

Immunotherapy of Neuroblastoma Targeting GD2 and Beyond



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Abstract Neuroblastoma is the most common extracranial solid tumor in children. Although low-risk neuroblastoma is mostly curable, the outcome of high-risk neuroblastoma, which comprises ~50% of neuroblastoma, is dismal. Abundant expression of disialoganglioside GD2 antigens is a feature of neuroblastoma. The success of immunotherapy targeting the GD2 antigen with dinutuximab has fueled the interest in developing improved immunotherapeutics including humanized anti-GD2 antibodies, GD2 mimotopes, cytokine-fused GD2-specific antibodies, GD2-specific chimeric antigen receptor T/NKT cells, GD2 vaccine, anti-GD2 idiotype monoclonal antibody, and anti-*O*-acetyl GD2 antibody. Advance in these active or passive cancer immunotherapies hold promises to further improve the outcome of neuroblastoma.

1 Introduction

Neuroblastoma is the most common malignancy in children aged under 1 year. It is a neuroendocrine tumor that originates from embryonal neural crest tissue. The common lesions of primary tumors are the adrenal medulla, neck, chest, and pelvis. About 8–10% of all childhood cancer cases are neuroblastoma. The incidence of neuroblastoma is 10.54 cases per million young children (<15 years old) per year. According to the International Neuroblastoma Risk Group classification system based on clinical and molecular features, the high-risk group, which represents about 50% of neuroblastoma, has a poor prognosis (Cohn et al. 2009). Genetic

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alterations are frequent in neuroblastoma. *MYCN* amplification, ranging from 100 kb to >1 Mb (Amler and Schwab 1989), is associated with the rapid progression of neuroblastoma (Seeger et al. 1985). *ALK* with gain-of-function mutations (Mosse et al. 2008) and paired mesoderm homeobox protein 2B (*PHOX2B*) with loss-of-function mutations (Trochet et al. 2004) are two predisposing factors for familial neuroblastoma. Lin-28 homologue B (*LIN28B*), which can suppress let-7 to increase *MYCN* expression, is an independent risk factor for high-risk neuroblastoma (Molenaar et al. 2012). On the other hand, altered glycosylation is a hallmark during tumorigenesis (Reis et al. 2010). GD2 is a tumor-associated carbohydrate antigen abundantly expressed on the surface of neuroblastoma cells with $\sim 10^7$ GD2 molecules per neuroblastoma cell (Wu et al. 1986), but limited expression in normal tissues, making it an attractive target for the development of immunotherapies against neuroblastoma.

Disialoganglioside GD2 is a b-series ganglioside consisting of GalNAc β 1-4 (NeuAc α 2-8NeuAc α 2-3)Gal β 1-4Glc β 1-1ceramide. Several enzymes are involved in the synthesis of GD2, including galactosyltransferase I, GM3 synthase, GD3 synthase, and GM2/GD2 synthase (Furukawa et al. 2002; Kolter et al. 2002). The biological functions of GD2 have not been fully explored. GD2 ceramide inhibited proliferation and induced apoptosis of T cells (Ladisch et al. 1992; Biswas et al. 2009). Recently, Siglect-7 has been identified to be a ligand for GD2 (Theruvath et al. 2022). Upon binding to GD2, Siglect-7 might suppress immune cell activity through its cytoplasmic immunoreceptor-tyrosine-based inhibitory motif domain. In normal tissues, GD2 expression is weak and restricted to the central nervous system, peripheral pain fibers, and skin melanocytes (Svennerholm et al. 1994; Yuki et al. 1997). In contrast, GD2 is overexpressed in a variety of cancers that include neuroblastoma, small cell lung cancer, melanoma, glioma, osteosarcoma, rhabdomyosarcoma, and Ewing sarcoma (Schulz et al. 1984; Heiner et al. 1987; Tsuchida et al. 1987; Kailayangiri et al. 2012). In addition, expression of GD2 has been reported on cancer stem cells of breast cancer (Battula et al. 2012; Liang et al. 2013) and malignant phyllodes tumors of the breast (Lin et al. 2014). Thus, GD2 is an ideal target for cancer immunotherapy.

Prior to 2015, the standard therapy for high-risk neuroblastoma is chemotherapy, surgical resection, myeloablative therapy, autologous hematopoietic stem cell transplantation, and isotretinoin. Addition of immunotherapy with anti-GD2 (dinutuximab), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2) to maintenance phase of treatment significantly improved the 2-year event-free survival from 46% to 66% (Yu et al. 2010), leading to the regulatory approval of dinutuximab in 2015. Thus, immunotherapy with dinutuximab has since been considered as the standard of care for high-risk neuroblastoma. In this chapter, we will focus on the immunotherapeutic strategies including immune checkpoint blockade, adoptive cellular therapies, antibody therapies, and cancer vaccines for further improvement of the outcome of neuroblastoma.

2 Antibody Therapies

Several GD2-specific antibodies have been investigated for the treatment of neuroblastoma, such as murine monoclonal antibody, monoclonal antibody fused with cytokine, chimeric monoclonal antibody, humanized monoclonal antibody, and bispecific antibody.

