Immunotherapy of Neuroblastoma: Facts and Hopes

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ABSTRACT

While the adoption of multimodal therapy including surgery, radiation, and aggressive combination chemotherapy has improved outcomes for many children with high-risk neuroblastoma, we appear to have reached a plateau in what can be achieved with cytotoxic therapies alone. Most children with cancer, including high-risk neuroblastoma, do not benefit from treatment with immune checkpoint inhibitors (ICI) that have revolutionized the treatment of many highly immunogenic adult solid tumors. This likely reflects the low tumor mutation burden as well as the downregulated MHC-I that characterizes most high-risk neuroblastomas. For these reasons, neuroblastoma represents an immunotherapeutic challenge that may be a model for the creation of effective immunotherapy for other "cold" tumors in children and adults that do not respond to ICI. The identification of strong expression of the disialoganglioside GD2 on the surface of nearly all neuroblastoma cells provided a target for immune recognition by anti-GD2 mAbs that recruit Fc receptor–expressing innate immune cells that mediate cytotoxicity or phagocytosis. Adoption of anti-GD2 antibodies into both upfront and relapse treatment protocols has dramatically increased survival rates and altered the landscape for children with high-risk neuroblastoma. This review describes how these approaches have been expanded to additional combinations and forms of immunotherapy that have already demonstrated clear clinical benefit. We also describe the efforts to identify additional immune targets for neuroblastoma. Finally, we summarize newer approaches being pursued that may well help both innate and adaptive immune cells, endogenous or genetically engineered, to more effectively destroy neuroblastoma cells, to better induce complete remission and prevent recurrence.

Introduction

The cancer immunotherapy revolution is exemplified by the outstanding success of checkpoint inhibitors in melanoma (1) and certain adult carcinomas (2) and chimeric antigen receptor T (CAR-T) cells in both adult and pediatric hematologic malignancies (3, 4). In contrast, the majority of childhood solid cancers have seen few clinical successes from immunotherapy, with especially disappointing response rates to checkpoint inhibitors (5, 6).

Neuroblastoma, a cancer of the sympathetic nervous system that derives from neural crest cells, is the most common extracranial solid malignancy occurring in children and accounts for approximately 10% of pediatric cancer deaths (7, 8). Like many childhood malignancies, neuroblastoma is a disease of disordered development, meaning that the malignancy is driven by aberrant expression and regulation of developmental proteins (9). The core regulatory circuitry driving neuroblastoma consists of normal human proteins that are expressed during embryonic development but largely turned off postnatally in normal tissues. The adaptive immune system is thought to be unable to target these so-called oncofetal antigens because high-affinity, self-reactive T cells are deleted during thymopoesis to prevent autoimmunity. It is therefore tempting to adopt a reductionist viewpoint that childhood cancer is not immunogenic, and thus alternate therapeutic strategies should be prioritized. However, neuroblastoma stands out among pediatric solid cancers as the exemplar where immunotherapy (with anti-GD2 antibodies) has been incorporated into both front-line and relapse treatment protocols to significantly improve patient outcomes and increase cure rates.

The Facts

Immunotherapy for neuroblastoma

The relative success of immunotherapy with anti-GD2 mAbs raises the question of whether neuroblastoma is an immunogenic tumor. Here, care must be taken in the definition of terms. If immunogenicity refers to a cancer's rejection by an adaptive immune response, there is scant clinical evidence for this phenomenon in patients with neuroblastoma. Lack of consistent clinical responses to checkpoint inhibition (6, 10) or vaccination approaches is consistent with histopathologic evidence of a general lack of tumor-reactive infiltrating T cells in a majority of cases (11–14), although high-risk neuroblastoma with higher T-cell infiltrate has been associated with improved survival (15). Tumor mutational burden estimations place primary neuroblastoma among the least mutated of human cancers, consistent with an "immunologically cold" classification (16).

Despite the lack of evidence of an adaptive immune response, there is also ample evidence that neuroblastoma, in common with most human cancers, has immune evasion hardwired into its biology (12) through mechanisms such as downregulation of MHC class-I (17), infiltration by suppressive myeloid cells (13, 18–20), and production of inhibitory factors such as arginase-2 (21) and TGFβ (22). This raises the intriguing possibility that the relative coldness of neuroblastoma may reflect immune evasion as much as a lack of inherent danger, providing some encouragement for immunotherapeutic strategies.

While harnessing a native immune response in neuroblastoma has largely been unsuccessful, researchers have instead focused on engineering synthetic immune recognition to help activate a response by endogenous or laboratory-manipulated immune cells (**Fig. 1**; **Table 1**).

