancers

To date, the majority of clinical experience of cancer immunotherapy in the paediatric population has been with tumour-targeted immunotherapies. Such approaches employ mAbs or *ex vivo* expanded immune cells, which are given to the patient, such that there is little reliance on the patient's own endogenous anti-tumour response. There have been some notable successes with these, including anti-GD2 mAbs for neuroblastoma (NB) and CD19-CAR T cells for acute lymphoblastic leukaemia (ALL) (see Fig. 2)[55,56]. However, this approach is limited by the fact that it usually relies on targeting a single tumour antigen, which can lead to the eventual rise of antigen-loss tumour variants. Also, this transferred immunity may not be sustained in the patient and may not be able to provide long-term protection. In contrast, immune-modulatory immunotherapies like CPB aim to provoke an active anti-tumour immune response within the patient, potentially targeting multiple tumour antigens and generating immunological memory).

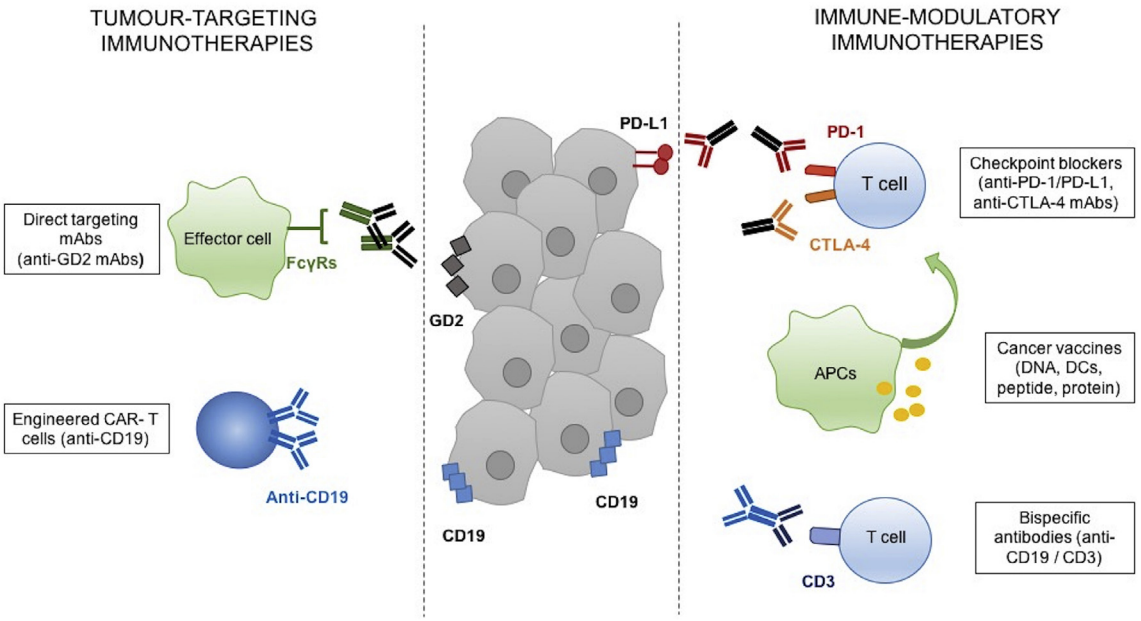

Fig. 2. ***Summary of immunotherapy approaches.*** Tumour-targeted immunotherapies use mAbs or *ex vivo* expanded cells (T-cell adoptive transfer, CAR-T cells) that directly target tumour-associated antigens. Tumour-targeting mAbs may rely on the engagement of Fc-γ receptors (FcγRs) present on immune effector cells to deliver their cytotoxic effect. On the other hand, immune-modulatory immunotherapies target lymphocytes or antigen presenting cells (APCs) to stimulate an immune response that can induce immunological memory. Examples of this include checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 mAbs, cancer vaccines and bi-specific mAbs directed both at a tumour-associated antigen and at CD3 on T cells.

3.1. Specific features of paediatric tumour immunology

Paediatric cancers differ from those in adults in many ways, bearing unique biological traits that need to be considered carefully when developing therapeutic strategies to treat them. Adult cancers are most frequently from an epithelial origin, arising as a consequence of continued DNA damage due to chronic exposure to environmental insults [57]. This origin often results in increased number of mutations and a more pro-inflammatory TME. In contrast, childhood malignancies generally arise from genetic abnormalities on cells of an embryonic or mesenchymal origin and have a shorter pre-neoplastic period, leading to lower mutational load [58–61]. Furthermore, paediatric tumours present with faster kinetics than most adult tumours, exhibiting rapid growth that could outpace the efficacy of immunotherapy, as these rely on the relatively slow and complex immune modulation of the TME. Despite these unfavourable features, several paediatric tumours have evidence of a natural anti-tumour immunity, and spontaneous remission has occasionally been seen in some paediatric patients [56,62–64].

