



# Immunotherapy targets in pediatric cancer

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Immunotherapy for cancer has shown increasing success and there is ample evidence to expect that progress gleaned in immune targeting of adult cancers can be translated to pediatric oncology. This manuscript reviews principles that guide selection of targets for immunotherapy of cancer, emphasizing the similarities and distinctions between oncogene-inhibition targets and immune targets. It follows with a detailed review of molecules expressed by pediatric tumors that are already under study as immune targets or are good candidates for future studies of immune targeting. Distinctions are made between cell surface antigens that can be targeted in an MHC independent manner using antibodies, antibody derivatives, or chimeric antigen receptors versus intracellular antigens which must be targeted with MHC restricted T cell therapies. Among the most advanced immune targets for childhood cancer are CD19 and CD22 on hematologic malignancies, GD2 on solid tumors, and NY-ESO-1 expressed by a majority of synovial sarcomas, but several other molecules reviewed here also have properties which suggest that they too could serve as effective targets for immunotherapy of childhood cancer.

**Keywords:** immunotherapy, tumor antigens, pediatric cancer, adoptive immunotherapy, chimeric antigen receptor, antibody therapy, tumor immunity

## PRINCIPLES GUIDING SELECTION OF TARGETS FOR IMMUNOTHERAPY OF CANCER

Improved outcomes for childhood cancer rests upon the development of new, targeted therapies to kill tumor cells. Much work has focused on targeting oncogenes mediating growth pathways to which the tumor has become “addicted” (Diehl et al., 2007; Sharma and Settleman, 2007; Weinstein and Joe, 2008). This strategy has yielded some impressive results, including the development of ABL inhibitors for chronic myeloid leukemia (Cortes et al., 2011), KIT inhibitors for gastrointestinal stromal tumor, and BRAF inhibitors for melanoma (Dhomen and Marais, 2009; Ribas and Flaherty, 2011). However, not uncommonly, oncogene inhibitors fail to mediate significant antitumor effects due to redundant growth/survival pathways that bypass the targeted pathway (e.g., FLT3 inhibitors in acute myeloid leukemia, EGF inhibitors in lung cancer; Diehl et al., 2007; Christoffersen et al., 2009; Normanno et al., 2009; Siena et al., 2009). For this reason, oncogene-inhibition has not yet assumed a major role in the treatment of pediatric cancers.

An alternative paradigm for targeted cancer therapy utilizes immune based therapies where the principles underlying target selection differ in some ways from those driving selection of oncogene-inhibition targets (Cheever et al., 2009). Most notably, while oncogene-inhibition targets must mediate non-redundant growth/survival pathways, immune targets need not play a significant role in the life or death of the cancer cell since they serve only to direct immune effectors to the tumor cell. Selection of antigen negative variants can occur when targeting non-essential molecules (Czerniecki et al., 2007; Sampson et al., 2010), but thus far this has not been the major factor limiting the effectiveness of immune based therapies (Smith, 2003). Thus, while it is desirable

for immune targets to be involved in tumor growth/survival, this is not a requirement.

A second area whereby target selection for oncogene-inhibition differs from immunotherapy relates to the importance of tumor-specific mutations. Mutated genes are compelling targets for oncogene-inhibition since they implicate the target as a “driver” in oncogenesis, and mutated kinases can be specifically targeted with small molecules. For immune based targeting, mutated targets present both opportunities and challenges. Tumor-specific mutations are predicted to be more immunogenic than non-mutated molecules, since immune tolerance to somatically mutated molecules is not induced during normal development. Consistent with this, early encouraging results have been reported with a vaccine targeting EGFRvIII, a mutated EGFR in gliomas (Sampson et al., 2010). But, the corollary is that the number of epitopes generated by most mutations is small, and the likelihood that any one epitope will be immunogenic is also very small. Because most mutated molecules in cancer reside intracellularly and can only be recognized by MHC restricted T cell receptors, the epitopes that are “immunogenic” will be so only in the fraction of the population expressing the appropriate MHC allele. For example, the breakpoint regions of PAX3-FKHR (van den Broeke et al., 2006) and SYS-SSX2 (Worley et al., 2001) are predicted to be immunogenic in HLA-B7<sup>+</sup> individuals, which comprise only approximately 15% of the population. Immune therapies that target one epitope present in only a small fraction of the population are difficult to standardize and translate into off-the-shelf therapies. Thus, very few tumor-specific mutations have been targeted successfully via immunotherapy.

Despite these challenges, the likelihood that immunogenic epitopes are present in any individual cancer increases with an

increase in mutation frequency. Carcinomas are estimated to carry, on average, 7–10 unique, novel mutations capable of being recognized by the immune system (Segal et al., 2008). Such “tumor-specific” immunogenicity was directly demonstrated in early studies of carcinogen induced sarcomas wherein tumor-specific immunity in individual tumor bearing mice was not found in genetically identical littermates bearing tumors of the same histology, presumably due to the presence of unique tumor-specific mutations in individual tumors (Wortzel et al., 1983). This concept of “private antigens” provides rationale for patient-specific immunotherapies such as whole cell vaccines, and/or broad based immunomodulators such as anti-CTLA4 or anti-PD1, which can augment immunity to “personal epitopes” and result in meaningful antitumor effects across populations, albeit to different epitopes in different individuals (Peggs et al., 2006; Ascierto et al., 2010).

A third point to consider when selecting immune based targets relates to exclusivity of tumor expression. Tumor exclusivity is desirable but rare, and target expression on some normal tissues is not necessarily a barrier to immune based targeting. Many successful immune targets display some normal tissue expression, including cancer–testis antigens that are also expressed on testicular germ cells, prostate specific antigen, and prostate acid phosphatase expressed in normal prostatic tissue, gp100, and tyrosinase expressed by normal melanocytes, and CD19, CD20, and CD22 expressed by normal B-cells. Normal tissue expression does however carry the risk of on-target, off-tumor effects, as evidenced by vitiligo following immunotherapy for melanoma due to damage to normal melanocytes (Turk et al., 2002; Luiten et al., 2005; Gogas et al., 2006), and loss of normal B-cells when targeting B-cell antigens (Kochenderfer et al., 2010). High-level expression can also limit the effectiveness of immunotherapy by inducing immune tolerance or by competing with the tumor target. Moreover, even limited non-tumor expression on vital organs can lead to unacceptable toxicity. Thus, optimal immune based targets do not necessarily require tumor exclusivity, but should have limited normal tissue expression that does not include cells required for maintaining vital tissue or organ functions.