2.1 Murine Anti-GD2 Antibodies

Monoclonal antibody (mAb) 14G2a (Fig. 1a) is an IgG isotype switch variant of a GD2-specific murine IgG3 14.18 (Mujoo et al. 1989). The mAb 14G2a could change morphology and increase apoptosis of GD2-positive mouse lymphoma EL4 cells (Doronin et al. 2014). Similarly, mAb 14G2a activated caspase 3 to induce apoptosis of human neuroblastoma IMR-32 cells (Kowalczyk et al. 2009). Moreover, mAb 14G2a decreased metalloproteinase-2 to inhibit invasion of SJSA-1, MG-62, and Saos-2 cells (Liu et al. 2014). Besides, many studies showed that mAb 14G2a could inhibit p70S6, Akt, 4E-BP1, mTOR, MYCN, Aurora kinases, an inhibitor of DNA binding 1, and T cell leukemia homeobox 2, and increase p53, PHLDA1, JUN, Ras association RalGDS/AF-6 domain family member 6, and supervillin (Wierzbicki et al. 2008; Horwacik et al. 2013, 2015; Durbas et al. 2015).

Another murine anti-GD2 IgG3 mAb 3F8 (Fig. 1a) has been extensively investigated by Cheng's group. It altered morphology of SH-SY5Y-TrkB cells by Src-dependent activation of *N*-methyl-D-aspartate receptor signaling (Tong et al. 2015). The mAb 3F8 also activated caspases 3, 7, and 8 and inhibited survivin and AKT, leading to apoptosis of human melanoma HTB63 cells (Tsao et al. 2015).

The mAb 14G2a not only induced apoptosis of cancer cells but also killed cancer cells by complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Mujoo et al. 1989). Moreover, mAb 14G2a suppressed the growth of M21 xenografts in athymic nude mice (Mueller et al. 1990). Phase I clinical trials of mAb 14G2a was conducted in patients with neuroblastoma or osteosarcoma. The dosage of mAb 14G2a was escalated up to 500 mg/m²/course. Infusion of mAb 14G2a caused pain, hypotension, fever, urticarial, hypertension, tachycardia, and hyponatremia (Saleh et al. 1992; Murray et al. 1994; Uttenreuther-Fischer et al. 1995a). The pain was attributable to the binding of mAb 14G2a to peripheral nerve fibers, which express GD2 (Svennerholm et al. 1994). Some patients with partial or mixed responses were observed in these early phase trials (Saleh et al. 1992; Murray et al. 1994).

In a phase I study, mAb 3F8 had similar side effects as mAb 14G2a (Cheung et al. 1987). A phase II clinical trial of mAb 3F8 showed long-term survival (79–130+ months) of three patients (3/16) with stage 4 neuroblastoma (Cheung et al. 1998). Other phase II studies showed 62% 5-year EFS for stage 4 patients in the first remission who received mAb 3F8 + subcutaneous GM-CSF + cis-retinoic acid