3.2. Translation of CPB into paediatric cancers

3.2.1. PD-L1 expression

In view of the role of tumour PD-L1 expression as a response biomarker in adult cancers, a number of studies have assessed PD-L1 expression in paediatric cancers. However, the results of these are very discordant, and hence difficult to interpret. Some studies report high expression levels across a broad range of paediatric malignancies [65], whereas other studies report very low expression levels [66–69]. These discrepancies may be partially explained by the lack of standardisation in technical aspects such as the use of different detection antibodies or the establishment of different thresholds to determine positivity [39]. Nevertheless there are some specific paediatric cancers where PD-L1 is reported in conjunction with PD-1 expression on infiltrating T cells, such as RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A) - fusion-positive ependymoma and NB [65,70], adding further support to the role of the PD-1/PD-L1 pathway in these cancers. Furthermore, PD-L1 expression is dynamic and responds to environmental cues in the TME such as cytokines or cell-to-cell interactions, and expression may change during the course of treatment or disease progression [19,21]. As such, PD-L1 expression may develop in paediatric tumours that show immune infiltration but not PD-L1 expression at the time of biopsy. In support of this theory, *in vitro* stimulation with IFN-γ up-regulates PD-L1 on tumour cells in some types of paediatric cancers [68,69,71–73]. This suggests that treatment-derived inflammation could result in the up-regulation of PD-L1 on tumour cells, thereby sensitising them to PD-1 blockade.

In addition to its expression on tumour cells, PD-L1 expression on myeloid populations is rapidly gaining greater notice. Activated CD4 helper T cells at the tumour site may sustain PD-L1 expression on tumour associated macrophages (TAMs), which could in turn contribute to the inhibition of cytotoxic T cells [44]. Myeloid cells expressing PD-L1 at the tumour-draining lymph nodes may also decrease the activation of tumour-reactive T cells and abolish the therapeutic effect of PD-1/PD-L1 blockade [45]. Although the relative contribution of PD-L1 expression in both tumour and myeloid cells is not yet clear, the high rates of PD-L1 in macrophages found in some paediatric cancers [66] suggests that these cells may play a role in PD-1 mediated inhibition.

3.2.2. Mutational burden

Paediatric cancers are generally accepted to carry fewer neoantigens and be less immunogenic than adult malignancies. A recent study showed that mutation frequencies in paediatric cancers varied between 0.02 and 0.49 mutation per Mb and were overall 14 times lower than in adult cancers, with significantly less mutated genes per tumour. Relapsed tumours, however, harboured significantly more mutations than primary tumours, and mutational burden increased with age [61]. As an exception, a small proportion of paediatric tumours (biMMRD) are hypermutated, with a mutational burden that exceeds that of CBP-sensitive adult malignancies [74]. Notably, the repertoire of genetic mutations and epigenetic alterations is specific to childhood cancers, and differs from that of adult tumours even in tumours with similar histology [75]. However, it is important to note that responses to CPB have been reported in adult cancers with a similar level of mutational burden (i.e breast cancer), evidencing the role of other factors in

determining clinical responses [58,76].

3.2.3. Immune contexture

As a consequence of their low immune infiltration, paediatric malignancies are often described as immunologically 'cold' tumours. In a cohort of 53 paediatric patients with solid tumours, low levels of TILs were described in all cancers except for germinomas and NB, with metastases harbouring larger infiltration of CD8 T cells [77]. Other studies have also confirmed that the number of tumour infiltrating lymphocytes appears to be significantly lower in paediatric tumours compared to adult malignancies [78]. Interestingly, in a study of patients with high-risk NB, T-cell infiltration into the tumour bed appeared to be a key limiting factor in the generation of effective anti-tumour immunity [62]. Whilst survivin-specific CD8 T cells could be detected in the blood of NB patients, very few T cells infiltrated the tumours [62]. A further insight into the type of immune infiltrate revealed that in paediatric cancers these are scattered within the tumour and are predominantly formed of macrophages that accumulate in areas of necrosis, with lower frequencies of DCs or lymphocytes as compared to many adult malignancies [78,79].

3.2.4. Additional factors

Given the nature of therapeutic responses after PD-1 blockade, engagement between the TCR and major histocompatibility complex (MHC) is essential for T-cell activation. Nevertheless, down-regulation of MHC-I molecules is a common escape mechanism that paediatric cancers exploit to prevent immune recognition [80–82]. Likewise, tumour cells can down-regulate alternative molecules like β-2-microglobulin that are involved in antigen processing and presentation [28,83]. Due to the intrinsic low immunogenicity of paediatric cancers, further reductions will strongly limit the activity of tumour-specific cytotoxic T cells, with the consequent loss in PD-1 blockade efficacy.

Another factor that may potentially challenge the translation of CPB therapies into paediatric patients is that many of these patients undergo very intensive multi-modal therapy as first line and salvage therapies, and are therefore likely to be very heavily pre-treated by the time they are considered for early phase trials of immunotherapies. The effect of such prior treatments on the patient's immune system is poorly studied and difficult to quantify, but warrants consideration. Similarly, intestinal microbiota has been demonstrated to influence the expansion of tumour-specific T cells and the activity of CPB [84,85]. However, microbiota is very variable across individuals, and changes with age and environmental factors like exposure to antibiotics and development stage of the mucosal immune system, further adding another level of complexity to CPB responsiveness [86].

