Unleashing the immune response against childhood solid cancers

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Abstract

Tumor immunotherapy has come to the fore fuelled by impressive clinical responses to checkpoint inhibitor antibodies in a range of adult malignancies, and by the success of CAR T cells targeting adult and pediatric B cell malignancies. Clearly, if appropriately fine-tuned, the immune system has the capability to seek out and destroy cancer. Studies in pediatric solid cancers have not so far shown the therapeutic potential checkpoint inhibitors described in adults cancers and this may reflect fewer tumor associated antigens or different immune evasion mechanisms. One potential approach to overcome these limitations will be combine interventions to undermine immune evasion mechanisms with engineered T cell adoptive transfer.

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The concept that the immune system can be used to recognize and destroy cancers is not new. In the early 20th century new York surgeon William Coley (1862-1936) inspired by his observation of cancer regression in patients recovering from septicaemia, injected cancer patients with live and then dead bacteria. Occasional responses are documented in his records although it is hard now to discern real cancer responses that could be attributed to immune activation. Nonetheless, the concept of cancer immunotherapy was born, and with it the belief that cancer might be inherently foreign to the host and hence amenable of detection and elimination. The degree of general acceptance of this belief has varied over the years but those who have kept the faith and made continuing scientific contributions to the field are to be credited for the resurgence of the field in the last 10 years. In 2013 Science hailed cancer immunotherapy as "breakthrough of the year". What has led to this exponential increase in scientific and clinical trial activity in the field? There are three main areas of scientific and clinical observation that taken together have led to the realization that we have at our disposal new insights and new tools to effect dramatic clinical responses in cancer patients. These are 1) increased understanding of the nature of immune evasion; 2) clinical breakthrough from interventions to overcome immunological checkpoints and 3) the blossoming of technologies for genetic modification of T cells into potent immune effectors.

Understanding of immune evasion

A prediction that follows from the theory that cancer can be seen as foreign is that individuals with defects of the immune system should have a higher incidence of cancer. For many years, the lack of experimental or observational evidence that the facts fitted this prediction led to widespread questioning of the underlying assumptions. A number of important breakthroughs have led to wholesale reappraisal. Firstly, in experimental animal models several groups have convincingly demonstrated that combining chemical carcinogenesis in combination with maneuvers to subvert the function of adaptive immunity leads to significant increases in the numbers of tumors [1]. Performing these sorts of experiments in humans is not ethically possible but certain experiments of nature can be observed to gain insight into human immune evasion. Interesting examples include the increased incidence of cancers associated with polymorphic variants diminishing innate immunity, higher incidence of cancer in the immunosuppressed, and the clinical observation of synchronous resurgence of cancer in multiple metastastic sites after long periods of remission. Evasion of immune destruction was adopted as a newly agreed hallmark of cancer in 2011 indicating the acceptance that it is a fundamental component of the mechanism of tumor development in virtually all cancers [2].

The molecular pathways that are integral in immune evasion are also coming into focus. Key mechanisms include TGF-beta, JAK2-STAT3 and IDO pathways, and these converge to create an immunosuppressive tumor microenvironment including for example, high concentrations of inhibitory cytokines (IL-10, IL-17), downregulation or hiding of cell surface danger signals for the immune response [3], polarization of immune effectors toward inhibitory or regulatory pheontypes, and depletion of essential growth factors or amino acids for immune effector cells [4].

These mechanism have been shown to be common in pediatric solid cancers [5]; evidence including the a predominance of immunoinhibitory genes in the transcriptional signature associated with adverse prognosis in neuroblastoma [6], the activation of STAT3 and inhibitory pathways by the oncogenic PAX3-FOXO1A fusion protein in alveolar rhabdomyosarcoma [7], production of TGF-β by high grade glioma [8, 9] and the inhibitory effects of gangliosides such as GD2 in a number of childhood solid cancers [10]. Immune privilege is the concept that the tumor creates its own "immune-priviledged site; in addition to the above mentioned pathways, there is

evidence in many cancers for stromal cells and the tumor vasculature acting as an effective barrier to the passage of T cells [11]

A number of trial approaches to target childhood solid tumors using immunotherapy have been developed. Table 1 lists representative trials and the general approaches are detailed below.

Clinical trials targeting immunological checkpoints.