Working from these fundamental principles, this manuscript will discuss candidate targets for immune based therapies of childhood cancer, including cell surface antigens suitable for non-MHC restricted targeting as well as MHC restricted T cell targets.

### CELL SURFACE ANTIGENS: NON-MHC RESTRICTED IMMUNE TARGETS IN PEDIATRIC CANCER

Immune based targeting of tumor cell surface molecules can utilize native antibodies, antibody-derived molecules linked to drugs, toxins, or radioactive isotopes, bi-specific antibodies that recruit effector immune cells (Baeuerle and Reinhardt, 2009), or T or NK cells genetically engineered to express a receptor that recognizes the antigen. Recently affinity-matured oligonucleotide structures, termed aptamers, have been used to target the tumor cell surface as well (Pastor et al., 2011). Regardless of the approach used, one compelling feature of targeting cell surface antigens in pediatric cancers is the lack of MHC restriction, which renders therapies applicable to all patients expressing the antigen on their tumor cells, regardless of MHC allele expression.

Historically, monoclonal antibodies, or their derivatives, could only passively target cell surface antigens, mediating antitumor

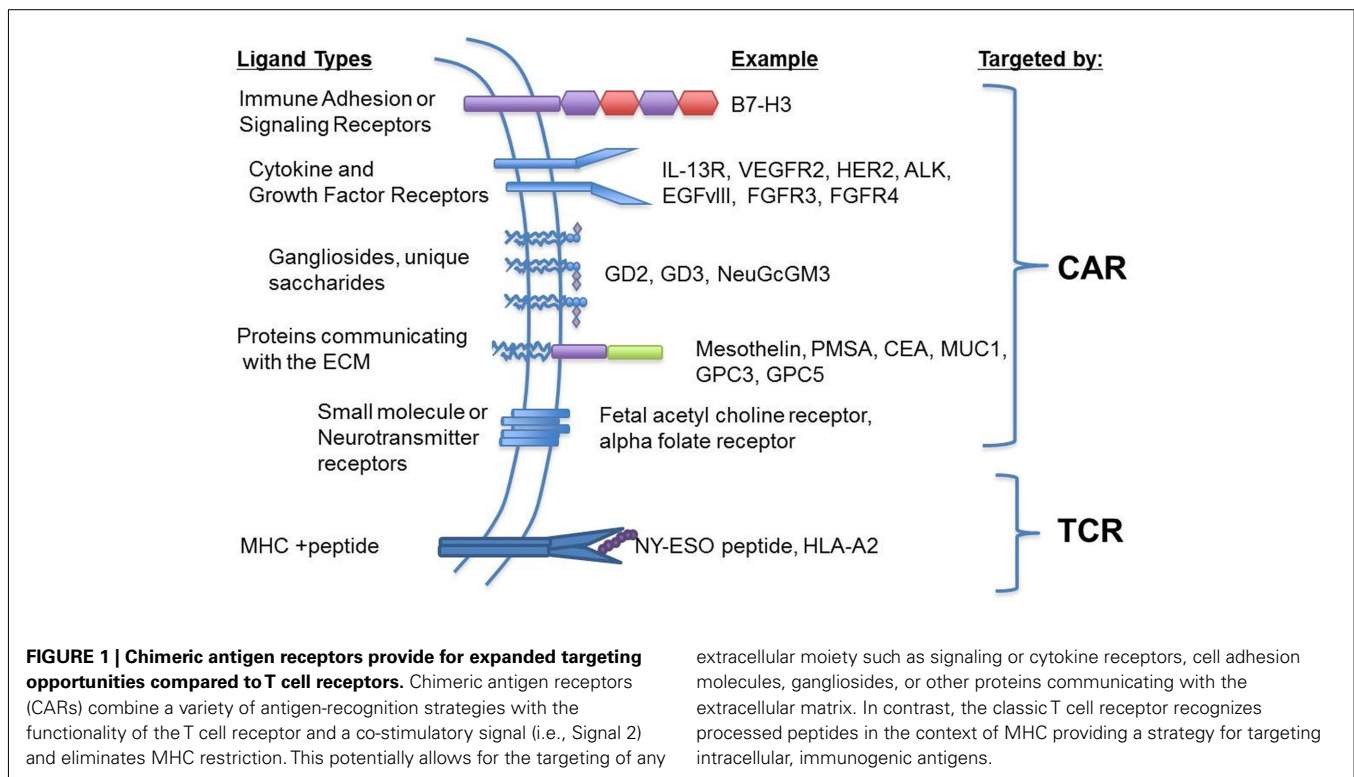
effects only as long as the antibody was present. T cell based therapies, in contrast could mediate active immunotherapy, since they had the potential for *in vivo* expansion, long-term persistence, and immune memory. These advantages of T cell based therapies, however, were offset by limitations posed by MHC restriction, where any particular antigen could only be targeted in individuals with a particular MHC allele. The development of chimeric antigen receptors (CARs), which endow T cells with the reactivity of a monoclonal antibody, combines the benefits of T cell based therapy but with MHC independence (Figure 1). For these reasons, many are optimistic that CARs will ultimately prove to be effective, off-the-shelf reagents with broad applicability in cancer (Urba and Longo, 2011). The fact that academia can generate these therapies, without the large scale pharmaceutical investment required for small molecule oncogene inhibitors, further enhances the attractiveness of this approach for targeting rare cancers, such as those occurring in children. For a detailed discussion of the current strategies and challenges facing CAR-based immunotherapy, please see Lee DW et al., *Clinical Cancer Research* (In Press). Below is a summary of cell surface molecules expressed by pediatric cancers that are candidates for immune based targeting. Those targets that are the focus of substantial current research are discussed in the text, while a more complete listing is provided in Table 1.

### CANDIDATE CELL SURFACE IMMUNE TARGETS EXPRESSED ON HEMATOLOGIC MALIGNANCIES OF CHILDHOOD

#### CD19

CD19 is an ideal immune target, being universally expressed by acute lymphoblastic leukemia, the most common malignancy of children, whereas expression on non-tumor tissues is restricted to B-cells and their progenitors but not hematopoietic stem cells (Nadler et al., 1983, 1984). Despite this favorable tissue distribution, early studies with unconjugated anti-CD19 monoclonal antibodies and anti-CD19 immunotoxins against CD19<sup>+</sup> lymphomas were ineffective (Stone et al., 1996; Furman et al., 2011; Schindler et al., 2011). Recently however, a variety of new therapies targeting CD19 have shown promise. Blinatumomab (anti-CD19 BiTE) is a bi-specific antibody that binds CD19 and CD3, thus activating T cells in close proximity to CD19<sup>+</sup> lymphoblasts. This agent showed antitumor activity in adults with B-cell lymphoma (Bargou et al., 2008), and in adult ALL patients treated with minimal residual disease, 16/21 patients had clearance of leukemia after blinatumomab monotherapy (Topp et al., 2011). Of four patients who suffered a relapse following blinatumomab, two were in sanctuary sites (testis, brain), and two relapsed with CD19<sup>-</sup> ALL, suggesting that tissue penetration and the development of antigen negative variants could pose a challenge for monotherapy with this agent (Topp et al., 2011). Reversible neurotoxicity was also observed in two patients (Topp et al., 2011). A Phase II trial enrolling adult patients with relapsed/refractory adult ALL with at least 5% bone marrow blasts is currently underway (NCT01209286), but pediatric studies have not yet been initiated.