A final challenge is that, in general, paediatric cancers grow rapidly compared to adult cancers. The generation of therapeutic responses with CPB may be relatively slow compared to responses to conventional therapies e.g to chemotherapy, and there may even be initial pseudo-progression as a result of initial immune infiltration. If the growth kinetics of the tumour exceed the generation of anti-tumour immunity then the patient will become compromised, and this will be considered treatment failure. Careful patient section must therefore be considered, with caution exercised in treating patients with rapidly progressing disease. All in all, the immune context of childhood cancers is incredibly variable and depends on many factors including tumour type, age, schedule of prior treatment and genetic alterations, as well as other less studied elements like gut microbiota. The specific features of paediatric cancers (summarised in Fig. 3) make them unlikely to benefit from PD-1 blockade as a monotherapy, and support their use in combination with other existing therapies.

4. Immune checkpoint blockade in childhood cancers

4.1. PD-1 blockade: clinical experience to date

Following on the huge breakthrough in adult cancers, the possible application and efficacy of CPB is currently being explored in paediatric cancer patients. One of the first clinical trials of CPB in childhood malignancies evaluated Ipilimumab in patients aged 2–21 years old with recurrent solid tumours [87,88]. Despite no significant objective responses, an increase in the number of proliferating T cells in peripheral blood was seen, and immune-related toxicities were similar to those seen in adult patients. Interestingly the development of immune related AEs correlated with improved survival, suggesting some therapeutic effect of the CPB [87]. In view of the suggested increased efficacy of PD-1 blockade [11,12], a plethora of clinical trials are currently investigating its efficacy in multiple childhood malignancies, as summarised in Table 1 and Table 2 with initial results suggesting that safety and tolerability are similar to in adult cancers [89,90].

Despite the growing number of clinical trials assessing PD-1 blockade in childhood cancers (Tables 1 and 2), single agent studies in most tumour types have shown little therapeutic activity. Administration of Pembrolizumab showed no clinical efficacy in a cohort of 5 paediatric patients with progressive primary brain tumours [91] or in a larger cohort of 121 children screened for PD-L1-positive tumours (16 central nervous system; 5 lymphomas; 45 advanced solid tumours), where only 4 partial responses were reported [92]. Similarly, Nivolumab as a monotherapy did not show anti-tumour activity in 20 patients with Ewing sarcoma (EWS) and OS [93]. In a study by Geoerger et al., which included 74 paediatric patients (12 OS, 11 EWS; 11 NB; 10 rhabdomyosarcoma (RMS); 10 non-RMS soft tissue sarcoma; 6 Wilm's tumour (WT); 5 Hodgkin's lymphoma (HL); 1 non-HL lymphoma; and 8 other tumour types), showed partial responses in only 2 HL and 1 atypical rhabdoid tumour after monotherapy with PD-L1 blocking antibody Atezolizumab [94].

Although over 300 children have been treated with PD-1 blocking antibodies, significant clinical activity has only been shown in paediatric HL and a few other case-report studies of EWS and biMMRD glioblastoma. In HL, adult phase I trials of Nivolumab and Pembrolizumab showed durable disease control, achieving an overall response rate of 83% and 65%, respectively [95,96]. Despite previous autologous stem cell transplant and targeted therapy, complete responses were seen in more than 15% of the patients [89]. Similarly, Pembrolizumab achieved an overall response rate of 69% and a complete response of 22% in a phase II trial [90]. Classical HL tumours treated with conventional therapies show strong PD-L1/L2 expression due to frequent copy number alterations in the PDL1/L2 locus, which is further increased in relapsed tumours after PD-1 blockade [97–99]. This may contribute to the high rate of response to PD-1 blockade in HL tumours despite not having a particularly high mutational burden compared to other cancers [58]. Although the number of paediatric patients with HL treated with CPB is limited, preliminary results from single agent studies appear similar to the adult experience [100,101].

Paediatric biMMRD glioblastoma is potentially favourable for CPB therapy in that it exhibits a higher mutational load and increased predicted number of neoantigens compared to sporadic gliomas and other childhood cancers [102]. In a case report, two children with biMMRD glioblastoma refractory to conventional therapies were treated with Nivolumab and showed remarkable reductions in tumour burden followed by durable remission [74]. In another case report, a heavily pretreated patient presenting with recurrent metastatic EWS achieved sustained remission after 9 cycles of Pembrolizumab [103]. Nevertheless, these responses to CPM monotherapy appear to be the exception rather than the rule, and few responses are reported in most paediatric tumours, as detailed above. While ongoing clinical trials are still in early stages, focus should be put on elucidating the best combination of therapies that could synergise with PD-1 blockade, and further trials