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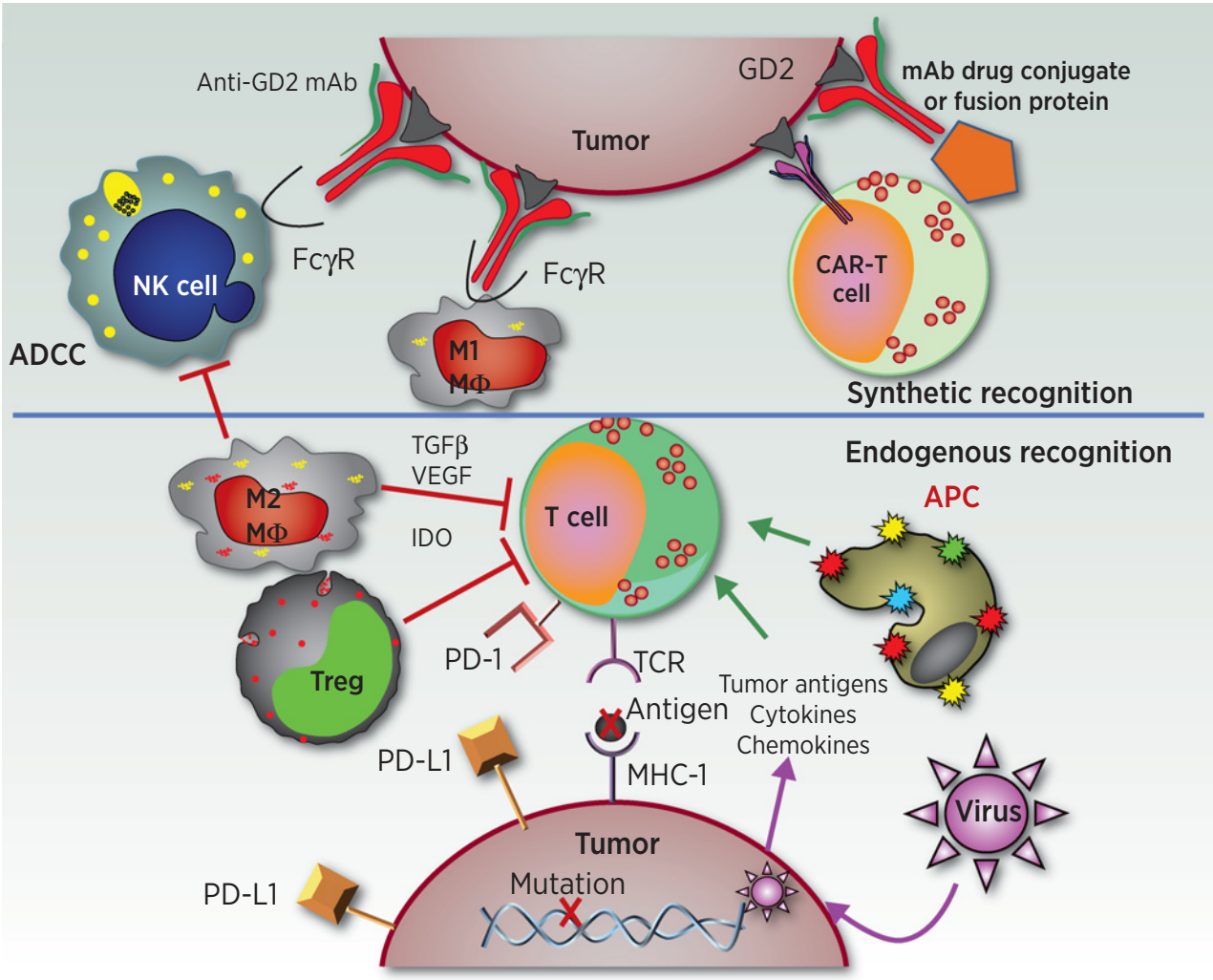

Figure 1.

Endogenous and synthetic recognition involved in immunotherapies for neuroblastoma. This simplified schematic shows some of the relationships between immune cells, molecules, and cancer cells involved in current and developing immunotherapies for neuroblastoma. The synthetic recognition pathways are shown above the horizontal line, and are all shown here as mediated via mAb-induced tumor recognition. The mAb-based tumor-recognition components, shown at the top, include an intact anti-GD2 mAb (at top left) binding to GD2 on the tumor engaging the Fcγ receptor (FcγR) on the NK cell or on the M1 macrophage (MΦ) to activate ADCC. At the top right is that same anti-GD2 mAb, now carrying a "payload." This payload can be a drug, as an ADC; an immune activator as in a fusion protein, such as an IL2-linked immunocytokine; a radionuclide; or a toxin. To its left is a CAR-T cell that utilizes the ScFv of the anti-GD2 mAb to provide anti-GD2 recognition for the genetically modified T cell. Below the horizontal line are the pathways involved in endogenous recognition, with a central role given to effector T cells. At the bottom of the endogenous T cell is its T-cell antigen receptor (TCR), which on clonally derived T cells can recognize tumor-associated peptides presented by the MHC molecules on the tumor surface. This recognition and T-cell activation can induce effector functions, including cytokine release, activation of innate immune antitumor cells, and direct T cell–mediated tumor cell lysis. Those tumor-associated peptides can be mutation-driven neoantigens (shown here) or germline controlled proteins that have restricted expression to tumor cells, with little or no expression on normal postnatal tissues. To the left of the T cell are endogenous cells that can interfere with T-cell function. One such inhibitory cell is an M2 macrophage (MΦ), which can interfere with antitumor immunotherapy via many pathways, including release of TGFβ, VEGF, and indoleamine 2,3-dioxygenase (IDO). Other myeloid elements, such as myeloid-derived suppressor cells (not shown), can also interfere with effector immune function. Regulatory T cells (Treg) are normally FoxP3$^+$ CD4$^+$ T cells that can directly kill or inhibit the functions of effector T cells. These inhibitory cells can also interfere with NK-cell function (not shown). To the right of the T cell is an antigen-presenting cell (APC), normally a dendritic cell, that picks up and processes tumor antigens and then presents them to T cells to induce an endogenous adaptive immune response. Cytokines and chemokines can help recruit immune cells into the tumor. Certain oncolytic tumor viruses are being injected in some trials to infect the tumor, release more chemokines, and recruit additional immune cells to the tumor microenvironment. The immunosuppressive PD-L1 ligand is one of several checkpoint molecules expressed by tumor cells (and shown here). PD-L1 activates the immune-inhibitory PD-1 receptor on the T cell (shown) and some NK cells (not shown). Not depicted is how anti-PD1 or anti–PD-L1 mAbs (forms of immune checkpoint blockade) can block these inhibitory interactions, enabling T and NK cell functionality in the suppressive tumor microenvironment.