Anti-CD19 CAR therapies have also induced robust clinical responses in CD19 expressing malignancies. Remissions have now been documented in refractory B-cell lymphoma and CLL after administration of lymphodepleting chemotherapy followed by infusion of T cells transduced to express anti-CD19 CAR (Kochenderfer et al., 2010; Kalos et al., 2011; Porter et al., 2011). These



studies provide proof-of-principle that anti-CD19 CAR therapies can mediate impressive antitumor effects. Yet many questions remain regarding the optimal methods for generating CAR T cells, optimal preparative regimens, whether concurrent cytokine therapy improves outcomes and the relative importance of specific T cell subsets in mediating antitumor effects. Two pediatric studies are currently using anti-CD19 CARs to treat ALL (NCT00840853 and NCT01430390). Both administer virus-specific T cells transduced with the anti-CD19 CAR, an approach that substantially extends the time it takes to generate the cells, but could translate into improved persistence as demonstrated using GD2-CAR T cells (Pule et al., 2008). While the elimination of normal B-cells has been observed in adult CD19 CAR trials similar to patients who receive the anti-CD20 monoclonal antibody, rituximab, patients tend to tolerate this on-target side effect with appropriate supportive care. In fact, the constant production of B-cells by CD19<sup>+</sup> progenitors may provide chronic, low-level stimulation to transferred anti-CD19 CAR T cells thereby potentially contributing to persistence and long-term surveillance for leukemia relapse. It is anticipated that several more trials will open soon using anti-CD19 CAR T cells to treat pediatric ALL, using a variety of CAR constructs, T cell populations and treatment schemas.

### CD22

CD22 is also universally expressed on pediatric ALL blasts and its expression is also restricted to the B-cell compartment (Campana et al., 1985; Gudowius et al., 2006). Epratuzumab, an unconjugated anti-CD22 antibody, was not sufficient as a single agent to achieve clinical responses in pediatric ALL, but when combined with four-drug re-induction chemotherapy 9/18 patients achieved a CR with seven of those nine MRD

negative (Raetz et al., 2008). Phase II studies of epratuzumab alone and in combination with other chemotherapy regimens and haploidentical NK cell infusions are underway. Because CD22 is efficiently internalized (in contrast to CD19; Chan et al., 1998) and since internalization is important for the efficacy of immunotoxins, there has also been substantial interest in the development of anti-CD22 immunotoxins. Moxetumomab pasudotox, a high-affinity anti-CD22 antibody conjugated to pseudomonas exotoxin, has induced complete responses in 4/17 (24%) patients with refractory and relapsed pediatric ALL and most patients showed evidence for antitumor activity with little toxicity (Wayne et al., 2010; NCT00659425). Inotuzumab ozogamicin is another anti-CD22 immunotoxin also under study in patients 16 years of age and older with ALL and B-cell lymphoma (NCT01134575). This immunotoxin uses the same calicheamicin derivative found in Mylotarg (gemtuzumab ozogamicin).

Combotox, a 1:1 mixture of anti-CD19 and anti-CD22 antibodies conjugated to deglycosylated ricin-A chain has recently shown hematologic activity in 10/17 children with refractory ALL, of which three were complete responses (Herrera et al., 2009). Similarly, hematologic activity was observed in adults with ALL, although dose limiting capillary leak syndrome was observed (Schindler et al., 2011). Combotox is currently being investigated in adult ALL to determine the MTD when co-administered with 3 days of high-dose cytarabine (4 g/m<sup>2</sup>/day; NCT01408160). DT2219ARL is another bi-specific antibody targeting CD19 and CD22 that is conjugated to diphtheria toxin and is also currently under study in pediatric ALL (NCT00889408; Vallera et al., 2009). Finally, anti-CD22 CAR T cell therapy is being actively pursued with some preclinical activity reported (James et al., 2008).

**Table 1 | Candidate cell surface targets for MHC non-restricted immunotherapy of pediatric tumors.**

IT target	Tumor expression	Normal expression	Comments	References
CD20	PTLD, B-cell lymphomas, CLL	Late pro-B-cells through B-cells Not before late pro-B-cells or on plasma cells	May be upregulated on pre-B ALL with chemotherapy, steroids NCT01363128; NCT01279707	Coiffier et al. (2002), Thomas et al. (2006), Kumar et al. (2011), Meinhardt et al. (2010), Woyach et al. (2011), Evens et al. (2010), Jeha et al. (2006), Piccaluga et al. (2010), Gaipa et al. (2005), Dworzak et al. (2008)
CD19	Pre-B ALL, B-cell lymphomas, CLL	Early pro-B-cells through mature B-cells Not before early pro-B-cells or on plasma cells	Promising early results using CARs NCT01209286; NCT00840853; NCT01430390	Kochenderfer et al. (2010), Nadler et al. (1983, 1984), Kalos et al. (2011), Porter et al. (2011)
CD22	Pre-B ALL, B-cell lymphomas, CLL	Early pro-B-cells through mature B-cells Not before early pro-B-cells or on plasma cells	Internalization facilitates toxin delivery NCT00659425; NCT01134575; NCT00889408	Campana et al. (1985), Gudowius et al. (2006), Chan et al. (1998)
CD30	Hodgkin's lymphoma, ALCL	Activated T and B-cells	NCT01192464; NCT01316146	Stein et al. (1985), Leoncini et al. (1990), Kennedy et al. (2006)
CD52	100% T cell ALL 81% Pre-B ALL (except MLL)	B-cells, T cells, NK cells, monocytes, macrophages, dendritic cells, T cell progenitors	Alemtuzumab has significant on-target, off-tissue toxicity NCT00061945; NCT00988528	Piccaluga et al. (2010), Tang et al. (1996)
CD70	Hodgkin's and diffuse large B-cell lymphomas Renal cell carcinoma Glioblastoma EBV+ undifferentiated nasopharyngeal sarcoma	Activated T and B-cells, dendritic cells	TNF superfamily, cell associated ligand for CD27 CARs using CD27 result in "inherent costimulation" NCT00944905; NCT01015911	Herbst et al. (1996), Lens et al. (1999), Agathangelou et al. (1995), Junker et al. (2005), Law et al. (2006), Wischhusen et al. (2002), Chahlavi et al. (2005), Shaffer et al. (2011)
CD33	AML, MDS APL CML JMML ALL (18%)	Myeloblasts, promyelocytes, myelocytes, monocytes, dendritic cells, macrophages	Gemtuzumab ozogamicin removed from market in 2010 AAML03P1-2 doses in 350 children with AML comparable toxicity, response AAML0531 – Phase III in frontline AML therapy, results pending	Cooper et al. (2011), Andrews et al. (1983), Matutes et al. (1985), Lucio et al. (2001)
CD47	Pre-B ALL T cell ALL AML	Highly expressed in brain (requires therapeutic that is excluded from CSF)	Downregulates innate immune responses via engagement of SIRP $\alpha$ on phagocytes Independent poor prognostic factor in ALL and AML	Chao et al. (2011), Majeti et al. (2009)
IL-7 Receptor $\alpha$	Pre-B ALL, B-cell lymphomas 15% TALL	B-cell progenitors, T cell progenitors, nearly all mature T cells, some dendritic cells	Mutated IL7R $\alpha$ serves as oncogene in 10% of TALL and more rarely in Pre-B ALL	Shochat et al. (2011)