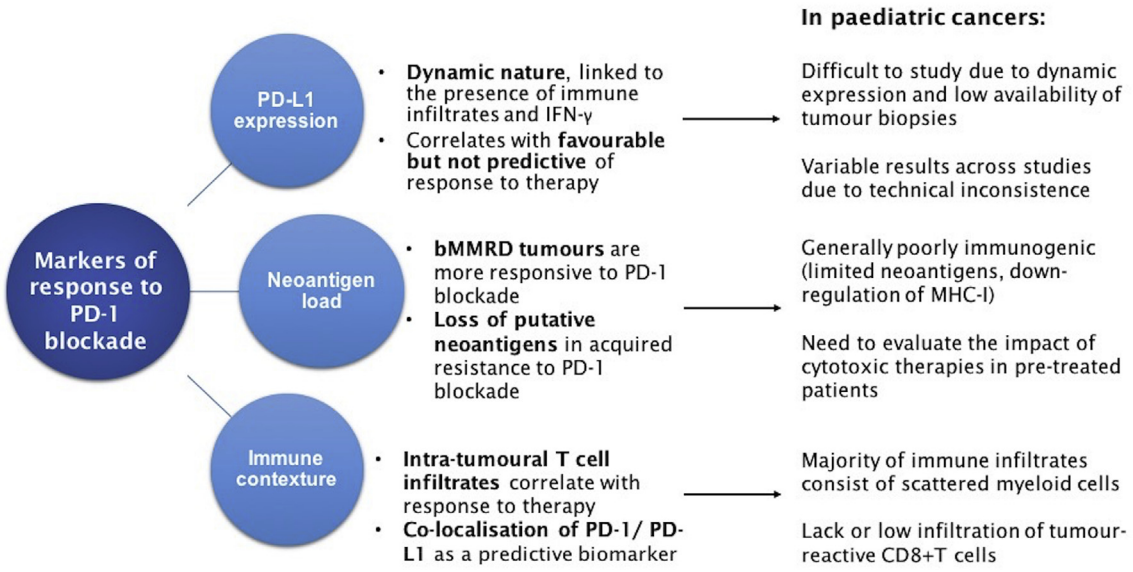

Fig. 3. ***Biomarkers of response to PD-1 blockade in adult cancers and their translation to paediatric malignancies.*** Favourable traits that predict response to PD-1 blockade in adult cancers include expression of PD-L1 at the tumour microenvironment (TME), high neoantigen load due to increased mutational burden and presence of PD-1-T cells co-localised with PD-L1 expression at the tumour site. Translation of these biomarkers into the paediatric population needs specific consideration due to their different biology and immunological landscape.

Table 1
Clinical trials with PD-1/PD-L1 blockade as a monotherapy in childhood malignancies.

Drug	Target	Study phase	Age	Cancer types	Study ID
Nivolumab	PD-1	Phase II	> 1 yr	GBM	NCT02550249
	PD-1	Phase I/II	1–18 yrs	biMMRD tumours	NCT02992964
	PD-1	Phase I/II	1–21 yrs	Solid tumours	NCT02901145
	PD-1	Phase I/II	1–40 yrs	Sarcoma, Solid tumours	NCT03465592
	PD-1	Phase II	> 6 months	Relapse/refractory ALK + anaplastic large-cell lymphoma	NCT03703050
Pembrolizumab	PD-1	Phase II	1–29 yrs	biMMRD brain tumours excluding HGG, DIPG	NCT02359565
	PD-1	Phase I/II	0.5–17 yrs	Melanoma, solid tumours, lymphoma (PD-L1 +, progressive)	NCT02332668
	PD-1	Phase II	> 14 yrs	NK/T-cell lymphoma	NCT03107962
	PD-1	Phase II	> 12 yrs	Bone and soft tissue sarcoma	NCT02301039
	PD-1	Phase II	> 12 yrs	Bone and soft tissue sarcoma	NCT03092323
	PD-1	Phase II	> 16 yrs	Early stage triple negative breast cancer	NCT03145961
	PD-1	Phase III	> 12 yrs	Advanced Merkel cell carcinoma	NCT03783078
	PD-1	Phase III	> 12 yrs	High-risk stage II melanoma	NCT03553836
Atezolizumab	PD-L1	Phase I/II	0–30 yrs	Solid tumours	NCT02541604
	PD-L1	Phase II	> 6 yrs	Metastatic Alveolar Soft Part Sarcoma	NCT03141684
Durvalumab	PD-L1	Phase I	1–17 yrs	Solid tumours, lymphoma, CNS tumours	NCT02793466
Avelumab	PD-L1	Phase I/II	< 18 yrs	Lymphoma, relapsed or refractory solid tumours	NCT03451825
	PD-L1	Phase II	12–49 yrs	Osteosarcoma	NCT03006848
	PD-L1	Phase II	> 16 yrs	Classical HL	NCT03617666
Cemiplimab	PD-1	Phase I/II	> 12 yrs	Lymphoma	NCT03769181

*Accurate as of January 2019. Glioblastoma (GBM); biallelic mismatch repair (biMMRD); diffuse intrinsic pontine glioma (DIPG); high-grade glioma (HHG); central nervous system (CNS).

of single agent CPB in most paediatric patient groups are probably not warranted.

