Peer Review File

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Reviewer Comments

In this manuscript, Shalita et al provide with a comprehensive review on immunotherapy for the treatment of pediatric brain tumors. The authors used a systematic approach to discern between studies relevant for the topic, including immunotherapies such as dendritic cell vaccines, oncolytic virotherapy/viral immunotherapy, CAR T cell therapy, peptide vaccines, immunomodulatory agents, among others. Importantly for systematic reviews, methods section as well as the flowchart and tables are written clearly, containing most of the relevant information. However, we would like to address several points of improvement.

Major comments:
1. It is common to put the initials behind the authors who extracted the data and the third peer for inclusion/exclusion of the articles. Did you use any program to select the articles (such as Rayyan screening tool) or was it all performed manually? Please include this information in the Methods section.

Authors Response:
The initials have been added behind the author who extracted the data and the appropriate third peer has been stated as requested. We did not use any program to select articles such as a screening tool and have added this information as requested for clarity. The screening was done manually by two reviewers who are also listed with initials.
Change to Text: "Search results were compiled in EndNote (Clarivate, Philadelphia) and imported into Covidence (Melbourne) for screening (SK)." Page 5, paragraph 2, lines 137-139.

2. The authors state that 'The goal of this systematic review is to report and summarize the completed pediatric immunotherapy clinical trials for primary CNS tumors.' Hence, the goal is foremost to summarize safety and efficacy. Therefore, the reader would first want to see how the studies were conducted per intervention (How many patients were included? Characteristics?). What do the combined articles tell us related to the patient outcome? E.g. are dendritic vaccines safe and effective? The reason why a therapy can fail would better be placed in the discussion.
For example, the authors may consider to start each piece with how many studies were found for this specific intervention (e.g. with the sentence: 'Four published pediatric specific studies utilized autologous dendritic cells pulsed with variations of tumor lysate or RNA from surgical resections.'), how many patients were included and excluded (and what was the reason for exclusion, e.g. dropouts due to toxicity or another reason) resulting in the x total number of patients that completed the study (what was their outcome: completed the study/passed away/no immune response could have be obtained/etc), how was immune response defined in each of the studies (e.g. were there clinically objective responsive rates, such as tumor shrinkage on MRI?). This information is not clear for all types of therapies described in this review. Lastly, was the treatment safe and effective? End with the final result if you were to combine these studies and how many trials are there still needed to be before a final conclusion can be made regarding the specific treatment.

Authors Response:
Thank you for your suggestion on concisely including patient characteristics, summarizing safety and efficacy and any relevant patient outcomes and clinical responses. In response to the reviewer we have completed the following: (1) included any relevant and available descriptive patient characteristics to all included studies, (2) ensured every section includes a clear statement about the

intervention safety/feasibility and any additional secondary outcomes, (3) as suggested we also specifically stated the number of included studies found for a specific intervention and any details of why any patients were excluded, (4) we specifically described whether a response to treatment was measured radiographically or through other means of immunologic measurement (5) ended each review with the final result namely a statement on efficacy or safety and whether there are more studies in progress (6) A statement on next steps needed before a final conclusion can be made regarding the specific treatment.

Change to text:

"Descriptive characteristics include: 50% female and age range (7 to 18 years)." Page 9, paragraph 1, lines 259-260.

"Descriptive characteristics include: age range (7-17 years)." Page 9, paragraph 1, line 265.

"Overall both studies demonstrate oncolytic therapy as preliminarily safe with only one case of grade 3 headache. Next steps include a larger multi-institutional clinical trail currently under progress." Page 9, paragraph 1, lines 267-269.

"Descriptive characteristics include: 1 female, 2 males, age range (19 to 26 years)." Page 10, paragraph 1, lines 311-312.

"Secondary objective assessing CAR T-cell distribution and disease response demonstrated positive cytokine levels in the CSF of patients consistent with immune. activation. Overall, this small sample demonstrated the feasibility of producing HER2-specific CAR T-cells that are well tolerated and mediate a localized immune response." Page 10, paragraph 1, lines 314-317.

"Twenty-six patients were included, 12 patients with newly diagnosed brainstem or high-grade glioma treated with radiation and concurrent chemotherapy and 14 patients with newly diagnosed brainstem glioma treated with irradiation. Descriptive characteristics include: 50% female, age range (2.2 to 17.9 years). There were no dose related toxicities reported nor grade 3 or higher systemic toxicities. Preliminary clinical outcome data assessed radiographically with MRI demonstrated". Page 11, paragraph 2, lines 338-344.