Table 1. Types of immune recognition potentially relevant to neuroblastoma immunotherapy.

Immune recognition	Type	Caveat (requires)	Example	Clinical relevance and application
Endogenous	T-cell receptor	Peptide presented by MHC on cell surface	NY-ESO-1 presented by MHC on NBL cells to autologous T cells (71)	Endogenous T-cell responses of patients with HR-NBL are weak, due to the substantial immunosuppressive chemotherapy received. No effective vaccine to stimulate yet tested in NBL.
	Antibody	Cell surface molecule	1. Antibody seen in OMS sees neuroblastoma (111) 2. GD2/GD3	1. Patients with NBL and opsoclonus-myoclonus syndrome (OMS) have induced an endogenous antibody against their neuroblastoma and also to normal CNS, causing this autoimmune syndrome. 2. Vaccination to these gangliosides is inducing antibody to them that may delay/prevent relapse
Synthetic	mAb	Cell surface molecule	GD2 (23)	Three separate mAbs to GD2 ganglioside have been approved for clinical use and have shown antitumor benefit in preventing relapse for patients in remission, for inducing responses for relapsed disease, and for antitumor effects when combined with chemotherapy for relapse, with early data indicating benefit when included with chemotherapy during induction.
	CAR	Cell surface molecule	GD2 (recognized by mAb ScFv) (49, 50, 80)	Several trials are testing CAR-T cells with CARs directed at GD2 through mAb technology, with some showing early signs of antitumor benefit.
	CAR	Cell surface MHC presenting a tumor peptide	PHOX2B peptide presented by HLA-I (95)	Even though PHOX2B is an NBL "driver" expressed only in cytoplasm and nucleus, its peptides are presented on the surface by MHC-I. A mAb against the PHOX2B peptide/MHC-I complex has been put into CAR-T cells and mediates potent tumor destruction *in vitro* and *in vivo* in PDX models.
	T-cell receptor	Peptide presented by MHC on cell surface	NY-ESO-1 (69, 112–115)	Using *in vitro* binding and selection processes, T-cell receptors specific for the NY-ESO-1 antigen (seen on some neuroblastomas and several tumors in adults) can be cloned from lymphocytes and transfected into cells of a cancer patient to get autologous tumor killing *in vitro*. Clinical testing in other diseases is proceeding.

Note: Different immune recognition mechanisms of different types, each with separate caveats for translation, are indicated for the antigens exemplifying their use, and with mechanistic clinical considerations for each.
Abbreviations: NBL, neuroblastoma; PDX, patient-derived xenograft.

By engineering therapeutics that can recognize neuroblastoma cells, scientists have been able to create a new immune response in tumors that otherwise appear impervious to native immune recognition. Such immunotherapies that use synthetic immune recognition are typically based on mAbs. mAbs recognizing the disialoganglioside GD2, overexpressed on most neuroblastoma cells, have revolutionized the care of neuroblastoma, increasing event-free survival by up to 20% (23). Other synthetic recognition agents can be further engineered from antibody derivatives, including CAR-T cells and antibody–drug conjugates (ADC). These therapeutics have begun to demonstrate signs of preclinical and early clinical efficacy, indicating that the immunotherapy revolution is poised to further alter the neuroblastoma treatment landscape.

Anti-GD2 antibodies

Evidence-based therapy for high-risk neuroblastoma prior to 2009 relied on combining surgery, local radiotherapy, and gradually more aggressive combination chemotherapy regimens, supplemented with supralethal chemotherapy-based "consolidation" regimens requiring autologous hematopoietic stem cell rescue. While this approach prolonged survival for some, fewer than 40% of patients survived for more than 5 years without relapse; relapsed disease could only rarely be cured (24). Only a decade after the original description of mAb selection and production, separate studies led by Reisfeld and by Cheung identified murine neuroblastoma-reactive mAbs 14.18 (later class switched to generate 14G2a) and 3F8, respectively, shown to recognize disialoganglioside, GD2 (25, 26). These mAbs could recognize GD2 on a variety of cancers, including some melanomas, small cell lung cancers, osteosarcomas. They were particularly able to recognize neuroblastomas, which appeared to show relatively uniform, high-level expression on virtually all tumor cells from nearly all patients. Preclinical studies demonstrated that anti-GD2 antibodies were effective and that their major mechanism of activity was via antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 27).