4.2. Combinatorial strategies

In view of the poor immunogenic nature of childhood malignancies, the combination of PD-1 blockade with cytotoxic or direct targeting therapies holds great promise thanks to their ability to reduce tumour burden and potentiate the release of tumour neoantigens. Whilst there is a plethora of potential combinatorial approaches, here we review some of the current strategies being assessed in paediatric patients. As summarised in Table 2, there are already multiple trials testing anti-PD-1 mAbs with cytotoxic (chemotherapy, radiotherapy) and direct targeting immunotherapies. Due to the encouraging clinical results that dual CPB showed in adult patients [104], this strategy is also being explored for paediatric cancers. Nevertheless, clinical trials in children are hampered by the low prevalence of paediatric tumours, limiting the numbers of recruited patients. This hinders the investigation of multiple combinatorial regimens within the paediatric population, highlighting the need to design better pre-clinical models that allow the screening of a broader range of combinatorial approaches, in the interest of guiding the design of clinical studies and identifying potential response biomarkers.

4.2.1. Chemotherapy

Cytotoxic chemotherapy is still the first line of treatment in most childhood cancers. Chemotherapy may cause temporary functional impairments in the immune system by depleting immune cells and inducing myelosuppression [105,106]. Nevertheless, the idea that chemotherapy-induced immunosuppression would diminish the efficacy of immunotherapy has been challenged by recent findings. Although several studies report a combined additive effect, the precise

Table 2
Clinical trials of combination therapies including PD-1/PD-L1 blockade in childhood malignancies.

Drug	Target	Study phase	Age	Cancer types	Study ID
Nivolumab + Ipilimumab	PD-1 + CTLA-4	Phase I/II	1–30 yrs	Solid tumours, sarcomas	NCT02304458
	PD-1 CTLA-4	Phase II	6 months - 21 yrs	High grade primary CNS tumours	NCT03130959
	PD-1 CTLA-4	Phase II	> 12 yrs	Advanced or metastatic solid tumours (TMB-H)	NCT03668119
Nivolumab + Relatlimab	PD-1 LAG-3	Phase II/III	> 12 yrs	Melanoma	NCT03470922
	PD-1 LAG-3	Phase II	> 12 yrs	Advanced chordoma	NCT03623854
Nivolumab + Blinatumomab ± Ipilimumab	PD-1 CD19/CD3 CTLA-4	Phase I	> 16 yrs	CD19-positive precursor B-lymphoblastic leukaemia	NCT02879695
Nivolumab + Brentuximab	PD-1 CD30	Phase II	5–30 yrs	Classic HL	NCT02927769
	PD-1 CD30	Phase I/II	> 15 yrs	Non-HL	NCT02581631
	PD-1 CD30	Phase II	> 16 yrs	Classic HL	NCT03712202
Nivolumab + EBV virus specific T cells	PD-1 EBV-ST cells	Phase I	> 12 Kg	EBV-positive lymphomas	NCT02973113
Nivolumab + Dinutuximab beta +131-I mIBG	PD-1 GD-2 RT	Phase I	1–18 yrs	Neuroblastoma	NCT02914405
Nivolumab + cyclophosph. ± radiotherapy	PD-1 CT RT	Phase I/II	< 18 yrs	Solid tumours	NCT02813135
Nivolumab + Stereotactic radiosurgery	PD-1 RT	Phase I	> 15 yrs	Chordoma	NCT02989636
Nivolumab +BMS-986205	PD-1 IDO1	Phase III	> 12 yrs	Advanced melanoma	NCT03329846
Nivolumab + Cyclophosph. + Vinblastine + Capecitabine	PD-1 CT	Phase I/II	4–18 yrs	Refractory/relapsing solid tumours and lymphoma	NCT03585465
Nivolumab + Axitinib	PD-1 Tyr-K inhibitor	Phase II	> 12 months	Metastatic/unresectable TFE/Translocation RCC	NCT03595124
Nivolumab + Azacitidine	PD-1 CT	Phase I/II	< 39 yrs	Recurrent osteosarcoma	NCT03628209
Nivolumab + NKTR-214	PD-1 IL-2R	Phase III	> 12 yrs	Inoperable/metastatic melanoma	NCT03635983
Pembrolizumab + ABVD/COPDAC	PD-1 CT	Phase II	3–25 yrs	Classical HL	NCT03407144
Pembrolizumab + Doxorubicin	PD-1 CT	Phase II	> 12 yrs	Soft tissue sarcoma	NCT03056001
Pembrolizumab + Decitabine + Radiotherapy	PD-1 CT RT	Phase I	12 months–40 yrs	Relapsed and refractory solid tumours or lymphoma	NCT03445858
Pembrolizumab + Aldesleukin	PD-1 IL-2	Phase II	> 15 yrs	Stage III/IV melanoma	NCT02748564
Pembrolizumab + iC9-GD2	PD-1 GD2 CAR-T cell	Phase I	All ages	Relapsed/refractory Neuroblastoma	NCT01822652
Pembrolizumab + Cisplatin + Radiotherapy	PD-1 CT RT	Phase II	> 16 yrs	Head and neck	NCT03532737
Pembrolizumab + Tabelecleucel	PD-1 EBV-CTLs	Phase I/II	> 12 yrs	EBV + nasopharyngeal carcinoma	NCT03769467
Pembrolizumab + Blinatumomab	PD-1 CD19/CD3	Phase I	12 months–40 yrs	Relapsed/refractory Acute B-cell leukaemia or lymphoma	NCT03605589
Pembrolizumab + Gemcitabine + Vinorelbine + Doxorubicin	PD-1 CT	Phase II	> 10 yrs	Hodgkin lymphoma	NCT03618550
Pembrolizumab + Induction CT + Brentuximab	PD-1 CT CD30	Early phase I	1–31 yrs	Advanced non-anaplastic T-cell or NK lymphoma/leukaemia	NCT03719105
Cemiplimab + Radiotherapy	PD-1 RT	Phase I/II	> 25 yrs	Relapsed/refractory solid/CNS tumours + recurrent glioma	NCT03690869
Durvalumab + Tremelimumab	PD-L1 CTLA-4	Phase II	> 16 yrs	Advanced rare tumours	NCT02879162