The clinical responses in adult cancer patients have fed into biomarker discovery programmes, which are now starting to identify the predictive biomarkers of response and provide insights into mechanism of action of these drugs. For example in melanoma trials for Ipilimumab, whole exome sequencing of pre-treatment tumor tissue has identified mutational load and cytotoxic gene expression profile in the tumor environment to be correlated with clinical benefit [12]. Response rates to PD1/PD-L1 pathway blockade similarly correlate with mutational burden and the presence of tumor-reactive lymphocytes; but the relationship between expression of the PD-L1 (a possible marker for activity of the pathway) and response is less clear; ie. correlation with response in some series but not others. The reason for this variation in the literature probably reflects the fact that some cancers appear to have deregulated PD-L1 in tumor cells as an oncogenic event (eg secondary to genomic amplification) whereas in other cancers its expression is driven by a pro-inflammatory cytokine environment. In the latter scenario, some cancers with naturally occurring responder T cells might have low PD-L1 in resting state due to other mechanisms of immune evasion, but become PD-L1 bright following a pro-inflammatory immunotherapy, by an effect known as “adaptive immune resistance” in which the tumor protect itself by inducing PD-L1 in response to inflammatory cytokines and especially Interferon gamma [13]. Tumors showing adaptive immune resistance are potentially highly responsive to PD-L1 blockade as a component of a combination treatment. Taken together, a strong consensus has developed from the adult oncology field that response to checkpoint inhibitors as single or combination agents correlates very strongly with mutational burden, the presence of tumor-reactive tumor infiltrating lymphocytes, and a cytotoxic transcriptional signature. These three factors are closely related because mutational burden is directly related to neoantigen frequency. A tumor neoantigen is defined as a mutated amino acid sequence specific for a tumor cell, which can be recognized by cells of the adaptive immune system. In the case of T cells this occurs through the normal pathways of intracellular processing of endogenous proteins, which leads to peptides with high affinity for the binding pocket of MHC molecules to thereby be “presented” in a form that can be recognized by the T cell receptor. As the locations of mutations are entirely unrelated to putative MHC binding, whether a mutation is within a peptide fragment that happens to have sufficiently high affinity for self MHC-I to be displayed, is entirely a matter of chance, but the greater the number of tumor mutations, the greater the possibility that a neoantigen will be displayed [14].

Childhood cancers have significantly lower mutational frequency than adult epithelial cancers that generally arise as a result of DNA damage due to environmental exposures [15]. Does this imply that there will be much lower responses to the checkpoint inhibitors? So far the data from trials suggest that this is the case [16]. However there are exceptions including Hodgkin disease where response rates are high, possibly reflecting the presence of T cell responses against shared viral antigens [17], and rare childhood cancers with very high mutational burden, for example high grade glioma with a hyper-mutator phenotype [18]. The availablility of checkpoint inhibitor antibodies should also lead to a clinical trial re-evaluation of vaccine trial

approaches that were previously unsuccessful in pediatrics but might shown more effect when combined with immune-modulation.

Adoptive T cell transfer and Engineering a T cell response

The principle that infusion of tumor reactive T cells can effect rejection of vascularized solid tumors, including brain metastases was initially demonstrated using infusions of tumor infiltrating lymphocytes, especially in melanoma studies. Melanoma has a very high mutational load and the tumor antigen specificities of oligoclonal populations of TILs is emerging through studies utilizing next generation sequencing technologies [19, 20]. A vital new principle from the TIL studies is that conditioning chemotherapy prior to infusion of T cells greatly increases T cell proliferation and survival within the tumor microenvironment, as well as directly contributing to survival [21]. The mechanism is likely to be multifactorial and probably involves conversion of the tumor microenvironment from anti to pro-inflammatory, induction of immunogenic cell death, depletion of regulatory immune cells, and promotion of danger signals from the host microbiome [22].

Engineering of T cells provides one possible solution to the lack of naturally occurring T cell responses in most childhood cancers. Two broad approaches are the genetic modification of a T cell population with a T cell receptor (TCR) recognizing a tumor associated antigen (TAA), or with a Chimeric Antigen Receptor (CAR). TCR transfer has not been evaluated extensively in childhood solid cancer, largely because of lack of appropriate TAAs. CARs combine - within a single molecule - the antigen binding domain of a monoclonal antibody linked via a transmembrane sequence to primary and secondary stimulation domains that mimic potent T cells stimulation [23]. In this way, through transduction of a patient's T cells with CAR, a large T cell population can be redirected towards a cell surface tumor associated antigen leading to potent T cell effector function only in the presence of the tumor [21, 24, 25]. Because the response is driven by antibody, it is MHC-unrestricted and can be fine-tuned for potent responses. Moreover, through modulation of the intracellular signalling of the CAR, maximal T cell responses can be optimized. The ideal T cell response will lead to cytotoxicity but also proliferative response and production of proinflammatory cytokines resulting in sustained memory responses, leading to sustained cancer regression.