ADCC is mediated via Fc receptor–bearing cells: natural killer (NK) cells that can be activated with IL2 stimulation, and macrophages and other myeloid cells whose production can be stimulated with GMCSF. Preclinical data suggested *in vivo* antitumor efficacy was better realized in the face of microscopic, rather than bulky disease (28). The Children's Oncology Group (COG) ran a large randomized trial for patients in remission or partial remission from standard upfront chemotherapy, treating them with dinutuximab (a murine-human chimeric version of 14G2a bearing a human IgG1 Fc region) in combination with IL2 and GMCSF, added to the standard of isotretinoin (23). Patients receiving the immunotherapy showed improved event-free survival (EFS) and overall survival (OS) initially, and after nearly a decade of follow-up (23, 29). This led to the FDA approval of dinutuximab, as the the first mAb approved specifically for a pediatric cancer indication and the first effective mAb recognizing a lipid-based cancer molecule. A similar antibody, dinutuximab-beta (produced in

Chinese hamster ovary cells), is approved in Europe, although it was not tested in a randomized fashion (30, 31). A third anti-GD2 antibody, naxitamab, a humanized version of 3F8, was recently FDA approved based on its activity in regressing neuroblastoma in patients with relapsed or refractory disease limited to bone or bone marrow (32, 33).

Based in part on preclinical data, and on the clinical development of anti-HER2 mAb in combination with chemotherapy as breast cancer treatment, COG and the St. Jude Children's Research Hospital each independently began testing anti-GD2 mAb in combination with conventional chemotherapy, for relapsed and refractory neuroblastoma, including bulky disease (34, 35). The randomized COG trial ANBL1221 compared a combination of dinutuximab with chemotherapy (irinotecan/temozolomide, I/T) versus temsirolimus (a targeted agent) with I/T and found that chemoimmunotherapy was highly effective in patients, especially those with chemorefractory disease. Remarkably, patients with bulky disease experienced significant regressions of otherwise chemorefractory soft-tissue masses. A number of responses appeared durable (34, 36). For this reason, this anti-GD2 mAb + chemotherapy approach has become standard for patients with relapsed or refractory disease and was also incorporated into induction chemotherapy for patients with newly diagnosed neuroblastoma in a recent St. Jude study with promising efficacy (37, 38). COG is now also pursuing this strategy in larger trials (including the recently completed, but not yet published ANBL17P1 trial of dinutuximab incorporated into induction as well as maintenance phases).

The impressive progress with anti-GD2 antibody has also uncovered important challenges. First, many patients relapse despite having received anti-GD2 during upfront therapy, and the combination of anti-GD2 and chemotherapy induces responses in <50% of patients with relapse (23, 29, 34, 36). Second, some patients have decreased GD2 expression at relapse, suggesting *in vivo* antigen remodeling in response to anti-GD2 (39, 40). Third, the administration of anti-GD2 mAbs is associated with substantial neuropathic pain, driven by mAb binding to GD2+ myelin sheaths of nerve fibers (41, 42); this restricts the MTD of anti-GD2 far below the doses used (~1/10 on a mg/M^2 basis) for other approved tumor-reactive mAbs (23, 43–45), and requires substantial administration of narcotics and other analgesics even at these low doses. These three challenges emphasize the need to identify additional cell-surface antigens, other than GD2, that are selectively overexpressed on neuroblastoma and can be targeted with mAbs to overcome antigen-loss escape, devise additional anti-GD2 strategies that can overcome tumor cell resistance, and reduce the neuropathic pain associated with current anti-GD2 mAb-based therapy.

Anti-GD2 CAR-T cells

Building on their successes in leukemia, CAR-T cells have emerged as a promising approach for immunotherapy based on synthetic immune recognition for so-called "immune cold" solid cancers (46). The clinical validation of GD2 as a target antigen drove early adoption of CAR-T cells in neuroblastoma. In fact, neuroblastoma was the first pediatric cancer to be targeted with CAR-T cells in a clinical trial. A trial of first-generation CAR-T cells, containing the same antigen recognition domain as dinutuximab (but no embedded costimulatory domain), mediated several clinical responses, and demonstrated no signs of on-target, off-tumor neurotoxicity despite known expression of GD2 on peripheral nerves (47, 48). A subsequent trial with an altered GD2 CAR-T-cell design was disappointing due to lack of clinical responses, even when combined with checkpoint inhibition (49). However, recently published work employing a CAR with an alternate anti-GD2 binder (50) and an abstract from a trial with a next-generation 14G2a-based CAR-T cell (51) have both demonstrated signs of clinical efficacy, including multiple complete responses in the second trial. GD2 CAR-T cells have similarly demonstrated clinical efficacy in patients with the universally fatal brainstem tumor, diffuse intrinsic pontine glioma (52).

Of note, despite GD2 expression on neural tissues and a high incidence of infusion-related pain in patient receiving mAbs targeting GD2, patients have not experienced on-target, off-tumor toxicity in trials of GD2 CAR-T cells. The precise mechanistic reasons for the different off-tumor toxicity of GD2 CAR-T cells versus anti-GD2 antibody remain yet to be fully elucidated. However, some important conclusions can be made: (i) CAR-T cells demonstrate a therapeutic window when targeting antigens expressed at low levels on normal tissue (52, 53) and (ii) the mechanism of anti-GD2 antibody-associated allodynia/neuropathy may be specific to antibody-based therapeutics, with evidence of the role of complement recruitment potentially playing a role (42). In patients with neuroblastoma, reported toxicities of GD2-targeting CAR-T have so far been related to immune activation and cytokine release syndrome (47–51). As these and other ongoing GD2 CAR trials continue to mature, it is likely that further clinical advances will be achieved.