TSLPR (CRLF2)	Pre-B ALL (7%) Pre-B ALL in Down's syndrome (60%)	T cell progenitors, dendritic cells	Mutated TSLPR serves as oncogene in high-risk and Down's syndrome associated Pre-B ALL TSLPR overexpression found in several groups of high-risk Pre-B ALL Associated with t(X;14) or t(Y;14) and activates Jak2 ADVL1011 Phase I study of Jak inhibitor	Russell et al. (2009), Harvey et al. (2010)
ROR1	10% pre-B ALL (associated with t(1;19) CLL Mantle cell lymphoma	Hematogones Adipose and pancreas (low-level)		Hudecek et al. (2010), Broome et al. (2011)
GD2	Neuroblastoma Osteosarcoma Soft-tissue sarcoma Melanoma	GD2(+) neuronal tissue (incl. peripheral sensory nerve fibers) Melanocytes	Functions to maintain and repair nervous tissue Pain is a side effect in GD2-based antibody therapy A number of clinical trials are active	Ohmi et al. (2011), Kushner et al. (2011), Svennerholm et al. (1994)
IL-13R $\alpha$ 2	Glioblastoma Diffuse intrinsic pontine glioma Melanoma Various carcinomas, including mesothelioma	Inducible on keratinocytes, lung epithelium, fibroblasts, smooth muscle	CAR-based therapy and an IL-13-linked toxin therapy for glioma (NCT01082926, NCT00880061) Antibody-based therapy for Crohn's disease (NCT01316601)	Hecker et al. (2010), Kawakami et al. (2006), Takenouchi et al. (2011), Kasaian et al. (2011)
VEGFR2	Tumor vasculature	Normal endothelium CD4+FoxP3+ Treg cells	CAR-based trials (NCT01218867) and antibody-based trials with ramucirumab are open (NCT00917384) DVT was the initial toxicity in antibody-based trials	Suzuki et al. (2010), Chinnasamy et al. (2010), Spratlin et al. (2010)
HER2	Osteosarcoma Colon cancer Breast cancer	Low-level expression on lung parenchyma and high cell dose led to a severe adverse event in an adult CAR trial	Phase II antibody-based trial for osteosarcoma is completed (NCT00023998) CAR trial enrolling Her2-expressing advanced sarcomas (NCT00902044)	Ahmed et al. (2009), Morgan et al. (2010)
ALK	Neuroblastoma Neuroectodermal tumors Glioblastoma Rhabdomyosarcoma Melanoma	Expressed on rare cells in CNS	Must be full-length to be a target for antibody or CAR therapy (as opposed to a fusion protein)	Webb et al. (2009)
EGFRvIII	Glioma	None	Expression is tumor restricted	Bax et al. (2009), Heimberger et al. (2005)
FGFR4	Rhabdomyosarcoma	Expressed during myogenesis		Taylor et al. (2009)
B7-H3	Neuroblastoma Carcinoma cell lines	Mature dendritic cells	CAR and antibody reagents have been described	Modak et al. (2001), Bumbaca et al. (2011)
Glypican-3, -5	Wilim's tumor Neuroblastoma Rhabdomyosarcoma Rhabdomyosarcoma Hepatic carcinoma Melanoma	Rare in mature tissues		Nakatsura et al. (2004), Saikali and Sinnott (2000), Thway et al. (2011)
FOLR1 (alpha-folate receptor)	Rhabdomyosarcoma Osteosarcoma Carcinomas	Luminal cell membrane of some epithelial tissues (and therefore may be hidden from direct recognition in intact tissues)	Antibody, farletuzumab, is currently being tested in ovarian and lung cancer (NCT007386699, NCT01218516)	Clifton et al. (2011)

## CD20

Rituximab (Rituxan<sup>®</sup>), an anti-CD20 monoclonal antibody, was the first approved for use in human cancer and remains one of the most successful immunologics in cancer therapy. Rituximab is now integrated into standard therapies for B-cell lymphomas (Coiffier et al., 2002; Thomas et al., 2006; Meinhardt et al., 2010; Kumar et al., 2011), chronic lymphocytic leukemia (Woyach et al., 2011), post-transplant lymphoproliferative disorder (Evens et al., 2010), and various autoimmune diseases (Stasi et al., 2001; Leah, 2011). Despite substantial depletion of normal B-cells following rituximab therapy, immunosuppression has not limited its use and B-cell numbers return to baseline within 3–6 months of completion of therapy (Thomas et al., 2006; Griffin et al., 2009). Not surprisingly, a variety of anti-CD20 based radioconjugates (Zinzani et al., 2008; Witzig et al., 2011) and CARs have also been successfully developed for adult malignancies (Till et al., 2008). In pediatric B-cell malignancies however, anti-CD20 therapies have had a limited role since neither ALL nor pediatric B-cell lymphomas commonly express CD20. (Jeha et al., 2006; Piccaluga et al., 2010) Interestingly though, recent data suggests that CD20 may be upregulated on ALL blasts after induction chemotherapy or with steroids, providing rationale for renewed efforts at the immunotherapeutic targeting of CD20 in pediatric ALL (Gaipa et al., 2005; Dworzak et al., 2008; NCT01363128; and NCT01279707).