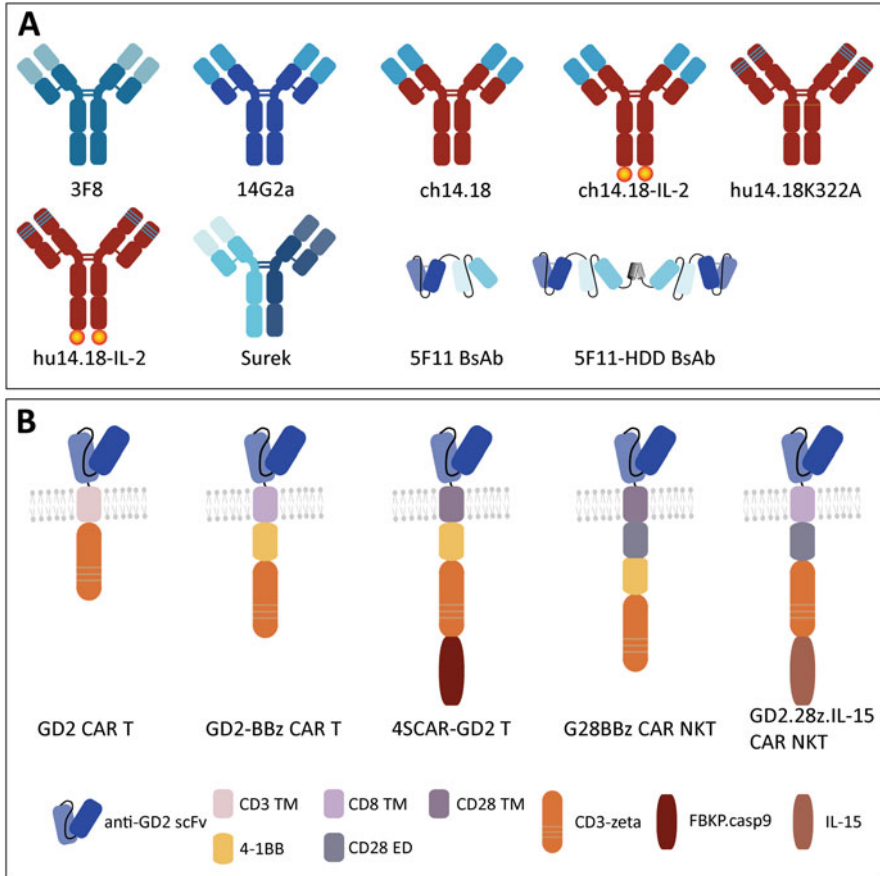


Fig. 1 Various GD2-specific antibodies and CAR constructs. (a) Various GD2-specific antibodies are shown: 3F8, 14G2a, chimeric 14.18 (ch14.18), ch14.18-IL-2, hu14.18K322A, hu14.18-IL-2, Surek (anti-GD2 x anti-mouse-CD3 trifunctional bispecific antibody), 5F11 BsAb (anti-GD2 x anti-CD3 bispecific antibody), and 5F11-HDD BsAb. (HDD: dimerization domain of human hepatocyte nuclear factor 1 α). (b) Various GD2-specific CAR constructs are shown: GD2 CAR, GD2-BBz CAR, and 4SCAR-GD2 for T cells and G28BBz CAR and GD2.28z.IL-15 CAR for NKT cells. (BB: 4-1BB, z: CD3 zeta signaling domain, 28: CD28, TM: transmembrane domain, ED: endodomain)

(Cheung et al. 2012a; Cheung et al. 2014). In these studies, patients with the FCGR2A (R/R) genotype had a better outcome (Cheung et al. 2006). ^{131}I -labeled mAb 3F8 showed anticancer efficacy in human neuroblastoma xenografts in mice (Cheung et al. 1986). A phase I trial of ^{131}I -labeled mAb 3F8 given by intrathecal injection was conducted in 3 patients with recurrent metastatic central nervous system neuroblastoma. Extended survival (43, 41, and 33 months) of these patients was observed (Kramer et al. 2010). However, ^{131}I -labeled mAb 3F8 at dose levels of 4 mCi/kg/day \times 5 days resulted in primary hypothyroidism (Bhandari et al. 2010).

Thus, to avoid potential adverse effects, protection of the thyroid gland will be needed for therapy of human neuroblastoma with ^{131}I -labeled mAb 3F8.

2.2 Chimeric Anti-GD2 Monoclonal Antibody

To reduce the induction of human antimouse antibodies, a chimeric mAb ch14.18 (Fig. 1a) was generated by fusing the variable regions of mAb 14G2a with constant regions of human IgG1-k (Gillies et al. 1989). The mAb ch14.18 triggered CDC and ADCC reactions to kill neuroblastoma cells in vitro and suppressed experimental liver metastasis in an NXS2 neuroblastoma animal model in vivo (Barker et al. 1991; Zeng et al. 2005). Moreover, the mAb ch14.18 displays 50–100-fold greater ADCC activity than mAb 14G2a (Mueller et al. 1990). In phase I studies, when compared to mAb 14G2a, mAb ch14.18 has a longer half-life (66.6 ± 27.4 h vs. 18.3 ± 11.8 h) (Handgretinger et al. 1995; Uttenreuther-Fischer et al. 1995a, b) but similar toxicity profiles (Handgretinger et al. 1995; Yu et al. 1998). The German Collaborative Neuroblastoma Study NB97 showed mydriasis and accommodation deficiency in 10/85 patients with high-risk neuroblastoma who received mAb ch14.18 treatment, but these ocular symptoms were reversible with time (Kremens et al. 2002). Notably, some patients with refractory/relapsed neuroblastoma in the phase I studies of mAb ch14.18 showed complete response (CR) and partial response (PR) (Handgretinger et al. 1995; Yu et al. 1998). To further enhance the anticancer efficacy, mAb ch14.18 was combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2, both of which enhance ADCC (Hank et al. 1990; Tarr 1996). Of 17 refractory/recurrent neuroblastoma, 5 CRs and 3 stable diseases (SDs) were observed in a pilot study of mAb ch14.18 + GM-CSF (Yu et al. 1995). Subsequently, a phase II study of mAb ch14.18 + GM-CSF showed 2 CRs, 2 PRs, and 1 mixed response (Yu et al. 1997). Subsequently, the MTD of mAb ch14.18 in combination with GM-CSF and IL-2 was $25 \text{ mg/m}^2/\text{d}$ for 4 days when administered following consolidation therapy and stem cell transplant (Gilman et al. 2009). The toxicity of the regimen of mAb ch14.18 + GM-CSF + IL-2 was manageable and reversible (Hank et al. 1990). Consequently, a randomized phase III trial (COG study ANBL0032) of mAb ch14.18 with GM-CSF, interleukin-2, and isotretinoin was conducted in children with high-risk neuroblastoma after completing stem-cell transplantation and radiotherapy. Two-year event-free survival and overall survival rates were superior in patients treated with immunotherapy, *that is*, ch14.18 (dinutuximab) + GM-CSF + IL-2 + isotretinoin, than those treated with isotretinoin only ($66 \pm 5\%$ vs. $46 \pm 5\%$, $P = 0.01$ and $86 \pm 4\%$ vs. $75 \pm 5\%$, $P = 0.02$, respectively) (Yu et al. 2010). Based on this data, US FDA and European medicines agency approved dinutuximab for the treatment of high-risk neuroblastoma in 2015. Since then, treatment with dinutuximab has become a standard of care for high-risk neuroblastoma. This is the first successful immunotherapy targeting a tumor-associated glycolipid antigen. Recently, a long-term follow-up (median 9.97 years) of the COG ANBL0032 study showed that mAb ch14.18 + GM-CSF + IL-

2 + isotretinoin significantly improved the 5-year EFS (57% vs. 46%) and OS (73% vs. 57%) of patients with high-risk neuroblastoma (Yu et al. 2021, 2022), although the benefits have decreased due to late relapses. Notably, immune correlative analyses revealed that patients with high affinity FCGR3A genotype, and KIR2DL2/C1 or KIR3DL1/HLA-B-Bw4-T80 genotypes had better outcome with anti-GD2 immunotherapy (Erbe et al. 2017, 2018; Desai et al. 2022).