Other immunotherapy targets in neuroblastoma

While the disialoganglioside GD2 is the most well-known and most highly expressed target in neuroblastoma, several other molecules have been identified as overexpressed on the surface of neuroblastoma cells for use in antibody-based immunotherapies (naked antibodies, antibody conjugates, bispecific antibodies, and CAR-T cells). Many of these have been targeted in preclinical models and early phase clinical trials. B7-H3 (CD276) is a checkpoint molecule (from the same family as PD-L1) that is broadly overexpressed on neuroblastoma and most other pediatric solid tumors, but has highly restricted expression on normal tissues. This molecule was first targeted by researchers at Memorial Sloan Kettering with an antibody named 8H9 before its exact target was even identified (54, 55). This antibody is currently being developed as a radioconjugate (omburtomab) for use in patients with neuroblastoma that has spread to the central nervous system (CNS) and other primary CNS malignancies (56–58). Another B7-H3–targeted antibody [MGA271 (59), enoblituzumab] has been tested in children with solid tumors including neuroblastoma (NCT02982941, results not published). B7-H3–targeted CAR-T cells have shown promise in preclinical models of pediatric cancer (60, 61), and have recently reached the clinic for patients with neuroblastoma (NCT04483778).

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is mutated or amplified in approximately 14% of patients with neuroblastoma (62) and is often expressed on the surface of neuroblastoma cells (63). Both ADCs and CAR-T cells targeting ALK have been described in preclinical studies (64, 65). GPC2 was recently discovered to be expressed on neuroblastoma tumors, particularly those harboring MYCN amplification (66). Both ADC- and CAR-targeting approaches for GPC2 have also been described previously (66, 67). In the case of both ALK and GPC2, the limited expression density on neuroblastoma (compared with the highly expressed GD2) may limit the efficacy of these therapeutics with the current generation of CAR-T cells (64, 68). Other targets identified in neuroblastoma include NCAM [preclinical studies describing an ADC (69)] and L1CAM (70) [L1CAM CAR is currently in clinical trials (NCT02311621)].

While early phase clinical trials are ongoing for several antibody-based therapeutics, other preclinical research has focused on targeting intracellular proteins that are specific to neuroblastoma. So-called cancer testes antigens, including NY-ESO-1 (71) and PRAME (72), have been identified as immunotherapy targets for neuroblastoma. These antigens, which are overexpressed in cancer but not in most normal tissues other than testes, have been safely and effectively targeted in patients with other malignancies using engineered T-cell receptors (73, 74), but these are yet to be clinically deployed in the context of neuroblastoma.

Hopes

As anti-GD2 antibodies have already proven an essential part of the anti-neuroblastoma armament, we anticipate that the role of immunotherapy in neuroblastoma will continue to grow as new targets are identified and newer targeting technologies are developed. Here, we explore the emerging data that we believe is poised to alter the trajectory of immunotherapy for neuroblastoma.

Innate immunity

While neuroblastomas demonstrate little evidence of T-cell infiltration (11–13) and T-cell checkpoint inhibition has thus far not worked well in the clinic (6), other cell types in the tumor microenvironment may also be harnessed for antitumor activity. Neuroblastoma tumors are well known to be infiltrated by innate immune cells, including NK cells and macrophages (20, 75). These cells are thought to be the major effectors involved in the efficacy of anti-GD2 antibody. In fact, patients inheriting certain NK-cell receptors (KIR) and their ligands are more likely to derive benefit from anti-GD2 antibody than those patients lacking their expression (76, 77). To further harness the activity of NK cells, researchers have attempted to administer *ex vivo* expanded NK cells with anti-GD2 antibody to patients with neuroblastoma. While some responses have been seen, it is unclear how much of this is attributable to the adoptive transfer of NK cells as opposed to the anti-GD2 antibody itself (78). More work is needed to understand whether administration of unmanipulated, or *in vivo* activated, NK cells can improve outcomes of patients with neuroblastoma. NK cell–mediated ADCC can be augmented via PD1 blockade, and this approach is now being tested with anti-GD2 (79). Researchers have also attempted to enhance the efficacy of NK cells by endowing them with CARs, including those recognizing GD2. Researchers at Baylor recently reported that endowing NKT cells (an innate immune cell type that shares features of NK cells and T cells) with a GD2 CAR resulted in NK-cell expansion and early signs of clinical activity (80).

Macrophages can be similarly harnessed for antitumor effects in neuroblastoma. Tumor cells express CD47, a macrophage checkpoint that suppresses tumor cell phagocytosis by macrophages (81). Recent clinical trials of anti-CD47 and anti-CD20 (rituximab) mAbs in patients with non–Hodgkin lymphoma indicate that the addition of anti-CD47 can overcome rituximab resistance (82). Preclinical studies have now demonstrated that anti-CD47 can similarly enhance the efficacy of anti-GD2, with potent synergy for the combination of these two antibodies. This synergy is driven by a newly uncovered role for GD2, a sialoglycan that was found to directly interact with Siglec-7, an inhibitory immunoreceptor expressed on both macrophages and NK cells (83). This approach has reached the clinic with a first-in-child/first-in-human clinical trial of combined anti-GD2/anti-CD47 for children with relapsed neuroblastoma (NCT04751383).