## CD52

Alemtuzumab (Campath<sup>®</sup>) is a humanized monoclonal antibody to CD52 approved for use in adults with chronic lymphocytic leukemia. CD52 is expressed on essentially all T cell ALL blasts and the vast majority of B-cell ALL cases also show high-level CD52 expression (Piccaluga et al., 2010). Despite this, alemtuzumab as a single agent has limited activity in patients with refractory pediatric ALL (Angiolillo et al., 2009). Furthermore, CD52 is widely expressed throughout the hematopoietic system, including B-cells, T cells, NK cells, monocytes, macrophages, dendritic cells (DC), neutrophils, and mast cells. As a result, treatment with alemtuzumab is myelosuppressive and immunosuppressive (Tang et al., 1996), which limits enthusiasm for combining it with other therapies. Nevertheless, trials are underway to explore the efficacy of adding alemtuzumab to intensification or clofarabine in adolescents and adults with ALL (NCT00061945; NCT00983528). Perhaps the most important role for alemtuzumab in pediatric hematology/oncology is as an adjunct to bone marrow transplantation, where it has been shown to enhance engraftment and diminish graft-versus-host disease (Flynn and Byrd, 2000; Shah et al., 2007).

## CD30

CD30 is expressed by anaplastic large cell lymphoma (ALCL) and Reed–Sternberg cells in Hodgkin lymphoma (HL; Stein et al., 1985; Leoncini et al., 1990). A member of the TNF receptor superfamily, CD30 is also expressed by activated and memory T cells, and activated B-cells, where it mediates a complex myriad of activation and regulatory signals via NF- $\kappa$ B (Kennedy et al., 2006; Angiolillo et al., 2009). Early attempts to treat HL and ALCL with unconjugated anti-CD30 antibodies were largely unsuccessful (Ansell et al., 2007; Forero-Torres et al., 2009). However, recently, Younes

and colleagues reported impressive results following treatment of patients with refractory CD30<sup>+</sup> lymphomas with brentuximab vedotin, an anti-CD30 antibody conjugated to the antitubulin agent monomethyl auristatin E. Brentuximab vedotin engages CD30 on the Reed–Sternberg cells and is internalized where its toxin is cleaved, arresting the cell cycle. 36/42 HL patients receiving brentuximab vedotin had tumor regression, of which 11 had complete responses while 2/2 additional complete responses were seen in ALCL patients (Younes et al., 2010). Together, this illustrates that even in the face of disappointing results with unconjugated antibodies, improved efficacy can be attained by using antibodies to direct the delivery of a toxic or cellular payload. To that end, CARs targeting CD30 are currently under study (NCT01192464; NCT01316146), but results have not yet been reported.

## CANDIDATE CELL SURFACE IMMUNE TARGETS EXPRESSED ON SOLID TUMORS OF CHILDHOOD GANGLIOSIDES

GD2's precise role in cell biology remains unclear, but it appears to represent a developmental antigen, since it is expressed on mesenchymal stem cells derived from bone marrow, adipose, and umbilical cord blood (Jin et al., 2006). The GD2 ganglioside is overexpressed on neuroblastoma, melanoma, and some sarcomas (Schulz et al., 1984; Navid et al., 2010) and recent, definitive studies demonstrated that anti-GD2 antibody therapy improves survival in high-risk neuroblastoma (Yu et al., 2010). GD2 on neuroblastoma has also been targeted in clinical trials using first generation CARs with some evidence for clinical activity (Park et al., 2007; Pule et al., 2008) and persistence (Louis et al., 2011). More recent preclinical studies demonstrated improved efficacy of GD2–CARs with the addition of chemokine receptors that improve homing to tumor (Craddock et al., 2010). In both monoclonal antibody and anti-GD2 CAR trials, targeting GD2 has been deemed safe.

Other gangliosides also represent attractive immune targets in pediatric oncology. GD3 is a disialoganglioside that is not expressed on normal tissues, but can be found on the surface of melanoma, soft-tissue sarcoma, and tumors of neuroectodermal origin (Hamilton et al., 1993). High tumor growth rates correlate with high-levels of GD3 synthesis (Ravindranath et al., 2007). First and second generation anti-GD3 CARs induce anti-tumor effects in animal models of melanoma (Lo et al., 2010). Finally, *N*-glycosylated gangliosides incorporate *N*-glycoyl, a molecule not produced by human cells but thought to be derived from diet, then incorporated into the tumor cell surface as a result of a response to hypoxia (Scursoni et al., 2010; Bernstein, 2011). *N*-glycoyl-GM<sub>3</sub> was recently described to be present on the surface of Wilms' tumor, neuroblastoma, and Ewing sarcoma (Mulens et al., 2010). Since *N*-glycosylated gangliosides are not expressed by any normal tissue, these molecules may provide an ideal target for toxin and/or CAR-based immunotherapies.

## GROWTH FACTOR RECEPTORS AND ONCOGENES

### IL-13R $\alpha$ 2

IL-13 is a Th2 cytokine that can bind to the low affinity IL-13R $\alpha$ 1 receptor, that signals in conjunction with IL-4R $\alpha$ , or a high-affinity IL-13R $\alpha$ 2 receptor that binds and internalizes the

cytokine on its own (Wynn, 2003). The low affinity receptor complex activates Jak–STAT pathways of signal transduction while the high-affinity receptor, the normal expression of which is limited to lung epithelial cells and fibroblasts at low-levels that increase with injury (Kawakami et al., 2006; Hecker et al., 2010), signals through the AP-1 transcription factor and induces production of TGF $\beta$  (Fichtner-Feigl et al., 2006). A wide array of tumors express IL-13R $\alpha$ 2, including 80% of glioblastomas, most diffuse intrinsic pontine gliomas as well as melanoma and renal cell, prostate, ovarian, and pancreatic carcinomas (Kioi et al., 2008; Takenouchi et al., 2011). Preferential expression on tumor cells has led to targeting by a variety of immunotherapeutics, including a fusion protein between IL-13 and pseudomonas exotoxin (cintredekin besudotox), which failed to show enhanced efficacy compared to a standard therapy when regionally delivered in glioblastoma multiforme (Kioi et al., 2008; Kunwar et al., 2010), but studies are continuing in pediatric diffuse intrinsic pontine glioma (NCT0088006). Jensen and colleagues reported antitumor effects in a glioblastoma xenograft model of CAR transduced T cells using an affinity enhanced IL-13 instead of antibody-based motifs to direct T cell targeting (Oshima et al., 2000; Kahlon et al., 2004).

#### VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2

VEGFR2 (Flk-1/KDR), a transmembrane tyrosine kinase, is the primary cellular receptor for vascular endothelial growth factor (VEGF). VEGFR2 is unique among tumor targets since it is expressed on tumor vasculature instead of the tumor itself, as well as on regulatory T cells (Brown et al., 1993). Mouse models of malignancy originally identified CD8<sup>+</sup> T cell clones specific for VEGFR2-derived peptides that could inhibit tumor angiogenesis and growth (Dong et al., 2006; Suzuki et al., 2010). VEGFR2-specific CARs have demonstrated antitumor activity in murine models (Chinnasamy et al., 2010) and a clinical trial is currently underway (NCT01218867). While the potential for targeting both T regulatory cells and tumor vasculature is compelling, caution in immune based targeting of VEGFR2 should be exercised as it is expressed at lower levels on normal blood vessels and other tissues and is upregulated on blood vessels during wound healing (Chinnasamy et al., 2010; Edelbauer et al., 2010). In addition, elimination of T regulatory cells may lead to autoimmunity.

#### HER2/Neu (ERBB2)

Monoclonal antibody-based targeting of the amplified human epidermal growth factor receptor 2 (*HER2/Neu*) oncogene with trastuzumab (Herceptin<sup>®</sup>), in breast cancer has been a major success of tumor immunotherapy (Gonzalez-Angulo et al., 2006). Although not clearly credentialed as an oncogene in pediatric cancers (Gilbertson, 2005), Her2/Neu is expressed on Wilms' tumor, medulloblastoma, and osteosarcoma but at levels that are not sufficient for antitumor effects using trastuzumab (Ragab et al., 2010). However, investigators have demonstrated activity of anti-Her2/Neu CARs created using the trastuzumab binding motif (Ahmed et al., 2009; Zhao et al., 2009). Thus the threshold of expression required for targeting cells with CARs may be lower than that for monoclonal antibodies. Unfortunately, the first clinical trial of an anti-Her2/Neu CAR in adult cancer was associated with a fatal adverse event (Morgan et al., 2010), likely related to a

combination of high T cell dose and low-level Her2/Neu expression in the lung, which resulted in massive cytokine release. This event does not necessarily preclude future immune targeting of ERBB2, as lower cell doses or different combinations of signaling motifs may ultimately prove to be safe. In fact, a phase I trial of anti-Her2/Neu CAR therapy in osteosarcoma using a cautious dose escalation scheme is currently underway and substantial toxicity has not been observed so far (NCT00902044).

#### ANAPLASTIC LYMPHOMA KINASE

Anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase in the insulin receptor superfamily, was first discovered as a partner with nucleophosmin in the oncogenic fusion protein found in ALCLs (Webb et al., 2009). During neuronal development, ALK is expressed as a transmembrane protein, then downregulated soon after birth. In mature animals, ALK shows restricted CNS expression, limited to rare neurons, pericytes, and endothelial cells (Pulford et al., 1997; Falini et al., 1999). The true ligand for ALK is still in question as some studies implicate pleiotrophin/midkine, while others do not (Moog-Lutz et al., 2005; Wellstein and Toretzky, 2011). In either case, a double knockdown of ALK and pleiotrophin blocks glioma tumorigenesis. (Grzelinski et al., 2009) Full-length, cell surface ALK expression is observed in a wide array of tumors including lung cancer, neuroblastoma, neuroectodermal tumors, glioblastoma, rhabdomyosarcoma, and melanoma (Webb et al., 2009) and recently ALK was identified as an oncogene in both familial and sporadic neuroblastoma (George et al., 2008; Mosse et al., 2008). Thus, ALK represents a promising target for both oncogene-inhibition and immunotherapy.

#### EGFRvIII

A mutant, constitutively activated form of the epidermal growth factor receptor, EGFRvIII, is overexpressed on the surface of approximately 20% of adult and pediatric gliomas (Heimberger et al., 2005; Bax et al., 2009). EGFRvIII has been targeted with T cell based immunotherapy as a vaccine (Sampson et al., 2010), and in addition, CAR vectors targeting EGFRvIII have been produced and validated *in vitro*, and clinical trials are awaited (Bullain et al., 2009; Ohno et al., 2010). Like ALK, the potential role of EGFRvIII in the growth/survival of these tumors makes it a promising target for both oncogene-inhibition and immunotherapy.

#### FIBROBLAST GROWTH FACTOR RECEPTOR 4

There are over 20 known fibroblast growth factors, the activity of which are mediated by four distinct receptors. Fibroblast growth factors play myriad roles in cell signaling, growth and differentiation, and mutations or alterations in this system have been described in a number of malignancies (Olsen et al., 2003; Wesche et al., 2011). Antibodies to fibroblast growth factor receptor 3 (FGFR3) are currently undergoing trials in multiple myeloma (NCT00866138). FGFR4 is a new target identified in pediatric solid tumors that is overexpressed by nearly all rhabdomyosarcomas with activating point-mutations observed in more aggressive disease (Taylor et al., 2009; Paulson et al., 2011) and with limited expression on normal myocytes. Thus, it also serves as a candidate target for both oncogene-inhibition and immunotherapy.

## OTHER CELL SURFACE MOLECULES

### 4IgB7-H3

Most adhesion receptors are broadly expressed, but some such as B7-H3 are expressed in a more restricted fashion, and thus offer the potential for immune targeting in cancer. B7-H3 has two isoforms; the 2Ig-B7-H3 isoform is expressed on mature DC and a number of carcinoma cell lines (Zhang et al., 2005) but has not been reported on pediatric tumors. However, the 4Ig-B7-H3 isoform is expressed on neuroblastoma (Castriconi et al., 2004) and has been reported to be the target of 8H9 (Xu et al., 2009), a monoclonal antibody which binds to a wide array of pediatric tumors, including sarcomas and most brain tumors (Modak et al., 2001, 2002). Masking of 4Ig-B7-H3 enhances NK cell lysis of neuroblastoma cell lines, suggesting that it may negatively regulate NK signaling (Castriconi et al., 2004). Clinical studies are currently underway using radioconjugated 8H9 for treatment of intraperitoneal desmoplastic small round cell tumor (NCT01099644) as well as for leptomeningeal spread of 8H9 binding tumors (NCT00089245). Due to concerns regarding hepatic expression of the 8H9 binding antigen, systemic administration of this monoclonal antibody has not been pursued. It remains unclear whether hepatic expression of 4IgB7-H3 will preclude systemic targeting of this antigen.