In the last 10 years many of the rules concerning optimal CAR T cell design have been learned from experience of treating patients with B cell malignancies in which CD19 has been identified as a very effective target antigen [25-27]. It has been found that second generation endodomains containing CD3 zeta as well as a costimulatory domain are optimal; and that CD28 provides potent activation, whilst TNF superfamily domains such as 41BB or OX40 lead to slower but more sustained responses. It has been established that conditioning of the patient with chemotherapy drugs such as fludarabine and cyclophosphamide (Flu/Cy) greatly improved T cell engraftment, and that the combination is more effective that cyclophosphamide alone [28]. Hence in acute B cell leukaemia trials using second generation CD19 CAR T cells and conditioning of patients with Flu/Cy, unprecedented complete response rates of 80-90% have been described in patients with chemotherapy-resistant disease [25].

Encouraged by the results of the leukaemia studies, several groups have run trials using second generation CAR T cells in solid cancer in which equivalent lymphodepleting conditioning has been given. Despite good antigen expression in the tumor and good apparent functionality in vitro, these CAR T studies have not led to significant regression of solid cancers [29-31] .

Inhibitory cells of the tumor microenvironment

The term myeloid derived suppressor cells (MDSC) is used to describe cells invariably found both in the blood and intratumorally in cancer patients and tumor-bearing

animals. Several subsets in humans have been proposed, with a consensus phenotyping of HLA-DR negative CD33 positive cells being subdivided into MDSC of a more granulocytic lineage (CD15) or monocytic lineage (CD14) [32], the latter associated with production of inhibitory cytokines, arginase and iNOS [33]. MDSC function by a number of mechanisms including depletion of the amino acids L-arginine and L-cysteine needed for T cell function [34, 35]. The depletion results from production of the enzymes arginase and inducible nitric oxide synthase-2 (iNOS2) consequent to local inflammatory cytokines. ARG and iNOS convert arginine to metabolites ornithine/urea and nitric oxide/citrulline respectively, which leads to downregulation of CD3-ζ component of the T cell receptor and loss of T cell function. MDSC also produce high concentrations of TGF-β, which polarizes T cells toward a regulatory phenotype (regulatory T cells, or Treg) characterized by expression of FOXP3 in CD4 cells. Tregs in the tumor environment in turn inhibit effector functions of cytotoxic and helper T cells [36-38]. COX2 expression has been documented within MDSC in both mouse and humans and appears to be a targetable molecule to inhibit MDSC function [39, 40]. Multiple lines of evidence have correlated the presence of MDSC with aggressive phenotype, advanced stage, resistance to immune therapies, and poor prognosis in many cancer types [41-43]. Other tumor infiltrating stromal cells may play important roles in immune evasion. However tumor infiltrating macrophages (M2 macrophages), which play an important role in promoting tumor growth and angiogenesis, may have immune suppressive properties and can be difficult to distinguish from MDSCs. Several other stromal cell types have been identified as protecting tumors from immune attack including tumor associated fibroblasts, which appear to have a functional role of chemoattracting T cells to themselves and away from the areas of transformed cells in the tumor niche.

Neuroblastoma as a model disease for strategies to overcome the inhibitory tumor microenvironment

Neuroblastoma is a childhood tumor where some of the pathways controlling the mechanisms of immune evasion have been carefully worked out, providing an opportunity for immune targeting in combination therapies, for example with gene modified T cells or vaccines. A 14 gene signature derived from expression array analysis of neuroblastoma tumors associated with poor prognosis, which was validated on independent data sets, contains 5 genes reflecting the tumor microenvironment (CD14, CD33, IL-10, CD16, IL-6R) [6]. Collectively these genes suggest activation of inhibitory cytokine pathways within myeloid cells (CD14, CD33) within the tumor, for example activation of a STAT3-IL-6-IL-10 axis as has been described previously in neuroblastoma [44-46]. MDSC have been shown to be integral to neuroblastoma tumor growth. Neuroblastoma patients [47, 48] and tumors have heavy infiltration of myeloid cells [49] which produce arginase and iNOS [50] through an IL-6/STAT3 pathway which inhibits effector T cells function and drives production of regulatory T cells through production of inhibitory cytokines IL-10 and TGF-β .