Next-generation antitumor mAb-based therapy

Recent advances in protein engineering have enabled creation of next-generation antibody-based off-the-shelf agents. ADCs are showing strong preclinical activity, including in neuroblasoma (66, 84), and clinical testing is moving forward for some in a variety of cancers. Bispecific T-cell Engager (BiTE) antibodies link a tumor-specific mAb or mAb fragment to an anti-CD3 mAb or mAb fragment. This architecture enables selective binding and bridging of tumor cells to a T cell and then subsequent activation of the T cell to kill the tumor. Blinatumomab is a bispecific CD19 x CD3 antibody that FDA approved for B-cell acute lymphoblastic leukemia, that can be effective even in the face of lymphopenia or immunedeficiancy (85, 86). Multiple analogous or similar constructs are being studied for various solid tumors. While BiTEs activate T cells, other mAb-based constructs bind to and activate other effectors cells such as NK cells, (so-called BiKEs) and can also be engineered to incorporate cytokines (so-called TriKEs; ref. 87). However, despite potent *in vitro* destruction of tumor cells by these bifunctional and trifunctional agents, their potency in mice or patients bearing solid tumors has not yet matched their potency against leukemia; possibly implicating the immune-excluded/immune-suppressive tumor microenvironment of many solid tumors, including neuroblastoma (20). Unique engineering strategies are also being deployed to reduce or avoid pain associated with anti-GD2 mAb, including use of alternative or mutated mAb isotypes to avoid pain-inducing complement activation (88, 89), or mAb strategies that require corecognition of two separate tumor antigens that are coexpressed simultaneously on the same tumor cells, but not coexpressed on cells from normal tissues (90). In addition, refinement of the antigen-binding component of the Fab (or ScFv) of the antitumor antibody, can identify more advantageous binding kinetics to facilitate improved interactions with the tumor cell surface for any antibody-based therapeutic modality (mAbs, ADCs or CARs).

The potential for endogenous immune-mediated destruction of neuroblastoma

In contrast to the "synthetic" immune recognition of antitumor-based mAbs and their engineered derivatives, the activity of T-cell checkpoint blockade depends entirely on the ability of endogenous immune cells to recognize and destroy autochthonous cancer, without a need for synthetic immune recognition. For the most part, this involves an adaptive T-cell response recognizing immunogenic tumor neoantigens (91, 92).

As most pediatric cancers have a very low tumor mutation burden, children likely have few if any actionable, mutation-generated, immunogenic tumor neoantigens (16, 93). Even so, they may still have some targetable MHC-associated tumor antigens that are expressed only at very low levels on a restricted number of normal tissues. These could include cancer-testis antigens, and other embryonic or differentiation antigens expressed during development and on pediatric cancers but not on normal postnatal tissues (94). Recently, researchers identified that members of the core regulatory circuitry driving neuroblastoma, such as PHOX2B, have peptides that are displayed on the surface of neuroblastoma cells by thier MHC molecules. These so-called oncofetal proteins, expressed during embryonic development but then silenced in normal tissue after birth, may be ideal targets for T cell–based immunotherapies because of their restricted expression outside of the tumor. Proof-of-concept preclinical studies utilizing a CAR-recognizing PHOX2B as presented by the MHC demonstrate the potential power of this approach (95).

Thus, while there may be limited neoantigen expression in neuroblastoma due to its relatively low tumor mutational burden,

developmental antigens may instead be a focus for T cell–based immunotherapy. Because high-affinity T-cell receptors (TCR) against self-antigens are generally deleted during thymic development, engineering of high-affinity TCRs or CARs recognizing peptide as displayed in MHC may have to be employed. Investigators will need to be wary of the potential for antigen cross-reactivity given the high sensitivity and potency of some of these receptors as has been previously observed with certain engineered TCRs (96, 97). It may also be possible to activate endogenous tumor-reactive T cells in a patient by giving agents that augment the immunogenicity of the tumor, activate antigen presentation, expand the endogenous tumor reactive T cells, and block the immunosuppressive tumor microenvironment. This experimental approach seeks to immunize the tumor-bearing individual with their own tumor, functioning as an *in situ* vaccine (98–101).

For these approaches to be effective, neuroblastoma cells will require at least some low level of surface MHC expression. For patients with low MHC expression due to "soft" (namely reversible, epigenetic) downregulation, this may be possible via epigenetic modification. For patients with no MHC expression due to "hard" genetic mutations in MHC or other antigen presentation machinery, strategies that rely on MHC recognition are not applicable.