"Pollack et al. then published their next study focused on pediatric patients with recurrent low-grade gliomas. This study included 14 patients, 2 patients were excluded one patient for grade 3 urticaria and one patient due to progressive disease. Descriptive characteristics include: 50% female, age range (1.9 to 19.0 years). Aside from grade 3 urticaria, no other regimen limiting toxicities were observed. Preliminary clinical outcome data was assessed radiographically with MRI one child had asymptomatic pseudoprogression noted at 6 weeks after starting the treatment regimen, followed by dramatic tumor regression >75% shrinkage. Three children had sustained partial responses lasting greater than 10, 31 and 45-months respectively, and one patient had a transient response. Median progression-free survival was 9.9 months and overall survival was 100% with an average follow-up of 42 months. ELISPOT analysis showed GAA responses in all patients including 3 to IL-13Ra2, 11 to EphA2, and 3 to survivin." Page 12, paragraph 2, lines 385-395.

"Finally, in 2016 Pollack et al. published their most recent peptide study focused on pediatric patients with recurrent high-grade gliomas. This study included 12 patients, 6 with. glioblastoma, 5 with anaplastic astrocytoma and one patient with malignant gliomatosis cerebri. Descriptive characteristics include: 50% female, age range (2.3 to 23.3 years). There we no reported dose-limiting toxicities reported or grade 3 or high toxicities. Preliminary clinical outcome data was assessed radiographically with MRI, one child had symptomatic pseudoprogression response to treatment, 1 child had a partial response. Median progression-free survival from the start of vaccination was 4.1 months and median overall survival was 12.9 months. At 6 months, progression-free survival was 33% and overall survival was 73%. ELISPOT analysis showed GAA responses in 9 patients including 4 to IL-13Ra2, 9 to EphA2, and 3 to survivin." Page 12, paragraph 3, lines 397-407.

“Overall, the three published studies utilizing peptide vaccination in the treatment of pediatric brain tumors have demonstrated safety with only one case of grade 3 urticaria and no dose limiting toxicities. Additionally, these studies also demonstrated feasibility in eliciting an immunologic response several patients showed evidence of T-cell responses against vaccine targeted GAA epitopes on ELIPSOT analysis. Thus, while these data are promising, larger studies are needed to further assess the benefits of this strategy through a multi-institutional setting.” Page 13, paragraph 2, lines 409-415.

“Two studies have been published by Fangusaro et al. including a phase I and phase II utilizing pomalidomide to treat pediatric brain tumors.” Page 13, paragraph 3, lines 424-521.

“Four patients were excluded total, one due to insufficient labs to monitor for toxicity, and three due to progressive disease. Patient characteristics include: 55% female, age range (5.4 to 20.8 years), 8 patients with astrocytoma, 11 different brain tumor types (Table 1).” Page 14, lines 525-528.

“Descriptive characteristics include: 36.5% female, age range (4 to 18 years). Of the patients a few of the listed reasons included screening failure, and one patient never receiving treatment. The primary endpoint if this study was either objective response or long-term stable disease both assessed radiographically via standard MRI.” Page 14, paragraph 2, lines 542-563.

“Ultimately, the results of these two studies of pomalidomide failed to demonstrate a clinically meaningful level of efficacy as a monotherapeutic regimen in pediatric patients with brain tumors, though the grade 3 and 4 toxicities were consistent with previous studies. While the phase II sample size was small, this study overall reinforces the need for further evaluation of tumor resistance against pomalidoamide.” Page 15, paragraph 1, lines 571-575.

“One study published in 2018 by. Fried et al. published a study utilized pidilizumab, in children with diffuse intrinsic pontine glioma (DIPG). This study included 9 enrolled children with DIPG. Descriptive characteristics include: age range (3 to 18 years). The primary objective of this study was assessment of efficacy and toxicity, secondary objectives included event-free survival and overall survival.” Page 15, paragraph 2, lines 583-590.

“Overall, as this is one of the first studies investigate feasibility and toxicity of immune modulating antibodies in pediatric brain tumors, the authors demonstrated both feasibility and safety with a low toxicity profile. However, further studies are needed to confirm these preliminary findings.” Page 16, paragraph 1, lines 595-598.

“. Two published studies utilized immune checkpoint inhibition through PD-1 and or CTLA-4 blockade.” Page 16, paragraph 2, lines 609-612.

“The primary outcomes reported included both adverse events and toxicities, tumor mutation burden, survival and clinical response assessed radiographically by MRI. A total of 10 patients were included in this study had received and failed multiple standard therapies for their specific disease before nivolumab treatment initiation.” Page 17, paragraph 2, lines 633-642.