Other lines of evidence point to multiple pathways by which neuroblastoma avoids immune detection. Neuroblastoma cells characteristically have very low expression of MHC class I [3]. They are known to secrete a number of factors to contribute to immune evasion; including soluble form of MICA/MICB, the receptors for the NK cell and T cell receptor NKG2D; these soluble forms acting as receptor antagonists [51, 52].

Approaches for Targeting Myeloid derived suppressor cells in combination studies with adoptive transfer of tumor-reactive T cells.

MDSC have been shown to be inhibitory to the function of CAR T cells [50, 53]. Several therapeutic approaches have been proposed for blocking function, reducing numbers

of MDSC, and direct cytotoxicity. Such therapeutics could be used in combination treatments with adoptively transferred T cells or other immunotherapies and so could represent a novel approach to target mutationally quiet pediatric solid cancers (Table 2).

Blockade of MDSC function

Sunitinib is a multi-target receptor tyrosine kinase inhibitor that abrogates a number of intracellular signaling pathways including those leading to activation of the STAT3 transcription factor. As well as its direct anti-cancer effects, sunitinib has been shown to decrease function and numbers of MDSC and regulatory T cells in cancer patients [43] and in preclinical models and to be synergistic with immunotherapy [54]. Sunitinib has also shown synergy with tumor irradiation to decrease intratumoral MDSC number and function [55]. In patients treated with sunitinib there has been documented expansion of intratumor lymphocytes and decrease in MDSC [56] as well as decrease in circulating MDSC and regulatory T cells [57]

Arginase production by MDSC is a central mechanism for their suppressive functions. *Inhibitors of arginase* are being developed and entering early phase evaluation in adult cancer patients. A number of existing drugs also inhibit arginase and other suppressive pathways in MDSC and could be redeployed as combination agents with immunotherapy. *Nitroaspirin* is an aspirin molecule covalently linked to nitric oxide and is an inhibitor of both iNOS and ARG [58]. It has an acceptable toxicity after trial in diabetes but there is no previous clinical trial experience in cancer. The COX-2 pathway is known to be targetable in neuroblastoma [59, 60]. COX-2 has been proposed to be a mechanism of arginase expression in MDSC through production of prostaglandin E2 [61-64]. COX-2 inhibition can be achieved pharmacologically by a number of agents but the greatest experience is with *celecoxib* and there is emerging clinical data for its potential to inhibit MDSC and function in combination with immunotherapy [40, 62, 65-67]. Another potential inhibitor of prostaglandin signaling is *sildenafil*, which targets PDE-5 in MDSC and has been shown to augment antitumor immunity [68-70].

A further naturally occurring compound *curcumin*, which is a component of turmeric, has a number of biochemical properties including inhibition of COX2, which is thought to be mediated by inhibition of JAK2/STAT3 signaling [71, 72], and leads to differentiation of immature myeloid cells and enhanced immunotherapy in murine [73] and human [74, 75] tumor models. Curcumin, has excellent safety profile and has been used in pediatric cancer patients [76] but its pharmacodynamics for MDSC suppression has not been described. *Indoleamine 2:3 dioxygenase* is produced by MDSC as well as tumor cells and has multiple immune-inhibitory functions that appear to stem from tryptophan catabolism; so far however there has been limited success in translating IDO inhibitors into the clinic [77].

Decrease of MDSC numbers.

It has been recognized that vitamin A is an essential factor of the differentiation of immature to mature myeloid cells [78, 79], and the use of vitamin A analogue *All trans retinoic acid (ATRA)* in acute promyelocytic leukemia mirrors this differentiation function. In animal studies administration of ATRA leads to decrease in MDSC numbers due to their differentiation into mature proinflammatory myeloid cells [80-82]. Indeed, use of 1μM ATRA to differentiate them into dendritic dells was one included in one of the early studies that defined human MDSC in cancer patients [83]. However, the role of ATRA on MDSC numbers has not been evaluated in human studies, and there are theoretical concerns regarding the ability of ATRA to induce regulatory T cells

[84]. The excellent safety profile and plentiful experience of ATRA in pediatric cancer studies make it an attractive agent to investigate in combination with adoptive T cell transfer.
Another agent with intriguing MDSC differentiating properties is *Polyphenon-E* a component of green tea. Importantly, its function has been demonstrated in neuroblastoma models [47, 48]. However, dosages and scheduling needed for effective combination therapies in humans have not yet worked out. Similarly, *withaferin A*, derived from withania root extract, has anti tumor activity which is through to be derived from STAT3 inhibition and tumor microenvironmental effects [85, 86]. Studies to determine immunomodulation in clinical studies are warranted.
A number of small molecule inhibitors and antibodies targeting the *macrophage colony stimulating factor-1 (CSF-1)* have been developed and evaluated in early phase trials. MDSC and tumor infiltrating macrophages express CSF-1 suggesting a potential combination approach with T cell transfer and other immunotherapies [87].