To improve the efficacy of dinutuximab by combining with chemotherapy, COG conducted a randomized phase II selection design trial in patients with relapsed/refractory neuroblastoma. Eligible patients were randomly assigned to irinotecan and temozolomide plus either temsirolimus or dinutuximab + GM-CSF. Irinotecan–temozolomide–dinutuximab–GMCSF (I/T/DIN/GM-CSF) showed superior antitumor activity with 9/17 (53%) objective responses, versus 1/18 (6%) patients treated with I/T/temsirolimus (Mody et al. 2017). The trial was expanded to recruit additional 36 patients nonrandomly assigned to I/T/DIN/GM-CSF. In total, 22/53 patients showed objective responses (41.5%) (Mody et al. 2020). These findings provided the rationales for the ongoing development of a COG phase III study of combining dinutuximab with induction chemotherapy in high-risk neuroblastoma.

In view of the limited access of mAb ch14.18 produced by murine myeloma SP2/0 cells in Europe, the International Society of Paediatric Oncology European Neuroblastoma Group (SIOPEN) uses mAb ch14.18 generated by Chinese hamster ovary cells, designated as mAb ch14.18/CHO (dinutuximab beta) for clinical investigations. As compared to mAb ch14.18/SP2/0, similar toxicology and comparable pharmacokinetics were observed in phase I bridging study of mAb ch14.18/CHO in patients with neuroblastoma (Ladenstein et al. 2013). Subsequently, a randomized SIOPEN study of mAb ch14.18/CHO with or without IL2 showed similar efficacy for high-risk neuroblastoma (Ladenstein et al. 2018), but the combination of IL-2 with mAb ch14.18/CHO resulted in more toxicities than mAb ch14.18/CHO alone. In 2017, mAb ch14.18/CHO received regulatory approval by the European Medicines Agency, and designated as Dinutuximab-beta.

2.3 Humanized Anti-GD2 Antibody

Humanization of murine antibodies can reduce immunogenicity, enhance effector function, and increase half-life in the serum. A humanized mAb 14.18 (hu14.18) antibody was generated by CDR grafting of mAb ch14.18 V regions onto variable regions of human IgG1 heavy and kappa light chains (Metelitsa et al. 2002). In addition, a point mutation K322A at the Fc region was introduced to reduce complement activation, which causes neuropathic pain induced by fixation of C5a complement on anti-GD2-binding neuron (Sorkin et al. 2010). Furthermore, YB2/0 rat myeloma cells, which have low fucosylation activity leading to increased ADCC (Niwa et al. 2004), were used to produce the mAb hu14.18K322A (Fig. 1a). Indeed, the mAb hu14.18K322A displayed ADCC activity but induced negligible CDC and less allodynia than mAb ch14.18 in a rat model (Sorkin et al. 2010). A phase I

clinical trial of mAb hu14.18K322A showed that the maximum tolerated dose was 60 mg/m²/d for 4 days (Navid et al. 2014). Reversible ocular toxicities were seen in patients treated with mAb hu14.18K322A (Tse et al. 2015). A phase II study of mAb hu14.18K322A in combination with 6 courses of chemotherapy was conducted in patients with newly diagnosed high-risk neuroblastoma. After the first two chemo-immunotherapy cycles, an impressive 67% PR or better responses were noted in 42/63 patients (Furman et al. 2019). At the end-of-induction 60 of 62 patients (97%) had a partial response or better. Updated results of the induction chemotherapy with hu14.18K322A have demonstrated that 3-year EFS and OS were 73.7% and 86%, respectively (Furman et al. 2022). In addition, treatment of children with recurrent/refractory neuroblastoma with hu14.18K322A in combination with chemotherapeutic agents and haploidentical NK cell therapy resulted in 3 CRs, 1 very good partial response, 4 PRs, and 5 stable diseases (Federico et al. 2017).

In 2012, a humanized mAb 3F8 (hu3F8) was generated, and its binding activity to GD2 was similar to murine mAb 3F8. When compared to murine mAb 3F8, mAb hu3F8 had more potent ADCC but lower CDC activity (Cheung et al. 2012a, b). Based on in silico assisted design, a D32H (located at the L-CDR1) mutation was introduced in mAb hu3F8 to enhance the binding affinity to GD2. Although the binding affinity and ADCC of mAb hu3F8D32H were increased in vitro, its antitumor efficacy in IMR-32-bearing mice was not further improved (Zhao et al. 2015). A phase I trial of mAb hu3F8 in combination with GM-CSF for patients with resistant neuroblastoma demonstrated reversible neuropathic pain, low immunogenicity, and prolonged progression-free survival (Cheung et al. 2017; Kushner et al. 2018). Two single-arm, open-label trials (NCT03363373 and NCT01757626) were conducted to evaluate the antitumor efficacy of mAb hu3F8 in patients with relapsed/refractory neuroblastoma in the bone or bone marrow. The overall response rate for NCT03363373 and NCT01757626 was 45% and 34%, respectively (FDA 2020). These findings led to the FDA approval for mAb hu3F8 (naxitamab) for the treatment of relapsed/refractory high-risk neuroblastoma in 2020.