*Accurate as of January 2019. Chemotherapy (CT); Radiotherapy (RT); Central nervous system (CNS); Epstein Barr virus (EBV); Hodgkin's lymphoma (HL); Cytotoxic T lymphocytes (CTL); High tumour mutational burden (TMB-H); Renal cell carcinoma (RCC).

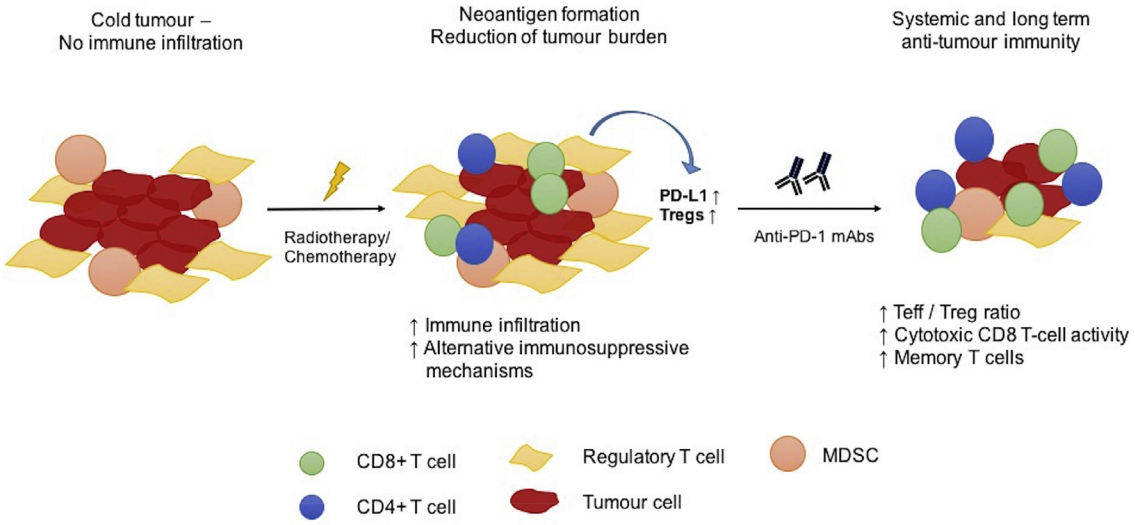

Fig. 4. ***Rationale for combining chemotherapy and radiotherapy with PD-1 blockers***. Treatment with cytotoxic agents can turn "cold" tumours into more immunogenic by triggering neoantigen release, as well as reducing tumour burden. Nevertheless, these treatments lead to the up-regulation of alternative immunosuppressive mechanisms such as PD-L1, thereby inhibiting newly primed immune cells. In this context, treatment with anti-PD-1 mAbs could release the inhibition and lead to increased infiltration of cytotoxic CD8 T cells and a more effective anti-tumour immunity.

mechanism of action is not well defined [107–112]. Chemotherapy can potentially modify the local TME in various ways, such as depleting Tregs, increasing antigen cross-presentation or inducing immunogenic cell death (ICD), see Fig. 4 [107,108]. In regards to PD-1 blockade in adults, the combination of Nivolumab and platinum-based chemotherapy increased overall survival in patients with NSCLC in a non-randomised clinical trial [109]. Additional pre-clinical studies showed that chemotherapeutic drugs sensitised tumours lacking immune infiltration to CPB, and promoted CD8 T cell-driven anti-tumour immunity [110]. The mechanism underlying this combined effect might be related to the up-regulation of PD-L1 that some types of chemotherapy like paclitaxel induce in tumour cells [111]. Alternatively, the decrease in Treg numbers induced by metronomic doses of cyclophosphamide could be driving the synergy with PD-1 blockade in some tumour models [112]. Paediatric cancers are extremely sensitive to chemotherapy, and can lead to clinical responses in the majority of children, even in the context of relapsed tumours. In order to enable a short-term impact in childhood oncology, immunotherapy should perhaps aim to enhance the efficacy of these existing treatments to achieve greater efficacy with lower doses, rather than aiming to replace them.