Cytotoxic killing of MDSC

A number of approaches have been suggested for targeting these cells through their cytotoxic elimination or inhibition of their key functional pathways. Markers of immature myeloid cells such as CD33 could potentially be targeted with antibody or CAR T cells to decrease MDSC numbers in the tumor. Such an approach could be envisaged in combination with an immunotherapy targeting of a tumor antigen. Several antibodies or antibody/drug conjugates such as Gemtuzumab ozogamicin have been evaluated in AML and could potentially be investigated for their ability to kill MSDCs [88]. However lysis of normal myeloid cells might cause unacceptable toxicity or undermine functionally important cells for immunotherapy responses. Use of CAR T cells jointly targeting tumor antigen and myeloid antigen such as CD33 remains a possibility albeit with risk of toxicity through normal myeloid inhibition. Use of *TRAIL targeting antibodies* has been suggested due to the high expression of TRAIL receptor on MDSC [89].
Hitherto, clinical studies with adoptively transferred T cells have focused on the use of lymphodepleting agents such as fludarabine, which has limited effect on myeloid cells, and cyclophosphamide which has been shown to increase MDSC numbers [90-93]. Alternative chemotherapies which are toxic to MDSC as well as lymphodepleting, such as *gemcitabine* [94, 95] and *5-fluorouracil (5-FU)* [96] could be considered as conditioning agents. There is contrary data on 5-FU with one study suggesting it increases MDSC function [97]. However there is no previous published experience of the use of gemcitabine for conditioning adoptive cell transfer in pediatrics and doses would need to be extrapolated from adult studies, for example using Gemcitabine with fludarabine in reduced intensity conditioning [98].

Use of checkpoint inhibitors.

Antibodies blocking the PD1:PD-L1 pathway might inhibit some of the functions of MDSC by blocking the PD-L1 ligand, which is present on tumor infiltrating myeloid cells [99], and thereby preventing inhibitory signaling in the T cells via PD1. *Blockade of CTLA-4*, for example through use of Ipilimumab, might abrogate function of MDSC in at least 2 ways: by interfering with engagement of CTLA-4 on T cells by CD80 and CD86 on MDSC, and by direct cytotoxicity of regulatory T cells that are likely to be present in the tumor environment due to the TGF-β produced by MDSC [100]. Interestingly responses to ipilimumab in melanoma patients have been shown to correlate with decrease in circulating MDSC [101, 102].

Improve entry of T cells into tumors

Interference with chemokine signaling: Evidence from adult malignancies such as pancreatic adenocarcinoma has identified mechanisms to concentrate lymphocytes in the vicinity of tumor stromal cells and prevent infiltration into the areas dense in malignant cells [11]. In pancreatic ductal adenocarcinoma and other tumors, tumor associated fibroblasts attract CXCR4 expressing T lymphocytes through upregulation of CXCL12, leading to a rim of T cell surrounding the tumor [103]. CXCL12 is also known to be a key chemokine for attracting MDSC into the tumor by a COX-2/PGE2 mechanism [65, 104]. Disruption of the interaction with the drug *Plerixafor* leads to T cell infiltration, and its use in combination with anti-PD1 antibody leads enhances tumor eradication in a mouse model [103]. Neuroblastoma stromal and vascular structures are known to express high levels of CXCL12, with weak expression in neuroblasts [105] and CXCL12 homing appears to be key for neuroblastoma metastasis [106].