2.4 Cytokine-Conjugated GD2-Specific Antibodies

Another strategy to enhance anticancer efficacy is to link an antibody with cytokine to generate immunocytokine fusion protein, thereby inducing high concentration of cytokine in the tumor microenvironment to stimulate cellular immune responses against cancer cells. A fusion protein of hu14.18 and IL-2, hu14.18-IL2 (Fig. 1a), prolonged survival of NXS2-bearing mice with the involvement of NK cells and T cells (Neal et al. 2004a, b). Furthermore, upon binding to tumor cells, hu14.18-IL2 remained on the surface of tumor cells for a prolonged time, leading to the recruitment of NK cells to kill tumor cells (Buhtoiarov et al. 2011). In a phase I trial of patients with recurrent/refractory neuroblastoma, MTD of hu14.18-IL2 (EMD 273063) was 12 mg/m²/day with expected toxicities similar to the combination of anti-GD2 and IL-2, and antitumor activity was observed in three patients (Osenga

et al. 2006). In a phase II study, hu14.18-IL2 resulted in 5 CR (5/23) for patients with disease evaluable by ^{123}I -metaiodobenzylguanidine (MIBG) imaging and/or bone marrow histology, while patients with measurable disease had no responses (Shusterman et al. 2010). Moreover, the mismatch between KIR and KIR-ligand was associated with better clinical response to hu14.18-IL2 therapy (Delgado et al. 2010).

2.5 Bispecific Antibody

It is an attractive therapeutic approach to combine fragments of two monoclonal antibodies to make a bispecific antibody that binds to two different antigens. Many bispecific antibody formats against GD2 have been generated and evaluated in vitro and in vivo. Generated by electrofusion of hybridoma cells, a trifunctional bispecific antibody TRBs07 was shown to redirect T cells to kill GD2-expressing melanoma cell lines (Ruf et al. 2004). Surek (anti-GD2 17A2 x anti-mouse CD3 Me361 with an intact Fc) (Fig. 1a), a trifunctional bispecific antibody generated by the TRION antibody platform technology, could not only prolong the survival of mice but also induce a long-term memory response to tumor cells (Eissler et al. 2012; Ruf et al. 2012). Interestingly, subcutaneous and intravenous injection of the Surek showed similar antitumor efficacy in mice; however, only subcutaneously injected Surek reduced CTLA-4 expression on CD4⁺ and CD8⁺ T cells (Deppisch et al. 2015). Subsequently, the anti-mouse CD3 in Surek was replaced with an antihuman CD3 to generate an Ektomun (anti-GD2 x anti-human CD3). Upon coculture of human neuroblastoma cells with PBMCs, Ektomun could activate effector cells to release proinflammatory cytokines and exert cytotoxic effects (Zirngibl et al. 2021). Another bispecific antibody anti-CD3 x anti-GD2 (3F8BiAb) generated by chemical heteroconjugation could redirect T cells to target neuroblastoma cells expressing GD2 (Yankelevich et al. 2012). A humanized hu3F8-BsAb showed GD2-specific cytotoxicity to neuroblastoma cells in vitro and anticancer efficacy in vivo (Xu et al. 2015). An MDX-260 BsAb prepared by crosslinking F(ab') fragments of mAb 7A4 (anti-GD2) and mAb 22 (anti-FcγRI) using bis-maleimide could trigger neutrophils to kill tumor in vitro (Michon et al. 1995).

A 5F11-BsAb (Fig. 1a) has been generated by genetic engineering with a gene construct comprised of VH-VL of anti-GD2 mAb 5F11, VHS44C and VLA100C mutations to stabilize disulfide bond, (GGGG)₃ linker to link anti-huCD3 scFv, and an affinity maturation mutation VHP104Y. In IMR-32-bearing BALB/cA-*Rag2*^{-/-}*Il2rg*^{-/-} mice where human PBMCs were used as effector cells, the 5F11-BsAb effectively suppressed tumor growth (Cheng et al. 2015). However, renal clearance of 5F11-BsAb was rapid due to its small size. Thus, to prevent the rapid renal clearance and enhance the potency of 5F11-BsAb, a dimerization domain (residues 1-32) of human hepatocyte nuclear factor 1α (HNF1α) was added to the C-terminus of the 5F11-BsAb to generate 5F11-HDD-BsAb (Fig. 1a). The HNF1α dimerization domain could increase molecular size and the avidity of 5F11-BsAb, without

introducing immunogenicity. Surface plasmon resonance assay showed that 5F11-HDD-BsAb had a better affinity to GD2 than 5F11-BsAb, while both had a similar binding affinity to CD3. Moreover, the 5F11-HDD-BsAb significantly reduced tumor growth and prolonged survival in the IMR-32-bearing animal model. Notably, serum pharmacokinetic analysis revealed that 5F11-HDD-BsAb displayed a significantly longer half-life than 5F11-BsAb (Ahmed et al. 2015).