### GLYPICANS

Glypican-3 is a cell surface peptidoglycan found on the surface of hepatocellular carcinoma and melanoma, and glypican-3 mRNA has been demonstrated on embryonal tumors including neuroblastoma and Wilms' tumor (Saikali and Sinnott, 2000; Nakatsura et al., 2004). Recently, a homologous molecule, glypican-5 was demonstrated to be amplified in rhabdomyosarcoma and to play a role in growth/survival of this tumor (Williamson et al., 2007). Thus, at least two glypican family of molecules represent potential targets in embryonal tumors.

### ALPHA-FOLATE RECEPTOR

A humanized antibody specific for alpha-folate receptor (FOLR1), farletuzumab, is currently in clinical trials for patients with ovarian carcinoma (Konner et al., 2010). The expression of this transmembrane folate receptor in normal tissues is limited to the luminal side of epithelium and therefore is not accessible to the vascular system and would not be targeted by monoclonal antibody therapies (Garin-Chesa et al., 1993; Ross et al., 1994). Analyses contained within the public tissue expression database<sup>1</sup> document overex-

<sup>1</sup><http://home.ccr.cancer.gov/oncology/oncogenomics/>

**Table 2 | Non-CTA, MHC restricted immune targets expressed in pediatric cancer.**

Class/target	Expression	Notes	References
<b>DIFFERENTIATION ANTIGENS</b>			
Proteinase-3 (PR-3)	AML (M2, M3 > M4 > M1)	PR-1 is a PR-3 derived peptide that has elicited immune responses in clinical trials	Dengler et al. (1995), Molldrem et al. (1996), Rezvani (2008)
Hyaluronic acid-mediated motility (RHAMM, CD168)	≈70% AML	Limited data on pediatric versus adult leukemias	Greiner et al. (2002, 2003)
STEAP (six-transmembrane epithelial antigen of prostate)	Ewing sarcoma	Data too limited to denote approximate percentage	Hu-Lieskovan et al. (2005), Hubert et al. (1999)
<b>ONCOGENES</b>			
Flt3-ITD	≈25% Pediatric AML	HLA-A1 epitope identified	Brown et al. (2004)
Her2/Neu	≈60% Osteosarcoma* ≈40% Medulloblastoma		Gilbertson (2005), Ahmed et al. (2009), Gorklick et al. (1999), Hughes et al. (2004), Ahmed et al. (2007), Tong et al. (2004)
WT1	≈70–80% AML ≈60–80% ALL ≈100% Rhabdomyosarcoma ≈50% ESFT	HLA-A1, A24, DP5, DR4 epitopes identified	Inoue et al. (1997), Rosenfeld et al. (2003), Ohta et al. (2009), Nakatsuka et al. (2006)
Survivin	All tumors	Fatricide reported by high-affinity survivin-specific CTLs due to T cell survivin expression	Leisegang et al. (2010), Coughlin et al. (2004, 2006), Rapoport et al. (2011)
Telomerase (hTERT)	All tumors		Rapoport et al. (2011)
BCR-ABL (p210)	100% CML	HLA-A2, B8, DR4, DR9 epitopes identified	Yotnda et al. (1998a)
PAX3-FKHR	≈90% Alveolar rhabdomyosarcoma	HLA-B7 epitope identified	van den Broeke et al. (2006)
SYT-SSX1, SSX2	100% Synovial sarcoma	HLA-B7 epitope identified	Worley et al. (2001)
ETV6-AML1	≈25% ALL	HLA-A2, HLA-DP5, HLA-DP17 epitopes identified	Yotnda et al. (1998b)
PML-RAR $\alpha$	100% M3-AML	HLA-DR11 epitope identified	Gambacorti-Passerini et al. (1993)

\*Expression in osteosarcoma is not associated with amplification as observed in Her2/Neu expressing breast carcinomas.



pression in some pediatric tumors as well, including osteosarcoma and Ewing's sarcoma. CAR-based cell therapies targeting FOLR1 are underway and could potentially be extended to FOLR1 pediatric tumors (Kershaw et al., 2006).

### MHC RESTRICTED IMMUNE TARGETS IN PEDIATRIC CANCER WILMS' TUMOR ANTIGEN

Wilms' tumor antigen (WT1) is expressed by several pediatric tumors, and appears to be immunogenic (Cheever et al., 2009). Some concerns remain regarding expression on hematopoietic stem cells, renal podocytes, and potentially other cycling populations, but the community remains optimistic that WT1 may prove to be an effective tumor antigen, as it was ranked the most promising of all tumor antigens by the NCI (Cheever et al., 2009). Several small, non-randomized studies have demonstrated WT1 specific immunity following peptide based vaccination and in some cases this was associated with evidence for anti-leukemic activity. An ongoing vaccine trial is underway for patients with recurrent leukemias and lymphoma at the NCI Pediatric Oncology Branch (NCT00923910).

### SURVIVIN

Survivin is an anti-apoptotic molecule that is overexpressed in most tumors, but also has widespread low-level expression in normal tissues. Survivin-specific T cells have been isolated from patients with cancer, including neuroblastoma, and some groups have demonstrated that survivin-specific T cells can mediate tumor lysis (Cheever et al., 2009). Based upon these results, several investigators sought to develop high-affinity survivin-specific T cells. Remarkably, such cells are able to lyse nearly all tumors but also mediate fratricide of normal T cells due to survivin expression following activation (Leisegang et al., 2010). Thus, normal tissue expression of survivin appears likely to limit the effectiveness of immunotherapies targeting this antigen and similar limitations could apply to other broadly expressed tumor antigens.

Several other non-cancer testis antigens that are candidate MHC restricted T cell targets expressed in pediatric tumors are shown in Table 2.

### CANCER-TESTIS ANTIGENS

Cancer testis antigens [also referred to as cancer germline genes (CGG)] are expressed in a wide array of cancers, with normal tissue expression limited to testicular germ cells and placental trophoblasts (see detailed reviews Fratta et al., 2011 and detailed listing of the genes at <http://www.cta.lncc.br>). The first tumor antigen identified, MAGE-A1, is a prototype and since its discovery the family has expanded to comprise 70 families with over 140 antigens. Over half of the CTAs are encoded by the X chromosome (X-CTA) and it is estimated that approximately 10% of the X chromosome encodes for these molecules. Expression is regulated in large part by epigenetic factors, although the BORIS transcription factor has also been shown to be a potent inducer of their expression (Fratta et al., 2011). The biologic function of CTAs has remained elusive and their scientific interest has been primarily based upon their inherent immunogenicity and their restricted tissue expression, which has made them compelling immune targets.