*Immunocytokine*s: Delivery of an inflammatory cytokine to the tumor niche through linkage to an antibody has the potential to achieve high functional concentrations at the tumor site with the avoidance of systemic toxicity [107]. One approach of interest in neuroblastoma is to effect local delivery of IL-2 for example by combination with the L19 antibody, which targets an alternatively spliced domain of fibronectin that is cancer specific and found in most solid tumors, and hence will localize to the extracellular matrix within the tumor niche. Phase I testing in adults by repeated iv infusion reveals significantly less toxicity than is observed with IL-2, and with evidence of clinical activity in renal cell carcinoma [108]. An immunocytokine comprising humanized ch14.18 anti GD2 antibody fused with IL-2 also has a good safety and toxicity profile, with some evidence of activity in neuroblastoma and could be used to deliver local IL-2 in combination with GD2-CAR T cells in view of the very high target antigen density in this cancer [109].

Other approaches: A large number of other theoretical approaches to decrease MDSC number of functionality in the tumor environment could be considered; for example use of hyperbaric oxygen to decrease hypoxic drive, MEK inhibitors to inhibit pathways in both MDSC and tumor cells

Concluding remarks

Pediatric solid tumor oncologists should be encouraged by the successful experience of immune checkpoint inhibitor antibodies in adult solid cancers, and adoptive transfer of tumor infiltrating lymphocytes in melanoma, or engineered T cells in leukemia. These studies have shown us respectively, that immune evasion can be overcome, that T cells have the potential to induce the lasting regression of solid vascularized tumors, and that engineering of T cell responses is safe and feasible. Moreover childhood cancers have a range of potential targets on the cell surface (table 3) that are ripe for therapeutic exploitation.

Table 1. Representative recent or current clinical trials in pediatric patients outlining the range of therapeutic approaches being developed.

Trial Title	Description	Clinical trials.gov ID
Antigen-targeting antibodies		
CH14.18 1021 Antibody and IL2 After Haplo SCT in Children With Relapsed Neuroblastoma	Antibody and cytokine in allogeneic setting	NCT02258815
Hu14.18-Interleukin-2 Fusion Protein in Treating Young Patients With Recurrent or Refractory Neuroblastoma	Immunocytokine	NCT00082758
Monoclonal Antibody 3F8 and Sargramostim in Treating Patients With Neuroblastoma	Anti-GD2 antibody (MSKCC group) in combination with GM-CSF	NCT00072358
Dinutuximab in Combination With Sargramostim in Treating Patients With Recurrent Osteosarcoma	Anti-GD2 antibody in combination with GM-CSF	NCT02484443
Cytokine based therapies		
Pilot Study of Zoledronic Acid and Interleukin-2 for Refractory Pediatric Neuroblastoma	Cytokine and agent to boost innate $\gamma\delta$ lymphocytes	NCT01404702
Immune checkpoint targeting		
Pilot Study of Nivolumab in Pediatric Patients With Hypermutant Cancers	PD (L)1 pathway blocking antibody for mutation-high gliomas	NCT02992964
A Single-Arm Study to Evaluate the Safety, Tolerability, Pharmacokinetics, Immunogenicity, and Preliminary Efficacy of MPDL3280A (Anti-PD-L1 Antibody) in Pediatric and Young Adult Participants With Solid Tumors	PD (L)1 pathway blocking antibody for solid tumours	NCT02541604
Enoblituzumab (MGA271) in Children With B7-H3-expressing Solid Tumors	B7- H3 is member of the B7 familiy with homology with PD-L1	NCT02982941
Pembrolizumab in Treating Younger Patients With Recurrent, Progressive, or Refractory High-Grade Gliomas, Diffuse Intrinsic Pontine Gliomas, or Hypermutated Brain Tumors	PD (L)1 pathway blocking antibody for brain tumours	NCT02359565
Vaccine –based studies		
A Pilot Study of Autologous T-Cell Transplantation With Vaccine Driven Expansion of Anti-Tumor Effectors After Cytoreductive Therapy in Metastatic Pediatric Sarcomas	Using ex vivo expanded autologous lymphocytes with a vaccine to boost effector cell function	NCT00001566
A Pilot Study of Tumor Cell Vaccine for High-risk Solid Tumor Patients Following Stem Cell Transplantation	Using the setting of resetting the immune system following transplant to vaccinate agasint tumour antigens	NCT00405327
Dendritic Cell Vaccine Therapy With In Situ Maturation in Pediatric Brain Tumors	Vaccine approach using immature DC	NCT01902771
Vaccine Trial for Clear Cell Sarcoma, Pediatric Renal Cell Carcinoma, Alveolar Soft Part Sarcoma and Children With Stage IV Melanoma	Vaccine made from autologous tumour cells and GM-CSF	NCT00258687
Therapy to Treat Ewing's Sarcoma, Rhabdomyosarcoma or Neuroblastoma	Using autologous tumour cells as vaccine	NCT00923351
Cell therapy		
Haploidentical Transplant With NK Cell Infusion for Pediatric Acute Leukemia and Solid Tumors	Ex vivo expansion and infusion without genetic modification	NCT00582816
Genetically modified T cells		
3rd generation gd2 specific chimeric antigen receptor transduced autologous natural killer t-cells for neuroblastoma (ginakit)	Novel approach to use CAR within cells with existing innate killing properties	NCT02439788
3rd Generation GD-2 Chimeric Antigen Receptor and icaspase Suicide Safety Switch, Neuroblastoma, GRAIN (GRAIN)	Third generation CAR for neroblastoma	NCT01822652
A Cancer Research UK Trial of Anti-GD2 T-cells (1RG-CART)	Second generation CAR for neuroblastoma	NCT02761915
A Phase I Trial of T Cells Expressing an Anti-GD2 Chimeric Antigen Receptor in Children and Young Adults With GD2+ Solid Tumors	Third generation CAR for all childhood cancers expressing GD2 neroblastoma	NCT02107963