4.2.2. Radiotherapy

Ionising radiation induces direct cell-death by activating DNA damage responses, but it is also proposed to induce ICD by activating intracellular signalling pathways, such as NF-kβ and PKC, that regulate immune responses [113–115]. Radiation-induced ICD is proposed to result in enhanced direct antigen presentation from tumour cells, favouring cross-presentation and priming of antigen-specific T cells by APCs [116]. However, radiotherapy also leads to the adaptive up-regulation of PD-L1 on tumour cells in response to environmental IFN-γ, thereby inhibiting T-cell responses (see Fig. 4) [117]. Pre-clinical reports show that the combination of radiotherapy and PD-1 blockade can lead to increased overall survival in various *in vivo* models, including poorly immunogenic cancers that were initially unresponsive to PD-1 blockade on its own. The combination regimen increased T-cell infiltration and provided long-term anti-tumour immunity that required cytotoxic CD8 T cells [117,118]. Notably, fractionated radiotherapy can lead to therapeutic effects outside the radiation field (the abscopal effect) when combined with anti-PD-1 and anti-CTLA-4 mAbs, supporting the presence of a systemic immune response [119,120]. Based on this evidence, diverse early-phase clinical trials are assessing the combination of radiotherapy, which increases T-cell infiltration and promotes neoantigen formation, with PD-1 blockade to expand the tumour-reactive T-cell clones in paediatric solid malignancies (NCT02989636, NCT02813135) or lymphoma (NCT03445858).

4.2.3. Direct targeting immunotherapy

Combination of PD-1 blockade with immunotherapies that directly target tumour cells could provide a similar synergistic effect to that of cytotoxic therapies, promoting tumour-cell death and antigen release [121]. In solid tumours, the efficacy of direct targeting mAbs or CAR-T cells is generally dampened due to the presence of a dense immunosuppressive TME and the rise of antigen loss tumour variants, limiting the therapeutic activity of such treatments as a monotherapy [56,122]. Moreover, recent studies describe an up-regulation of PD-1 ligands in the TME following therapy with direct targeting mAbs, further supporting the combination of both approaches [123]. Therefore, the combination of such approaches with PD-1 blockade could benefit children with solid tumours where PD-1 inhibition is a prominent pathway within the TME. The MiNivAN trial (NCT02914405), which has just opened in the UK, aims to exploit this in NB by combining Nivolumab with dinituximab beta (anti-GD2 mAb) as well as targeted molecular radiotherapy (131-I mIBG). The rationale for this combination is based on pre-clinical data suggesting synergy between immune/tumour -targeting monoclonal antibodies and radiotherapy, with the later promoting a more favourable immune microenvironment through depletion of Tregs [124]. Similarly, clinical trials are underway to investigate combination of CPB with the CD30-drug conjugate Brentuximab in HL (NCT02927769, NCT02581631).

4.2.4. Immune-modulatory immunotherapy

Blockade of CTLA-4 and PD-1 elicits anti-tumour immunity through different mechanisms. Whilst blockade of CTLA-4 is proposed to act during the priming phase in secondary lymphoid organs and by Treg depletion, PD-1 is thought to primarily act at a later stage to enhance effector function at the tumour site [11]. Given that blockade of one pathway alone may lead to the up-regulation of the unblocked pathway [125], dual targeting of CTLA-4 and PD-1 offers the possibility to trigger non-redundant pathways to promote effective anti-tumour responses [125,126]. In melanoma patients, this combination resulted in significantly higher response rates (61 vs 11% in CTLA-4 only) and progression-free survival. However, toxicity was markedly increased

alongside clinical responses, with over 80% of patients requiring the administration of immunosuppressive therapy to treat irAEs and a treatment-related drug discontinuation of 36–47% in the combination arm [127]. Later trials in other types of cancer support this highly toxic profile, with over 90% of patients experiencing AEs and 50% exhibiting grade 3–4 irAEs [37]. Therefore, caution has to be taken when designing combinatorial approaches that include one or more immune-modulatory therapy, due to the potent additive effect they may have on the host's immune system. Nevertheless, and based on the encouraging clinical responses achieved in adults, several clinical trials are looking at the effect of dual CPB in children.

As well as up-regulating the inhibitory checkpoint molecules, the TME possesses numerous other mechanisms to suppress immune responses and sustain tumour growth. Amongst them, the enzyme indoleamine 2,3-dioxygenase (IDO) is known to inhibit anti-tumour immunity by catalysing the depletion of tryptophan, an essential amino acid required for T and NK cell proliferation, as well as activating suppressive immune populations such as Tregs or myeloid-derived suppressor cells (MDSCs) [128–130]. Similar to PD-L1, IDO is expressed on tumour cells and monocytes upon exposure to IFN-γ and apoptotic cells [131,132]. Hence, up-regulation of IDO in the context of an inflammatory response could hinder the effective function of the newly PD-1-reinvigorated T cells, thereby decreasing the overall efficacy of anti-PD-1 mAbs [133]. Although the expression of this enzyme in paediatric cancers has not been largely studied, IDO expression has been reported in HL and OS, where it correlated with significantly lower 5-year overall survival [134,135]. Therefore, apart from the ongoing clinical trial in melanoma (NCT03329846), other studies exploring the combination of PD-1 and IDO blockade in children are likely to come.