3 Adoptive Cellular Therapies

Adoptive cellular therapy (ACT) is a rapidly emerging immunotherapy approach, fueled by the FDA approval of two CAR T-cell therapies in 2017, one for the treatment of children with [acute lymphoblastic leukemia](#) (ALL) and the other for adults with advanced lymphomas (Mullard 2021). CAR is a synthetic receptor that combines a tumor-antigen-specific single-chain variable fragment with a transmembrane domain and an intracellular signaling domain (Rafiq et al. 2020). Upon binding to cancer cells by single-chain variable fragment, the intracellular signaling domain of CAR can transduce a signal to activate CAR T cells. Thus, CAR T cells can eliminate cancer cells in a major histocompatibility complex class I-independent fashion. Several GD2-directed CAR-T therapeutics are under evaluation in clinical trials. Epstein-Barr virus-specific T lymphocytes expressing a CAR specific for the GD2 (Fig. 1b, GD2 CAR T) have been generated and used for the treatment of patients with high-risk neuroblastoma. Tumor necrosis or complete remission was observed in 2 and 1 patients, respectively, at 6 weeks after treatment (Pule et al. 2008). Moreover, a long-term follow-up showed that the patients with persistence of GD2-CAR-T cells (>6 weeks) had a prolonged time to progression (Louis et al. 2011). Furthermore, tumor cells infected with oncolytic viruses to express RANTES and IL-15 ectopically could enhance the antitumor function of the GD2-CAR-T cells (Nishio and Dotti 2015). Another anti-GD2-BB- ζ CAR T cells (Fig. 1b), which comprised of scFv of mAb 126 (anti-GD2 IgM), the transmembrane domain of human CD8a hinge, human 4-1BB, and signaling domain of human CD3- ζ , killed neuroblastoma cells in vitro and suppressed tumor growth SH-SY5Y-bearing NOD.scid mice (Prapa et al. 2015). In a phase I clinical trial, six patients with refractory/recurrent neuroblastoma showed stable disease 6 months after treatment with 4SCAR-GD2 T cells (Fig. 1b), which contained intracellular signaling domain of CD28/4-1BB/CD3 ζ -iCasp9, with an inducible caspase 9 gene in the anti-GD2-CAR construct (Yu et al. 2021, 2022). Adverse events associated with the 4SCARGD2 T-cell therapy, which included cytokine release syndrome and neuropathic pain, are manageable (Yu et al. 2021, 2022). In addition, the frequency of MDSC in human peripheral blood is a negative predictor of response to GD2-CAR-T (14G2a scFv-CD28-4-1BB-CD3 ζ) cell therapy (Tumino et al. 2021).

In addition to CAR-T cells, different forms of ACT are being actively pursued. Natural killer T (NKT) cells, which are a sublineage of T cells with V α 24 invariant T cell receptors, play a role in neuroblastoma (Metelitsa et al. 2004; Song et al. 2007;

Liao et al. 2021). A G28BBz-CAR construct, which consisted of mAb 14G2a scFv, CD28 transmembrane domain, CD28 endodomain, 4-1BB endodomain, and ζ chain endodomain (Fig. 1b), was used to transfect primary human NKT cells to generate CAR.GD2 NKT cells. The CAR.GD2 NKT cells could infiltrate into the tumor site to exhibit potent cytotoxic activity (Heczey et al. 2014). Another CAR.GD2 NKT is generated using CAR construct (GD2.28z.15), which consisted of mAb 14G2a scFv, CD8 hinge and transmembrane regions, CD28 endodomain, CD3 ζ signaling domain, 2A sequence of the equine rhinitis A virus, and IL-15 (Fig. 1b). It showed superior antitumor activity in CHLA-255 neuroblastoma-bearing mice (Xu et al. 2019). Thus, a first-in-human phase I trial of GD2.28z.15 CAR NKTs for children with relapsed/refractory neuroblastoma was conducted. An interim analysis of the first 3 patients showed that the GD2.28z.15 CAR NKTs could expand in vivo and migrate to tumor sites, and one patient had a partial response (Heczey et al. 2020). Thus, GD2-CAR T/NKT cell therapy will likely bring hope for the treatment of neuroblastoma in the near future. Nevertheless, it should be cautioned that it is still early days for CAR T cells and other forms of ACT. Whether they will ever be as effective against solid tumors as hematological malignancies remains to be demonstrated.

4 Cancer Vaccine

4.1 *Racotumomab Vaccine*

The majority (85%) of neuroblastoma expressed *N*-glycolyl GM3 (NeuGcGM3), regardless of NMYC amplification (Scursoni et al. 2011). NeuGcGM3 was undetectable in healthy human tissues (Irie et al. 1998), making it a potential target for neuroblastoma therapy. An anti-idiotypic murine mAb racotumomab (also known as 1E10) (Vazquez et al. 1998) against anti-NeuGcGM3 mAb P3 (Vazquez et al. 1995) has been generated. In B16 melanoma- and F3II mammary tumor-bearing animal models, vaccination with racotumomab decreased tumor growth (Vazquez et al. 2000). Treatment of neuroblastoma patients with racotumomab showed grade 1–2 toxicity at the injection site and induction of IgG and IgM against NeuGcGM3 in the phase I trial (Cacciavillano et al. 2015). A phase II of racotumomab for children with high-risk neuroblastoma is ongoing (NCT02998983). Importantly, progression-free and overall survival were significantly prolonged in patients with non-small cell lung cancer who were vaccinated with racotumomab in a randomized phase II/III trial (Alfonso et al. 2014). Racotumomab was approved for the treatment of patients with recurrent/advanced non-small cell lung cancer in Cuba and Argentina in 2013.