Table 2. Potential combination agents to use with gene modified T cells for childhood solid cancers.

Agent	Mechanisms of Action	Refs	Suitability for combination with 1RG-CART
Sunitinib	Reduces viability of MDSC in preclinical studies and clinical trials probably through targeting STAT3	[54, 56, 57, 110]	• Readily available and well tolerated in paediatrics • Clinical trial evidence of inhibition of MDSC[43]
Chemotherapy Gemcitabine	Cytotoxic/biologic agents with high degree of myeloid toxicity; Known to kill MSDC; alternative agents of conditioning agent pre adoptive transfer	[94, 95, 110]	• Readily available • Pediatric safety data available • Dose for MDSC depletion not known. • Potential to replace cyclophosphamide
Plerixafor	Disruption of CXCR4-CXCL12 interaction at the surface of tumor cells	[11, 65, 103, 105]	• CXCL12 known to be expressed on neuroblastoma stromal cells • CXCR4 expression on MDSC drives their migration to tumor
Celecoxib	COX2 inhibitor. COX2 stimulates Arginase production in MDSC through stimulation by PG-E2	[40, 61-67]	• COX 2 redegulated in neuroblastoma • Preclinical data for COX2 inhibitors in neuroblastoma • Celecoxib will tolerated and readily available • Clinical trial evidence of inhibition of MDSC function
Arginase Inhibitor CB1158	Reverses the effect of tumor-induced arginases that create an arginine-deplete local and systemic microenvironment	[50, 111]	• Drug currently in phase II in adults • Negotiation with pharma required
Sildenafil	targets PDE-5 in MDSC and has been shown to augment antitumor immunity	[68-70].	• Readily available • Good safety profile in paediatrics • No clinical trial data of its MDSC inhibitory effect
Ipilimumab +/- Nivolumab	Checkpoint inhibitor antibodies that target the CTLA-4 and PL1 T cell inhibitory pathways respectively. Nivolumab will block interaction of PD-L1 high MDSC and TAMs with CAR T cells. Ipilimumab blocks inhibition induced by CD80/86 on tumor infiltrating myeloid cells and is cytotoxic for regulatory T cells that are induced by MDSC. Responses to ipilimumab correlate with decrease in circulating MDSC	[101, 102, 112, 113]	• Anecodal use of PD1 blockade with third generation GD2-CAR does not improve CAR expansion • Single agent Ipilimumab and PD1 pathway inhibitor studies in paediatrics have limited clinical response • More activity for combination therapies than single agent in many cancer types • Ipi/Nivo combination possible through BMS-otherwise prohibitively expensive.
immunocytokines L19-IL-2 Hu14.18-IL2	Capable of local delivery of IL-2 to the tumor site	[108, 109]	• Pediatric phase I data for hu14.18-IL-2 but not for L19-IL-2 • Neither agent available commercially
All Trans Retinoic Acid	Reduces number of monocytic MDSCs and diminishes the suppressive potency of granulocytic MDSCs	[78-84, 114].	• Readily available and well tolerated in paediatrics • Animal data for MDSC inhibition • But no human proof of concept data
Curcumin	Anti inflammatory, anti Jak2/STAT3, COX2 inhibition	[71-76]	• Strong preclinical evidence • Minimal clinical proof of concept studies • Minimal toxicity • Scheduling and pharmacodynamics unknown.
Bevacizumab	Normalizes the vasculature to decrease tumor hypoxia, decrease pathway to promote MDSC, and allow greater entry of T cells into tumor	[115]	• Readily available but expensive • Good phase I data in paediatrics • The vascular normalization hypothesis for improved T cell function in humans has yet to be tested
Withaverin	Anti-tumor activitiy in many models Thought to function via STAT3 and inhibition of immune suppression in microenvironment	[85, 86]	• Well tolerated but needs clinical evidence of immunomodulatory activity in humans
Nitroaspirin	Increased the number and function of tumor Ag-specific T lymphocytes *in vitro* and *in vivo* by decreasing ARG and NOS activity in $CD11^+$ B lymphocytes	[58, 110]	• No previous clinical trial evaluation in adult of pediatric cancer patients
Gemtuzumab	Direct cytotoxicity of myeloid cells expressin CD33	[88]	• Safety and efficacy concerns in AML • No evidence that it is toxic/lytic to MDSC
CSF-1 inhibitor	Small molecules and antibodies to target tumor associated macrophages CSF-1R is expressed on MDSC	[87]	• Not evaluated in paediatrics • No preclinical data in neuroblastoma • Requires pharmaceutical collaboration
Hyperbaric oxygen	Tissue hypoxia drives MDSC	[116]	• No clinical trial evidence for its effect on T cell function • Requires oxygen chamber which is unacceptable in phase I pediatric setting • Evidence that HBO can drive tumor growth in some cancers
IDO inhibitor	IDO is produced by MDSC and by tumor cells and inhibits T cells function by several mechanisms including depletion of tryptophan in microenvironment.	[77]	• No published evidence for an IDO mechanism in neuroblastoma • 5-Methyl Tryptophan is the only agent tested in paediatrics but has limited clinical efficacy
MEK inhibitor	Trametinib Improves functionality of adoptively transferred T cells in Pmel model. MEK pathway activation in neuroblastoma	[117]	• Phase I data available in paediatrics and well tolerated • No clinical evidence of its effect on MDSC function or activity in neuroblastoma • Requires pharmaceutical collaboration
5-FU	Myelotoxic chemotherapy	[96, 97]	• No clinical evidence of its inhibitory function on MDSC • Contradictory data; potential for stimulation of MDSC
TGF-beta inhibition Fresolimab Galunisertib	TGF-β is a fundamental pathway of suppression by MDSC.		• Development and proof of efficacy of TGF-β inhibitors has been disappointing despite many years of effort
Lenolidamide and related inhibitors	Inhibitory effects on range of regulatory immune cells		Competing with other studies in pediatrics Little direct evidence for role in neuroblastoma Requires pharmaceutical collaboration