Engineering CAR-T cells

Although anti-GD2 antibodies engage NK cells and macrophages, they do not recruit T cells, which have shown themselves to be highly potent in regressing solid cancers in certain adult malignancies (102). Thus, researchers have focused on engineering CARs, synthetic receptors that harness the cytolytic capacity of T cells in a genetically unrestricted manner by employing an antibody fragment as the antigen-binding domain. Although many preclinical studies have demonstrated the promise of CAR-T cells to treat neuroblastoma (61, 68, 70) and some clinical data provide proof that these can translate (47, 48, 50–52), obstacles remain. Some major roadblocks that need to be overcome are insufficient CAR-T-cell expansion, and persistence and reduced functionality in the suppressive tumor microenvironment. Engineering CAR-T cells to overcome these barriers must be balanced against potential for causing toxicity; promotion of enhanced functionality can increase the risk immune overactivation (e.g., cytokine release syndrome) or on-target, off-tumor recognition of normal tissues. For instance, because GD2 is expressed on peripheral nerves and normal neurons in the CNS (41, 42), there has long been concern for GD2 CAR-mediated neurotoxicity, contributing to conservative design in terms of dose and even CAR-T-cell potency. However, GD2 CAR-T cells have now mediated significant clinical responses without evidence of on-target, off-tumor neurotoxicity, indicating that the CAR constructs being used fall within a therapeutic window in which they recognize high GD2 expression on tumor but not lower GD2 expression on normal tissue (47, 48, 50–52, 68). As next-generation CAR-T cells (**Fig. 2**) are being engineered to contain additional modules to enhance functionality through transcriptional reprogramming (103), avoidance of inhibitory molecules (104), and provision of cytokine signaling (105), it is possible that they may also recognize lower levels of antigen expressed by normal tissues and potentially cause significant on-target, off-tumor toxicity. Thus, as our ability to engineer and manufacture highly functional CAR-T cells matures, researchers may also need to deploy so-called Boolean logic gating strategies that can further improve specificity and prevent immune attack of normal tissues (106, 107), as is also being done for the improvement of mAb-based therapy (noted above).

Conclusion

Neuroblastoma is a childhood malignancy that is marked by aberrant development and, as opposed to the majority of adult malignancies, does not usually harbor a high mutational burden. The limited number of somatic, actionable mutations severely restricts the *de novo* immune responses that might be unleashed using checkpoint blockade (92, 93). Therefore, approaches to immunotherapy for neuroblastoma must differ significantly from those being successfully employed for many adult solid tumors. To date, virtually all active immunotherapies for neuroblastoma have relied on targeting GD2, a glycolipid overexpressed on the surface of neuroblastoma cells with only low-level expression on normal tissue. Such differentially expressed antigens may represent the best classes of immunotherapy targets in pediatric oncology and thus there has been an intense research focus on identifying similar targets. Those studies have begun to bear fruit with the identification of targets including GPC2, B7-H3, and ALK. Researchers have used synthetic immune recognition to engineer antibody-based immunotherapies which are now reaching clinical trials. In addition, scientists have recently discovered that the developmental origins of neuroblastoma may also serve as an Achilles heel because many developmental proteins are expressed in neuroblastoma cells but not healthy postnatal tissues and therefore may serve as unique and specific targets for immunotherapy.

Despite these exciting emerging approaches, the neuroblastoma tumor microenvironment remains hostile to endogenous immune elements, containing immune cells such as M2-polarized macrophages and myeloid-derived suppressor cells that can interfere with immunotherapeutic strategies. A focus on reprogramming the tumor microenvironment and reversing its suppressive activity with therapies such as radiation, chemotherapy, or CD47 blockade may improve the antitumor efficacy of endogenous immune cells or of genetically modified immune cells. This is perhaps the reason a chemoimmunotherapy approach combining cytotoxic agents with anti-GD2 has been successful for some children with neuroblastoma, and is currently considered the standard of care for children with relapsed or refractory disease (34–37).

In addition to identifying new immunotherapy targets and engineering therapeutics, researchers will also need to focus on developing additional rational combinations of traditional cytotoxic agents and radiotherapy, small-molecule inhibitors of oncogenic pathways, and immunotherapies. As next-generation small molecules, such as ALK inhibitors, aurora-A inhibitors, and CDK9/2 inhibitors (108–110) are being integrated into treatment for appropriate patients, their ability to potentially synergize with (or antagonize) combination immunotherapy regimens will require careful analyses in preclinical studies and clinical trials. Once these approaches have established clinical efficacy, it will become an important focus to reduce the reliance on high-dose radiochemotherapy to minimize acute treatment-associated toxicity and long-term late effects. Eventually, correlative lab testing may enable selection of somewhat personalized combination therapy regimens, based on analyses of tumor or host/immune factors measured at the time of diagnosis or relapse (75, 76).

The hope of the basic, translational, and clinical neuroblastoma research community is for efficacious treatment regimens that employ novel immunotherapies which enable effective cancer eradication while relying less on high-dose genotoxic radiochemotherapy; the goal is to minimize the long-term morbidity and mortality of the disease, and its therapy. Furthermore, the vast majority of children with other high-risk solid tumors are similarly plagued by (i) poor responses to current "conventional" immunotherapy being used to treat some cancers of adults, due to few actionable