The CTA family officially encompasses a vast number of molecules (Almeida et al., 2009), but a much smaller number have been

demonstrated to be immunogenic and thus are truly promising targets for immunotherapies (detailed in <http://www.cancerimmunity.org/peptidedatabase/tumorspecific.htm>). Furthermore, analysis of CTAs in pediatric tumors has been limited (Liu et al., 2000; Zendman et al., 2002; Foell et al., 2008; Jacobs et al., 2008; Oba-Shinjo et al., 2008; Grau et al., 2009; Pollack et al., 2011). A summary of the studies reporting CTAs in pediatric tumors is shown in Table 3, but several caveats should be emphasized. First, most studies involve limited numbers of tumors, which are not adequate to reliably define the percent of tumors of any specific histology expressing CTAs, and most studies have not been confirmed. Thus, the prevalence of expression listed for any histology must be considered an approximation at best.

Second, whereas NY-ESO-1 and PRAME each represent one gene, the rest of the CTAs shown in Table 3 represent gene families, for which expression is likely to vary across the specific genes

**Table 3 | Cancer testis antigens in pediatric tumors.**

Antigen	RNA expression <sup>∞</sup>	References
NY-ESO-1/ LAGE-2	≈70% Synovial sarcoma, ≈90% osteosarcoma, ≈30% neuroblastoma, ≈0% ESFT, ≈25% rhabdomyosarcoma, ≈40% ependymoma, ≈10% medulloblastoma	Jacobs et al. (2007), Robbins et al. (2011), Segal et al. (2005), Jungbluth et al. (2001)
PRAME	≈100% Synovial sarcoma, ≈80% medulloblastoma, ≈70% osteosarcoma, ≈40% pediatric acute lymphoblastic leukemia, ≈60% pediatric acute myeloid leukemia	Segal et al. (2005), Vulcani-Freitas et al. (2011), Toledo et al. (2011), Steinbach et al. (2006), Steinbach et al. (2002), Spanaki et al. (2007), Boon et al. (2003), Oberthuer et al. (2004)
MAGE A	≈0–70% Synovial sarcoma <sup>§</sup> , ≈80% neuroblastoma, ≈40% rhabdomyosarcoma, ≈50% ESFT, ≈100% osteosarcoma, ≈30–60% glioma, ≈20–60% medulloblastoma, ependymoma, choroid plexus	Jacobs et al. (2007), Kasuga et al. (2008)
SSX2	≈80% Synovial sarcoma	Segal et al. (2005)
GAGE	≈30% Neuroblastoma, ≈60% ependymoma, ≈20–80% medulloblastoma, ≈10% ESFT, ≈100% osteosarcoma	Steinbach et al. (2006), Kasuga et al. (2008)
XAGE-1	≈40–90% Ewing sarcoma	Liu et al. (2000), Zendman et al. (2002)

<sup>∞</sup> Documentation of protein expression is limited to NY-ESO-1 in synovial sarcoma, and MAGE-A1 and A4 in limited numbers of pediatric solid tumors (Jacobs et al., 2007) and brain tumors (Jacobs et al., 2008).

<sup>§</sup> MAGE-A1 expression is absent in synovial sarcoma by immunohistochemistry (Jungbluth et al., 2001).

within that family. For example, within the MAGE A family at least eight different immunogenic genes have been identified (A1-4, 6, 9, 10, and 12). Given that targeted immunotherapy trials typically focus on one antigen, the percent of tumors that could be targeted with any given vaccine will be limited to those tumors expressing that specific gene. Third, very few studies have incorporated immunohistochemistry or other approaches to confirm protein expression of CTAs in pediatric tumors. In the few that have, some have reported that mRNA expression correlated with protein expression (Jacobs et al., 2007), while others have noted that mRNA positivity is associated with patchy or very weak immunohistochemical staining (Oba-Shinjo et al., 2008). Together, one can conclude that CTAs are likely to be expressed in a sizable percentage of childhood cancers, but substantially more work is needed to accurately define the prevalence of CTA protein expression among pediatric histologies, to better characterize the intensity, distribution, and extent of homogeneity of CTA expression within tumors. Clarification of these issues is needed before one can accurately predict whether targeting CTAs for immunotherapy of pediatric cancer is likely to be effective.

One tumor where robust CTA protein expression has been clearly documented is synovial sarcoma. NY-ESO-1 expression is seen in approximately 70% of synovial sarcomas and is characterized by diffuse, intense protein expression. Furthermore, preliminary results demonstrate that meaningful antitumor effects can result from targeting NY-ESO-1 in these tumors, most recently using adoptive transfer of T cells genetically engineered to express a high-affinity NY-ESO-1 peptide specific T cell receptor (Robbins et al., 2011). Despite these exciting results, challenges in translating this therapy on a larger scale epitomize the challenges unique to T cell based immunotherapies. The TCR used in this therapy targets an HLA-A2 restricted peptide, and approximately 40% of

the Caucasian population expresses HLA-A2, while lesser percentages are found in individuals of Asian and African descent. There are approximately 800 new diagnoses of synovial sarcoma each year, of which approximately 200 small tumors (<5 cm) have an excellent prognosis with surgery alone<sup>2</sup>. Thus, fewer than 200 new cases of synovial sarcoma per year could benefit from this exciting emerging therapy. Given the rarity of this disease, it is easy to understand why executing trials and standardizing MHC restricted T cell based therapies for rare cancers is challenging.

#### FERTILE GROUND FOR NEW CANCER IMMUNOTHERAPEUTICS

We have discussed the major, current attractive targets for immunotherapy in pediatric cancer taking into consideration expression on normal or vital tissues. While much progress has been made in pediatric oncology, some subgroups, such as those with metastatic sarcomas and brain tumors, have not seen major improvement in survival over the past few decades despite intensifying chemotherapies. The long-term benefit of monoclonal antibody therapy has yet to be proven and none except anti-GD2 have become standard therapy in pediatric oncology. In addition to further developing monoclonal antibody therapy, the field is moving to use the target cell specificity of antibody or antibody-like molecules as a means to deliver toxins or immune effector cells to the tumor. Advances in small molecule technology, production of new forms of antibody, and innovations in cell production techniques are responsible for recent exciting results, albeit in small numbers of patients, mostly limited to adult oncology. Still, these techniques are just now becoming applicable to the clinic and are likely to be a fertile arena for clinical progress in pediatric oncology for the next decade.

<sup>2</sup>[http://sarcomahelp.org/learning\\_center/synovial\\_sarcoma.html](http://sarcomahelp.org/learning_center/synovial_sarcoma.html)

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