“Descriptive characteristics include: 40.0% female, age range (1.5 to 17 years).Grade 2 toxicities were observed without any dose limiting toxicities. Reported adverse events included leukopenia, transaminitis, hyperglycemia, hypoalbuminemia, pancreatitis, anemia, nausea, and vomiting. Tumor mutation burden, assessed by the total number of somatic mutations, was to intermediate (median 1.3, range 0 to 6.3).” Page 18, paragraph 1, lines 646-650.

“Overall, the results of this study demonstrate overall safety in the administration of nivolumab. While these early findings, the data suggests that future trials should consider stratification of pediatric patients based on tumor subtype and PD-L1 expression status.” Page 18, paragraph 1, lines

654-657.

“The aim of this study was to explore safety and feasibility in patients with brain tumors and to assess for evidence of clinical and immunologic response. This study enrolled 12 patients. Descriptive characteristics include: 42% female, age range (4 to 13 years).” Page 19, paragraph 1, lines 674-677.

“Clinical response was evaluated radiographically via MRI, nine patients showed no change following the last injection of Hsp70, one patient showed a complete response, one patient showed a partial response, and one patient showed disease progression.” Page 19, paragraph 2, lines 679-682.

“Overall, heat shock protein 70 is demonstrated to be feasible and safe with a low toxicity profile. However, while immunomodulation was observed via changes in T-cell mediated activity, further studies via randomized controlled trial are necessary to further understand anti-tumoral effects of Hsp70.” Page 19, paragraph 1, lines 690-693

“The aim of this study was to determine the safety, toxicity of treatment, survival and response assessed radiographically via MRI to the combined therapy. A total 32 pediatric patients were enrolled. Descriptive characteristics include: 50% female, age range (2 to 17 years).” Page 20, paragraph 1, lines 708-711

“The median time to progression from study entry was 5 months, and median time to death was 9 months. Overall, beta-interferon did demonstrate grade 3 and 4 toxicity including one case of severe neurotoxicity, though it was well tolerated overall. However, this study demonstrated little evidence of clinical efficacy and other immunomodulatory therapies should be considered alternatively.” Page 20, paragraph 1, lines 716-719.

“The primary outcome of this study was to assess the induction of an immune response against pediatric brain tumors. Secondary outcomes assessed for tumor recurrence via serial MR. Three patients were enrolled including two high grade gliomas and one ependymoma. Descriptive characteristics include: age range (0.4 to 17 years).” Page 21, paragraph 1, lines 734-737.

“The primary goal of this study. Was to assess the feasibility, immune response, and overall survival of this multimodal approach. Immune response was monitored via PanTum detect tests used to monitor mRNA expression level of PDL-1.” Page 21, paragraph 2, lines 751-756.

“Descriptive characteristics include: 32% female, age range (2 to 19 years). No major toxicities were reported. Disease progression was difficult to assess in this study due to patient heterogeneity and thus for this retrospective study, progression free survival was defined as the moment a new treatment strategy was implemented by a local oncology center.” Page 22, paragraph 1, lines (761-765).

“Additionally, there was a shift in immune response towards type 1 T-helper mediated response in PanTum Detect tests. Overall, this study reported safety with no major toxicities and feasibility of multimodal therapy and immune response monitoring via PanTum Detect testing.” Page 22, paragraph 1, lines 769-771.

“Nine patients were enrolled and received the therapy up to 3 infusions weekly with escalating doses up to 3 cycles. Descriptive characteristics include: 33% female, age range (8 to 18 years). There were no dose limiting toxicities or severe adverse outcomes. MRI imaging was used to assess for response.” Page 23, paragraph 1, lines 785-788.

“Overall, this study demonstrated a low toxicity profile and efficacy of intratumoral NK infusion. A follow-up study is in development to attempt NK cell delivery at longer intervals for more cycles.” Page 23, pargraph 1, lines 792-795.

3. Most of the relevant information is reported in this review, however the order of different parts per

treatment sometimes differs and thus can be improved and consistent throughout.

Authors response: Thank you again for the reviewers' comments on improving consistency. We have addressed this through the extensive changes shown above by ensuring every section includes descriptive characteristics, the number of studies included, specific primary and secondary objectives, brief and specific details summarizing any relevant primary and secondary results and a summary sentence for each section.
Changes to text: please see changes listed under Reviewer comment 2.