4.2 GD2 Vaccines

In general, carbohydrate or glycolipid molecular including GD2 are poor antigens for inducing immune responses. Several approaches have been applied to enhance the immunogenicity of GD2. For example, conjugation of GD2 to keyhole limpet hemocyanin (KLH) protein derived from the *Megathura crenulato*, which is a T-cell-dependent antigen, can stimulate the immune system to produce antibodies (Swaminathan et al. 2014). The use of a potent immunostimulatory adjuvant, such as QS21 isolated from the *Quillaja saponaria*, can activate dendritic cells and CD169⁺ macrophages to elicit strong immune responses. Another adjuvant, β -glycans, which are yeast cell wall carbohydrates, can activate immune cells and enhance ADCC (Cheung and Modak 2002; Dambuza and Brown 2015). GD2-KLH and GD3-KLH vaccines were produced by chemically crosslinking KLH to either GD2 or GD3. In a phase I trial, children with high-risk neuroblastoma in second or later remission were treated with GD2-KLH, GD3-KLH, a QS-21 equivalent OPT-821, and b-glucans. This regimen did not cause major toxicity and induced antibody responses against GD2/GD3 in 12 of 15 patients (Kushner et al. 2014). Subsequently, a phase II of the GD2-KLH/GD3-KLH/OPT-821/b-glucan in patients with high-risk neuroblastoma in remission was conducted. Multivariable analyses showed anti-GD2 IgG1 titer ≥ 150 ng/mL by week 8 could contribute to favorable PFS and OS (Cheung et al. 2021).

4.3 GD2 Mimotope

GD2 mimotopes are carbohydrate-mimetic peptides that stimulate immune response to induce GD2-specific T cells and antibodies. Using a decamer phage display library, Forster-Waldl et al. obtained GD2 peptide mimotopes that could compete for binding to mAb ch14.18 (Forster-Waldl et al. 2005). To enhance immunogenicity, the GD2 peptide mimotopes were linked to KLH. The GD2 mimotope-KLH vaccine could elicit IgG antibodies to GD2 (Riemer et al. 2006). Another GD2 mimotope 47-LDA obtained from X(15) phage display library had increased mimicry to GD2 by molecular modeling. The 47-LDA-encoded plasmid DNA vaccine could induce GD2 cross-reactive IgG antibody response in vivo (Bolesta et al. 2005). Moreover, the 47-LDA DNA vaccine could induce cytotoxic T lymphocyte response to prolong survival of syngeneic neuroblastoma NXS2-bearing mice (Wierzbicki et al. 2008). In addition, computer modeling of mAb ch14.18 was used to design a GD2 mimicry MD (C-DGGWLSKGSW-C). A pSA-MA vector encoding for T-cell helper epitope and GD2-mimicking MD peptide was generated and transfected into *S. typhimurium* SL7207. Induction of tumor protective immunity was observed in NXS2-bearing mice, which were immunized by oral gavage of SL7207 carrying the pSA-MA vectors. Notably, the vaccine could increase NK cell activity, IFN- γ secretion, CD4⁺ T cells, and CD8⁺ T cells (Fest et al. 2006). In summary, GD2

mimotopes can induce not only humoral response but also cellular response, providing strong evidence for further development.

4.4 *Anti-GD2 Idiotypic Monoclonal Antibody*

Danish immunologist Niels Kaj Jerne conceived the idiotypic network theory that antibodies (termed “Ab2” antibody) react to antigen-specific antibodies (termed “Ab1” antibody) (Jerne 1974). Anti-idiotypic (anti-Id) antibodies, which belong to Ab2, can bind to the idiotope of another antibody. An idiotope is a unique region within the Fv region of an antibody. Thus, the antigen-binding domain of anti-Id antibodies can structurally resemble the antigen epitope. The mAb 14G2a was used to generate an anti-idiotypic antibody mAb 1A7 (also known as TriGem), which mimics the GD2 antigen (Saleh et al. 1993). Besides, DNA vaccines encoding scFv of mAb 1A7 could induce immune sera against GD2 in vivo (Zeytin et al. 2000).

A phase I clinical trial required 47 patients with advanced melanoma to study the toxicity and immune response of TriGem. Minimal toxicity was observed, including mild fever, chills, and local reaction at the injection site. Induction of anti-GD2 IgG antibody (Ab3) was found in 40 patients (85.1%, 40/47). Notably, one complete response and 12 stable diseases occurred in 47 patients with TriGem therapy (Foon et al. 1998, 2000).

In addition, 31 patients with high-risk neuroblastoma in first or subsequent complete remission or very good partial remission after frontline therapy were enrolled in a clinical trial of mAb 1A7 vaccine mixed with QS21 (Yu et al. 2001). Adverse reactions were transient fever, chills, serum sickness, and local reactions. Anti-mAb 1A7 was detected in all patients. Immune sera from some patients lysed human neuroblastoma cells in the ADCC and CDC reactions. At a median follow-up of 6.8 years, 16/21 patients (76.1%) remained in first remission with no evidence of disease (Yu et al. 2001).