Table 3. Examples of potential target antigens for pediatric cancers. *Direct targeting with antibodies or CAR T cells is possible with molecules naturally resident on the cell surface and absent from most normal tissues. Tumor specific antigens arise as a result of a characteristic mutation. For such an antigen to be targeted by T cells the mutated amino acids must have the appropriate affinity for self MHC to be presented as a peptide fragment; for example the peptides spanning a chromosomal translocation breakpoint. Non membrane bound tumor associated antigens also require presentation on the cell membrane with MHC and are non mutated (self) peptides but distinguishable by the immune system from non-tumor tissues by virtue of low level expression on normal tissues. For detailed reference list see* [118, 119].

Antigen	Cancer
Directly Targetable cell surface molecules	
GD2	Neuroblastoma, sarcomas
ALK	Neuroblastoma, IMT, rhabdomyosarcoma
FGFR4	Rhabdomyosarcoma
B7H3	Pontine glioma, neuroblastoma
IGF1R	Ewing sarcoma, RMS
EGFR	Medulloblastoma, glioma, sarcoma
STEAP-1	Ewing sarcoma
Fetal Acetylcholine receptor	Ewing sarcoma, rhabdomyosarcoma
PDGFR	High grade glioma
Tumor-Specific Antigens	
PAX3/7-FOXO	Alveolar rhabdomyosarcoma
Histone H3 mutation	Midline high grade gliomas
Ewings translocations	Ewing family sarcomas
EWS-WT1 fusion	Desmoplastic small round cell tumor
Non-membrane bound Tumor associated antigens	
WT1	Wilms' rhabdomyosarcoma
NY-ESO	Synovial sarcoma
PRAME	Neuroblastoma, pontine glioma, Ewing, osteosarcoma, medulloblastoma

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