4. The discussion section is rather short. The results section should only contain the data which the researcher found for their patients. The rest can be moved to the discussion section (as mentioned in comment number 2).

Authors response: Agree with the reviewer and have removed information not pertaining to the results to the end of the article. In line with several recently published Narrative Reviews, we changed the "Discussion" section to "Implications for Future Research" which is more consistent with what has previously been published. It also allows for a better summary of the discussion items that were added as requested.
Changes to text:
Changes to text:
"Implications For Future Research". Page 23, paragraph 2, line 797.

"Autologous dendritic vaccines are currently undergoing phase II study to further evaluate the efficacy of the use of tumor lysate loaded dendritic cells vaccines in high grade gliomas in children and adults (NCT01213407). In addition, due to the power of dendritic cells in activating the immune system, they remain a focus of cancer immunotherapy and pediatric CNS tumor treatment with other tumor specific antigens. Trials investigating dendritic cells with other tumor specific targets are underway including CMV viral peptide in high grade glioma, (NCT03615404), stem cell loaded dendritic cells in recurrent high-grade glioma or medulloblastoma (NCT01171469), DIPG tumor neoantigens in newly diagnosed DIPG (NCT03914768), Wilms' tumor-1 antigen mRNA loaded DCs (NCT04911621), and total tumor RNA loaded DCs in recurrent medulloblastoma (NCT01326104), newly diagnosed DIPG (NCT04837547, NCT03396575), and high grade glioma (NCT03334305).

Oncolytic viruses for pediatric CNS tumors present an exciting advancement in cancer immunotherapy and early phase trials of direct intratumoral delivery show overall safety and feasibility as well as early encouraging survival benefit. Phase II studies are underway to better understand the efficacy and ongoing safety monitoring. In addition, there are ongoing trials evaluating other oncolytic viral therapy including a phase I study with adenovirus (DNX2401) in newly diagnosed DIPG (NCT03178032), measles virus (MV-NIS) in recurrent medulloblastoma and atypical rhabdoid/teratoid tumor (NCT02962167), reovirus in recurrent high-grade tumors (NCT02444546), and poliovirus (PVSRIPO) in recurrent high-grade glioma (NCT03043391).

CAR T-cells are continuing to be evaluated in both adult and pediatric CNS tumors. Similar adult glioblastoma targeted CAR T-cell studies with targets including IL13a2, HER2, and EGFRvIII have shown safety and feasibility as well as positive anti-tumor activity as well as some patients with radiographic response on MRI in some patients (78-80). CAR T-cell investigations with varying target antigens common in pediatric high-grade tumors are underway to continue to evaluate the safety, ideal dose and schedule, and ultimate efficacy. Current investigations include CAR T-cells targeting IL13a2 (NCT04510051), GD2 (NCT04196413, NCT04099797), HER2 (NCT03500991, NCT02442297), EGFR (NCT03638167), and B7H3 (NCT04185038). The early interim results of the first pediatric focused CAR T-cell therapy demonstrates the feasibility and early safety data with ongoing studies evaluating a multitude of different target antigen CARs in pediatric CNS tumors.

Lastly, there are currently several ongoing clinical trials utilizing peptide vaccines in the treatment of pediatric brain tumors. NCT03299309 (PRiME) is a phase I clinical trial using PEP-CMV, a peptide

vaccine that contains a long synthetic peptide, in the treatment of recurrent medulloblastoma and malignant glioma. NCT01795313 is an ongoing phase I clinical trial studying the use of HLA-AS restricted peptides in combination with Imiquimod in the treatment of recurrent ependymoma. NCT04749641 is a currently ongoing clinical phase I clinical trial the use of peptide vaccination targeting the H3.3.K27M neoantigen peptide in pediatric patients with diffuse intrinsic pontine glioma.

To date, many of these published studies were phase I and pilot studies focused primarily in establishing safety, maximum dose-tolerance, toxicity, and efficacy in utilizing these therapeutics in pediatric patients. However, as additional trials continue to develop, novel immunotherapies may help in delivering more specific and targeted therapy directed at tumor-specific features." Pages 23-25, lines 805-869.

Minor comments:
1. Figure 1 title and description are missing (line 770, page 26).
Authors Response: We have added a Figure Legends section after the Discussion to include:

Changes to text:
Figure Legends
Figure 1. Flow diagram detailing identification, screening, and selection of studies.

2. The wording 'narrative review' in the title: please consider to rephrase into 'systematic review'?

Authors Response: Thank you for your suggestion. On initial submission it was recommended that we submit this article as Narrative Review.