5. Further directions and challenges

Treatment of patients with advanced stage, relapsed or refractory tumours remains a major challenge in paediatric oncology, and new approaches are needed. Immunotherapy is the most promising strategy in the treatment of cancer, with immune checkpoint inhibitors as key players in this success. Tolerability of PD-1 blocking antibodies appears to be similar to that observed in adult patients; however, data gathered from clinical trials in children report little evidence of clinical activity as a monotherapy in most paediatric tumours, with the exception of HL and rare tumours with favourable biological features. Paediatric tumours generally have a lower mutational burden and are less immunogenic compared to adult malignancies. In addition to these traits, the more rapid tumour growth and the intensive conventional cancer therapies that most of these patients receive, make utilising CPB as a paediatric cancer therapy challenging. For all these reasons it is unlikely that CPB monotherapy will be beneficial for the majority of paediatric tumours, and combinatorial approaches are likely to be needed to achieve therapeutic success. For some patients it may be possible to use CPB to enhance the efficacy of immunotherapies with established benefit in paediatric malignancies (e.g. anti-GD2 mAb, CAR T cell therapies). However for the majority of paediatric tumour types, it will be necessary design of studies that take into account the specific biological features of paediatric tumours. In particular, approaches targeting innate elements of the immune system may be more successful than those focussing purely on the T cell response.

Given the predominance of TAM in paediatric tumours, strategies targeting macrophages should be considered, and TAMs have been shown to express PD-1 in both pre-clinical models and patients [136]. Pre-clinical studies in a murine metastatic osteosarcoma model, have demonstrated that anti-PD1 mAb can exert macrophage dependent therapeutic effects by redirecting TAM from an inhibitory (M2) phenotype to a more inflammatory M1 phenotype [137]. It may be possible to capitalise on this effect by combining anti-PD-1 antibodies with other agents with the potential to favourably modulate the TME. For example, agents targeting VEGF/VEGFR (e.g. bevacizumab, lenvatinib) are increasingly recognised to have immunomodulatory as well as anti-angiogenic effects, with reported beneficial effects on TAMs, MDSC, Treg, and cytotoxic T cells [138]. Lenvatinib has been demonstrated to have synergistic effects with anti-PD-1 In a range of murine tumour models, with reduction in TAMs and promotion of an anti-tumour CD8 T cell response [139], and this combination has shown promising results in an early phase trial in patients with advanced endometrial carcinoma [140]. Similarly, bevacizumab has been shown to reduce intratumoral Treg and TAMs in patients with glioblastoma, and to have synergistic therapeutic effects with anti-PD-1 in a range of pre-clinical models [141,142]. Such combinations are already being widely tested in adult malignancies, and paediatric studies are likely to follow shortly.

Another class of agents which are worthy of consideration are PI3-kinase γ (PI3Kγ) inhibitors. PI3Kγ is expressed by macrophages and high levels of expression have been correlated with suppressive activity of macrophages and infiltrating myeloid cells [143], leading to resistance to anti-PD-1/PD-L1 therapy in pre-clinical models. PI3Kγ inhibitors have been demonstrated to overcome this inhibition, and enhance responses to CPB [144]. Such combinations may therefore be beneficial in paediatric tumours, given the predominance of suppressive myeloid cells and TAMs.

An alternative approach may be blockade of colony-stimulating factor 1 receptor (CSF1R) on TAMs and MDSCs. CSF1R has a key role macrophage recruitment and differentiation, and inhibition with small molecules or antibodies has been shown to reduce the number of TAM and skew towards a more inflammatory M1 phenotype. Tumour responses were achieved in a spontaneous murine model of NB when PD-1 blockade was combined with a selective CSF1R inhibitor that was reported to reduce MDSCs [145]. Synergy has also been reported when such antagonists are combined with CPB in a number of different pre-clinical tumour models, and a number of early clinical trials in adult tumour types are in progress [146,147]. In a similar way, blocking the traffic of MDSCs to the tumour site by anti-CXCR2 mAbs synergised with PD-1 blockade and achieved more effective responses than single agent treatments in murine models of RMS [148].

Finally, encouraging results have recently been reported from a clinical trial combining CPB with a histone deacetylase (HDAC) inhibitor in patients with melanoma [149]. HDAC inhibitors appear to have effects on macrophage transcription factors such that they result in switching of TAMs to highly a phagocytic M1 phenotype, with consequent suppression of tumour growth in murine breast tumour models [150]. Combining the HDAC inhibitor with anti-PD1 antibody further enhanced this therapeutic effect.

There are many other approaches that may be taken to target the TME, and have the potential to synergise with CPB. The number of possible combinations is huge and therefore such studies should be biomarker driven, and carefully guided by knowledge of the paediatric TME.

Conflicts of interest

None declared.

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