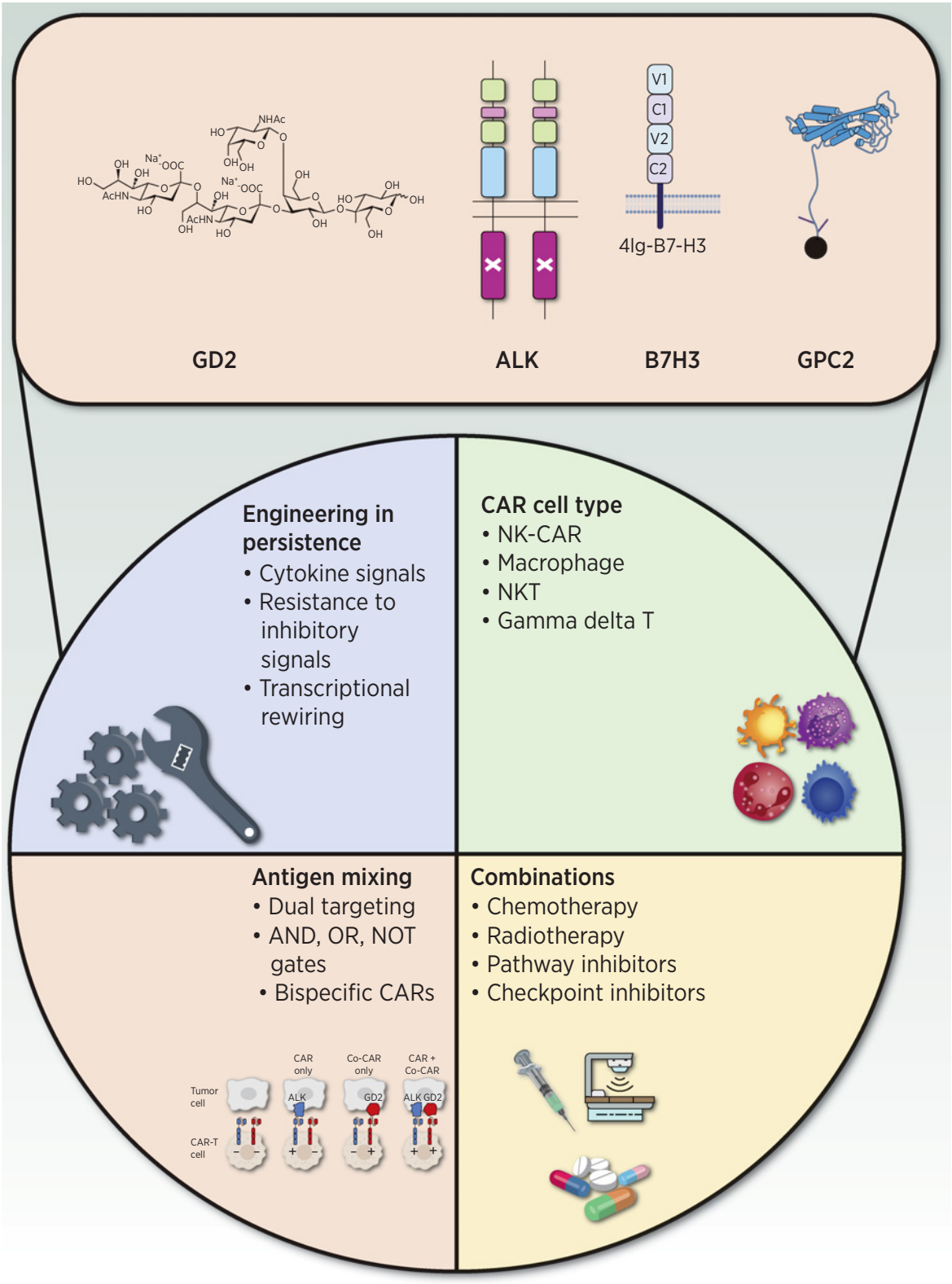

Figure 2.

Enhancing CAR-T cell sensitivity and specificity. Schematic of technologic solutions to enhance CAR-T cell sensitivity and specificity. Representative neuroblastoma tumor antigens that are currently under clinical and preclinical evaluation are indicated at the top. At the bottom are the categories of approaches for enhancing functionality of CAR-T cells that are relevant to neuroblastoma.

mutations/neoantigens, and an immunosuppressive tumor microenvironment and (ii) substantial acute and long-term treatment-induced toxicity due to high-dose radiochemotherapy. As such, the efforts of the neuroblastoma research community to address these hurdles in a combinatorial, rather than sequential, manner may translate into hope for the creation of similar strategies for other high-risk cancers. We hope that through greater collaboration and rapid adoption of technology, novel immunotherapies will move quickly towards the clinic and rapidly alter the treatment paradigm for children with neuroblastoma.

Authors' Disclosures

J. Anderson reports other support from TC Biopharm outside the submitted work. R.G. Majzner reports personal fees from Link Cell Therapies, Syncopation Life Sciences, Lyell Immunopharma, NKarta, Immunai, Aptorum Group, Arovella Therapeutics, Zai Lab, Illumina Radiopharmaceuticals, and Y-Mabs outside the submitted work; in addition, R.G. Majzner has multiple patents in the field of CAR-T cell therapy pending. P.M. Sondel reports grants and non-financial support from Invenra Inc. during the conduct of the study; in addition, P.M. Sondel has a patent for bispecific mAb held by the Wisconsin Alumni Research Foundation and Invenra Inc. pending to Wisconsin Alumni Research Foundation (WARF) and Invenra Inc. No other disclosures were reported.

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