Other idiotypic antibodies mimicking GD2 were generated. The mAb 4B5 was generated by fusing murine myeloma cell line Ag8 with peripheral blood lymphocytes of a patient who received mAb 14G2a treatment (Saleh et al. 1993). A clinical trial of human mAb 4B5 + GM-CSF was conducted in patients with stage III/IV melanoma (NCT00004184), but no results have been published. In addition, the mAb 3F8 was used to generate rat anti-3F8-idiotypic antibodies C2D8, Idio-2, AIG4, C2H7, C4E4, and A2A6. These anti-Id antibodies could inhibit the binding of mAb 3F8 to GD2 and induce GD2-specific antibodies in mice (Cheung et al. 1993). So far, these anti-Id antibodies have not been tested in a clinical trial.

In summary, immunotherapy with anti-Id GD2 vaccines can induce humoral responses to GD2-expressing tumor cells. Its therapeutic efficacy awaits verification in randomized clinical trials.

5 *O*-Acetyl GD2-Specific Antibody

A 9(7)-*O*-acetyl transferase can add an *O*-acetyl ester to the external sialic acid of GD2 to form an *O*-acetyl GD2 (Sjoberg and Varki 1993). An *O*-acetyl GD2-specific mAb 8B6 was generated by screening antibodies that bind to alkali-labile ganglioside *O*-acetyl GD2 after immunizing A/J mice with LAN-1 cells (Cerato et al. 1997). The mAb 8B6 could bind to many human tumor tissues, including neuroblastoma, melanoma, small cell line cancer, and renal tumors, but not pain nerve fibers (Alvarez-Rueda et al. 2011). Moreover, a human-mouse chimeric mAb c.8B6 not only exhibited ADCC and CDC activity in vitro but also reduced neuroblastoma liver metastasis in vivo. Importantly, the mAb ch14.18-inducing allodynia in a rat model was not observed in animals treated with the mAb c.8B6 (Terme et al. 2014). In addition, the mAb 8B6 could induce apoptosis and cell cycle arrest to inhibit the growth of GD2-expressing cancer cells (Cochonneau et al. 2013), as well as targeting *O*-acetyl GD2+ breast cancer stem cells to suppress tumor growth in vivo (Cheng et al. 2021). These promising preclinical findings provide a strong rationale for developing the *O*-acetyl GD2-targeting immunotherapeutics.

6 Immune Checkpoint Inhibitors

Blockade of immune checkpoint molecules cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) has shown remarkable therapeutic efficacy in some cancer patients. A few clinical trials of immune checkpoint inhibitors in neuroblastoma are now underway. Expression of programmed death-ligand 1 (PD-L1) in clinical specimens of neuroblastoma has been reported to range from 18.9% to 35% in several studies (Zuo et al. 2020) (Saletta et al. 2017). Another study showed 25% (5/20) and 65% (13/20) of neuroblastoma at diagnosis and after 5-cycles of chemotherapy, respectively, expressed PD-L1 (Shirinbak et al. 2021). The clinical relevance of PD-L1 expression to the efficacy of anti-PD-1/PD-L1 in neuroblastoma remains to be determined. In preclinical studies, mAb PD-1 alone and mAb PD-L1 alone had no effect on tumor progression in syngeneic mice bearing with PD-L1-expressing Neuro2a and NXS2 neuroblastoma cells (Rigo et al. 2017). In a phase 1-2 trial of nivolumab (3 mg/kg) in children with measurable neuroblastoma, five of ten patients had stable disease (Davis et al. 2020). In another phase I clinical trial of ipilimumab, one neuroblastoma patient had stable disease for 2 months after single dose of ipilimumab at 10 mg/kg (Merchant et al. 2016). To improve the efficacy of ICI inhibitor in neuroblastoma, anti-GD2 in combination with PD-1 blockade was shown to significantly reduce tumor volume in A/J mice bearing neuroblastoma NXS2-HGW cells (Siebert et al. 2017). Based on this preclinical finding, two patients with refractory neuroblastoma were treated with the combination of Nivolumab and dinutuximab beta. One patient attained complete remission for 6 months, and the other showed complete disappearance of soft tissue

lesions and regression of skeletal lesions (Ehlert et al. 2020). Another checkpoint molecule CD47, which is a potent “don’t eat me” signal to prevent phagocytosis by macrophages, is a potential therapeutic target for cancer immunotherapy. Combination of CD47 blockade and anti-GD2 showed significant antitumor efficacy in TH-MYC*N* syngeneic neuroblastoma model (Theruvath et al. 2022). In another study, inhibition of CSF-1R⁺ myeloid cells with a tyrosine kinase inhibitor BLZ945 was shown to improve the anticancer efficacy of blocking antibodies against both PD-1 and PD-L1 in spontaneous neuroblastoma TH-MYC*N* mice (Mao et al. 2016). These findings suggest that neuroblastoma patients may benefit from ICI in combination with immunotherapy or other immune-modulating agents.

7 Conclusions

Improved outcomes in high-risk neuroblastoma patient after postconsolidation immunotherapy with dinutuximab have fueled further interest in refining immunotherapy against neuroblastoma. Strategies that aim for ameliorating the toxicities of the dinutuximab, improving efficacy by combination with chemotherapy or ICI, or developing new immunotherapeutics targeting GD2, *O*-acetyl GD2, GD3, NeuGcGM3 are flourishing. These endeavors will likely further improve the outcome of neuroblastoma.

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